Metabolic Profiling in Chronic Venous Ulceration

A thesis submitted to Imperial College London.

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I, Rahul Velineni confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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be a darn nuisance and my hope is that the work will make a positive impact on a group of patients who do not necessarily have many options particularly when routine treatment is unsuccessful.

Abstract

Introduction

This thesis seeks to determine the potential utility of metabolic profiling in chronic venous ulceration of the lower limb. Venous ulceration is a final common pathway for legs subjected to abnormally sustained and elevated venous pressures either as a consequence of venous obstruction or reflux. What is not clear is how venous abnormalities contribute to the syndrome of tissue loss. This thesis has outlined the present understanding of the pathogenesis of venous ulceration and has sought to outline the role of hypothesis generating metabolic profiling that may be able to shed new light on venous leg ulceration.

Methods

After a pilot study and concurrent development of methods. A cohort of patients with venous ulceration was recruited with the appropriate ethical approval for sequential clinical assessments and sampling of urine, serum and ulcer fluid (captured by sterile filter paper discs). Global metabolic profiling using nuclear magnetic resonance and mass spectrometry was performed and multivariate statistical analysis was performed according to known clinical variables that have been able to predict ulcer healing in the literature.

Results

Multivariate statistical analysis revealed that it was possible to discriminate between clinical prognostic groups based on the global metabolic profile of urine, serum and ulcer fluid. Multiple potential metabolite markers were identified and will be studied in detail in future work in terms of relevance to the pathogenesis of chronic venous ulceration and to prediction of prognosis.

Conclusion

This novel work has demonstrated the utility of metabolic profiling in chronic venous ulceration of the lower limb. When stratified for clinical parameters related to prognosis or ulcer healing status a variable metabolic phenotype was identified. Future work will involve the detailed characterisation of the metabolite features responsible for difference between clinical groups, validate the results of this thesis in a larger cohort as a prognostic tool and consider the role of venous ulcer biopsy.

Abbreviations

ABPI - Ankle Brachial Pressure Index

ACN - Acetonitrile

CPMG - Carr-Purcell-Meiboom-Gill

CV - Coefficient of Variation

CV-ANOVA - Cross validated analysis of variance testing

CVI – Chronic venous insufficiency

CVU - Chronic venous ulceration

D_20 - Deuterium Oxide

HILIC - hydrophilic interaction chromatography

HPLC - High performance liquid chromatography

LC - Liquid chromatography

MMP/MMPs - Matrix Metalloproteases

MS - Mass Spectrometry

MTBE - methyl tert-butyl ether

NHS - National Health Service

NMR - Nuclear Magnetic Resonance

OPLS - Orthogonal partial least squares (analysis)

OPLS-DA - Orthogonal partial least squares: Discriminant Analysis

PCA - Principal Component Analysis

QC - Quality Control

RCF - Relative centrifugation force.

RP - Reverse Phase

STOCSY - Statistical total correlation spectroscopy

TGF-β - Transforming growth factor beta

TIMPs - Tissue inhibitors of matrix metalloproteases

TNF - Tumour necrosis factor

TSP - 3-(trimethylsilyl)-[2,2,3,3,2H4] propionic acid sodium salt

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Chapter 1: Introduction

1.1 Wounds

A wound is defined as a loss of epithelial integrity of the skin and for the purposes of this thesis is synonymous with an ulcer. Successful wound healing is defined as complete re-epithelisation of the wound.

1.1.1 Normal Wound Healing

The restoration of epithelial integrity involves a complex interplay of overlapping cellular and molecular events within a wound over a period of time. The evolutionary conserved process of wound healing is transferable with caveats within human systems and across species (Figure 1.1).

There are four defined overlapping phases of wound healing:

1. Haemostasis.
2. Inflammation.
3. Proliferation.
4. Remodelling.

1.1.2 Haemostasis

In response to vascular disruption, several mechanisms collude to restrict haemorrhage and form a bridgehead for subsequent phases of healing. The archetypal cell of this phase is the platelet which in turn is activated by the exposed

fibrillar collagen, fibronectin and assorted extracellular matrix proteins[1]. In the first instance, this provides temporary wound coverage.

Activated platelets demonstrated adhesive and aggregatory properties but perhaps the most important purposes are secretory. Vasoactive mediators are secreted in addition to adhesive proteins. This serves to further drive platelet aggregation, form a platelet plug and to generate a fibrin clot[2].

Subsequently, the coagulation cascade is also activated by the activation of circulating factor XII by platelet aggregation culminating in the conversion of fibrinogen to fibrin. An alternative route of fibrin generation is the 'extrinsic' pathway that can be stimulated by the release of tissue factor from a variety of sources including damaged tissue and endothelial cells[2].

In addition to the coagulation role, platelets upon aggregation release a range of growth factors including transforming growth factor beta, platelet derived growth factor to recruit neutrophils, macrophages and fibroblasts[3].

1.1.3 Inflammation

The overall aim of this phase is to recruit appropriate cells to the site of injury. The key cells involved in this phase are mast cells, neutrophils and macrophages[3]. Neutrophils predominate in early inflammation with macrophages predominating in late inflammation.

Mast cells release vasoactive amines and histamine to promote the cardinal features of acute inflammation (erythema, pain, swelling and heat). Neutrophils are driven to the site of injury by increased vascularity, attracted from the circulation by the endothelial expression of selectins in turn driven by platelet secreted factors and by-products of the coagulation cascade[2]. The role of the neutrophil is to remove foreign particles from the wound including bacteria which can either be extruded in wound fluid or phagocytosed by macrophages[1, 3].

Circulating monocytes are attracted to the wound by similar factors that attract neutrophils and breakdown products of the extracellular matrix and specific monocyte chemoattractants including monocyte chemoattractant protein 1 and macrophage inflammatory protein 1. Monocytes settle within tissue spaces and transform into macrophages.

The primary role of the macrophage is to phagocytose, kill and digest pathogens, scavenge tissue fragments and remove any remaining neutrophils as the wound enters the late inflammatory phase[2, 4, 5]. An important secondary role is to consider the macrophage as a factory for various factors critical for healing: fibronectin to attract fibroblasts and several cytokines namely; transforming growth factor alpha and beta, platelet derived growth factor, vascular endothelial growth factor and fibroblast growth factor.

1.1.4 Proliferation

This phase is characterised by the inward migration of fibroblasts and endothelial cells with the generation of extracellular matrix, granulation tissue and ultimately formation of epithelium[6].

Fibroblasts are attracted to a wound at 2-4 days post injury by the expression of transforming growth factor beta and platelet-derived growth factor in addition to other factors. Fibroblast proliferation and expression of fibronectin, hyaluronan and collagen then occurs to construct the extracellular matrix[6].

Keratinocytes and the host of inflammatory cells involved with the wound secrete vascular endothelial growth factor to attract endothelial cells, stimulate proliferation and to generate increased vascularity through the development of capillary tubes[5].

Keratinocytes initially migrate from the free edges of the wound and various factors promote migration such as the extracellular matrix protein, integrin receptors, matrix metalloproteinases, growth factors and the initial matrix generated in the haemostasis phase[2].

Once migration is complete, possibly by a contact inhibition process[2], proliferation takes place in order to complete wound closure especially in larger wounds where closure may not be possible with migration alone driven by transforming growth factor alpha, transforming growth factor beta and epidermal growth factor[4].

1.1.5 Remodelling

This phase takes place throughout the wound healing process with a prime example being the transformation of the fibrin clot matrix into granulation tissue during the proliferative phase and finally into a collagenous scar[2]. In larger wounds where keratinocytes alone cannot provide coverage, fibroblasts differentiate into myofibroblasts to effect closure by wound contraction[4].

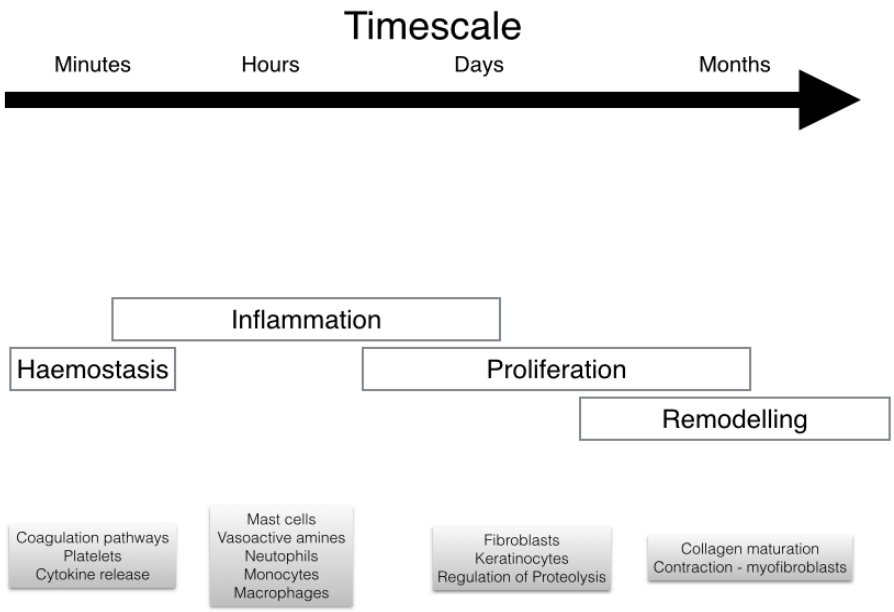


Figure 1.1 - Wound healing timescale, the sequential biological steps.

1.1.6 Abnormal Wound Healing

Most wounds will heal rapidly as a consequence of the preserved molecular cascade. However, some wounds will either heal slowly or not at all. Chronic wounds pose a significant burden to humanity in terms of patient morbidity and healthcare resources

[7]. Commonly the reason for wound healing 'failure' is a contribution from many factors which can be divided into local and systemic factors.

1.1.7 Local Factors

These include[6]:

- Arterial or venous insufficiency.
- Presence of non-viable tissue.
- Ongoing active infection - cellulitis or osteomyelitis as examples.
- Excess mobility or increased skin tension.
- Pressure.
- Trauma.
- Neuropathy.
- Malignancy.

1.1.8 Systemic Factors

1.1.8.1 Diabetes Mellitus

Diabetes can impart a negative effect of wounds by a multitude of mechanisms including peripheral vascular disease, neuropathy and infection[7]. In addition, multiple alterations in wound milieu can occur including sub-optimal angiogenesis, decreased nitric oxide species formation, impaired macrophage function, impaired fibroblast function, reduced collagen deposition and limited epithelial cell progenitor migration [8].

1.1.8.2 Nutritional Deficiency

In addition to deficiencies of glucose and protein, deficiency of Vitamin C, Vitamin E, iron, arginine, glutamine, zinc and magnesium have postulated roles in impaired wound healing[9].

1.1.8.3 Other Systemic Factors

Further implicated systemic factors include insufficiency of the hepatic or renal system, immune deficiency and connective tissue disorders[7].

1.1.9 Chronic Wound Healing Pathophysiology

A chronic wound represents, in simplistic terms, the culmination of a final common pathway from multiple sources resulting in a wound that will not heal (beyond its normal expected time)[6]. Typically, these wounds at a cellular level may have subtle differences from acute wounds in cellular function, cytokine signalling, extracellular matrix components or the activation or regulations of MMPs. Specific details relating to CVU will be discussed later in this chapter.

1.1.9.1 Prolonged and Altered Inflammation

Despite the heterogeneity of local and systemic factors that can lead to a chronic wound, a common feature is the propensity for these wounds to 'remain' in the inflammatory phase which in itself is compared to normal acute inflammation[6]. There is evidence to suggest that neutrophils from biopsies taken from chronic wounds, release more reactive oxygen species than compared to healthy controls and that the

lymphocyte balance is altered[10, 11]. At a dermal level, there is a persistent histological infiltration of T-lymphocytes and macrophages which may serve to over stimulate the influx of neutrophils in a chronic wound[12].

1.1.9.2 Proteolysis

The polymorphonuclear neutrophil is a hallmark cell type in early acute inflammation which is attract to a wound by markers of injury, traverses the endothelium and enters the extracellular matrix to initiate debridement and to maintain a defensive posture against bacterial infection. The movement of the neutrophil through the endothelial wall and extracellular matrix is facilitated by the production of protease enzymes and oxygen free radicals[13]. Biopsies suggest a preponderance of neutrophils in chronic wounds associated with oedema and evidence of proteolysis[14].

In wounds three specific groups of compounds relate to proteolysis[13];

1. Reactive oxygen species
2. Matrix metalloproteases
3. Anti-protease systems.

In chronic wounds, there is evidence to suggest that the proteolytic environment is overactive and unregulated leading to the inadvertent destruction of healthy tissue and possibly the loss of growth factors implicated in normal wound healing[15-18].

1.1.9.3 Biofilms

One factor that may contribute to the ongoing unregulated inflammatory phase in chronic wounds is the presence of bacteria. By definition a wound with its epithelial

defect provides an attachment site for bacteria which may then aggregate and transform from a planktonic phase to a chronic aggregated clump of cells with modified growth characteristics and an ability to evade host defences and resist antibiotics[19].

As a consequence of this 'survival' mechanism, the biofilm can modulate multiple aspects of the host inflammatory response, potentiating and prolonging it. There have been documented effects on neutrophils, macrophages, innate immunity, cytokines, growth factors and the proteolytic systems[20]. The net effect of this modulation may prolong inflammation in chronic wounds.

1.2 Ulceration of the Lower Limb

Chronic ulceration of the lower limb is defined as a breach in the epithelial integrity of the skin between the knee and the ankle of greater than 6 weeks duration and the aetiology can be broad[21]. Around 90% of lower limb ulceration (Figure 1.2) can be ascribed to vascular and neuropathic causes although other causes include trauma, vasculitis, malignancy, pyoderma gangrenous and lymphoedema[22].

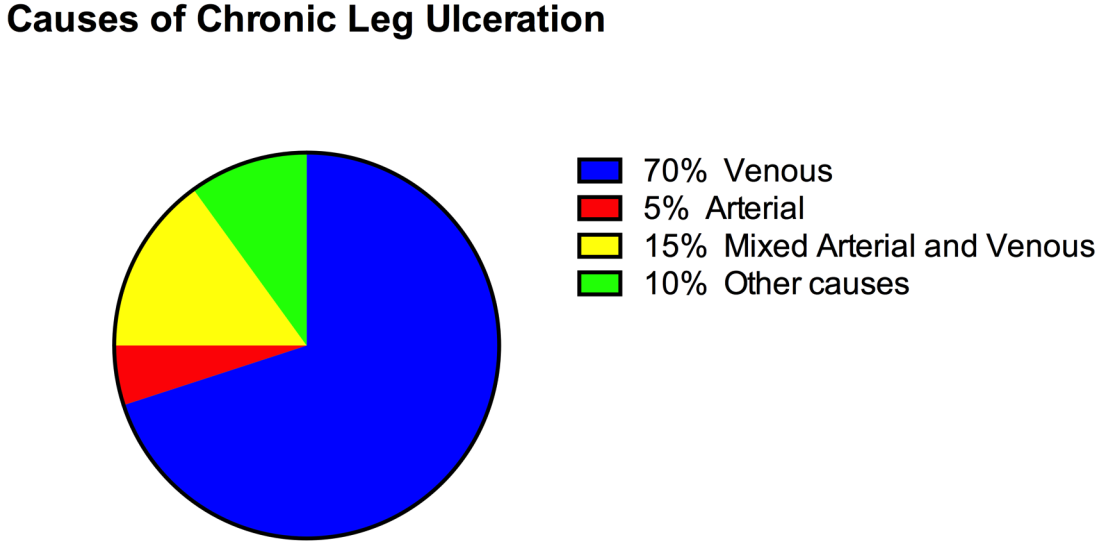


Figure 1.2 Causes of leg ulceration expressed as percentages.

1.3 Chronic Venous Ulceration of the Lower Limb

1.3.1 Definition

For the purposes of this thesis, chronic venous ulceration of the lower limb is defined as an open wound between the knee and the ankle with an ankle brachial pressure index of greater than 0.85 typically with evidence of venous reflux or obstruction[23].

1.3.2 CEAP Classification

In order to classify venous disease appropriately, the CEAP system was introduced by a consensus group in 1996[24] and revised in 2004 incorporating the clinical, etiologic(sic), anatomical and pathophysiological features (Table 1.1). It is used in the standardised reporting of venous disease in the literature.

Clinical	
C0	No visible or palpable signs of venous disease
C1	Reticular veins / telengectasia
C2	Varicose veins
C3	Oedema
C4a C4b	Pigmentation/eczema Lipodermatosclerosis/atrophie blanche
C5	Healed venous ulcer
C6	Active venous ulcer
Etiologic (sic)	
Ec	Congenital
Ep	Primary
Es	Secondary
En	No venous cause
Anatomic	
As	Superficial veins
Ap	Perforator veins
Ad	Deep veins
An	No venous location identified
Pathophysiologic (sic)	
Pr	Reflux
Po	Obstruction
Pr,o	Reflux and obstruction
Pn	No venous pathophysiology

Table 1.1 – CEAP classification variables.

1.3.3 Epidemiology

Multiple attempts have been made to chart the incidence and prevalence of chronic venous ulceration. These studies vary greatly in terms of study methodology and reporting standards.

1.3.3.1 Incidence

In a cohort of 880 patients examined sequentially over 13 years in Edinburgh, the 13 year incidence of active or healed venous ulceration (C5, C6) in this population was 0.6% (confidence interval 0.2 - 1.6%), complicated varicose veins (C3, C4) had an incidence of 8.6% and uncomplicated truncal varicose veins (C2) had an incidence of 18.2% [25]. In a study in Newcastle-upon-Tyne, a survey of district nurses revealed an annual incidence of 3.5/1000 patients over 45 years[26].

1.3.3.2 Prevalence

An accepted estimate of the prevalence of active venous ulceration (C6) is of 0.3% of the population having an open wound and 1.0% of the population having a healed (C5) ulcer[27, 28].

1.3.4 Risk Factors for Venous Disease

Many risk factors are implicated in varicose vein disease including positive family history, increase age, female gender, increasing parity, and exposure to the hormones

oestrogen, progesterone and relaxin[29]. Obesity, cigarette smoking and diabetes mellitus are thought to possibly increase risk but this is not universally agreed[29, 30].

However, not all subjects with early stage venous disease will progress to chronic venous insufficiency (CEAP 3-6) and it is thought that the changes in venous haemodynamics and microcirculation act on the glycocalyx and endothelium to promote an inflammatory response[30].

1.3.5 Normal Venous Function

The goal of the lower limb venous circulation is return blood to the heart and to act as a capacitance reservoir to store blood. In the lower limb, there are two systems; the deep and superficial veins which communicate at the sapheno-popliteal junction and sapheno-femoral junction. In addition, there is a system of communicating perforator vessels that permit further transmission of blood from the superficial to the deep systems. In normal conditions, the deep venous system has a high pressure and the superficial system has a low pressure[30].

In addition to onward forward momentum generated by the heart, venous return to the heart is also promoted by the calf and foot muscle pump (reducing the calf pressure from 90mm Hg on standing to 30 mm Hg on walking) [31] and a system of endothelial derived valves to prevent retrograde flow and to prevent flow from the high pressure deep system to the low pressure superficial system via the perforating vessels.

1.3.5 Abnormal Venous Function

Abnormal venous circulation within the lower limb can be the result of defects in the deep, superficial or perforating systems and possibly failure of the calf muscle pump. The most common cause of sustained venous hypertension is superficial venous reflux presumed secondary to valvular reflux, although post thrombotic syndrome typical as a sequel of deep venous thrombosis and its destruction of deep venous valves can also be a cause [32] and the calf muscle pump may fail typically because of underuse in immobility[31].

From a haemodynamic perspective, the resting venous pressure in the foot of a patient with normal or abnormal veins is typically 90mm Hg and on walking, the pressure in the normal foot can be reduced to usually 30mm Hg. However, this response is attenuated in the abnormal limb leading to more sustained higher venous pressures[33, 34].

In the context of limbs with venous ulceration compared to normal limbs, it was seen that no ulceration was present in limbs with an ambulatory venous pressure of less than 30mm Hg whilst 100% of subjects with an ambulatory venous pressure of greater than 90 mmHg had venous ulceration present[35].

If the perforating vein valves fail, pressure generated by the calf pump can be transmitted to the superficial system in addition to high pressure from the deep system. This may in turn cause failure of valves in the superficial system[30].

1.3.6 Rationale for Clinical Assessment and Management

Individual patient assessment is critical to plan appropriate clinical management and is usually the start of a long-term relationship between the patient and provider of healthcare irrespective of location. The following factors should be covered in the initial assessment:

- Aetiology.
- Identification and targeting of specific factors that may preclude healing.
- Avoidance of complications.
- Selection of optimal treatment.
- Determine the effect of ulceration on the patient.

1.3.6.1 Clinical Features

In the history, the symptoms of venous disease (prominent varicosities, limb swelling and skin changes) should be sought, in addition the presence of previous episodes of ulceration may hint towards a venous aetiology. The presence of arterial symptoms such as intermittent claudication, night pain or rest pain may point towards arterial insufficiency.

The examination requires an assessment of perfusion typically gleaned from skin temperature, skin colour at rest and on elevation and clinical assessment of lower limb pulses at the common femoral, popliteal, dorsalis pedis and posterior tibial.

Finally, a formal assessment of the actual ulcer is required. An estimation of ulcer area, the shape of the edge, ulcer location and whether the ulcer is clean, granulating or sloughy can help distinguish between arterial, venous or mixed aetiologies.

1.3.6.2 Investigations

1.3.6.2.1 Ankle Brachial Pressure Index (ABPI)

It is important to exclude an arterial cause for leg ulceration because the application of compression bandaging in a limb with arterial compromise will be deleterious to the limb. In addition, arterial compromise if detected can be treated by many means including conservative, endovascular and open surgical approaches. Clinical examination alone may be an unreliable method to detect arterial disease and thus an ABPI may be useful as a validated, reliable and reproducible marker for the presence of arterial disease [36].

For the purposes of this thesis, an ABPI < 0.85 was deemed to be suggestive of significant arterial disease. This definition was used in a large randomised trial comparing open surgery against compression alone for the treatment of CVU[37].

1.3.6.2.2 Colour Duplex Ultrasonography

A colour venous duplex scan in the lower limbs can evaluate the superficial and deep venous systems of the lower limb. Normally venous blood will drain towards the heart and from superficial veins to deep veins. Chronic venous insufficiency can arise from

venous occlusion, thrombosis or valvular incompetence all of which can be determined using ultrasonographic investigation.

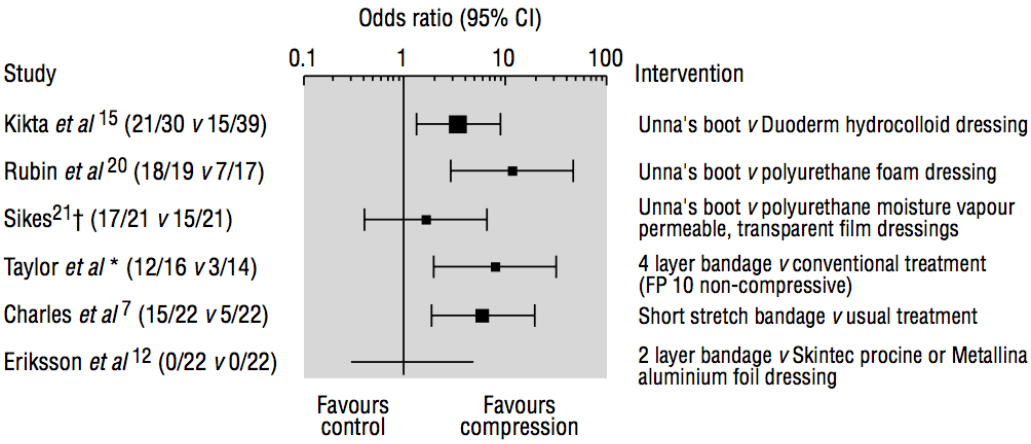
In the context of CVU, a confirmation of chronic venous insufficiency (CVI) is required as is a 'roadmap' of venous abnormalities to plan further treatment.

1.3.7 Treatment of Venous Ulceration

1.3.7.1 Conservative Measures

Exercise and Leg Elevation: Supervised exercise programmes exist for patients with peripheral arterial disease in the United Kingdom but there is not an analogous system for CVU. This probably relates to a lack of evidence indicating effectiveness, a systematic review looked in detail at 16 publications with the conclusion that there may be some benefit to exercise in CVU although the precise relationship has not been elucidated[38].

Compression Bandaging: The aim of banding in CVU is to promote venous return in the lower limb and to reduce the syndrome of sustained elevated venous pressure with a target application pressure upper limit pressure of 60mm Hg. At present, compression bandaging is the most efficacious treatment available to patients with CVU. Detailed reviews on the effectiveness of compression bandaging have been published but in summary, a systematic review has demonstrated that in direct comparison between compression or primary dressing alone, a greater proportion of CVU wounds healed with compression [39]. Successful application of bandaging is a difficult skill to master requiring significant training.



* Unpublished.
† Analysis based on multiple ulcers rather than patients, so analysis is inappropriate.

Fig 1 Estimate of effect of compression treatment versus no compression (complete healing, after varying lengths of treatment)

Figure 1.3 - Odds ratio comparing compression to primary dressing alone - outcome complete ulcer healing. Reproduced with permission from BMJ group [39].

1.3.7.2 Medical Treatment

Pentoxifylline: The exact mechanism of Pentoxifylline is unknown but it is thought to have an anti-inflammatory effect, a reduction in blood viscosity, platelet aggregation and fibrinogen levels. A meta-analysis updated in 2012 suggests that Pentoxifylline alone and in combination with compression bandaging may improve venous ulcer outcome in terms of healing but there was marked heterogeneity between studies[40].

Aspirin: As an irreversible modulator of the cycloxygenase system mediating a reduction of prostaglandin E2 and thromboaxane A2 which are key inflammatory mediators, first principles would dictate that a role for aspirin in CVU does exist. However, efficacy has not been demonstrated in a randomised controlled trial with sufficient participants and sound methodology[41].

Other agents: A randomised double blind placebo controlled trial from the Philippines suggests that healing rate is increased, healing time is decreased and quality of life scores are improved when Simvastatin 40mg is administered. Although promising, the trial does not clearly state whether these patients were on a cholesterol lowering medication before the trial started[42]. Stanozolol is a steroid with fibrinolytic activity which may in theory should improve ulceration in the fibrin cuff paradigm proposed by Browse and Burnand, however the literature does not support this statement[43].

1.3.7.3 Superficial Venous Disease Treatment

With compression bandaging established as the gold standard in the treatment of CVU, a randomised trial comparing bandaging and open venous surgery with compression bandaging alone in 500 patients was undertaken. At 24 weeks, 65% of ulcers had healed in both groups but at 12 months, recurrence in the surgical group was 12% with 28% recurrence in the bandaging alone group[23]. Moreover, 47 patients in this study assigned to surgical treatment refused to undergo an operation and this may have underestimated the potential benefit. Endovascular techniques which are now widely offered as treatment for superficial venous disease may be more appealing to patients than open surgery[44] and a large randomised controlled trial is currently in progress[45].

1.3.8 Clinical Outcomes in Venous Ulceration

1.3.8.1 Wound Healing

In patients with open venous ulceration treated with four-layer compression bandaging in standardised studies, an estimate of wound closure at 26 weeks lies between 74-76% leaving approximately 24-26% unhealed [46, 47].

1.3.8.2 Factors Affecting Healing Time and Recurrence

Although multiple studies have attempted to examine the factors related to delayed healing in CVU, there is marked heterogeneity between different studies relating to study performance, subject selection and definitions of 'delayed' healing[48].

Ulcer Duration: Increasing ulcer chronicity (usually recorded in months) has been identified as a factor with an association with delayed wound healing during a defined time period and also on recurrence after healing[46, 49].

Ulcer Dimensions: Initial increased wound area has been found to predict delayed healing in multiple studies[49-51]. In particular, an initial ulcer size <20cm2 was predictive of healing within 52 weeks of compression bandaging[50].

Distribution of Venous Disease: Taking into account the changes in diagnosis and definitions of disease, deep venous abnormalities appear to impart a negative effect on ulcer healing and ulcer recurrence after healing[48].

Other Factors: Interestingly there is no clear consensus in the published literature regarding other factors relating to patient demographics, mobility, movement of the ankle joint and nutrition.

1.4 Biological Concepts in Chronic Venous Ulceration

Chronic venous ulceration of the lower limb is incompletely understood since it is not entirely clear how chronically sustained elevations in venous pressure translate into the clinical syndrome of tissue loss and poor healing. This section will outline the historical background in the biological study of venous ulceration, the key theories that have emerged and outline the key factors studied.

1.4.1 Pathophysiology of Chronic Venous Ulceration

Active chronic venous ulceration represents the most severe manifestation of chronic venous insufficiency. Chronic venous insufficiency ultimately can develop from multiple mechanisms of failure[52]:

1. Valvular failure in the deep or superficial system.
2. Perforator incompetence.
3. Obstruction.
4. Calf muscle pump insufficiency.
5. A combination of factors 1,2,3 and/or 4.

These factors will ultimately combine to lead to sustained venous hypertension[35] most typically pronounced whilst standing. Unabated, sustained venous hypertension in larger vessels can translate to sustained microcirculatory changes[53]. Ultimately, this

will lead to the clinical syndromes seen in latter CEAP clinical stages (3-6) namely skin changes, ulceration and poor and disorderly healing. How the sustained circulatory changes translate into skin changes is incompletely understood at present although several theories have been generated.

1.4.2 Connecting Chronic Venous Hypertension to Ulceration

Various theories have been proposed linking sustained elevation of lower limb venous hypertension to the clinical syndrome of tissue loss and abnormal healing seen in CVU. They range from the historical concept of stasis or arteriovenous shunting and more recently the fibrin cuff formation, white cell and cytokine trapping.

1.4.2.1 Stasis

It has been well recognised for over a century that sustained venous hypertension ultimately leads to the syndrome of tissue loss, ulceration and impaired healing manifested by chronic venous ulceration. Homans[54] suggested the first prevalent intermediate theory postulating that sluggish flow in varicose veins led to stasis. This was supported by the work of De Takats[55] who found that in varicose veins the oxygen content was indeed lower than that of similar venous blood from the antecubital fossa. However, Blalock put the oxygen content difference as a function of dependency. Furthermore, further work has suggested that in fact, oxygen content in venous blood from affected limbs actually shows a higher oxygen concentration [56].

1.4.2.2 Arteriovenous Shunting

With the finding of increased oxygen content in the venous blood from the lower limbs of CVI patients, it was suggested that in fact arteriovenous shunting (and thus faster

circulation times) could be the underlying cause of CVU[57]. However, the presence of arteriovenous shunts have not been identified in vivo using radioisotope marker studies[58].

1.4.2.3 Fibrin Cuffing

Landis' work from the 1930s demonstrated that increased venous pressure in the limb markedly increases capillary bed luminal pressure to a degree 5-6 times greater than the corresponding rise in arterial pressure. Further work demonstrated that in CVI patients, the dermal capillary bed was enlarged compared to controls and Skin biopsies from patients with CVI and lipatodermatosclerosis showed an increased number of capillaries compared to controls[59] and pericapillary fibrin was noted in patients with an impaired ability to reduce venous pressure with exercise[60].

In an attempt to unify these findings, Browse and Burnand[61] suggested that increased capillary pressure in turn secondary to sustained elevated limb venous pressure enlarged inter-endothelial pores allowing the release of fibrinogen which in turns polymerises to fibrin around capillaries. It was postulated that the newly formed 'fibrin cuff' acts as a diffusion block to nutrients and oxygen hence leading to CVU. Although the potential to 'block' in vitro has been demonstrated, there is no sufficient evidence to suggest that this occurs in vivo.

1.4.2.4 White Cell Trapping

White cells have been shown to accumulate in dependent legs and reduced capillary loops have been demonstrated in patients with CVI which is somewhat at odds with previously reported work. Taking this observations into account, Coleridge-Smith and

colleagues[62] argued that elevated lower limb venous pressures manifest in reduced capillary network intramural pressure which promotes the accumulation, margination and extravasation of white blood cells. The argument is that firstly, white cells block capillary loops preventing adequate flow and that the extravasation of white cells from the local circulation promotes the activation of the proteolytic cascade and release of oxygen radicals which initiate and promote tissue destruction and endothelial damage (which in turn increased capillary permeability).

However, these changes can be seen in patients without CVU[63] and thus the question is whether white cell trapping is the initiator of CVU.

1.4.2.5 Growth Factor Trapping

The most predominant current theory suggests that the combination of venous hypertension and endothelial injury possibly brought about by white cell mediated injury allows the extravasation of macromolecules such as fibrinogen and α2-macroglobulin[64] which in turn sequester factors critical to wound healing such as TGF-β and TNF which is supported by the inhibition of CVU fluid on in-vitro fibroblasts, endothelial cells and keratinocytes[64]. However, compelling this theory, active cytokines can still be detected in CVU fluid[65-68].

1.4.3 Methods of Biological Study in Chronic Venous Ulceration

Multiple methods have been utilised to sample the micro-environment of CVU which are summarised in Table 1.2. These include:

1. Biopsy of the ulcer edge or base[67, 69-93].

2. An occlusive dressing technique that involves covering the ulcer with a non-permeable transparent sheet, allowing fluid to accumulate over a defined period and the fluid is then aspirated typically with a needle [65, 68, 77, 94-102].

3. Direct elution of fluid from wound dressings or material acquired at the time of wound debridement[103-107].

4. Application of filter paper (or discs) to the CVU wound surface, retrieval after a defined period and extraction of the fluid for analysis [108].

5. Sampling of local blood from the affected limb[109].

Technique	Summary
Biopsy	A biopsy usually performed under local anaesthesia of the ulcer edge or base.
Occlusive dressing	The ulcer is covered using a non-permeable transparent dressing with wound fluid allowed to accumulate over a defined period. Fluid is then aspirated typically using a needle.
Direct elution of fluid	Fluid taken from routine wound dressing or material taken from time of debridement.
Application of filter paper	Application of absorbent filter paper to the wound, retrieval at a defined time with subsequent extraction of fluid at a later time and date.
Blood sampling	Typically sampling of venous blood from the affected limb.

Table 1.2: Methods of CVU sampling

1.4.3.1 Difficulties in Study

Although animal models exist to simulate diabetes, ageing and venous haemodynamic abnormalities, no such model exists to simulate CVU[110]. Thus without a validated animal model, biological study of CVU by definition requires samples from humans with ulceration.

Wound fluid, local blood and tissue biopsies have been utilised and although useful knowledge has been obtained, the variation in patient selection, sample collection and assay techniques may limit the validity and reproducibility of studies. In addition, most

studies are small, which may reflect difficulties in recruitment given that in general, patients with CVU tend to be elderly and frail.

1.4.4 Proteolysis

Metalloproteinases are a group of endopeptidases that utilise a zinc or calcium ion within their active site and 24 types have been found in mammals[111]. They can be broadly categorised into four groups interstitial collagenases, gelatinases, stomolysins and membrane type metalloproteinases[112]. Four types of tissue inhibitors of metalloproteinases (TIMPs) in addition to other factors such as alpha-2-macroglobulin inhibit the activation of MMPs by binding to the active site[111]. In wound healing, MMPs serve to cleave various components of the extracellular matrix in order to promote remodelling when an optimal balance of MMPs and their inhibitors exist, and a balance between new protein formation and cleavage must be achieved in order to achieve epithelial integrity.

In a comparison of chronic wound fluid obtained using the occlusive dressing technique in six participants and of fluid obtained from the drains of ten women who underwent mastectomy it was seen that in chronic wound fluid that the levels of MMP-2 and MMP-9 were ten times higher and demonstrated increased levels of pro-enzyme and activated enzyme activity as demonstrated using gel zymography[113]. Again comparing CVU fluid from ten patients to acute wound fluid from six patients total collagenase (MMP-2 and MMP-9) and gelatinase (MMP-2) activity was higher ulcer fluid[114]. Somewhat contrary to other work, cell culture of fibroblasts from CVU and

patient matched ipsilateral thigh demonstrated reduced MMP-1 and MMP-2 expression and activity with increased TIMP-1 and TIMP-2 activity in venous ulcer fibroblasts although a similar conclusion of disordered proteolysis was reached[115]. Regarding collagen turnover, this was assessed by the measurement of collagen III N terminal peptide and degraded collagen with assessment of total MMP, MMP-1 and MMP-3 levels demonstrating the CVU biopsies demonstrated increased MMP activity and collagen turnover. Furthermore, increased collagen turnover and MMP-1 activity differentiated fast healing ulcers compared to slow healing ulcers[74].

ELISA assay of wound fluid demonstrated that the concentrations of MMP-9 are higher in wound fluid from patients with CVU compared to wound fluid obtained from operations in control groups and that the levels of MMP-9 in CVU fluid decrease over time with this effect more pronounced in rapid healers as opposed to slow healers and non-healers[17] following surgical treatment.

In summary MMPs tend to be elevated in concentration and activity in the CVU microenvironment however results are not entirely concordant. However, the concept of disordered proteolysis can be seen from the present body of knowledge and this may be driven by the pro inflammatory environment in CVU.

1.4.5 Angiogenesis

Angiogenesis is defined as the formation of new capillaries which form a significant part of the healing cascade. Capillary density, tortuosity and leakiness correlates with

the severity of venous disease as determined by histological analysis[60]. Further histological analysis demonstrates that capillary basement membranes are abnormal in venous disease[116]. Vascular endothelial growth factor (VEGF) is an angiogenic factor which can promote the formation of new capillaries and increase the permeability of capillaries. Increased VEGF in plasma has been noted in patients with chronic venous insufficiency compared to normal patients using sandwich ELISA[117].

The elevated expression of VEGF and its receptors Flt-1 and KDR was confirmed in chronic wounds with VEGF mRNA strongly expressed in the wound edge although the expression of epidermal VEGF protein was relatively low. Chronic wound fluid appeared to have a strong degradation of VEGF in-vitro and this process is inhibited by protease inhibitors[118]. Venous ulcer fluid obtained using an occlusive dressing technique inhibited in vitro angiogenesis using in comparison to acute wound fluid [98]. Acute wound fluid and tissue compared to venous ulcer fluid and tissue demonstrated similar quantities of vascular endothelial growth factor and its receptor as demonstrated by sandwich enzyme linked immunoassay and reverse transcriptase polymerase chain reaction, with slightly higher level to be seen in less chronic venous ulcers[97]. In addition to the inhibitory effect of CVU fluid on an in-vitro angiogenesis model, an inhibitor of MMP-2 and MMP-9 can negate the inhibitory effect of CVU fluid on angiogenesis[105].

In lipodermatosclerosis of the leg secondary to venous disease, VEGF, angiopoetin and VEGF receptor mRNA and protein expression are unregulated in comparison to normal skin. This is in contrast to the results seen with venous ulceration where protein

expression appears relatively sparse suggesting that an imbalance within the angiogenesis may promote the formation of ulceration[119] which may ultimately be related to proteolysis[119].

1.4.6 Aberrant Cellular Behaviour

IL-24 administered to immortalized human keratinocytes reduces cell migration (fully reversed by an AKT pathway inhibitor) and by analysis of transcription and immunohistochemistry, CVU biopsies show increased expression of IL-24 and its receptor compared to acute wounds with the majority of IL-24 localised to the suprabasal keratinocyte layer[120].

Under the assumption that the CVU microenvironment is hypoxic, fibroblasts cultured from normal skin and ulcers were isolated and cultured. Thereafter, the fibroblasts were exposed to varying degrees of hypoxia in vitro and protein synthesis measured. Both normal and CVU fibroblasts produce fibronectin and collagen with only collagen synthesis reduced in hypoxic conditions with normal fibroblasts showing more sensitivity[92].

Punch biopsies from the edge of ulcers and ipsilateral normal thigh skin in seven CVU patients with subsequent fibroblast cell culture demonstrated decreased growth rates in ulcer fibroblast culture, increased senescence associated beta-galactosidase in ulcer fibroblasts and significantly higher expression of fibronectin protein and mRNA[80]. Furthermore, neonatal foreskin fibroblasts exposed to CVU fluid were driven into a

senescent state in a dose dependent as measured by reduced growth curves and the increased expression of senescence associated beta-galactosidase. This effect was replicated by the addition of exogenous TNF-α or TGF-β1[121].

Neonatal foreskin fibroblasts in cell culture exposed to pressure in-vitro show increased senescence associated beta-galactosidase in a dose dependent manner that is not affected by exogenous TGF-β[82].

Comparing four patients with CVU (CEAP 6) with four patients with superficial venous reflux alone (CEAP 2), proximal thigh fibroblasts from both groups, ulcer edge fibroblasts and medial malleolar fibroblasts (reflux group) were cultured. Assessment of cellular kinetics demonstrated increased doubling time in ulcer fibroblasts and that there was marked increase in senescence associated beta-galactosidase in ulcer fibroblasts over in-vitro passage. The conclusion being that ulcer fibroblasts appear to be a more advanced state of cellular senescence[121].

Further confirmation of the senescent phenotype measured by cell culture growth rates has been demonstrated with the observation that senescent ulcer fibroblasts can be treated with bFGF to stimulate cell division in culture[122]. Further work demonstrated that bFGF application downregulates p21 expression and upregulates phosphorylated pRB expression in venous ulcer fibroblasts hence overcoming S phase cell cycle arrest [123]. Contradictory evidence suggests that in culture, fibroblast proliferation and senescence associated β galactadiosodase activity from normal and CVU fibroblasts

appears to be similar, however the P-value was quoted as 0.1 and sample preparation was different to the contradicted studies [115].

The negative effect of CVU fluid on fibroblast proliferation was determined to be due to a combination of inhibition of a Ras mediated pathway, reduced expression of hyperphosphorylated retinoblastoma tumour suppressor gene and reduced expression of cyclin D. This was evidenced by the finding that permanently activated Ras negated the effects of added CVU fluid whereas an inhibitor of downstream MEK, PD 98059 replicated the effects of CVU fluid in fibroblast culture [106].

CVU fibroblasts consistently over express P38 and by the addition of its inhibitor in-vitro (SB203580), fibroblast culture growth rates are increased. The addition of bFGF to CVU fibroblast culture can reduce the expression of p38 with the possibility of promoting cell cycle progression[73].

1.4.7 Cytokines

As major components of cell-cell signalling and thus coordinating the cellular responses in wound healing, the role of cytokines in CVU has been studied.

Cyclo-oxygenase 1 and 2 isoforms were studied in normal skin and CVU biopsies. COX-1 is ubiquitous in both skin types whereas significantly more COX-2 was

expressed in CVU biopsies. Radioimmunoassay confirmed increased COX activity in CVU tissues suggesting an inflammatory phenotype exists[86].

Eighteen patients with CVU were stratified by healing status and ELISA study of PDGF, GM-CSF, Il-1α, Il-1β, Il-6 and B-FGF was undertaken. In addition, protein and collagenase concentrations were measured. There was no significant difference in concentrations between the two groups[66].

In ten patients, effluent veins draining ulcers were cannulated and the leg was kept horizontal, then dependent and finally elevated to recreate conditions of local ischaemia and reperfusion. PAF, IL-6, IL-1RA and P-selectin were studied but no change was seen in venous concentrations with alterations in leg position. This may reflect the fact that cytokines were assayed from local veins [109].

Whilst demonstrating a preponderance of CD68 cells and HLA-DR positive cells in immunohistochemical analysis of CVU tissue compared to a mixture of acute and chronic wounds of other types, it was observed that CVU tissue had an excess of Il-10 with a paucity of GM-CSF staining suggesting that cell mediated immunity may not function effectively in CVU[89].

Ten patients with CVU were compared with ten patients with a mix of other chronic wounds or acute wounding presenting to a plastic surgery service. Wound biopsies were taken with donor site wound biopsies and underwent fixation and immunostaining. Although there were increased numbers of CD68 (inflammatory cells)

in the CVU tissue, there was a marked elevation in IL-10 (anti-inflammatory) without a similar rise in GM-CSF (pro-inflammatory) which led to authors to suggest that there was an insufficient inflammatory stimulus in CVU 201[89]

TNF-α was initially detected in the venous ulcer bed together with fibrin [124]. With the hypothesis that excessive TNF-α antagonises CVU healing. CVU fluid was collected from patients with non-healing and healing CVU with immunoassay demonstrating significantly increased levels of TNF-α in non-healing ulcers. However, bioactivity measured using a rhabdomyosarcoma cell line showed no difference in bioactivity of TNF-α sourced from the two wound types. This may be explained by increased concentrations of soluble TNF-α receptor p75 in non-healing ulcers negating the effect of excessive TNF-α[102].

TNF-α bioactivity and immunoreactivity in wound fluid was assessed in 21 patients with CVU admitted for wound care management in hospital. Absolute concentrations of TNF-α were seen to decline as the wound transitioned from non-healing to healing but the bioactivity remained the same during the two phases[102].

By combination of ELISA and bioassay techniques, multiple cytokines were screened for in CVU fluid obtained by occlusive dressing. TNF-α was seen to have an inverse relationship to the concentrations of PDGF/EGF, a direct relationship was seen between the concentrations of bFGF and IL-1α and IL-6. No statistical relationship was seen between cytokine levels and ulcer size. PDGF, EGF, bFGF, TGF-β1 did not alter between healing and non-healing phases. The concentrations of pro-

inflammatory cytokines Il-1, Il-6 and TNF-α were significantly higher in the wound fluid of non-healing venous ulcers [68].

Eighty patients underwent occlusive dressing for sampling of CVU fluid at two separate time points and using sandwich ELISA determined the concentrations of cytokines involved in inflammation (TNF-α, IL-1), angiogenesis (bFGF and VEGF) and fibroblast proliferation (TGF-β1). No clear relationship between cytokine levels and healing was established in most cases with the only significant associations being that decreased concentrations of TGF-β1 in ulcer fluid correlated with healing and that bFGF concentrations were proportional to ulcer size[65].

Using tissue biopsies from the edge of areas of CVU and controls from the ipsilateral thigh taken before and after the use of compression therapy, 22 cytokines with roles in inflammation were assessed using a multiplexed array in addition to TGF-β1 that was assessed by a sandwich ELISA. All cytokines bar IL-1α had higher levels in CVU tissue opposed to healthy skin and all cytokine levels fell after compression therapy. When correlated with healing status, rapidly healing ulcers had significantly higher levels prior to compression of IL-1α, IL-1β, IFN-γ, IL-12p40 and GM-CSF with high Il-1 Ra after compression. IFN-γ levels decreased in rapidly healing patients [125].

A study of five patients with the hypothesis that increased CVU levels of TNF-α correlate with recalcitrant ulcers. Biopsies were taken from the edge of CVU and sun exposed skin from the forearm. Qualitative immunostaining was performed and the

levels of TNF-α staining in CVU tissue were greater than controls, with higher levels in ulcers of a longer duration[71].

Fluid was sampled from eight patients with CVU using polyurethane foam covered in a transparent dressing and compared with fluid obtained from 25 patients with open granulating wounds one week post pilonidal excision. A microarray was created for the quantification of cytokines, somewhat contrary to other work; no significant difference in cytokine activity was seen between ulcer fluid and acute wound fluid. The only significant difference was that S100A8/A9 had a lower level in ulcer fluid [126].

In a study demonstrating the utility of debridement material from CVU, ELISA cytokine analysis was performed demonstrating the presence of TNF-α, Il-1α, Il-6 and Il-8 (pro-inflammatory) and in turn that these concentrations were higher than the mediators Il-10 and TGF-β1 (anti-inflammatory). CVU fluid was shown to stimulate fibroblast migration and in an artificial skin substitute able to stimulate the production of chemokines that promote healing [127]

1.4.7.1 Transforming Growth Factor – Beta (TGF-β)

Immunohistochemistry of CVU biopsy material encompassing the ulcer base and edge and comparing it against normal tissue demonstrated TGF-β1 immunoreactivity in the perivascular fibrin cuffs but not in the provisional wound matrix [64]. ELISA analysis of the fluid from the ulcers of eighty patients demonstrated that increased ulcer size and ulcer healing correlated with increasing concentrations of TGF-β1[65]. However, in a study of non-healing CVU, ELISA did not detect a significant temporal TGF-β1 concentration change[68].

Despite the presence of TGF-β in CVU, its effectiveness may be subdued in the CVU microenvironment. Cultured fibroblasts from the thigh and CVU were shown to have no difference in the production of TGF-β mRNA or protein; however, whereas normal fibroblasts were able to mount a twofold increase in collagen production in response to TGF-β, this response was absent in CVU fibroblasts[90].

An explanation for the reduced effectiveness of TGF-β may be found in signalling deficits. CVU tissue demonstrated decreased expression of TGF-β I and III receptor whilst immunohistochemistry demonstrated reduced staining for TGF-β I, II and III whilst transcriptional analysis showed loss of regulation of TGF-β transcriptional targets[70].

1.4.8 Summary

Multiple efforts have been documented to decipher the translation between sustained elevated venous pressure in the lower limb and the syndrome of tissue loss and dysfunction. However, many studies have evaluated a small number of subjects with significant variation in the sampling and analytical methods used. Furthermore, many studies have been 'hypothesis' driven testing the effect of a given variable.

Nonetheless, significant strides have been made in the study of CVU biology with a note made of the role of cytokines, cellular senescence, metalloprotease activity and

angiogenesis which act to deviate healing away from the ‘normal’ acute wound healing cascade.

Metabolic profiling has not been mentioned previously as a conduit for the study of CVU biology and although a comprehensive review into the biology of CVU has been presented, it is still clear the genesis of CVU is unclear.

Metabolic profiling of samples from participants with CVU may be able to reveal more information in a hypothesis ‘generating’ manner to identify hitherto unrecognised components of CVU biology. This concept will be expanded in further sections of this thesis.

1.5 Metabolic Profiling

1.5.1 Overview

Since the completion of the human genome project multiple '-omic' disciplines have been conceived including genomics, transcriptomics and proteomics. Metabonomics is one of the more recent additions having first being coined in 1999 from within Imperial College London. Metabonomics is defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification." [128]

When conceptualised as a system, the cascade of '-omic' disciplines represent the link between genotype and phenotype in such that the genetic material contained within the nucleus represents the potential of the cell, the transcriptome represents "what appears to be happening," the proteome represents what "makes it happen" and the metabolome (the net sum of all sub-1000 Dalton molecules within a biological system) what has actually happened within cells [129].

Despite the fact that genomics, proteomics and transcriptomics have rapidly advanced in the last twenty years, it is felt that these disciplines do not provide a complete enough view of biological system function and interest in developing knowledge in the metabolic profile of biological systems has risen greatly [130].

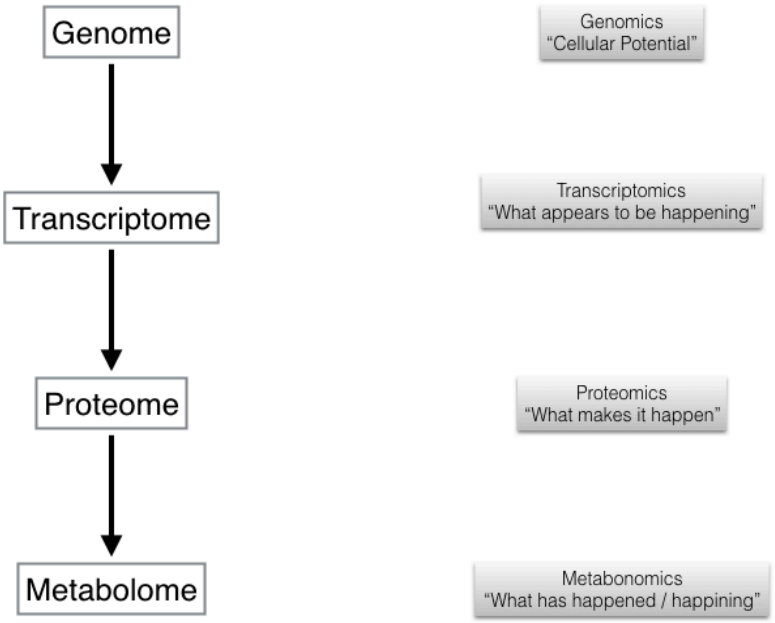


Figure 1.4 'The -omics cascade,' adapted from Dettmer (2007)[129].

1.5.2 Analytical Platforms in Metabolic Profiling

Metabolites demonstrate a great degree of diversity in terms of molecular structure that include hydrophilic sugars, organic acids, amino acids, nucleotides, volatile alcohols, bile acids, fatty acids, triglycerides, phospholipids and other forms of lipids. A single analytical platform would be unable to detect and quantify these species with the net result that multiple analytical platforms are required.

To provide as comprehensive a view as possible, two complementary platforms are used in metabolic profiling studies to maximise the pick-up of multiple metabolites in biological samples; nuclear magnetic resonance and mass spectrometry[131, 132].

1.5.3 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR is widely used in chemistry to determine molecular structure and relies on the principle that nuclei with a mismatch between the number of protons and neutrons will demonstrate 'spin.' In a magnetic field, the spin aligns with field direction and with the application of an energy pulse, the spins jump to the higher energy level deflecting the magnetisation against the direction of the field. Once the radiofrequency pulse is discontinued, the spins relax to the default lower energy level and these processes can be detected as an NMR signal[133]. By generating energy pulses of multiple frequencies to excite multiple nuclear, multiple nuclear spin sets can be stimulated. The raw data is visualised as intensity over time and using a Fourier transformation, a frequency domain spectrum can be obtained reflecting a multitude of metabolites in a given biological system.

With respect to metabolic profiling, the technique is non-destructive, reproducible, relatively quick with high daily throughput and accurate with minimal sample preparation and can give information on molecular structure, dynamics and concentration. Multiple biological samples can be analysed including various body fluids, tissue and in-vitro systems[134].

Given its ubiquity within biological molecules, ^{1}H-NMR spectra are visualised frequently in metabolic profiling studies and a typical ^{1}H-NMR spectrum of urine and

serum will contain a multitude of high and low molecular weight compounds giving rise to a diverse range of signal linewidths[135]. In biological samples, water is found in high quantities and its signal can be supressed using a standard NMR solvent suppression pulse to allow the unmasking of molecules of interest which may be present at lower levels of intensity[136]. To make sense of the data, a reference compound is used to allow identification of peaks based on their frequency shift. In aqueous fluids, the common reference compound is usually the sodium salt of 3-trimethylsilylpropionic acid (TSP) with a deuterated methylene peak and to allow quantification of biomarkers of interest, a compound of known concentration can be inserted[134].

The most common use of NMR in metabolic profiling is working with biofluid samples or homogenised tissue specimens. Urine samples are treated with a small amount of phosphate buffer to eliminate signal variation due to pH differences. Similarly, dilution with deionised water, 0.9% NaCl and buffer (0.1 phosphate/D^2O) is required for serum and plasma samples[134-136].

Intact tissue samples can be analysed without any pre-treatment using the NMR technique of magic angle spinning. This involves spinning the sample at typically 4-6 kilohertz at an angle of 54.7^o relative to the applied magnetic field to avoid the loss of information inherent to non-liquid material[134, 137].

1.5.4 Mass Spectrometry (MS)

Put in simple terms MS provides a very sensitive weighing scale at the molecular level and has a multitude of uses in metabolic profiling. It is a very sensitive and specific technique. The key difference from NMR analysis is that a more extensive sample preparation process is required to remove proteins which may have a significant effect on the results obtained.

The principle of MS is the measurement of the mass to charge ratio of a molecule to determine its structure and polarity. If a separation of metabolites is required, this can be achieved with chromatography. The instrument set up in MS requires five components:

1. Injection apparatus - This can be direct or coupled with a separation technique.
2. Ion generation source - Allows ionisation and transformation into the gas phase
3. Mass analyser - Separation of ions based on the mass/charge ratio
4. Detector
5. Recorder

Urine has the inherently beneficial property of being physiologically devoid of protein (assuming a functioning glomerulus) and only requires centrifugation to remove particulate matter and dilution with water[138]. With tissue samples, homogenisation of the material is required followed by centrifugation. The supernatant is dehydrated and then suspended in methanol and water to provide an aqueous extract. The solid precipitate following homogenisation is treated with dichloromethane and methanol,

homogenised again and following centrifugation is dissolved in methanol and water to obtain the organic extract[139].

There are several variants of MS that depend on the form of chemical separation used which include gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE).

GC-MS was the first MS method used in analysis of the metabolome primarily within the study of plants and mammalian toxicology study. This technique has a high resolution, specificity, sensitivity and well-stocked databases to facilitate metabolite identification. GC-MS does require an adequate vapour pressure with analyte thermal stability. Polar metabolites require a derivatization step at the functional group to reduce polarity and to increase thermal stability[129]. However, the derivatization step (including the methoximation of carbonyl groups and the silylation of active hydrogens) in functional groups may compromise analysis because of the bias due to the formation of by-products and incomplete derivatization.

LC-MS has been increasing in popularity in metabolic profiling studies[132]. The key advantages over GC-MS are the reduced sample preparation required, the lack of a derivatization step and the shorter time required for analytical runs. Although liquid can be directly injected into the spectrometer, a separation phase can reduce the competing ion flow[138].

Commonly LC-MS is used with a reversed-phase (RP) gradient chromatography separation method coupled to electrospray ionisation operating in positive and negative modes. However, RP separation is poorly suited for the analysis of polar and ionic compounds (such as organic acids and amino acids)[140]. An approach to improve coverage of the metabolome is the use of hydrophilic interaction chromatography (HILIC) which can separate polar and ionic compounds more efficiently[141]. The technique of ultra-high performance liquid chromatography has been developed to improve separation and chromatographic separation has been pioneered using smaller particle size packing column material to improve flow rate and delivery pressure[138, 139].

Capillary electrophoresis (CE) allows the separation of charged metabolites according to size offering high efficiency, resolution in a small sample volume. It is useful for the separation of polar and charged compounds[142]

Given that no one MS method is all encompassing in terms of metabolite coverage, it is common to use more than one variant to obtain maximal metabolite profiling.

1.5.5 Data Processing and Analysis

MS and NMR have been in use for decades but metabolic profiling is a relatively recent tool because of the large amounts of raw data generated and to make sense of the large amounts of raw data, rigorous mathematical and statistical approaches have been developed[143, 144].

A data set with samples from a large number of subjects obtained using metabolic profiling platforms will be a multidimensional entity, however a large number of variables between subjects may show no variation and hence it would be useful to reduce the dimensionality of a given data set to identify variables responsible for difference between groups. Principal component analysis (PCA) is an unsupervised technique that can visualise data sets in a two or three-dimensional space without human operator labelling, presenting principal components as vectors that attempt to explain the variation in the presented data. Each principal component is orthogonal and is thus independent from other principal components[131] and will be suffixed with a percentage to encompass how much of the data can be seen to vary because of that component. PCA score and loading plots can help to determine outliers within a data set to drive hypothesis generation. In addition, a PCA score plot can be highlighted so each plotted value can represent which group it comes from (Figure 1.5)

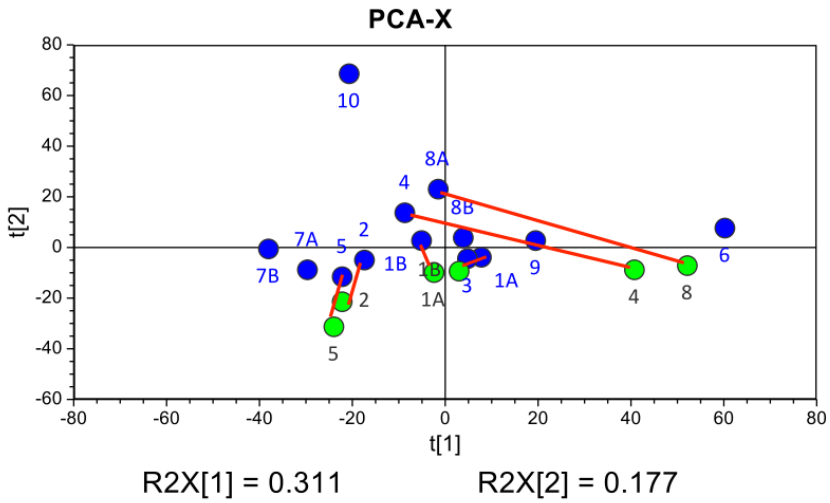


Figure 1.5 – An example PCA plot with manual highlighting of points to demonstrate their origin in green and blue.

Partial least squares regression (PLS) is a multivariate regression method to correlate two data matrices (X and Y for this example) and hence in the future can predict the outcome of variable Y whilst only knowing the results of variable X. Interestingly, PLS can be used to examine the influence of time on a data set. A variant of PLS, discriminant analysis (PLS-DA) can be used to determine how best to discriminate between classes but cross validation should take place to ensure that the model is not over fitted[131, 145]. In simplistic terms this involves removing a portion of data from the data set to see if it is accurately predicted.

1.6 Applications of Metabolic Profiling

1.6.1 Arterial Diseases

1.6.1.1 Atherosclerosis

In vascular metabolic profiling, a significant body of work has examined the biology of atherosclerosis. The following table (1.3) details the various findings of multiple studies attempting to characterise systemic biomarkers of atherosclerotic disease.

Author	Year	Animal	Tissue	Markers
Wurtz et al.[146]	2012	Human	Plasma	Tyrosine, glutamine
Chen et al.[147]	2010	Human	Plasma	Palmitate, stearate
Mayr et al.[148]	2009	Human	Atherosclerotic plaque microbubbles	Taurine
Jove et al.[149]	2013	Hamster	Plasma	Glycerophospholipids, sphingolipids, glycerolipids, free fatty acids, sterols, ceramide, docosahexaenoic acid, taurocholic acid
Leo et al.[150] Mayr et al.[151]	2009 2005	Mice Mice	Aorta, urine Artery	Xanthine
Ou et al.[152]	2012	Mice	Plasma	Fatty acids, lysophosphatidylcholines, bile acids
Zhang et al.[153]	2009	Rats	Plasma, urine	20 different metabolites indicating aberrant metabolism of phenylalanine, tryptophan, bile acids, amino acids
Cheng et al.[154] Mayr et al.[151] Messner et al.[155]	2010 2005 2008	Mice Mice Human	Plasma, urine Aorta Atherosclerotic lesion	Metabolites indicating aberrant metabolism of choline, fatty acids, and disruption of TCA cycle
Thukkani et al.[156, 157]	2003	Human	Atherosclerotic lesion	Alpha-chloro fatty aldehydes, unsaturated lysophosphatidylcholine

Table 1.3: Metabolic profiling studies in atherosclerosis.

1.6.1.2 Cerebrovascular Disease

Atherosclerotic disease of the carotid bifurcation and internal carotid artery can lead to disabling cerebral vascular events secondary to embolisation from an unstable atherosclerotic plaque. The treatment of the atherosclerotic lesion is driven by risk of stroke resulting from treatment versus the risk of stroke without treatment. A biomarker that may identify risky disease may inform the surgical decision making process.

An animal model of transient ischaemic attack versus defined stroke demonstrated a decreased level of decreased levels of glutamate, N-acetyl-aspartate, and taurine using NMR analysis of brain tissue[158]. Metabolic profiling of diabetic plaques demonstrated increased levels of non-esterified fatty acids in the plaques of diabetic subjects although no change was detectable in serum[159].

An animal model of acute stroke was developed with serum samples taken before and after the cerebral event demonstrating a reduction in a number of branched chain amino acids and interestingly the same branched amino acids were reduced in human patients after an acute stroke demonstrating the possibility of acute stroke detection[160].

1.6.1.3 Abdominal Aortic Aneurysm (AAA)

Intraluminal thrombus harvested from aneurysmal aortas compared to normal diameter aortic thrombus analysed using LC-QTOF-MS demonstrated that AAA tissue is associated with a leukotriene related compound (either leukotriene B5 or keto-leukotriene B4) which may support a chronic inflammatory component to AAA formation, whilst fatty acid amides (for example anandamide and oleamide facilitate cell-cell communication, modulate cell death and evoke platelet aggregation) were

also elevated in AAA tissue as was hippuric acid (a gut commensal related metabolism product)[161].

A follow-on study using plasma from patients with large AAA, small AAA and normal aortas and analysed with LC-QTOF-MS demonstrated an ability to classify samples based on plasma metabolic profile correctly. Interestingly, the one sample that was classified wrongly by the model actually had an incorrect aortic size documented clinically[161]. Similarly, plasma metabolic profiles were shown to correlate with small AAA (3-5cm), large AAA (>5cm) and normal aorta (<3cm) using NMR and GC-MS with partial least squares analysis[162].

1.6.1.4 Venous Disease

Venous insufficiency and varicose veins represent another area of study with potential for metabolic profile. A study by Anwar et al. was the first to metabolically characterise varicose veins[137]. They took lower extremity varicose vein samples and compared them to non-varicose great saphenous vein using an NMR metabonomics approach. The researchers indicated several biomarkers for varicosity including creatine, myo-inositol and lactate. These are likely implicated in varicose veins via smooth muscle hypertrophy, inflammatory pathways, and hypoxia respectively.

In venous thromboembolism (VTE). It was hypothesised that dyslipoproteinemia may cause, or be associated with VTE[163]. 49 male venous thrombosis patients were compared to a group of age-matched patients as controls. The patients were taken three months or more after a diagnosis of DVT and the plasma samples were analysed

with NMR. The venous thrombosis patients had significantly lower levels of HDL particles, large HDL particles, HDL cholesterol and apolipoprotein A1. They also had significantly higher levels of LDL particles and small LDL particles. The venous thrombosis patients also had an elevated ratio of apolipoprotein B/apolipoprotein A1[163].

1.6.1.5 Wound Healing

Tentative steps have been made in the metabolic profiling of wound healing models. A targeted assay for uric acid precursors (purines, the metabolism of which reflects anaerobic energy production in a 'hypoxic' cell) utilising HPLC-MS was carried out. 29 patients with chronic venous ulceration were studied sequentially with samples of wound fluid as captured by a modified occlusive dressing with 14/19 wound classed as healed within the 24 week study period and wound severity was assessed using the PUSH tool. Interestingly, wounds classed as severe by the PUSH tool had a paucity of uric acid precursors whilst uric acid levels were elevated in the same group[164]. A relationship between uric acid levels and healing status was not observed in this study. The authors suggest that this represents increased xanthine reductase activity in vivo which causes; the depletion of purine precursors that are required for wound healing, the generation of monosodium urate crystals that instigate an inflammatory process and the generation of reactive oxygen species in turn leading to DNA damage, lipid peroxidation and protein modification[165].

1.7 Thesis Aims

The aims of this thesis are:

1. To determine the utility of metabolic profiling in chronic venous ulceration.

2. To identify whether differential metabolic profiles can be identified from sample subsets of chronic venous ulceration to determine:

a. Whether biomarkers predictive of healing can be identified?

b. Whether further insights into the translation between sustained venous pressure elevation and tissue damage in venous disease can be identified?

1.8 Hypotheses

- That the determination of the metabolic profile of biological fluids obtained from patients with chronic venous ulceration is feasible and reliable (Chapter 2).

- That a differential metabolic phenotype can be obtained from varicose vein tissue from participants of differing clinical severity according to the presence of absence of skin changes (Chapter 3).

- That a differential metabolic phenotype of ulcer fluid, serum and urine from participants with CVU can be obtained using metabolic profiling techniques according when stratification according to clinical factors known to predict healing outcome (Chapters 6, 7 and 8).

- That candidate biomarkers can be identified from the metabolic profiling of ulcer fluid, serum and urine related to clinical factors known to determine prognosis in CVU (Chapters 6, 7 and 8).

Chapter 2: Preliminary Chronic Venous Ulcer Metabolic Profiling: Proof of Concept?

2.1 Introduction

The main premise of this thesis is to explore the potential utility of a systems biology model typified by metabolic profiling in CVU. This would represent a novel application of metabolic profiling in a novel biofluid. As a prelude to a larger scale study, a pilot study was undertaken to demonstrate the feasibility of recruitment, sample collection, sample transport and storage, sample processing and the acquisition of meaningful metabolite information. This chapter reports the outcome of the pilot phase study that was carried out in 2013.

2.1.1 Summary of CVU Biology

It is accepted that chronic venous insufficiency is the underlying cause of CVU but the understanding of how sustained elevations of venous pressure precisely translates into the syndrome of tissue loss and impaired healing although multiple theories have been postulated including fibrin cuffing[60], white cell trapping and cytokine trapping[166]. Chronic wounds fail to progress through the well-defined healing cascade of haemostasis, inflammation, proliferation, with CVU being thought to remain within the inflammatory phase persistently. Much of the literature examining the biology of CVU has focused on single pathways or groups such as inflammation, angiogenesis or specific cellular function such as fibroblast activity or senescence. Most studies examining the biology of chronic venous ulceration and venous disease in general have tended to focus on specific factors such as individual cytokines, enzymes or specific aspects of cellular behaviour such as cell division or apoptosis, and assessing wound fluid's potential to inhibit angiogenesis[97, 98].

2.1.2 The Role of Systems Biology

Utilisation of systems biology methods may reveal further insights into the genesis and progression of CVU as highlighted recently in a comprehensive review looking at the multiple analytical methods as applied to wound fluid[30]. With the use of systems biology techniques, a hypothesis generating study (as compared to hypothesis driven studies) may help to determine hitherto unrecognised pathways or potential biomarkers. In brief, systems biology techniques utilise global profiling platforms combined with a robust data mining method. Studies of the genome, transcriptome and proteome have revealed a strong association between chronic venous disease and gene mutation, gene expression and protein expression.

Despite the relative plethora of genomic, transcriptomic and proteomic studies there is a dearth of biochemical information particularly the characterisation of low molecular weight metabolites (below 2000 Da) in the wound microenvironment. For example it has been shown that the amino acids in wound fluid regulate the effect of epidermal growth factor on fibroblast replication[167], but there is lack of data regarding the role of amino acids in the leg venous ulcer microenvironment. A more recent study illustrated the correlation of elevated uric acid with wound severity [165]. These findings support the need of the application of metabolic profiling approaches in the understanding of wound microenvironment and healing progression, as information can be captured about metabolites with low molecular weight in a given biological system. According to the '-omics' cascade; genomics represents the potential of a cell, transcriptomics represents what is about to happen, proteomics represents how something is going to

happen whereas metabolites represent what has happened[129]. Metabolic profiling techniques have been successfully applied to the study of varicose veins[137], colorectal cancer[168] and drug discovery[169].

2.1.3 Chronic Wound Fluid

Chronic wound fluid is thought to comprise an amalgam of extravasated material, locally synthesized factors and possibly the results of interaction with local colonizing bacteria[100]. This may be a route of gleaning further mechanistic information in wound biology. Wound fluid can be captured using multiple techniques, the most prominent being the use of the occlusive dressing method which involves placing an adherent clear film over the wound of interest and using a needle to extract the pool of fluid that has accumulated over a defined period of time[18, 68, 170]. The use of sterile filter paper discs placed directly onto a wound to absorb fluid with subsequent extraction and analysis has also been described[108].

2.1.4 Aims

1. Determine the feasibility of wound fluid capture using the occlusive dressing and filter disc techniques.

2. Validation of the above techniques to provide metabolic profiling data as a prelude to a larger study.

2.2 Materials and Methods

2.2.1 Regulatory Approval

Ethical approval was obtained from the UK National Research Ethics Service - East Midlands (Derby) Committee (reference number 13/EE/0011 - "Investigating the Genome-Phenome Correlations for Human Vascular Disease") in February 2013. The study was registered with Research and Development at Imperial College NHS Healthcare Trust and the Joint Research Office at Imperial College London. All specimens collected were anonymised and stored according to the rules set out in the Human Tissue Bill (2004). Informed written consent was obtained from all patients involved in this study. This study was devised with advice from the supervisors of this thesis, Professor AH Davies and MS Gohel. In addition, scientific advice was obtained from the Section of Computational and Systems Medicine at Imperial College London (Professor E Holmes and Dr K Spagou).

2.2.2 Patient Selection

For this study, ten patients with chronic venous ulceration were recruited between June and July 2013. Chronic venous ulceration was defined as epithelial discontinuity between the knee and the ankle with an ankle brachial pressure index of greater than 0.85.

All patients were recruited from the vascular surgery outpatient clinics at Imperial College NHS Trust, London (Charing Cross Hospital and St Mary's Hospital). All patients had been clinically evaluated for venous disease including with a colour venous duplex ultrasound scan.

2.2.3 Sample Collection

The current dressing was removed from the lower limb to be studied and a maximum of three 10mm diameter sterile filter discs were placed on the wound (Sigma-Aldrich, Missouri, USA) and simultaneously, a sheet of clear adhesive film (Tegaderm - 3M, Minnesota, USA) was placed to encompass the entire wound (Figure 2.1). Patients were asked to keep the study leg dependent for between 60-90 minutes and after the completion of this time period, any accumulated fluid was extracted from under the clear dressing using a 2.5ml syringe and 18g hypodermic needle [18, 68, 170]. Thereafter, the filter discs were retrieved from the wound. Once all samples were collected, a specialist nurse with an interest in leg ulcer management applied four-layer compression bandaging[171, 172]. All samples were placed into specimen tubes and frozen at -80°C until analysis.



Figure 2.1: Ulcer fluid 'capture' - three filter paper discs are placed in the wound with an occlusive clear dressing covering the entirety of the wound.

2.2.4 Metabolite Extraction

A biphasic extraction protocol to extract non-polar and polar metabolites from filter discs and ulcer fluid was employed based on prior validation of metabolic profiling studies for biological fluids[134, 139]. This involved the addition of 700µL MTBE:water:methanol solvent mixture (3:3:1) to sample tubes either containing ulcer fluid or ulcer fluid contained within filter discs. Three tubes with blank filter discs (not exposed to ulcer fluid) were prepared in parallel. In addition, three blank samples containing only solvents were prepared to identify contamination potentially introduced by the addition of solvent or use of sample tubes. Samples were then vortexed for one minute, added into an ultrasound bath for ten minutes and finally centrifuged at 20000g

for 30 minutes (Eppendorf, Centrifuge 5417R, Germany) at 4°C. Subsequently the organic and aqueous phases were isolated with a pipette and the extraction step was repeated one more time as described above to enhance the efficiency of extraction.

Aqueous extracts were dried in a speed vacuum at room temperature and stored at -stored in -40°C freezer pending for NMR analysis whilst organic extracts were allowed to evaporate overnight in a fume cupboard and stored at -40 °C pending MS analysis.

All solvents detailed below used for the extraction of metabolites from the ulcer fluid and filter discs were obtained from Sigma-Aldrich (Missouri, USA) and were of HPLC Analytical Grade. Further solvent information is listed in the appendix of this thesis.

2.2.5 NMR Profiling - Aqueous Extracts

This was carried out according to protocols for biofluid developed at Imperial College[134]. Pure ulcer fluid and filter disc aqueous extracts were prepared by the addition of 600 µL phosphate buffer made up in deuterium oxide containing 1 mM (TSP) as an external reference and 2 mM sodium azide as a bactericide. This was followed by centrifugation (Eppendorf, Centrifuge 5417R, Germany) at 20000g for 10 min at 4 °C. 1H NMR spectra were acquired with a Bruker Avance 600 MHz spectrometer operating at 600.13 MHz for 1H at 300 K. It was equipped with a 5 mm broad-band inverse configuration probe. Samples were randomly analysed in automation with a B-ACS 60 sample changer system. For each aqueous extract a standard one-dimensional NMR spectrum was acquired with water peak suppression using a standard pulse sequence (recycle delay (RD)-90o –t1- 90o –tm -90o- acquire

free induction decay (FID)). The RD was set at 2s, the spin echo delay at 400 μs, the acquisition time per scan was 1.36s and, for each sample, 8 dummy scans were followed by 256 transients. The spectra were collected into 64K data points using a spectral width of 20 ppm.

2.2.5 RP-UPLC-MS lipid profiling of the organic extracts

The organic extracts of ulcer fluid and filter discs were reconstituted in a mixture of isopropanol/ACN/water (2:1:1, 200 µL), vortexed for 30sec, placed in an ultrasound bath for 5min and vortexed for 30sec. This was followed by centrifugation at 20000g for 30 minutes at 4°C. The supernatant from each sample was transferred into glass inserts contained in specific LC-MS vials. A quality control sample was prepared with 30µL of each sample to assess the analytical reproducibility of the method[173].

The experimental run comprised of column conditioning by injection of the pooled quality control sample until the data showed adequate stability. Thereafter, the QC sample was injected after three samples to monitor the instrument's performance. The run was completed with the analysis of extraction and finally with blank solvent samples.

Lipid profiling was performed on an Acquity UPLC system (Waters Corp, USA) coupled to a QTof Premier MS system (Waters MS Technologies, UK). Chromatography was performed using an Acquity UPLC CSH C18 2.1x100mm, 1.7um, column (Waters Corporation, USA) held at 55°C. Separation was achieved

using gradient elution with 0.1% (v/v) formic acid in acetonitrile/water (60:40) (A) and 0.1% (v/v) in isopropanol/ACN (B) (90:10) at a flow rate of 0.4 mL/min. In both mobile phases ammonium formate (LC-MS grade, Fluka, USA) was diluted to 10mM. Starting conditions were 60% A and 40% B for 2.0 min, changing linearly to 43% B over the next 2 min, and then to 50% B within 0.1 min and to 54% B over the next 10min, when it was changed to 70%B within 0.1 min and finally to 99% B over the next 6 min. Afterward the solvent composition returned to starting conditions over 0.1 min, followed by re- equilibration for 2 min prior to the next injection.

Mass spectrometry was performed using electrospray in both positive and negative ESI ionisation modes. The capillary voltage was 1.5 kV (ESI+) and 2.4 kV (ESI–), cone voltage was 35 V, desolvation temperature was 350 °C, and source temperature was 120 °C. The cone gas flow rate was 5 L/h, and desolvation gas flow rate was 1000 L/h.

The QTof Premier was operated in sensitivity mode with a scan time of 0.2 s and interscan delay of 0.01 s. For mass accuracy, a LockSpray interface was used with a 20 µg/L leucine enkephalin (555.2645 amu) solution (50/50 ACN/H2O with 0.1% v/v formic acid) at 15 µL/min was used as the lock mass. Data were collected in centroid mode with a scan range of 50–1000 m/z, with lockmass scans collected every 30 s and averaged over 4 scans to perform mass correction.

2.2.6 Data Processing and Statistical Analysis

2.2.6.1 NMR Data Extraction

Data [-1.0 to 10.0 ppm] were imported into MATLAB 7.0 software (MathWorks, Natick, MA), where they were automatically phased, corrected for baseline distortions and referenced to the TSP signal at δ 0.00. The region between δ 4.67- 5.0 containing the residual water resonance was removed from all spectra. Assignment of endogenous ulcer fluid metabolites was made by reference to published literature data and in-house databases.

2.2.6.2 MS Data Extraction

Raw MS data were converted to netCDF format using the DataBridge tool implemented in MassLynx™ software (Waters Corporation, Milford, USA). The data were processed using the open source XCMS package in R programming software[174, 175] and an output table was obtained comprising pairs of m/z_RT and intensity values of the detected metabolite features in each sample. The dataset then was normalised with the media fold change method in R with an in-house script [176].

NMR acquired spectra were imported into Matlab (R2013a, Mathworks, Natick, MA) using in-house routines (MetaSpectra, Dr O. Cloarec) for full resolution introduction of the spectra into Matlab, and a vector running from 0 to 10 ppm, in increments of 0.00025 ppm, was formulated. After removal of the area corresponding to residual water (4.40–5.2 ppm), the remaining data were aligned and then probabilistic quotient normalisation was applied using Matlab routines developed within Imperial College.

2.2.6.3 Statistical Analysis

Multivariate data analysis was performed using the SIMCA package (version 13.0.2, Umetrics, Umeå, Sweden) with the assistance of a postdoctoral researcher at Imperial College London. Metabolite features were subjected to Pareto scaling prior to modelling with PCA to visualise data and assess the reproducibility of the methodologies.

2.2.6.4 Metabolite Assignment

Metabolite identification using MS data was conducted by matching accurate m/z measurements of detected chromatographic peaks to theoretical value from in-house databases and on-line databases such as the human metabolite database (HMDB, http://www.hmdb.ca/), KEGG (http://www.genome.jp/kegg/ligand.html), LIPID MAPS (http://www.lipidmaps.org/tools/index.html), METLIN (http://metlin.scripps.edu/). Tandem MS fragmentation patterns were then obtained for further structural elucidation. Assignment was confirmed by the comparison of retention times and MS/MS data with the authentic standards.

NMR assignments were carried out according to the literature and in-house database[135]. A series of two-dimensional (2D) spectra were acquired for a number of samples to assist or confirm the assignment of peaks in the 1D spectra to metabolites. Also, statistical correlation spectroscopy analysis (STOCSY) was used to display the correlation of the intensities of different peaks in the sample and, in this way, aid the identification of molecules[177]

2.3 Results

2.3.1 Clinical Data

The following table (Table 2.1) presents patient demographics and clinical characteristics.

No of Samples Gender	10 Male 8, Female 2
Age	22-87 years (median 62)
Hypertension	2/10
Cardiac disease	0/10
Peripheral arterial disease	0/10
Statin use	2/10
Diabetes Mellitus	0/10

Table 2.1: Participant characteristics

2.3.2 Assessment of Filter Disc Capacity to Absorb Fluid

This study primarily uses an alternative approach to capturing ulcer fluid to that devised in the mid 1990s by Trengove et al [18, 68, 170]. Tarlton in Bristol devised the utilisation of sterilised Whatman 54 filter paper to capture fluid although the time placed on the wound was not noted[108]. In this study, a hybrid technique was undertaken to maximise fluid capture, as stated in the previous chapter, ulcer fluid discs were placed on the wound and a sterile transparent adhesive dressing was also placed to allow the capture of any fluid accumulated directly with a needle puncture of the film after 90 minutes.

2.3.2.1 Dry Filter Disc Weight

Given the findings of the pilot study where filter discs were seen to be more efficacious at capturing ulcer fluid, filter discs were the mainstay of this study. In a variation to the Tarlton published technique, sterile filter discs for microbiology were used that were already manufactured with a fixed diameter of 1cm. Figure 2.2 refers to a section of dry filter discs to ascertain variability of weights with a majority lying within one standard deviation of the mean.

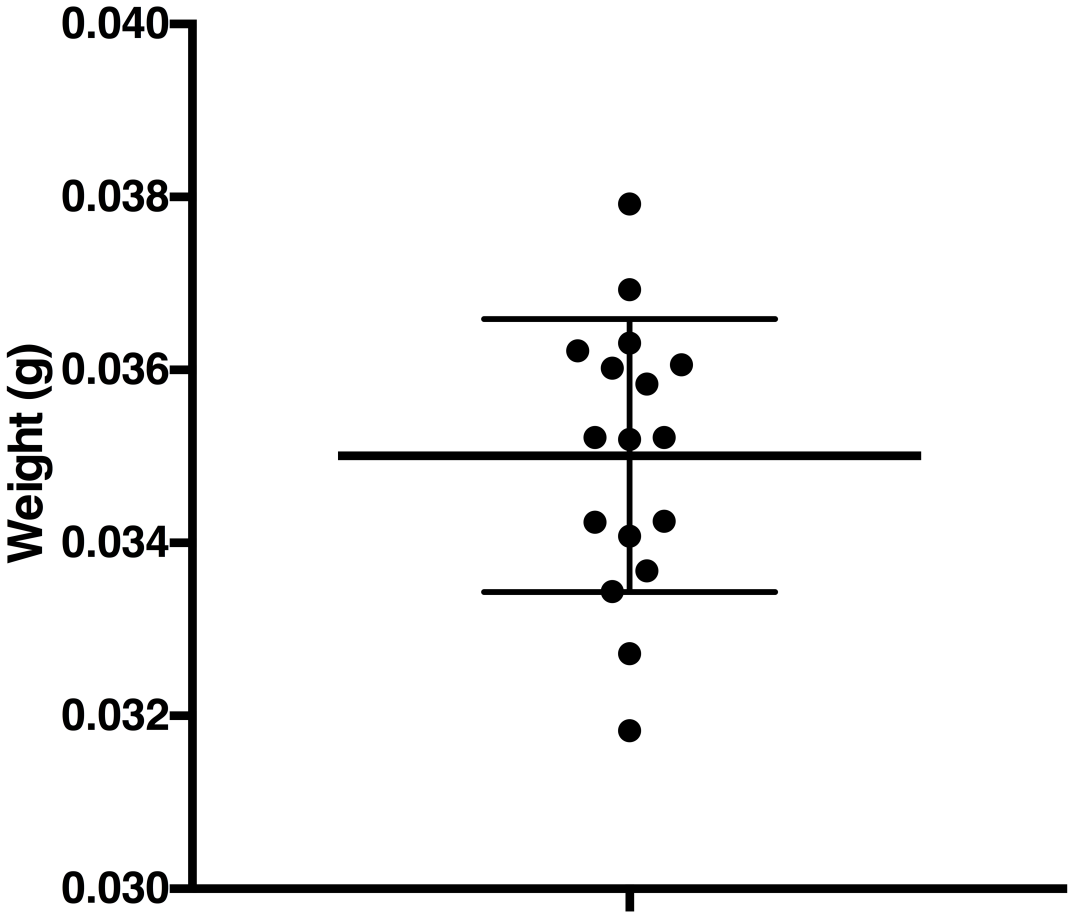


Figure 2.2- Sample of dry filter discs plotted by weight.

2.3.2.2 Filter Disc Absorptive Capacity

To test the absorptive capacity of filter discs, a cohort of discs were individually laid out on plastic weighing dishes with 1ml of sterile water. Three discs were exposed for 30 minutes, three discs exposed for 60 minutes and three discs exposed for the 90 minutes. This takes into account that the filter discs and occlusive dressing was laid on the wound for between 60-90 minutes (Figure 2.3).



Figure 2.3 - Filter disc exposure to sterile water.

In test conditions, all nine discs absorbed fluid during their respective period of exposure ranging between 0.08g and 0.11g as demonstrated in Figure 2.4. ANOVA one way testing did not demonstrate any significant difference between groups as determined by exposure time ($p = 0.4887$). The inference is that disc absorbance is not affected by time after a certain period which is almost certainly less than 30 minutes.

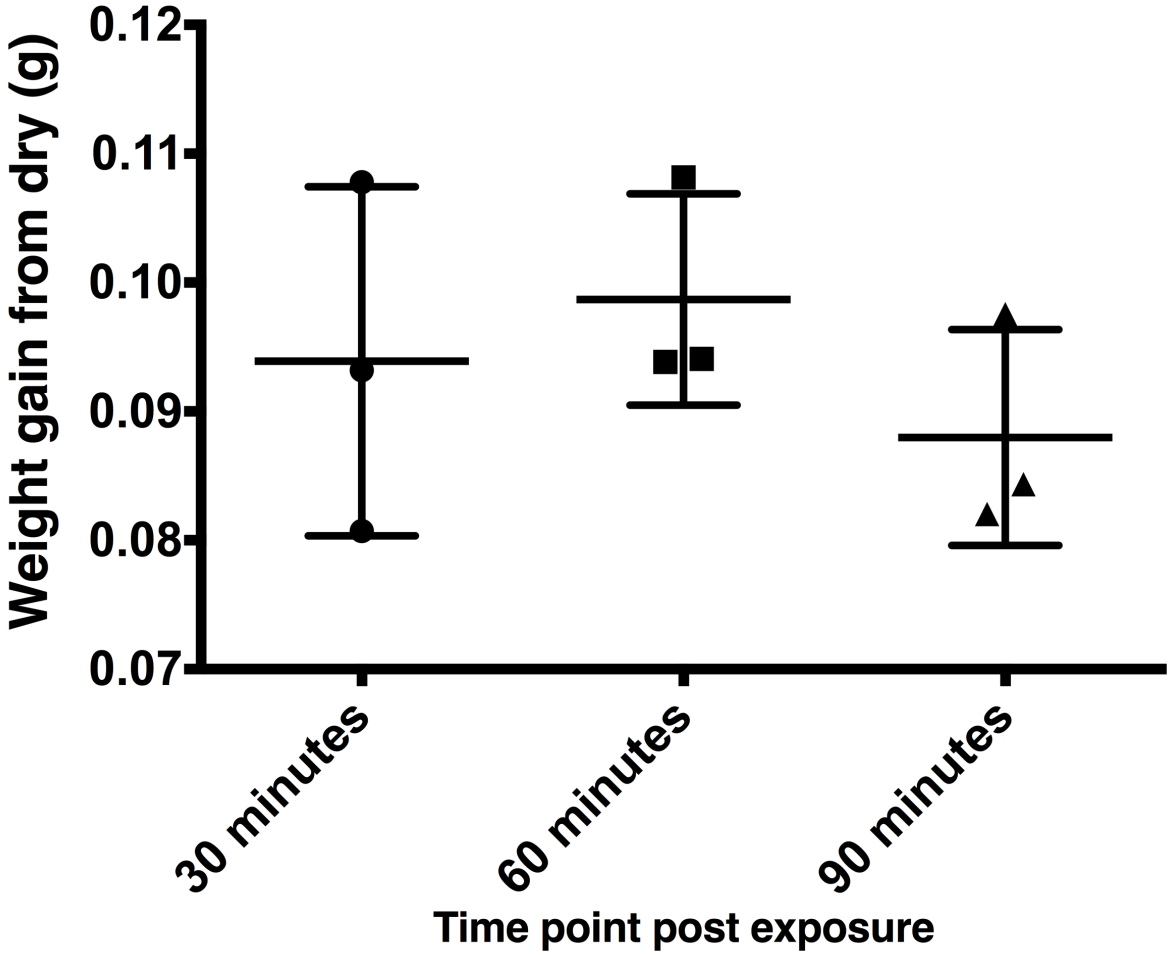


Figure 2.4 - Filter disc absorbance over time.

2.3.3 CVU Ulcer Fluid Sampling

Table 2.2 refers to the sample collection in each patient in this study.

Participant number	Number of filter discs	Volume of fluid from occlusive dressing (mL)
1	3	0.1
2	1	0.1
3	1	0
4	2	0.1
5	1	0.6
6	2	0.02
7	6	0.02
8	3	0.1
9	3	0.5
10	1	0.02

Table 2.2: Ulcer fluid obtained from each participant

There were no technical failures in such that a sample of ulcer fluid was collected but the number of filter discs that could be placed on the wound was limited by the area of the ulcer. The minimum was 1 filter disc with a maximum of 6 with a median of 2 discs placed per wound. Ulcer fluid using simultaneous occlusive dressing placement on the whole was less rewarding. Although two patients generated ulcer fluid samples of 0.5ml and 0.6ml respectively, the median was 0.06ml.

2.3.4 Metabolic Profiling

Ulcer fluid filter discs were used to generate two extracts; organic for MS lipid profiling and aqueous for NMR. For three patients, two filter discs were analysed to determine reproducibility.

Ulcer fluid directly obtained from beneath occlusive dressings was typically of a volume that was insufficient to undertake both MS and NMR. Given that MS is a more sensitive technique, this was the first line assay. The organic extract of fluid was extracted for patients with at least 0.1ml of ulcer fluid.

Figure 2.5 demonstrates individual chromatograms obtained from RP-UPLC-MS derived from the organic phase of fluid extracted from filter discs. A blank disc without exposure to ulcer fluid has also been analysed. Every disc demonstrated peaks which may correlate with metabolite features. To ensure the reproducibility of filter discs placed on the same wound (figure 2.6) and it can be seen that the profiles obtained are broadly similar.

With regard to RP-UPLC-MS of ulcer fluid obtained from the occlusive dressing method, figure (figure 2.8 lowest chromatogram) which demonstrates the ability to generate a metabolic profile with peaks that may represent metabolite features. Similarly, figure 2.12 demonstrates that a profile (demonstrated by a base peak chromatogram) could be demonstrated in the ulcer fluid from six out of ten patients in

this study. Of note, the profiles obtained are distinct from that of the solvents without ulcer fluid alone.

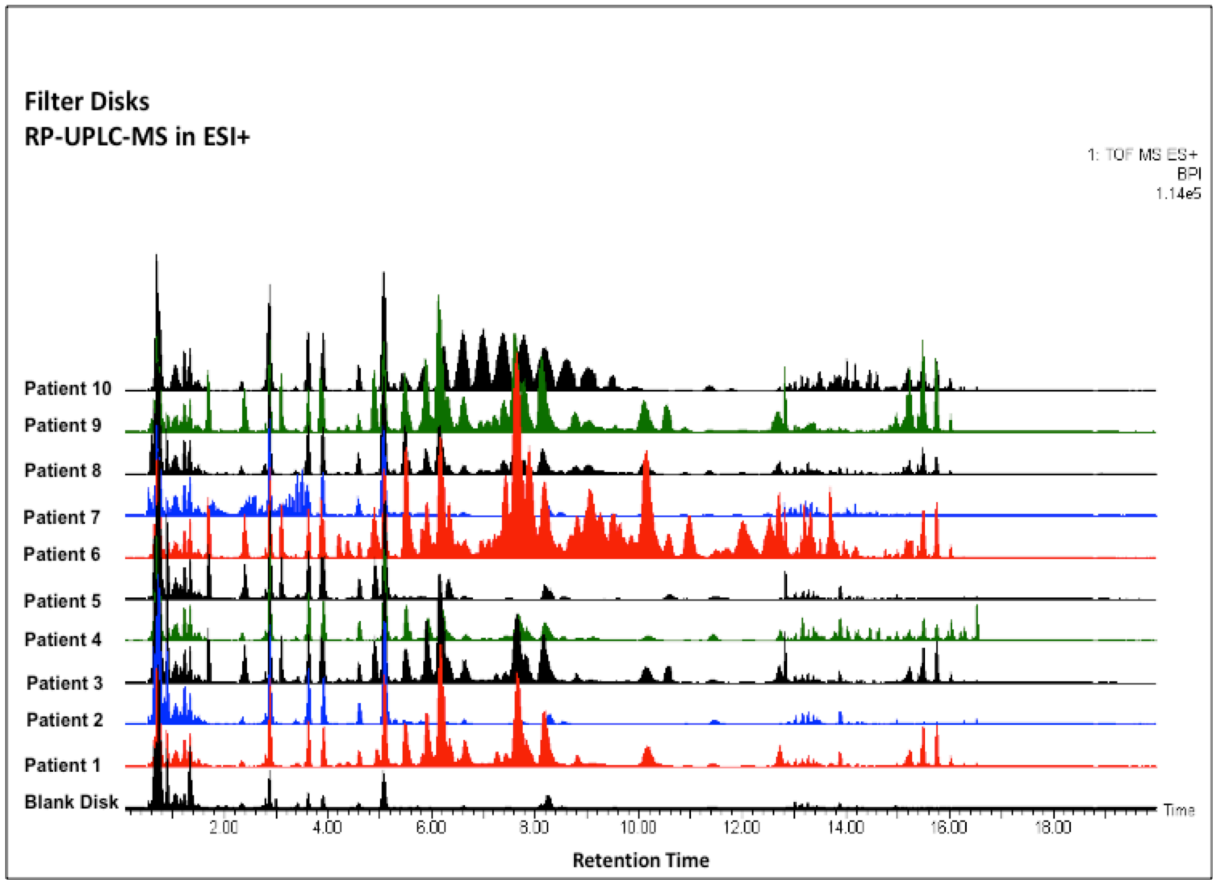


Figure 2.5: RP-UPLC-MS in electrospray positive mode for all patients in the pilot study with a blank disc for comparison.

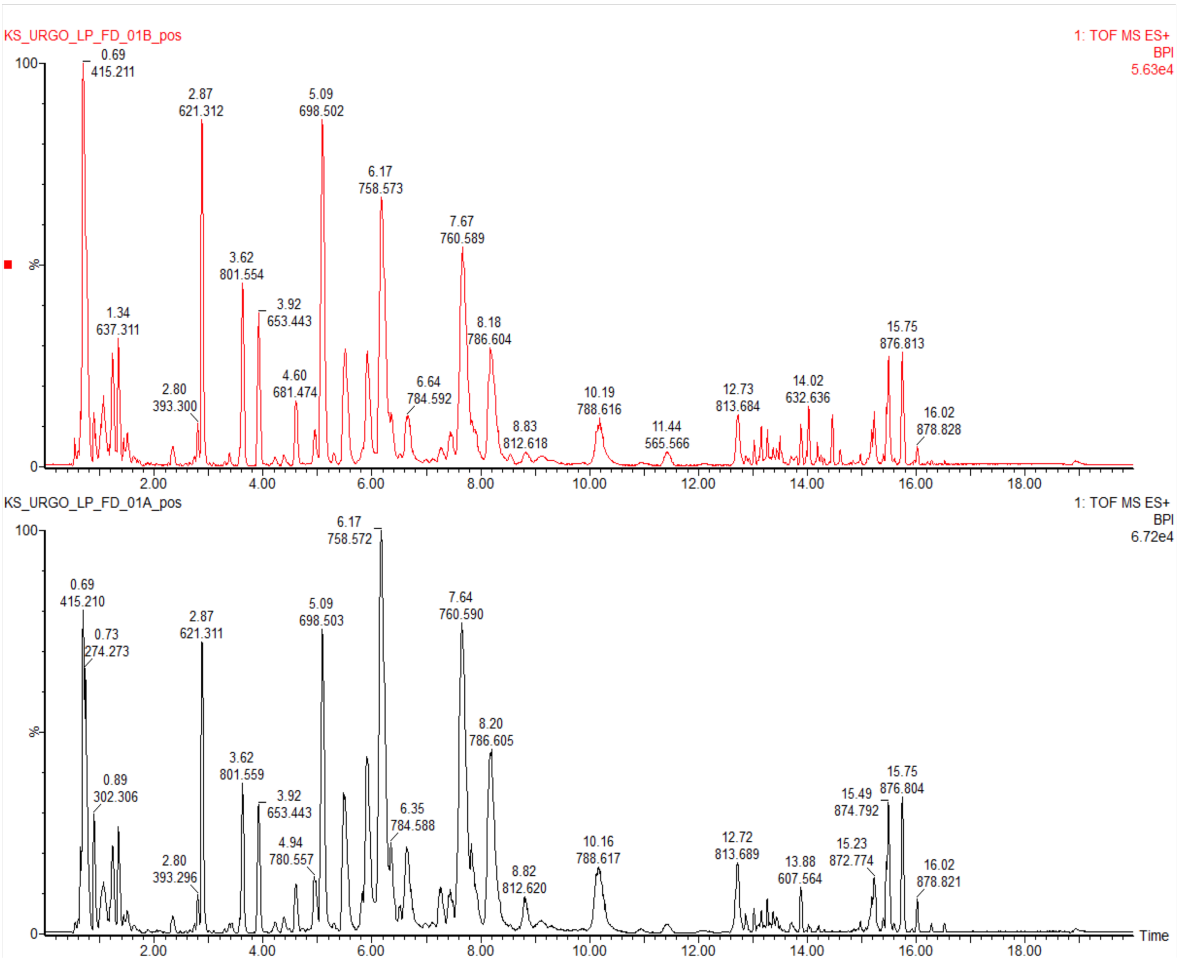


Figure 2.6: Individual chromatograms from one participant in the pilot study (participant 1) demonstrating the broad similarity between two discs placed on the wound bed.

2.3.5 Statistical Analysis

In metabolic profiling studies, it is crucial to generate data with excellent quality, thus obtaining reproducible results and increasing the potential for meaningful biological interpretation. With NMR analysis, there are numerous studies that have proven the intra- and inter- reproducibility of the analytical platform [134, 178]. However, LC-MS analytical system changes in chromatographic retention time and signal intensity during the analysis of a batch of samples, which can lead to irreproducible results[173, 179]. This platform variability can arise from variations in sample injection, LC conditions such as column degradation, contaminant build-up, intrinsic or extrinsic

pressure/temperature changes and MS conditions such as source contamination, ionisation efficiency fluctuations, matrix effect, decrease of detector sensitivity. To monitor such changes and evaluate the reproducibility of generated data it is a common practice in metabolic profiling studies to use the widespread strategy of QC, whereby pooled QC samples, representative of the entire sample batch typically by combining equal aliquots from all samples in the study, are used to monitor the analysis[138, 173, 179].

The datasets from the ulcer fluid and filter discs were initially examined in terms of reproducibility using multivariate statistical analysis methods. Clustering of QC samples was assessed using PCA to reveal if platform stability had been achieved. A PCA scores plot of all organic and aqueous ulcer fluid samples and QCs analysed in both MS and NMR is shown in figure 2.7 The QC samples are tightly clustered, indicating good reproducibility of the data and providing confidence for the quality of the data obtained from both analytical platforms. The amount of variation in the dataset explained by the first two PCs was 32.5% and 30.4% for the organic extracts ulcer fluid samples analysed by lipid profiling UPLC-MS assay in positive and negative ESI mode respectively.

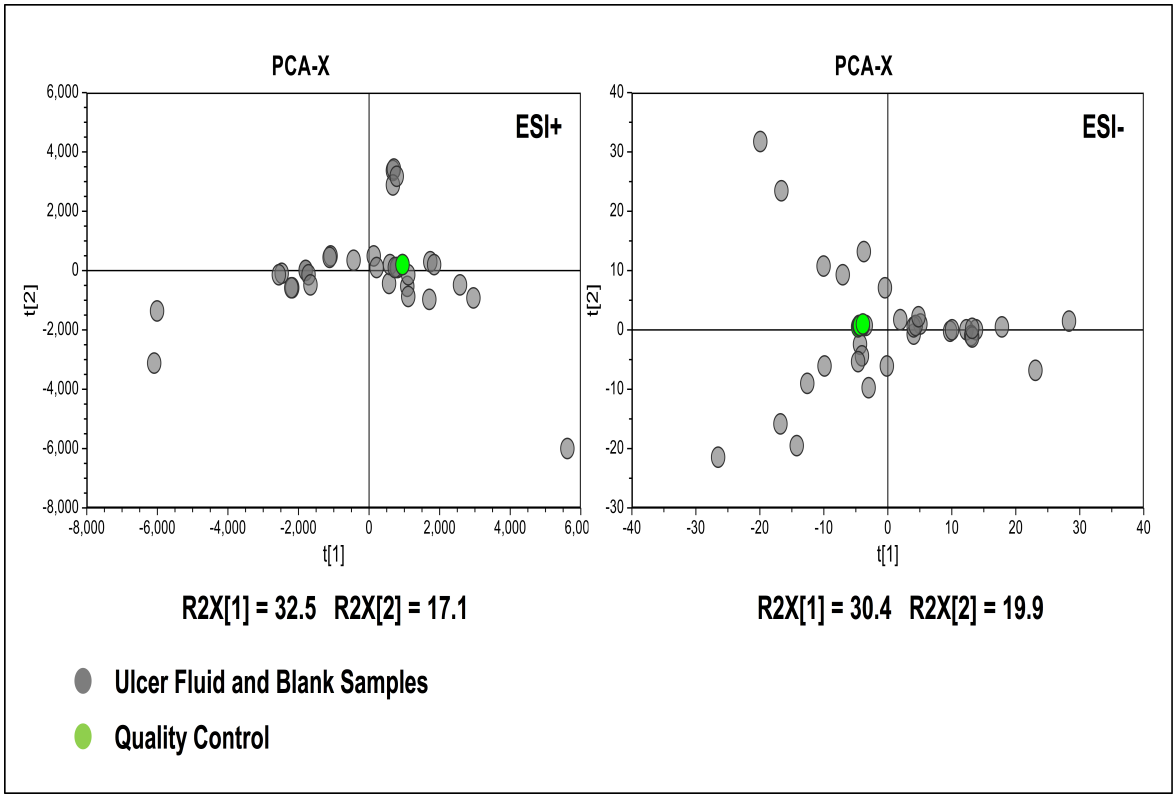


Figure 2.7 All samples of ulcer fluid from the occlusive technique and filter paper discs, each sample is represented by a grey dot. The green dots represented summated quality control samples.

The CV (standard deviation/mean) values were calculated for all the intensities of metabolite features (mz/Rt) in the QC samples analysed within the run. In the generated data sets the features presented a CV lower than 30%, a lower than 20% in replicated injections of the QC samples running within the run.

To assess the reproducibility of the collection protocol, replicates of consecutive discs with ulcer fluid were collected from the same ulcer wound and the analytical workflow as described was applied in both batches of samples generating organic and aqueous extracts which were analysed by MS and NMR analysis respectively.

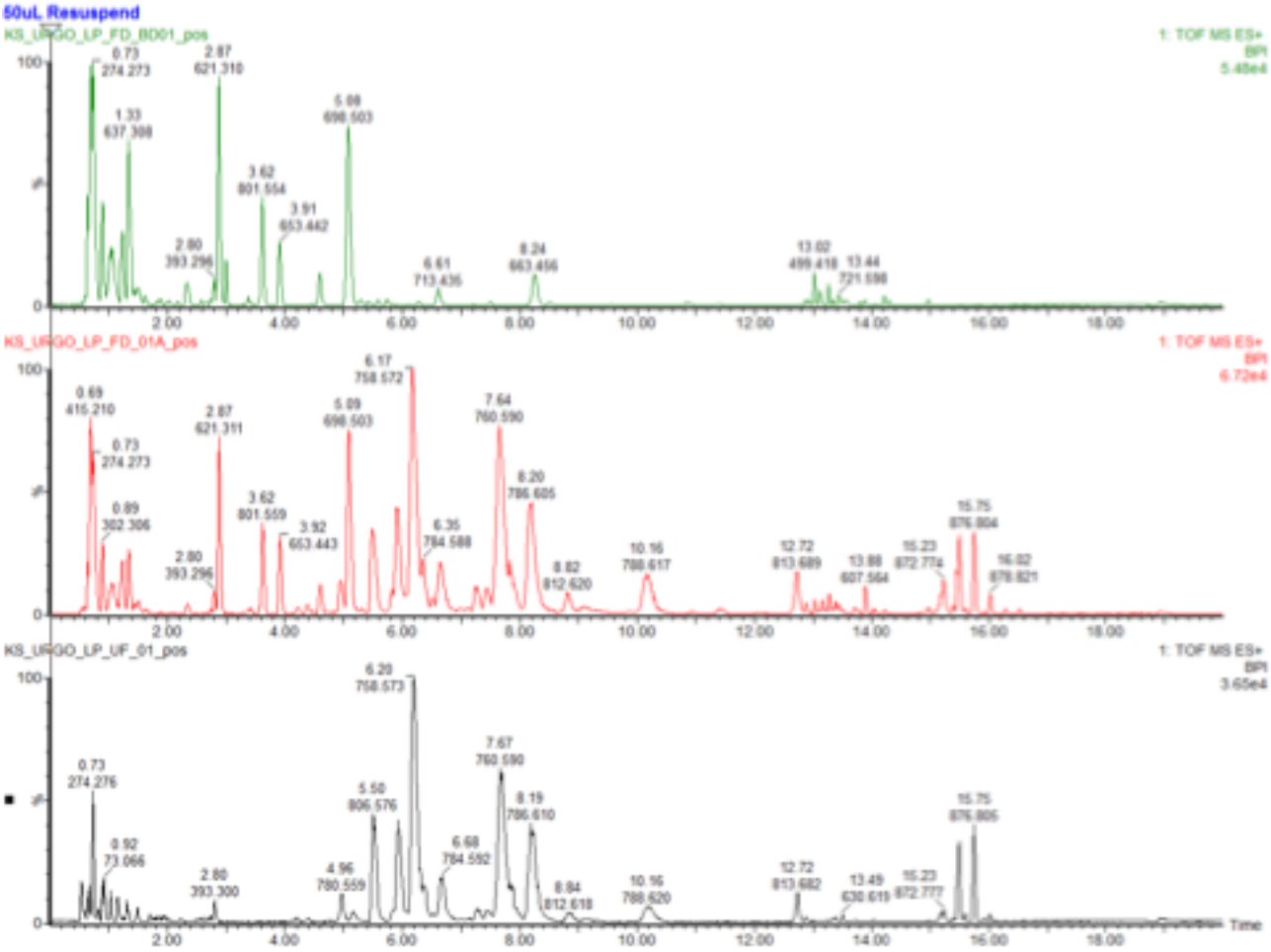


Figure 2.8 - One participant - multiple base peak intensity plots, upper plot demonstrates a blank disc, middle plot demonstrates a filter disc from the participant's ulcer and the lower plot demonstrates the analysis of fluid from the ulcer obtained from the occlusive dressing.

The metabolic profiling datasets from the two different collection protocols were also compared. Given the paucity of fluid available from direct aspiration of occlusive dressings, it was only possible to undertake MS analysis of the organic extracts of the ulcer fluid, which was directly compared with the organic extract profiles obtained from the fluid absorbed into filter paper discs. Considering the small number of patients in this pilot study and the relative paucity of fluid obtained using the occlusive dressing technique it is difficult to directly compare the merits of occlusive dressing or filter discs in terms of efficacy of fluid capture. However Figure 2.8 demonstrates the

chromatogram of a sample of ulcer fluid from a blank disc, ulcer filter disc and ulcer fluid.

Blank discs without exposure to ulcer fluid have also been included. These discs were extracted and analysed with the same protocols as the ulcer wound fluid in order to monitor impurities that can be introduced from the discs, the sample preparation procedure and solvents. Comparing all three plots in figure 2.5 and subtracting the influence of a blank disc, the profiles from the fluid are broadly similar.

Figure 2.9 demonstrates the RP-UPLC-MS lipid profiling analysis of the organic extract a single disc from a patient in this study and clearly demonstrates key separation of the common lipid classes found in the biological matrix including phospholipids, glycerols, sphingomyelins, cholesterol esters, triglycerides and free fatty acids.

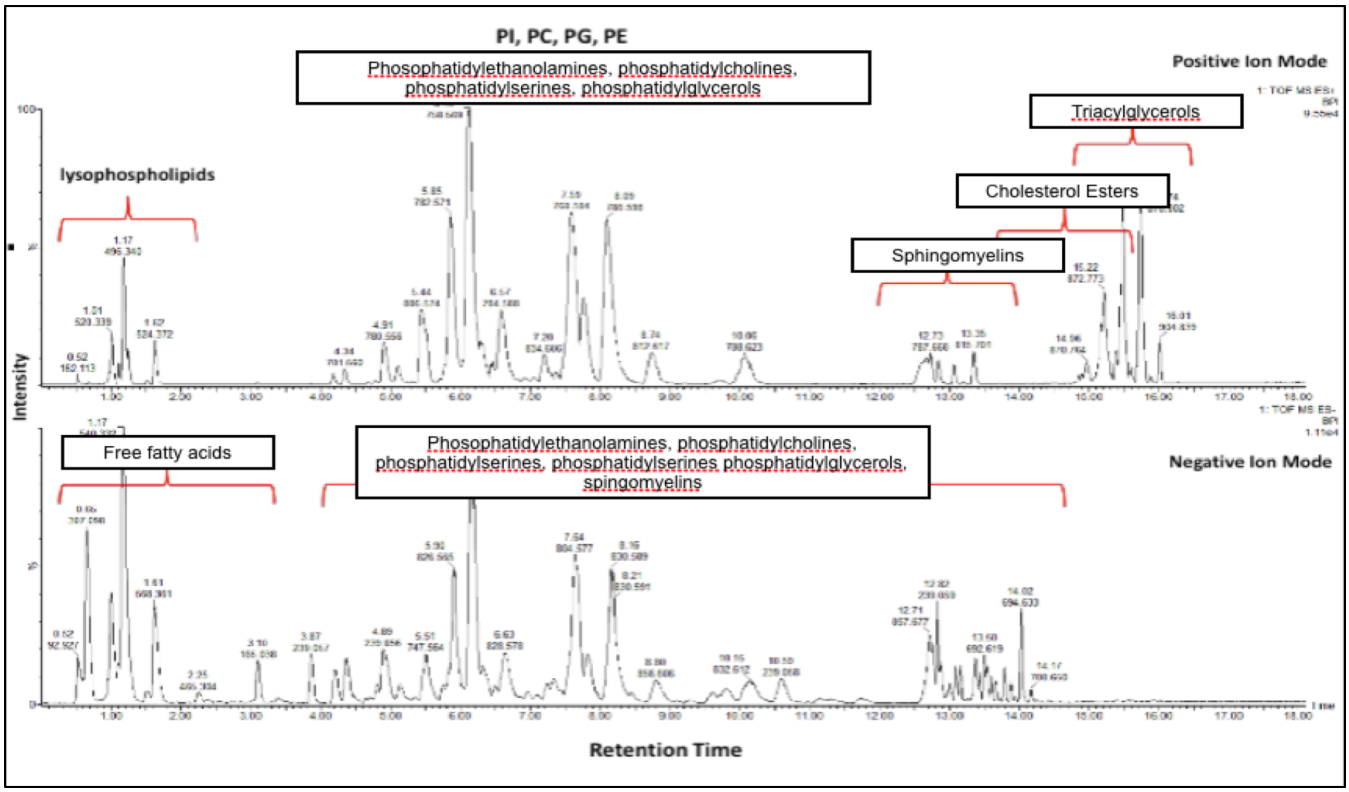


Figure 2.9 - Global metabolic profile of one disc from one participants demonstrating the lipid groups detected.

The NMR metabolic profiling analysis of the aqueous extracts obtained from the same filtered disc above revealed the presence of smaller molecules in the wound fluid microenvironment. The spectra illustrated in Figure 2.7 demonstrates the detection of multiple metabolites in the wound fluid including glucose, taurine, phenylalanine, tyrosine and histidine.

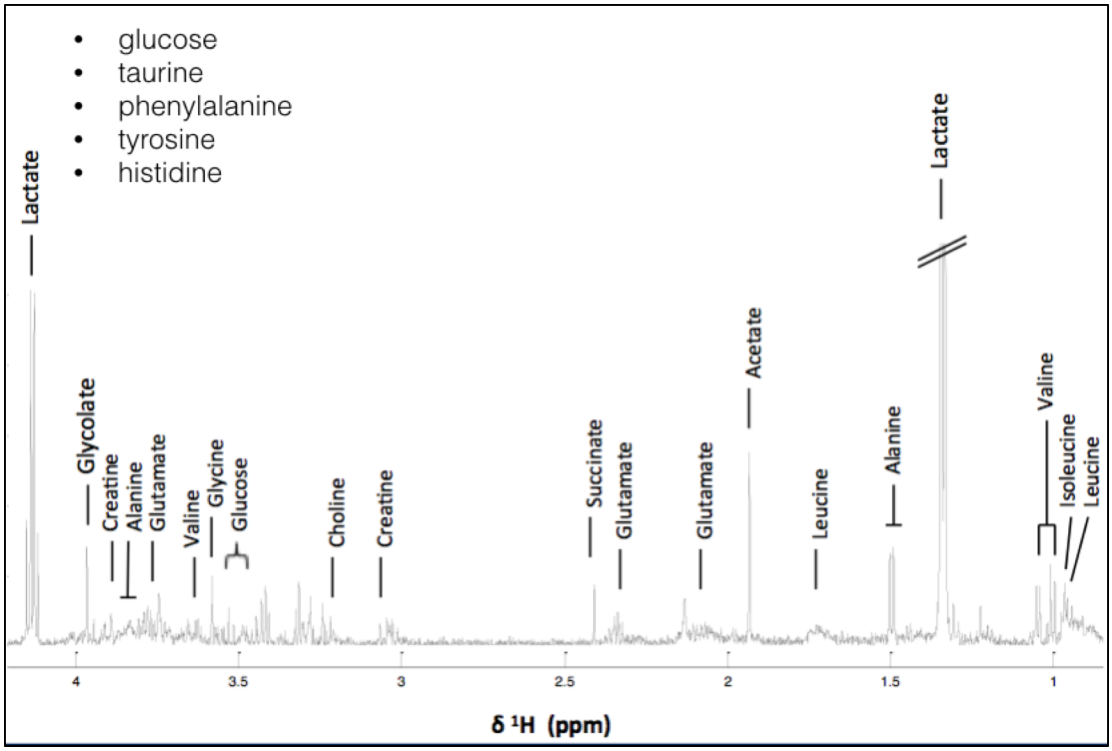


Figure 2.10 - Identification of metabolite features from an ulcer fluid sample obtained from a filter disc from a 1H-NMR analytical workflow.

Given that two forms of ulcer fluid capture were evaluated in this pilot study, it was sought to evaluate the concordance between the two techniques. Figure 2.11 demonstrates a PCA-X plot , although the low numbers in this cohort preclude meaningful statistical analysis it can be seen that broadly where a sample from the

occlusive dressing and filter disc exist, they are broadly comparable. However, the caveat is that this is a simple observation of the distribution of the points on the chart.

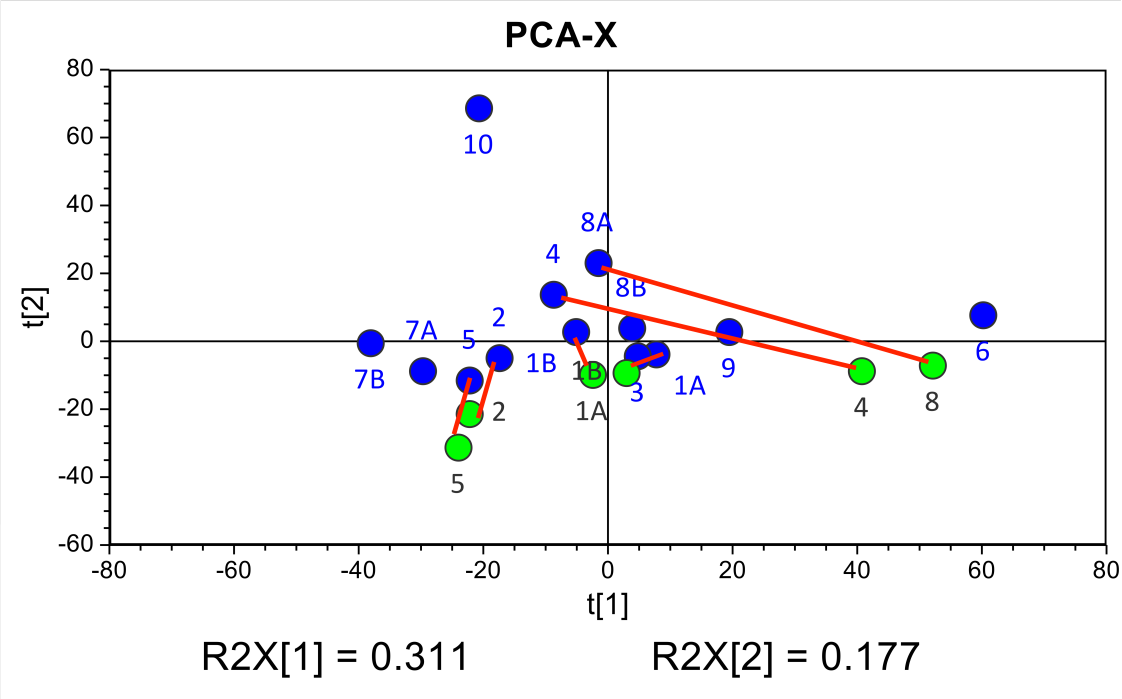


Figure 2.11 PCA-X modelling of ulcer fluid obtained from discs and occlusive dressing. Points in blue represent discs and points in green represent fluid from the occlusive dressing.

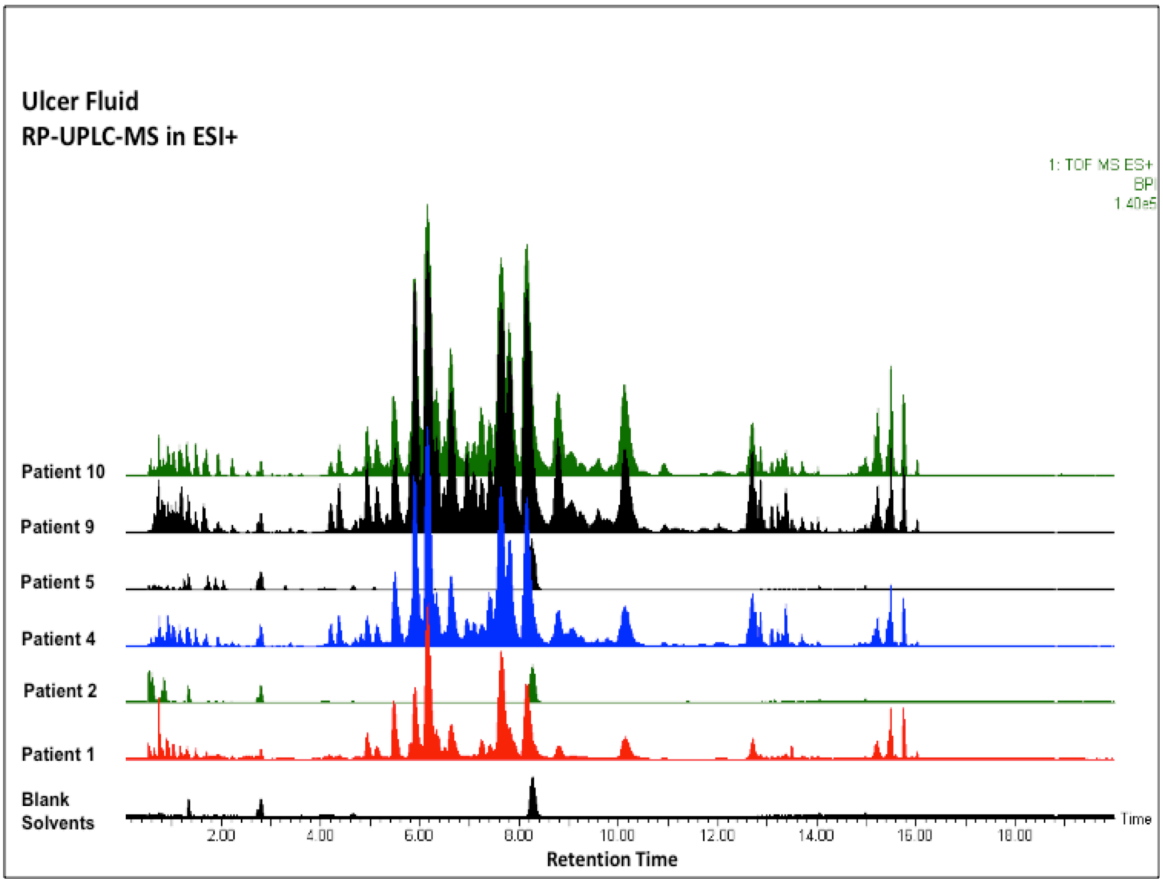


Figure 2.12: Base peak intensity chromatograms from participants in the study in whom it was possible to obtain sufficient ulcer fluid for analysis.

2.4 Discussion

This pilot study demonstrated the utility of wound fluid collection from patients with CVU by use of the sterile filter paper and occlusive dressing techniques which have been reported previously[18, 108]. It is possible to apply widely used techniques of NMR and MS to undertake metabolic profiling analysis which is a novel application in the context of CVU and chronic wounds in general.

The collection of wound fluid from participants with CVU can be difficult as demonstrated by the volume collected after a period of occlusive dressing in this study and the number of patients in whom RP-UPLC-MS analysis was possible with fluid. It was promising that filter paper discs appeared able to capture fluid from wounds in a manner more effective than the occlusive dressing and that the analysis was able to generate a metabolic profile from more patients. The difficulty of fluid collection using the occlusive dressing technique has been documented previously with particular reference to failure of the dressing to remain adherent, some wounds being too dry and too small[18, 65, 170]. In addition, filter discs appear to be a more effective method of fluid capture from CVU wounds with an easier application and method of sampling compared to the occlusive dressing technique; furthermore, more filter disc samples demonstrated meaningful chemical results than the corresponding samples from the occlusive dressing technique.

It would be interesting to note if any metabolites of interest could be identified in a systemic biological fluid such as urine or blood.

It is also difficult to recruit patients with CVU into clinical studies and trials although this can be mitigated by a multi-centre approach. For the purposes of this study in a single institution, broad inclusion criteria were selected to maximise the availability of suitable participants who would represent the population at large of CVU.

Typically, previous studies in CVU and chronic wounds has been hypothesis driven and hypotheses have been generated from the concepts of acute wound healing and its failure. Therefore, progress has been made in the understanding of cytokines[65] and related pathways, fibroblast senescence, angiogenesis and proteolysis[180]. With appropriate stratification such wounds that heal and do not heal over a defined time period it may be possible to apply the top down systems biology approach to CVU in order to generate hypotheses for further study or validation. This present study demonstrated that the feasibility of this approach to CVU although there were no identified potential biological markers of interest given the lack of stratified groups. It is clear though that in establishing feasibility that multiple small metabolites and lipids have been identified within the biological substrate analysed.

2.5 Conclusion

This study demonstrated the potential of metabolic profiling in CVU and feasibility of further ongoing work. It suggested that it may be more feasible to use ulcer fluid soaked onto filter discs. In the following sections of this thesis, a prospective study including ulcer fluid, filter paper discs, serum and urine from CVU patients will be described.

Chapter 3: Metabolic Profiling of Patients with Differing Severity of Venous Disease

3.1 Introduction

It is fairly well accepted dogma that the underlying cause of CVU is the failure of the superficial and/or the deep venous system resulting in a cascade that results in the syndrome of abnormal skin changes, tissue loss and abnormal healing[35, 112]. As described in Chapter 1, skin changes are thought to be predominantly a function of abnormal cellular behaviour partly modulated by aberrations in inflammation and regulation of proteolysis. An ideal therapeutic target at this biological level would seek to either accelerate ulcer healing or perhaps even prevent ulceration in an 'at risk' limb with venous skin changes.

However, falling back to a more global view of CVU and with the prevalent theory that chronic venous hypertension is the ultimate cause, an ideal therapeutic target would perhaps prevent the sequelae of venous disease by curtailing or preventing progression.

In a summation of 1249 ulcerated limbs, reflux was seen in 1153 (94%) with the superficial system alone accounting for 45% of limbs, mixed superficial and deep disease 43% and the deep veins alone 12%. Taking this into account, one can surmise that varicose veins may indeed account for many cases of CVU[46].

3.1.1 Primary Varicose Vein Aetiology

There are many predominant theories explaining the genesis of varicose veins with the major point of divergence as to whether 'valve failure' is a primary or secondary phenomenon[181]. They include:

- Congenital or acquired valve dysfunction
- Structural vein wall weakness
- Impaired venous tone
- Endothelial activation
- Increased arterial inflow - Abnormal arteriovenous communication

In particular, histological abnormalities have been detected in the vein wall with increased expression of phosphorylated retinoblastoma protein, smooth muscle cells disorganised collagen layout and a change to a secretory phenotype[182]. Type I collagen synthesis is increased in venous smooth muscle cells from varicose vein tissue relative to type III collagen suggesting a synthetic and possibly proteolytic imbalance[183]. There is also evidence to suggest the extracellular matrix is abnormal and this may be in part due to alterations in MMP expression[184].

In summary, multiple hypothesis driven studies have demonstrated abnormalities in the venous wall, smooth muscle, proteolysis cascade, inflammation and thrombosis but no overarching prevalent explanation for the development of varicose veins.

3.1.2 Metabolic Profiling in Superficial Venous Disease

Preliminary work has been undertaken using magic angle spinning nuclear magnetic spectroscopy comparing healthy vein to varicose vein tissue samples. Spectra from

all samples analysed are presented together in the figure below demonstrating a qualitative difference between the two groups.

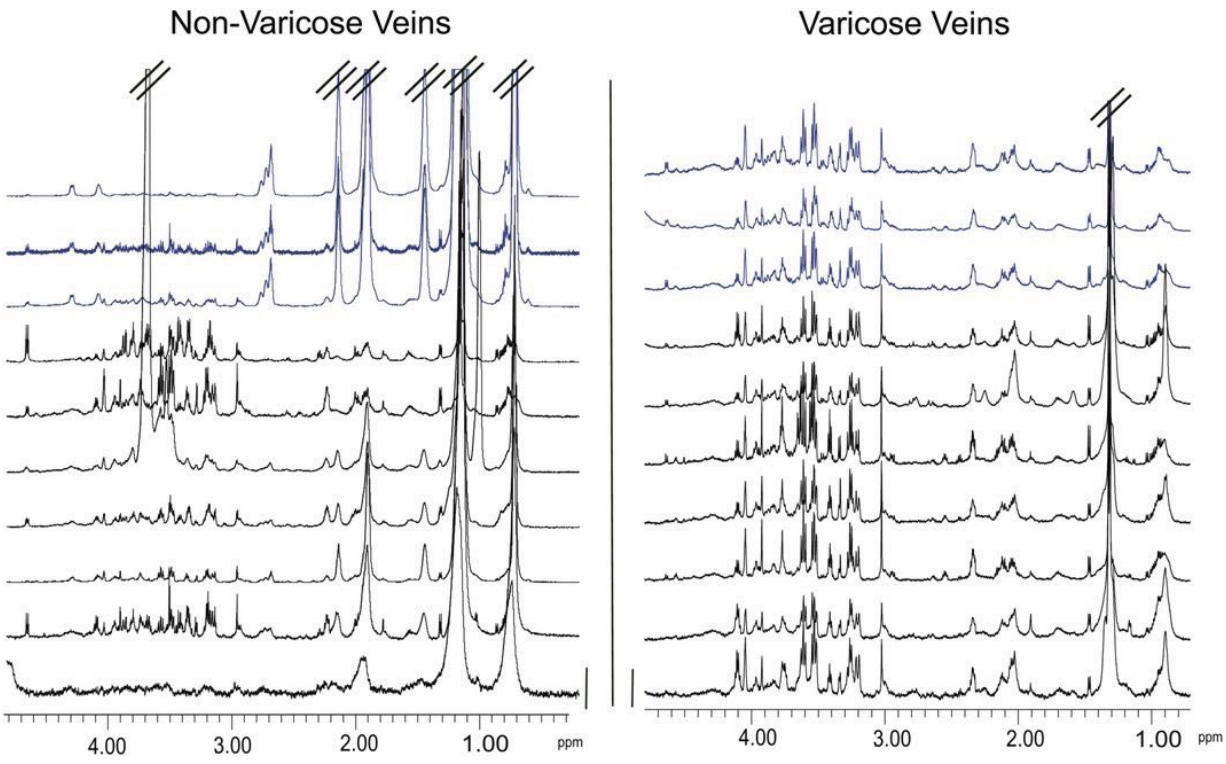


Figure 3.1: Normal vein versus varicose vein tissue samples - NMR spectra for each sample. With permission from Anwar et al (2013)[137]

An OPLS discriminant analysis scores plot shown below demonstrated that the two groups (varicose and non-varicose veins) were separated from each other in a model that was predictive and significant.

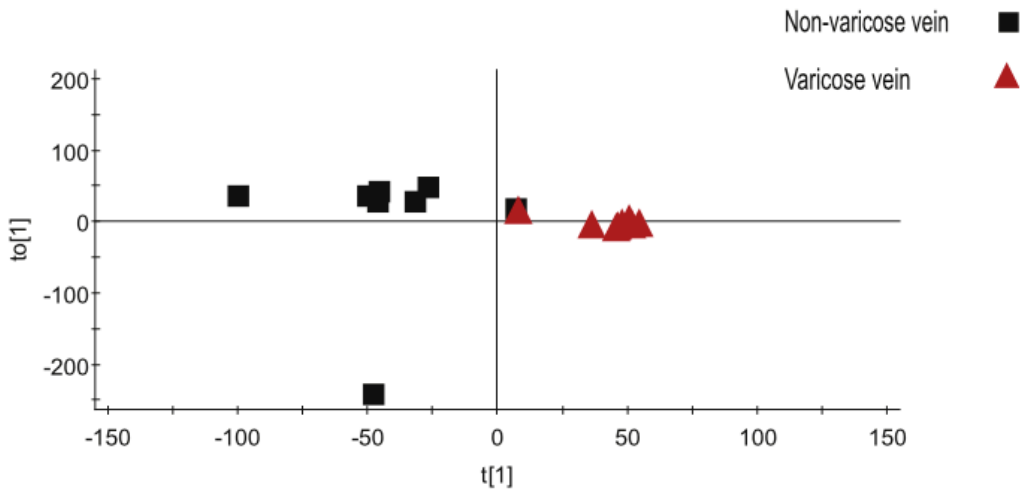


Figure 3.2: OPLS demonstrating separation between the two groups. $Q^2 = .406$ suggesting a strong relationship between the model and the clinical variation between normal and varicose veins. With permission from Anwar et al (2012)[137]

Furthermore, on identification of the metabolites responsible for the difference in the model, STOCSY demonstrated that the intensity of lactate, myo-inositol and creatine was higher in varicose vein samples whereas triglyceride intensity was higher in normal venous tissue.

Pathway analysis of the implicated metabolites has hypothesised that the difference between varicose and normal veins at a cellular pathway level integrates with the HIF-2 pathway (instigated by hypoxia and pressure) and may impart an effect on the control of cell survival, inflammation and apoptosis [185].

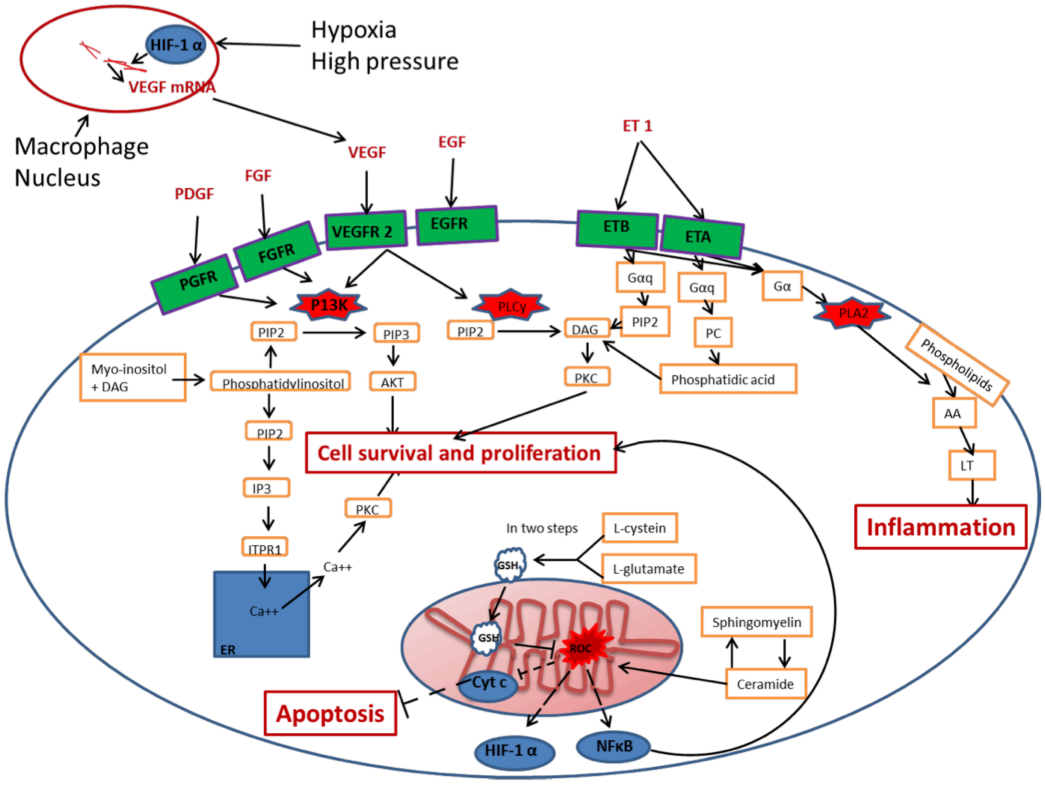


Figure 3.3: Pathway analysis integration relating to varicose veins. Adapted from MA Anwar et al (2017) [185].

What is not apparent however is whether metabolic profiling can generate any insights into the progression of venous disease. The clinical manifestations of venous disease are recorded as part of the CEAP classification but there is no overt consensus to describe who is at risk of progressing from early stage disease to the more severe manifestations of skin change or ulceration. The benefits in this approach are that biomarkers predictive of progression may be identified prompting early and efficacious treatment or potential therapeutic markers to prevent or at least slow down progression may be identified. Furthermore, although local metabolic changes have been identified, it would be interesting to see if any systemic manifestations of venous disease are apparent.

3.1.3 Aims of This Study

Building on previous preliminary work in metabolic profiling, the overt aims of the study presented in this chapter were twofold:

1. Determine if the there is a difference between vein tissue classified by clinical severity of disease as recorded by the clinical component of the CEAP classification.
2. Determine if markers of venous disease can be demonstrated in systemic biological fluids - serum and/or urine.

3.2 Methods and Materials

3.2.1 Regulatory Approval

Ethical approval was obtained from the UK National Research Ethics Service - East Midlands (Derby) Committee (reference number 13/EE/0011 - “Investigating the Genome-Phenome Correlations for Human Vascular Disease”) in February, 2013. The study was registered with Research and Development at Imperial College NHS Healthcare Trust and the Joint Research Office at Imperial College London. All specimens collected were anonymised and stored according to the rules set out in the Human Tissue Bill (2004). Informed written consent was obtained from all patients involved in this study. This study was devised with advice from the supervisors of this thesis, Professor AH Davies. In addition, scientific advice was obtained from the Section of Computational and Systems Medicine at Imperial College London (Professor E Holmes and Dr K Spagou).

3.2.2 Patient Selection

Consecutive patients attending the Local Anaesthetic Varicose Vein Unit for treatment that included superficial vein avulsions through stab incisions at Charing Cross Hospital in London were approached with the details of the study. If after discussing the study the patient agreed to participate, full informed written consent was obtained.

Patients attending for procedures had been referred to the unit by their own general practitioner with a view to diagnose, characterise and treat venous disease. As a part of the clinical work up they were reviewed in the vascular surgery clinic and all patients

had a colour venous duplex scan to confirm the presence and distribution of superficial venous disease.

3.2.3 Sample Collection

For this study, samples of urine, serum and avulsed vein were obtained. Serum and urine were obtained from the patient prior to their venous procedure and vein samples were obtained from stab avulsions obtained by the surgeon performing the procedure.

Pre-procedure a fresh mid-stream urine sample was obtained and blood obtained from a superficial antecubital fossa vein of the non-dominant arm. Urine was immediately divided into three aliquots of 1ml and placed on dry ice to freeze whereas serum was allowed to stand in its collection tube for 30 minutes at room temperature, spun at 2500 RCF and then divided into three aliquots of 0.4ml prior to being placed on dry ice.

All patients attending Charing Cross Hospital underwent venous procedures under local anaesthetic and stab avulsions were performed as decided by the operating surgeon. As soon as the avulsion had taken place, the vein was immediately placed into a plastic container and placed on dry ice.

Samples were placed in definitive storage in the dedicated freezer at the Section of Computational and Systems Medicine, Imperial College London, South Kensington Campus at -80°C until analysis.

3.2.4 Clinical Stratification

For the purposed of analysis in this study, patients were divided into two groups based on clinical manifestations according to the clinical stage of the CEAP system. The first group comprised samples from patients without skin damage (C2-C3) and the second group comprised samples from patients with skin damage (C4-C6).

3.2.5 Metabolic Profiling

3.2.5.1 NMR Profiling of Urine

An aliquot of 540µL for each urine sample was mixed with 60µL of 0.075M phosphate D2O buffer (17% D2O, pH 7.4) containing 0.1% TSP. The samples were vortexed and allowed to stand for 10 min prior to centrifugation at 14,000 rpm for 10 min at 4 °C to remove insoluble material. Aliquots of the supernatants (550 μL) from each urine sample were pipetted into 5mm NMR tubes for 1H-NMR analysis.

1H-NMR spectra were acquired with a Bruker Avance 600 MHz spectrometer operating at 600.13 MHz for 1H at 300 K. It was equipped with a 5 mm broad-band inverse configuration probe. Samples were randomly analysed in automation with a B-ACS 60 sample changer system. All samples were analysed using a CPMG relaxation-editing sequence with presaturation. The sequence attenuates the signal the signal of any remaining large molecules (proteins). It was run with 256 transients, 8 dummy scans, 64k time domain points, a spectral width of 20 ppm., 2 s relaxation delay, an acquisition time of 1.36s, 80 loops, and a spin echo delay of 400 μs. Irradiation is at the water peak (~ 2800 Hz) during the relaxation delay.

3.2.5.2 MS Profiling of Urine

To maximise metabolite coverage in urine, two complimentary chromatography methods were utilised. HILIC offers better retention of polar compounds whereas RP-LC retains highly polar and ionic analytes.

Urine samples for the HILIC-UPLC-MS analysis were diluted with ACN (1:3), vortexed for 30 seconds and centrifuged at 4 °C for 10 min at 14,000 rpm. For RP-UPLC-MS

analysis neat urine samples were vortexed and centrifuged without any dilution. Afterwards, 150µL of the supernatant was transferred into LC-MS vials with glass inserts. The UPLC setting was assembled using an Acquity UPLC system coupled to a SYNAPT® G2 High Definition MS™ System for the HILIC-UPLC-MS analysis and a XEVO G2 Q-TOF system for the RP-UPLC-MS obtained from Waters (Waters Corporation, Milford, U.S.A.). The details of the methods used for the metabolic profiling of the urine with both approaches in both positive and negative ESI mode are summarised in the appendix:

The instrument set up (accurate mass and calibration) was performed prior to analysis, using sodium formate. For mass accuracy, a Lockspray interface was used with a 2 mg/L leucine encephalin (555.2645 amu) solution (50/50 ACN/H2O with 0.1% v/v formic acid) at 10 µL/ min as the lock mass. Lockmass scans were collected every 30 s with a scan time of 0.2 sec and averaged over 20 scans to perform mass correction.

As described in Chapter 2, A QC approach was used with a pooled sample injected after every third sample to ensure that analytical variation due to machinery or ambient conditions was noted.

3.2.5.3 NMR Metabolic Profiling of Serum

For serum analysis, 300 μL of serum was added to 300 μL of 0.9% NaCl and centrifuged at 14,000 rpm for 10 min at 4°C. Aliquots of the serum solution (550 μL) from each sample were transferred to 5mm NMR tubes for 1H NMR analysis. The serum samples were analysed using CPMG relaxation-editing sequence with presaturation as described above. Moreover, serum samples were also analysed

using water suppressed 1D NMR spectrum using the NOESYPRESAT pulse sequence (256 transients). Irradiation of the solvent (water) resonance was applied during presaturation delay (2.0s) for all spectra and for the water suppressed 1D NMR spectra also during the mixing time (0.1s). The pulse sequence parameters including the 90° pulse (~ 12 μs), pulse frequency offset (~ 2,800 Hz), receiver gain (~ 90), and pulse powers were optimised for each sample set run. The spectral width was 20 ppm for all spectra. The NMR was processed with an exponential line broadening of 0.3 Hz prior to Fourier transformation, which were collected with approximately 64 k real data points.

3.2.5.4 MS Metabolic Profiling of Serum

For serum, excellent metabolite coverage can be obtained from one modality of MS alone. Therefore, RP-UPLC-MS was the modality of choice with serum samples.

Serum samples are prepared by isopropanol precipitation. Serum, QC and Blank (high purity water) samples were simultaneously prepared by adding chilled isopropanol in a ratio of 3:1; isopropanol: serum, then vortexed and stored cold at -20˚C overnight. The next day the samples were centrifuged at 14,000 rpm for 30 minutes at 4°C and then aliquot 150 µl supernatant to glass inserts in MS vials. A QC sample was prepared with 50 µl of each sample and was used to assess the analytical reproducibility of the method. A “blank sample” was also prepared to identify potential contamination from the preparation process.

Chromatographic separation was conducted using an Acquity UPLC system (Waters Ltd, 26 UK) coupled to a Waters Q-TOF premier analyser (Waters Corporation, Milford,

U.S.A.) using an ESI interface. The details of the method used for the RP-UPLC-MS lipid profiling of the serum in both positive and negative ESI mode are summarised in the appendix:

The instrument set up (accurate mass and calibration) was performed prior to analysis, using sodium formate. For mass accuracy, a Lockspray interface was used with a 2 mg/L leucine encephalin (555.2645 amu) solution (ACN:H2O 50:50 with 0.1% v/v formic acid) at 20 µL/ min as the lock mass. Lockmass scans were collected every 30 s with a scan time of 0.2 sec and averaged over 3 scans to perform mass correction.

3.2.5.4 Generic Tissue Preparation for Metabolic Profiling

In general, the vein tissue samples were processed in accordance with the work outlined in the thesis of my predecessor MA Anwar and subsequently published [185]. An extraction protocol was devised to extract non-polar (aqueous) metabolites followed by polar (non-aqueous) metabolites. [186]. The precise order of metabolite extraction and combination of chemicals used was sequentially examined previously to determine which provided the largest yield of metabolite information.

To extract non-polar metabolites, 1.5ml of 75% MTBE and 25% methanol was added to an 2m microtube (VWR) containing the vein sample and 1mm zirconium beads (BioSpec USA). Samples were frozen on dry ice and run on a bead beater (Bertin Technologies, Precellys France) for 4 cycles at 6500Hz for 40 seconds whilst maintained on dry ice during the breaks. Samples were Centrifuged (Eppendorf 5810, US) RCF of 17949.49 x g rpm for 20 minutes at 4°C.

Content from each beading tube was then transferred into 5 glass vials with PTFE seal (Fisher, UK) each containing 200µl. Glass vials containing non-polar metabolites were dried overnight in a fume hood at room temperature and then stored in -80°C freezer. 1.5ml of 50% water: 50% methanol was added in each 2ml microtube containing the sample which was then run on bead beater for 2 cycles at 6500 Hz speed each lasting 40 seconds. This was followed by Centrifuged (Eppendorf 5810, US) RCF of 17949.49 x g for 20 minutes at 4°C temperature. The supernatant from each aliquot was transferred into a new Eppendorf tube for subsequent extraction of polar metabolites (non-aqueous). Eppendorf tubes with non-polar metabolites dried in a speed vacuum overnight at room temperature and then stored in -80°C freezer.

3.2.5.5 NMR Metabolic Profiling of Vein Tissue

Dried non-polar extracts were suspended in deuterated TSP phosphate buffer solution at pH 7.4. After 1 minute vortexing, 5 minute sanitation and a further 1 minute vortex samples were centrifuged at 18000 RCF 4°C temperature. Thereafter, samples were transferred into NMR analysis tubes and loaded into the NMR autoloader to be run.

Non-polar extracts were analysed using a 600 MHz 1H-NMR spectroscope (Bruker BioSpin, Rheinstetten, Germany). Standard 1D pulse sequence with water suppression applied during RD of 2 s. A total of 512 scans were acquired into 64K data points. Spectrum width was δ 20.00 with relaxation delay of 2 sec, acquisition time 1.36 sec, spin-echo delay t=400µs and total echo time of 64 ms for all aqueous experiments. On TOPSPIN 3.0 software (Bruker BioSpin, Rheinstetten, Germany), aqueous spectra were phased and calibrated using chemical shift of sodium 3-trimethylsilyl-1- [2,2,3,3,-2H4] propionate (TSP) at δ 0.00. The base line was also

corrected manually. Spectra were imported into MATLAB R2009b (Mathworks™, 2009) using an Imperial College in-house model built on MATLAB. The region containing the water resonance (from δ4.68 to δ5.24) was removed from all spectra. In addition, the resonance of TSP (from δ-1 to 0.2 δ) was removed from all spectra. All spectra were subsequently aligned and normalised using probabilistic quotient normalisation.

3.2.5.6 MS Metabolic Profiling of Vein Tissue

Dried organic metabolites were resuspended in isopropanol and were vortexed and sonicated for 5 minutes at 4°C, 200 µl of each sample was put inside the 1.8mm LCMS glass vials and the remaining 50 µl of each sample was added together to form the summated QC sample.

Samples were analysed using an Acquity UPLC System (Waters, Manchester), coupled to a Q-Tof Premier mass spectrometer (Waters, Manchester) in both positive (ESI+) and negative (ESI-) electrospray ionization modes. Chromatography was carried out at 40°C on a Waters Acquity UPLC HSS T3 column (1.8µm, 2.1x100mm). Separation was performed with ACN/H2O (60:40) with 10mM ammonium formate and 0.1% formic acid (A), and isopropanol/acetonitrile (90:10) with 10 mM ammonium formate and 0.1% formic acid (B). ESI conditions were set with the source temperature 120°C, desolvation temperature 400°C, cone gas flow 25L/hr, desolvation gas flow 800L/hr, capillary voltage 3000 V for ESI+ and 2500V for ESI-, cone voltage of 30 V for ESI+ and 25 V for ESI-. The instrument was set to acquire over the m/z range 50-1200 in V mode with scan time of 0.2 s and an inter-scan delay of 0.02 s. Data were collected in centroid mode. Leucine/Enkephalin (200pg/µL, in ACN: water 50:50, 0.1%

formic acid) was used as a mass lock with an analyte-to-reference scan ratio of 4:1. The instrument was calibrated before analyses using 0.5mM sodium formate solution. The order of injection of samples was randomised. QC sample was injected regularly throughout the run to monitor the stability of the of the UPLC-MS platform. Samples were run using a random sequence and a QC was set to run after every 10 samples. Data were pre-processed using MassLynx version 4.1. Three dimensional LC-MS data was processed and aligned into a table of time aligned features with their retention time and m/z intensity. Isotopes, peaks and fragments and adducts were treated as a separate feature

3.2.5.7 Data Processing and Statistical Analysis

SIMCA-P+ 12.5 statistical software (UMETRICSTM, Sweden) and MATLAB R2009b (MathworksTM, 2009) using in-house model built on MATLAB were used to analyse the data acquired from NMR and LCMS. Both PCA and OPLS-DA analysis were performed. This has been fully described in Chapter 2 (section 2.6.6.4).

3.3 Results

3.3.1 Clinical Recruitment and Population Characteristics

This study recruited patients between July 2013 and January 2014 with a target of forty patients as per previous metabolic profiling of vein tissue study[137, 185]. The following table lists information about the group and details of stratification

N	44
Gender	18 male 26 female
Age range	Range 24-84y Median 58y
Group 1 (CEAP 2-3)	20
Group 2 (CEAP 4-6)	24

Table 3.1 - Study participant characteristics

3.3.2 Metabolic Profiling Against Clinical Severity

All samples were successfully analysed using NMR and MS techniques, raw spectra were successfully pre-processed, processed and using an in-house algorithm, peak data was extracted [138 187].

Based on the two clinical groups, multivariate statistical analysis was undertaken and an OPLS-DA model created. There is a weak difference between the groups when vein was analysed using NMR, urine with MS and serum with MS. However, none of these weak models demonstrated significance. No variation was detected when spectra were analysed using other modalities. The Q values and p values where

appropriate are presented in the table. It is prudent to bear in mind that the numbers of participants

OPLS-DA analysis		
Samples	NMR	MS
Vein	Q2=0.06 p-value=0.94	Q2<0
Urine	Q2<0	Q2=0.1 p-value=0.14
Serum	Q2<0	Q2=0.06 p-value=0.7

Table 3.2 - Details of OPLS-DA analysis comparing the spectra of the two groups

3.3.3 Detection of Implicated Metabolites in Varicose Veins in Systemic Biofluids

Previous work suggests that the following metabolites are increased in varicose vein tissue compared to normal vein tissue [185]:

- Myoinositol
- Phosphatidylcholine 18:0/20:3
- Phosphorloethanolamine 20:4/18:2
- Sphingomyelin 18:0/24.0
- Sphingomyelin 18.1/24.0
- Glutamate
- Taurine

Serum MS analysis revealed that all the implicated lipids in varicose vein tissue are present and the figure below (figure 2.3) demonstrates a sample from a single subject.

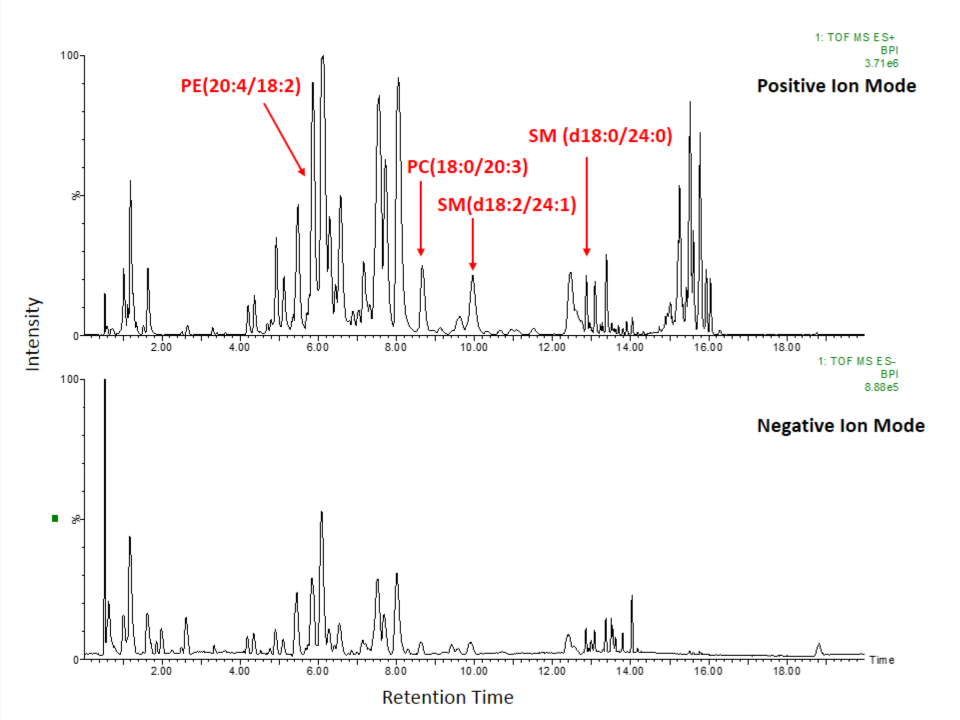


Figure 3.4: Representative serum sample which demonstrates the presence of the specific lipids implicated in varicose vein tissue:

- PE 20.4/18.2: Phosphorloethanolamine 20:4/18:2
- PC18.0/20.3 Phosphatidylcholine 18:0/20:3
- PE 20:4/18:2 Phosphorloethanolamine 20:4/18:2
- SM 18.0/24/0 Sphingomyelin 18:0/24.0
- SM 18.1/24.0 Sphingomyelin 18.1/24.0

Serum NMR demonstrated the presence of glutamate (Figure 3.5) and urine NMR demonstrated the presence of taurine (Figure 3.6)

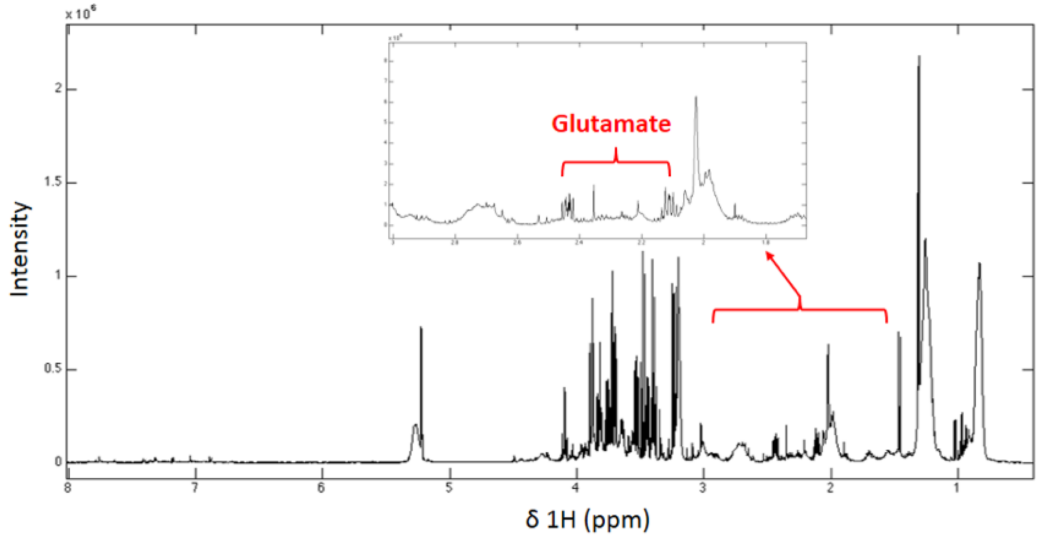


Figure 3.5: Representative serum NMR spectra with glutamate demonstrated.

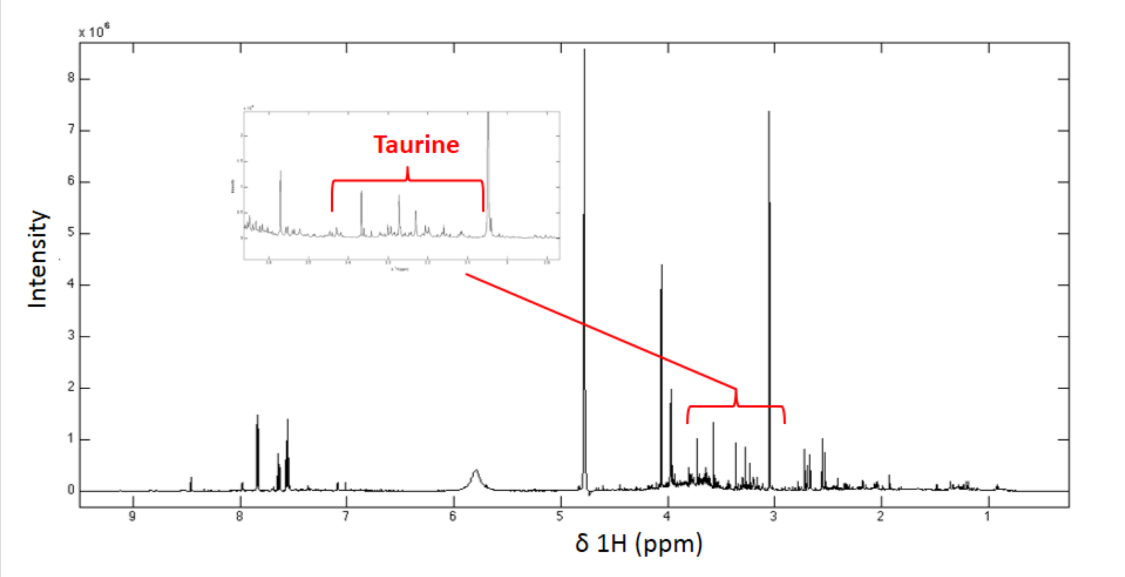


Figure 3.6: Representative serum NMR spectra with taurine demonstrated.

3.4 Discussion

This study confirmed that varicose veins demonstrate a different metabolic phenotype compared to normal veins[137]. Furthermore, metabolites detected in varicose vein tissue with a greater intensity were consistently detected in urine and serum from participants in this study.

A trend has been demonstrated when comparing groups based on the presence or absence of skin damage in the NMR analysis of tissue, MS analysis of serum and MS analysis of urine with the caveat that no analysis reached statistical significance. Furthermore, no difference was demonstrated when vein tissue was analysed with MS, urine with NMR and urine with NMR. These findings may reflect that the sample size of 44 patients may not be large enough to detect significant differences. The absence of metabolic profiling studies thus far in the systemic biofluids of patients with varicose vein disease made it difficult to calculate an ideal sample size but future studies may be able to use this data to plan the optimal sample size.

The CEAP classification predominantly describes venous disease as a function of clinical progression, aetiology and anatomical distribution. However, clinical disease progression towards the higher clinical stage is a process that is recorded by the presence of skin disease (C4 - venous skin changed, C5 - healed ulceration and C6 - active venous ulcer) and it is not entirely clear how up migration from the early to the latter clinical stages happens at a biological level. It may be possible that once the primary abnormality in the vein wall occurs, skin changes may in fact be related to an

inherent abnormality in the skin such as cellular behaviour, inflammation or proteolysis as outlined in the Chapter 1 of this thesis.

With reference to the detection of implicated varicose vein metabolites, this finding must be regarded with a degree of caution as this study does not have controls without documented venous disease. On this basis any future study that seeks to establish the difference between the biofluids in patients with and without venous disease would be prudent to confirm the presence of venous disease with duplex ultrasonography[188].

If the presence of systemic biomarkers could be confirmed the implications would be two fold. Firstly, it may aid in the prognostication of patients with venous disease, for example in subjects who are asymptomatic or in those with minimal symptoms. Secondly, it may redefine venous disease as a systemic disease with manifestations in the venous system of the leg with the potential for a top down systems biology approach with the aim of 'reverse engineering' of the syndrome to identify and develop potential therapeutic targets. Nonetheless, these points remain conjecture at this point.

Chapter 4: Clinical Study Design

4.1 Introduction

As the continuation and follow up of the pilot study described in Chapter 2 of this thesis, a more comprehensive metabolic profiling study in CVU was planned and in this chapter the principles of the study design and requisite planning will be outlined comprehensively.

4.2 Process of Study Design

Prior to commencing the study, a significant period was dedicated to the design, principally by the author of this thesis with significant consultation with the two supervisors and nurses with an interest in vascular disease. Significant scientific advice regarding metabolic profiling was taken from the Section of Computational and Systems Medicine at Imperial College London. Furthermore, the study prior to submission for regulatory approval was fully scrutinised by the Joint Research Office of Imperial College London and Imperial College NHS Healthcare Trust, London.

4.3 Regulatory Approval

Ethical approval for the main study was granted by the Cambridge South Committee (13/EE/0137) in July 2014.

The study was also adopted onto the NIHR Portfolio and local research and development approval was obtained from the following English healthcare organisations:

- Imperial College Healthcare NHS Trust
- Cambridge University Hospitals NHS Foundation Trust

- West Suffolk NHS Foundation Trust and
- Central London Community Healthcare Trust

4.4 Clinical Settings

4.4.1 London

In London, patients were recruited from Imperial College NHS Healthcare Trust and Central London Healthcare NHS Trust. At Imperial, patients with CVU were referred to the Vascular Surgery Outpatient clinic having been initially managed in the community either with their own General Practitioner or community nurses and were referred with a view to confirmation of diagnosis or advice on ongoing management. Patients attending St Charles' Hospital in London (Central London Community Healthcare Trust) usually had the aetiology of their CVU confirmed already and were being reviewed regularly to facilitate the ongoing management of CVU with regular clinical wound review and dressings.

4.4.2 East Anglia

Patients in East Anglia were recruited from Cambridge University Hospitals NHS Foundation Trust, Cambridge and West Suffolk NHS Foundation Trust, Bury St Edmunds. Typically, General Practitioners or community nurses in the area would refer patients to the local vascular service to either confirm the aetiology of leg ulceration and/or to advise on ongoing clinical management. Furthermore, certain patients with leg ulceration were referred to the Joint Leg Ulcer Clinic in Cambridge run by Vascular Surgery in conjunction with Dermatology.

4.5 Clinical Assessment

Typically, all patients underwent clinical assessment, ABPI and colour venous duplex to exclude concomitant arterial disease, confirm a venous aetiology and exclude other causes of leg ulceration. A management plan for the treatment of CVU was drawn up and communicated to the patient's own General Practitioner.

4.6 Clinical Recruitment

This study opened for recruitment on the 1st December 2013 for patients in London (Charing Cross Hospital and St Charles' Hopspital), 1st May 2014 for patients in Cambridge (Addenbrooke's Hospital) and 1st June 2014 for patients in Bury St Edmunds (West Suffolk Hospital).

Suitable patients were identified from electronic record systems and clinic lists and if the inclusion criteria were met, information about the study was provided. Thereafter patients were contacted one week after the initial meeting to confirm that they wanted to take part in the study and if agreeable, a date for assessments was set. At the first meeting in the study, written informed consent was obtained. The inclusion and exclusion criteria were kept broad to maximise patient recruitment into this study.

4.6.1 Inclusion Criteria

- Male or female over the age of 18 years

- Chronic venous ulceration – Defined as wound of greater than four weeks in duration between the foot and the ankle with an Ankle Brachial Pressure

Index greater than 0.85.

- Colour venous duplex evidence of chronic venous insufficiency showing either reflux or obstruction.

4.6.2 Exclusion Criteria

- Acute infection in the studied lower limb within the last four weeks.
- History of malignancy in the lower limb to be studied.
- History of connective tissue disease.
- Patients on medications that can cause immunosuppression – Corticosteroids, chemotherapy or radiotherapy for cancer and recombinant immunological medications.

4.7 Patient Assessment

Individual patients were studied at multiple time points to allow cross referencing of metabolic profile data with clinical data. The time points were chosen as week 0, 1, 2, 4, 8, 12 and 20. These points were chosen to chart the progress of the wounds with the possibility of redundancy if not all assessments could be attended which is a strong possibility in a group of patients who tend to be frail and elderly.

Initial Assessment (Week 0)
This comprised the following steps and procedures:

- Completion of written informed consent.
- Collection of relevant clinical details (principally venous history, medications and ulcer history).
- Removal of wound dressing.
- Collection of ulcer fluid
- Ulcer size measurement (digital photography and manual planimetry).
- Collection of mid stream urine sample.
- Collection of venous blood from the antecubital fossa.
- Redressing of the wound.

Subsequent Assessments (Week 1, 2, 4, 8, 12, 20)

- Confirmation of consent.
- Collection of relevant clinical details (principally venous history, medications and ulcer history).
- Removal of wound dressing.
- Collection of ulcer fluid
- Ulcer size measurement (digital photography and manual planimetry).
- Collection of mid-stream urine sample.
- Collection of venous blood from the antecubital fossa.
- Redressing of the wound.

Patient endpoints for study participation were either:

1. Ulcer healed - defined as complete wound closure.

Or

2. Twenty-week period complete.

During the study, no changes were made to the patients' routine dressing regimen and all appointments with community and specialist services were maintained. The dressing regimen had been applied prior to the study for a variable amount of time. Consultation between the author and the supervisors of this thesis was made to determine if participants should not be in compression for a defined period prior to enrolling into the study however this was felt to be impractical and unethical.

4.8 Study Participant Summary of Procedures

First Meeting	**Introductions, discussion and consent**
Initial Assessment Week 0	Preliminary evaluation – Interview about medical problems, physical examination. Blood, urine and ulcer fluid sampling. Wound dressing.
Week 1,2, 4, 8 and 12	Clinical review Blood, urine and wound ulcer fluid sampling
Week 20	Clinical review Blood, urine and wound ulcer fluid sampling If wound has not healed, apply Urgostart® dressing to wound dressing. If wound has healed – end of study.
Week 21, 22, 23 and 25	Clinical review Blood, urine and wound ulcer fluid sampling Continue dressing with Urgostart®
Week 28	Clinical review Blood, urine and wound ulcer fluid sampling Completion of Urgostart® dressing End of study

Table 4.1 – Study procedures for clinical evaluation.

4.9 Ulcer Size Measurement

Ulcers were measured using two methods. For the purposes of analysis of samples in this thesis, manual planimetry was utilised as the primary measurement tool to determine the size of the ulcer.

4.9.1 Manual Planimetry

A specialised two ply tracing grid with 1cm^2 grid delineation (Convatec, Deeside, UK) was placed upon the wound. A 0.7mm diameter indelible pen was used to outline the extent of the ulcer. The transparent layer of the systems was discarded leaving the overlay grid to be placed onto the supplied piece of opaque hard paper sheet. This was marked with the patient's study number and time point. Thereafter completed grids were scanned using a document scanner with an autofeed mechanism (Fujitsu Scansnap S1300i Duplex Colour Scanner, Fujitsu, Minato-Ku, Tokyo, Japan). All files were imported into the JPEG format.

JPEG files were opened using an open source. Java based image manipulation tool ImageJ[189]. The image was size calibrating by referencing to a known length measurement (the 1cm markers of the tracing grid), the ulcer was marked using the polygon tool and the measurement function was selected to provide an area and perimeter measurement. All data recorded in a spreadsheet for future cross referencing with ulcer metabolic profiling data.

4.9.2 Digital Photography

A Sony Cyber-shot DSC-WX60 16.2 Megapixel Digital Camera was used with flash enabled but all other image pre-processing macros disabled. A 3cm calibration strip with marked 1cm squares placed in field of vision of camera but not obscuring wound edge. The camera positioned 15cm from wound perpendicular to midpoint. Image captured. If the wound could not be captured in one single image, the wound was divided into two or more segments and images summated.

As described for measuring grids, all images were processed in the JPEG format and measurement was undertaking using ImageJ with all measurements transposed to a spreadsheet.

4.10 Sample Collection and Storage

4.10.1 Serum

Blood was collected from the right or left antecubital fossa after standard antiseptic precautions without the use of alcohol based skin preparation. 10mls of venous blood was drawn using an 18g needle and 10ml syringe. Thereafter, blood was transferred into plain blood tubes without any additives and allowed to rest at room temperature for 30 minutes[178, 190].

Sample tubes were centrifuged at 2500 RCF for ten minutes at room temperature and the supernatant was extracted to generate three aliqouts of 0.4 ml which were placed into storage at -80^{o}C until required.

4.10.2 Urine

Patients were asked to provide a mid-stream urine sample into a sterile container at the time of their assessment. This was used to generate three 1ml aliquots of urine for storage at -80^{o}C until required.

4.10.3 Ulcer Fluid

Based on the pilot data in a previous chapter, the preference for efficacious fluid capture in this study was from filter discs placed on the wound however despite the

inherent difficulty in obtaining ulcer fluid from the occlusive dressing technique both techniques would be used to obtain ulcer fluid for this study.

General Procedure for Wound

- Wound cleansed in 0.9% NaCl.
- Dried with plain sterile gauze.
- Three filter discs placed on wound (Sterile filter discs for microbiology - Sigma Aldrich, St Louis, USA)
- Wound covered with adhesive dressing (Tegaderm sheet, 3M, St Paul, Minnesota, USA).
- Limb with wound left dependent for 60-90 minutes.
- Any accumulated fluid removed with 18g needle obtained through occlusive sheet. Fluid collected placed into sample tube.
- Filter discs removed and placed into sample tubes.
- All samples placed on dry ice pending transfer to definitive -80^{o}C storage.

4.10.4 Sample Labelling and Storage

All patients on entering the study had a unique identifying number and samples were suffixed with an appropriate designation depending on sample type (ulcer fluid, filter disc, urine and serum). All samples were ultimately placed in secure storage in the Imperial College South Kensington Campus (Sir Alexander Fleming Buillding) according to Human Tissue Authority guidance. Samples on collection. Transportation to South Kensington of samples was undertaken with the samples placed on dry ice.

Blood serum	B
Urine	U
Wound fluid – Occlusive dressing	fX
Wound fluid – Filter disc	fN

Table 4.2 – Sample designations for storage.

4.11 Data Handling

4.11.1 Clinical Data

The initial assessment comprising history, examination, routine blood investigations, colour venous duplex result, venous clinical severity score, CEAP classification and assessment of wound status were recorded onto a clinical report form. Subsequent assessments shall be recorded onto addenda of the clinical report form.

Clinical data were placed into a computerised database and each patient will be given a unique study identifying number. Clinical data documentation were placed into the study master file as designated by the protocol for the study, research ethical approval and research and development approvals.

Unique identifying numbers will be held at Imperial College and at the relevant clinical sites.

The study master file remains within the secure vascular clinical research office at Imperial College's Charing Cross Campus.

Chapter 5: Flow Through Clinical Study

5.1 Screening and Recruitment

Recruitment to the study took place between December 2013 and February 2015 in the following sites:

- Imperial College Healthcare NHS Trust - Charing Cross Hospital.
- Central London Community Healthcare NHS Trust - St Charles' Hospital.
- Cambridge University Hospitals NHS Foundation Trust - Addenbrooke's Hospital.
- West Suffolk NHS Foundation Trust - West Suffolk Hospital.

55 patients were identified in these units as suitable for the study and 32 agreed to take part. 28 patients managed to complete a successful week 0 visit. Recruitment was broadly stable throughout the study with an increase in the latter half with sites in Cambridge and West Suffolk opening. This was a function of staggered local NHS trust approval dates.

The reasons for participants declining to take part included:

- Unable to commit to a significant period of follow up of 20 weeks.
- Too frail to make multiple extra visits to hospital.
- Inability to contact patient (wrong address, phone number)
- Appointments would interfere with other activities.

Of the four patients who agreed to take part but were unable to attend, one ulcer had healed, one ulcer was on a limb with a fractured bone with plaster covering the ulcer and two patients developed serious illnesses requiring admission acutely to hospital.

Most patients were already under clinical review by the respective NHS clinical services at the above NHS trust and research assessments were carried out whilst the patient was followed up normally by NHS services be that a vascular surgery or wound clinic.

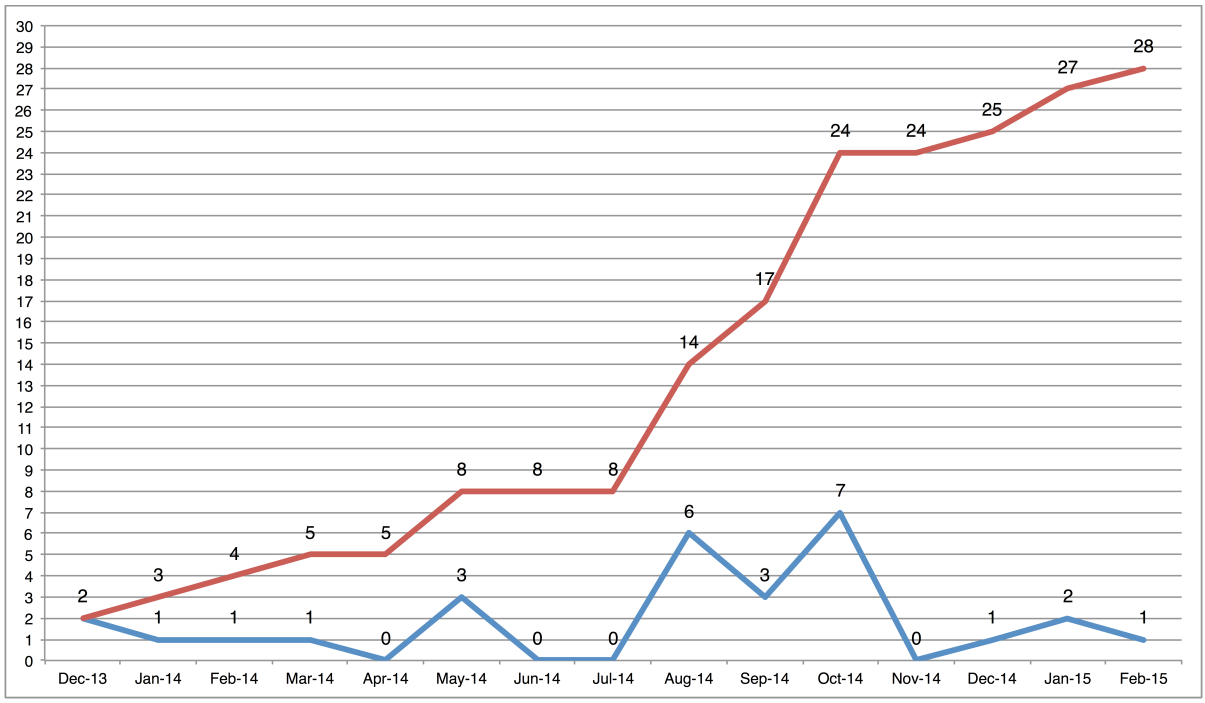


Figure 5.1 - Monthly and Cumulative Recruitment

5.2 Population Demographics

The general details about patients recruited into the study is listed in the table below.

The median ulcer duration was 24.5 months with a median patient age of 71 years.

5.2.1 Adverse Events

As stated in the ethical and regulatory approvals, any adverse events were to be logged and reported to the study sponsor (Imperial College London) and the relevant research ethics committee (Cambridge South). In this study no adverse events were recorded.

Total number of subjects	N = 28
Age range (years)	37 - 96 (median 71)
Gender split	Male 18: Female 10
Ulcer chronicity	2 - 156 months (median 24.5 months)
Treated arterial hypertension	4/28
Diabetes	5/28
Current smoker	7/28
Venous disease distribution	Superficial venous alone 17/28 Deep venous alone 10/28 Mixed superficial and venous 1/28

Table 5.1: General study population.

5.3 Clinical Outcome

28 patients recruited into the study made 110 visits for study purposes including their initial visit. Within 20 weeks of commencing the study, 15 patients healed and 13 patients were unhealed. From a pragmatic perspective, many patients opted provide notification of ulcer healing without attending a research appointment and in cases of doubt this was verified with the clinical provider who regularly reviewed the ulcer. The

pictures below outline examples of healing, static wounds and deteriorating wounds during the study.



Figure 5.2 - Participant with a healing wound



Figure 5.3 - Participant with a mainly static wound.



Figure 5.4 - Figure with rapidly improving wound over 4 weeks.



Figure 5.5 - A wound clearly deteriorating over a twenty-week period.

Four patients withdrew from the study. One patient was admitted to hospital for an unrelated illness and could not commit to further appointments within the 20 week period, two patients could not attend further assessments because of other commitments and one patient moved away from the area. However, it was possible to ascertain their healing status at week 20 and their samples were included in metabolic profiling analyses discussed later in this thesis.

5.3.1 Ulcer Size and Healing Rate

Descriptive statistics for the initial ulcer sizes are presented in table 5.3. A range of ulcer sizes on commencement of the study. These values will be utilised later in this thesis in the interpretation of metabolic profiling data.

	Area (cm^2)
Minimum size	0.325
25% percentile	2.767
Median aize	12.51
75% percentile	28.33
Maximum size	244.2
Mean	25.69
Standard deviation	46.67

Table 5.3 – Descriptive statistics reflecting ulcer size at week 0..

As stated above, 15 patients out of the total of 28 had a healed ulcer within the 20 week period that commenced at the first assessment (designated as week 0). As a further stratification, the change in area was calculated as the area of the ulcer at week 4 as a percentage of the ulcer area as calculated at week 0. Participants verified as

healed prior to week 4 were accordingly recorded as 0% wound area and the 4 participants known to be unhealed by week 20 who did not attend further appointments were excluded from this analysis. The data are summarised in Table 5.4 and visualised in Figure 5.6.

Minimum (representing ulcer closure)	0
25% percentile	11.75
Median	53
75% percentile	98
Maximum (representing ulcer expansion)	201
Mean	61.25
Standard deviation	52.47

Table 5.4 – Descriptive statistics reflecting the percentage change in ulcer area between week 0 and week 4.

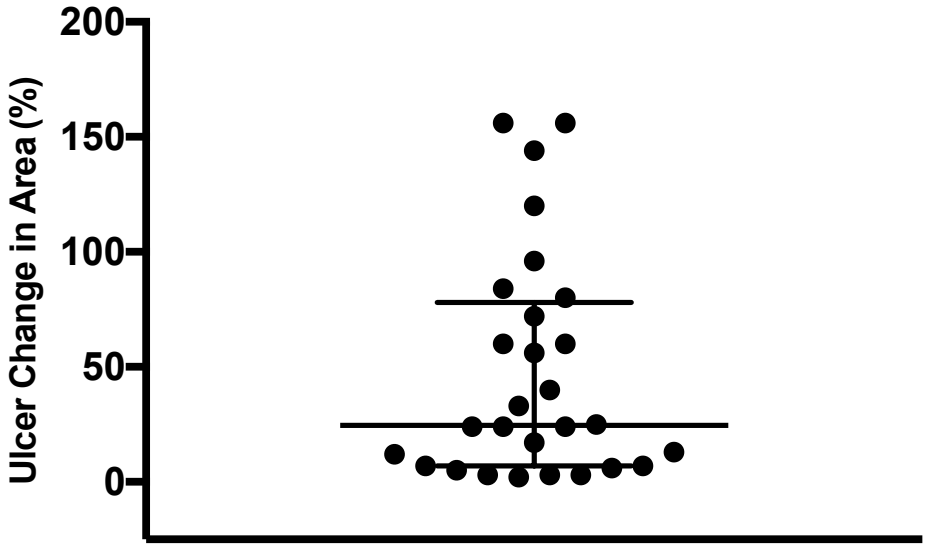


Figure 5.6 – Scatter plot representing the change in area from week 0 to week 4 as a percentage of original ulcer size. Median and interquartile ranges marked.

5.3.2 Ulcer Chronicity

The chronicity of the studied ulcer was defined as the time in months between the first appearance of the ulcer and the first assessment (week 0). Descriptive statistics are outlined in table 5.5.

	Ulcer Chronicity (months)
Minimum	2
25% percentile	7
Median	24.5
75% percentile	78
Maximum (representing ulcer expansion)	156
Mean	28.62
Standard deviation	66.52

Table 5.5– Descriptive statistics reflecting the duration of ulceration at week 0.

5.3.3 Ulcer Fluid Collection

108/110 visits demonstrated an active ulcer and were all able to generate an ulcer fluid filter disc. Aliquots of ulcer fluid obtained from the occlusive dressing were obtained in 19/110 visits.

5.3.4 Serum Collection

Serum was obtained from the centrifugation of whole blood obtained from the antecubital fossa. This was successful in 80/108 visits reflecting that sampling of

venous blood was unsuccessful either for technical reasons or that sampling at a specific visit was declined by the participant.

5.3.5 Urine Collection

A midstream urine was obtained in 84/108 visits. A fresh sample was asked for at the time of the participant's visit to minimise the delay between production and definitive stable storage. The inability to obtain a urine sample at all visits probably reflects the inability to produce urine on demand or certain difficulties in producing a urine sample in a hospital environment.

5.4 Clinical Stratification Parameters

With the dataset that will be explored fully in subsequent chapters for samples sourced from various biofluid compartments (ulcer fluid, serum and urine), it is imperative that stratification parameters are applied to the dataset to determine 'differences' between groups. For the purposes of this thesis, the determination of 'prognostic' biomarkers in CVU is the main aim and thus accepted clinical correlates predictive of ulcer healing have been applied to the dataset.

1. Ulcer chronicity - 'Age' of the ulcer at week 0 of the study, recorded in months.

2. Initial size - A measurement in cm^2 based on manual planimetry at week 0

3. Healing rate - Calculated as percentage of active ulceration remaining at week 4 compared to week 0.

4. Healing status at week 20 - whether the ulcer was completely closed by week 20.

5.4.1 Rationale for Selection

Factors that were easily accessible were extracted from the clinical data from the group. For the factors selected, there is a basis in the literature supporting their use.

Ulcer chronicity has been demonstrated to be an independent risk factor for delayed CVU healing[46, 48, 49]. Ulcer area again was based on findings in the literature suggesting that larger ulcers in terms of defined surface area do have increased time to healing[48, 49, 51]. A healing status at week 20 was the main outcome measure of the study and thus was included as a permutation tested in the following chapters. Finally, the ulcer healing rate between weeks 0 and 4 was included as to differentiate ulcers based on wound behaviour in a part of the study top loaded with assessments.

Chapter 6: Global Metabolic Profiling of Ulcer Fluid

6.1 Introduction

During the course of the main study carried out as part of this thesis. 147 aliquots of filter disc fluid as absorbed onto sterile filter discs were generated from 28 patients as recruited over the course of their study follow up.

This chapter seeks to describe the global metabolic profile of CVU fluid as prepared using a protocol outlined earlier in this thesis. Thereafter subject clinical data previously known to affect CVU prognosis was transformed into sample metadata to determine whether the metabolic phenotype was altered in relation to stratification factors.

6.1 Methods

6.1.1 Sample Preparation and Metabolite Extraction

A two layer extraction strategy was employed in the extraction of metabolites from ulcer filter disc. A 0.7ml mixture of MTBE, sterile water and methanol in a 3:3:1 ratio was added to each filter disc within a single Eppendorf tube. Three blank filter discs were also prepared at this time to identify any contamination as part of the process.

Thereafter, all tubes were vortexed for 60 seconds, added to an ultrasound bath for 10 minutes, centrifuged at 20000g for 30 minutes at 4°C. At this point the organic and the aqueous extracts were discernible and separated. Another round of centrifugation of the extracts at 20000g for 30 minutes at 4°C was performed to improve the efficiency of the extraction process.

Aqueous extracts speed dried in a speed vacuum and stored at -40°C overnight pending preparation for NMR analysis. Organic extracts were allowed to evaporate at room temperature in a fume hood cabinet overnight and stored at -40°C pending MS analysis.

All solvents used for the extraction of metabolites from the ulcer fluid and filter discs were obtained from Sigma-Aldrich (Missouri, USA) and were of HPLC Analytical Grade.

6.1.2 NMR Metabolic Profiling of Aqueous Extracts

Ulcer fluid disc aqueous extracts were prepared by the addition of 600 µL phosphate buffer made up in deuterium oxide containing 1 mM 3-(trimethylsilyl)-[2,2,3,3,2H4] propionic acid sodium salt (TSP) as an external reference and 2 mM sodium azide as a bacteriocide. This was followed by centrifugation at 20,000 x g for 10 min at 4°C. 1H NMR spectra were acquired with a Bruker Avance 600 MHz spectrometer operating at 600.13 MHz for 1H at 300 K. It was equipped with a 5 mm broad-band inverse configuration probe. Samples were randomly analysed in automation with a B-ACS 60 sample changer system. For each aqueous extract a standard one-dimensional NMR spectrum was acquired with water peak suppression using a standard pulse sequence (recycle delay (RD)-90o-t1-90o-tm-90o- acquire free induction decay (FID)). The RD was set at 2s, the spin echo delay at 400 μs, the acquisition time per scan was 1.36s and, for each sample, 8 dummy scans were followed by 256 transients. The spectra were collected into 64K data points using a spectral width of 20 ppm.

6.1.3 RP-UPLC-MS lipid profiling of the organic extracts

The organic extracts of ulcer fluid and ulcer fluid-discs were reconstituted in a mixture of isopropanol/ acetonitrile/ water (2:1:1, 200 µL), vortexed for 30sec, sonicated for 5min and vortexed for 30sec. This was followed by centrifugation at 20,000 x g for 30 minutes at 4°C. The supernatant from each sample was transferred into glass inserts in the LC-MS vials. A QC sample was prepared with 30 µL of each sample and was used to assess the analytical reproducibility of the method[179]. First the column was conditioned by injecting the QC pooled sample, several times, until data showed

adequate stability. Afterwards the QC sample was injected every 3 samples to monitor instruments performance. The run was completed by the analysis of extraction and solvent blank samples.

A reversed-phase UPLC-MS method was applied for the untargeted lipid profiling of the organic extracts obtained from ulcer fluid samples. The method has shown excellent retention time repeatability and demonstrated separation of structural isomers. This method is also described in Waters application note “Lipid Separation using UPLC with Charged Surface Hybrid Technology”. The method was performed on an Acquity UPLC system (Waters Corp, USA) coupled to a QTof Premier MS system (Waters MS Technologies, UK). Chromatography was performed using an Acquity UPLC CSH C18 2.1x100mm, 1.7µm, column (Waters Corporation, USA) held at 55^{o}C. Separation was achieved using gradient elution with 0.1% (v/v) formic acid in acetonitrile/water (60:40) (A) and 0.1% (v/v) in isopropanol/acetonotrile (B) (90:10) at a flow rate of 0.4 mL/min. In both mobile phases ammonium formate (LC-MS grade, Fluka, USA) was diluted to 10mM. Starting conditions were 60% A and 40% B for 2.0 min, changing linearly to 43% B over the next 2 min, and then to 50% B within 0.1 min and to 54% B over the next 10min, when it was changed to 70%B within 0.1 min and finally to 99% B over the next 6 min. Afterward the solvent composition returned to starting conditions over 0.1 min, followed by re- equilibration for 2 min prior to the next injection.

Mass spectrometry was performed using electrospray in both positive and negative ESI ionization modes. The capillary voltage was 1.5 kV in both ESI+ and ESI–, cone

voltage was 30 V, desolvation temperature was 600°C, and source temperature was 120°C. The cone gas flow rate was 50 L/h, and desolvation gas flow rate was 1,000 L/h. The XEVO G2 Q-TOF was operated in sensitivity mode with a scan time of 0.2 s and interscan delay of 0.015 s. For mass accuracy, a LockSpray interface was used with at 9 ng/mL leucine enkephalin (555.2645 AMU) solution (50/50 ACN/H_2O with 0.1% v/v formic acid) at 10µL/min was used as the lock mass. Data were collected in centroid mode with a scan range of 50–2000 m/z, with lockmass scans collected every 30s and averaged over 4 scans to perform mass correction.

6.1.4 Data Processing and Statistical Analysis

Data processing and statistical data analysis were carried out exactly as outlined in Chapter 2 (Section 2.2.6.4).

6.3 Results

Three metabolic profiling datasets were generated from samples of ulcer fluid in this set of experiments. There was one dataset from NMR and two from MS. Prior to analysis against known prognostic variables, a 'quality control' approach was undertaken to ensure that variation was not due to changes in the environment or chemical standards.

6.3.1 Quality Control Assessment

The role of quality control in these experiments was to ensure reproducibility of the platforms especially given that there is a relative dearth in the application of CVU fluid analysis. A pooled sample from all participants was injected at the start of an analytical run as a conditioning primer and subsequently after every 10th sample to determine system suitability, analytical system stability and sample repeatability prior to any dataset analysis in these experiments

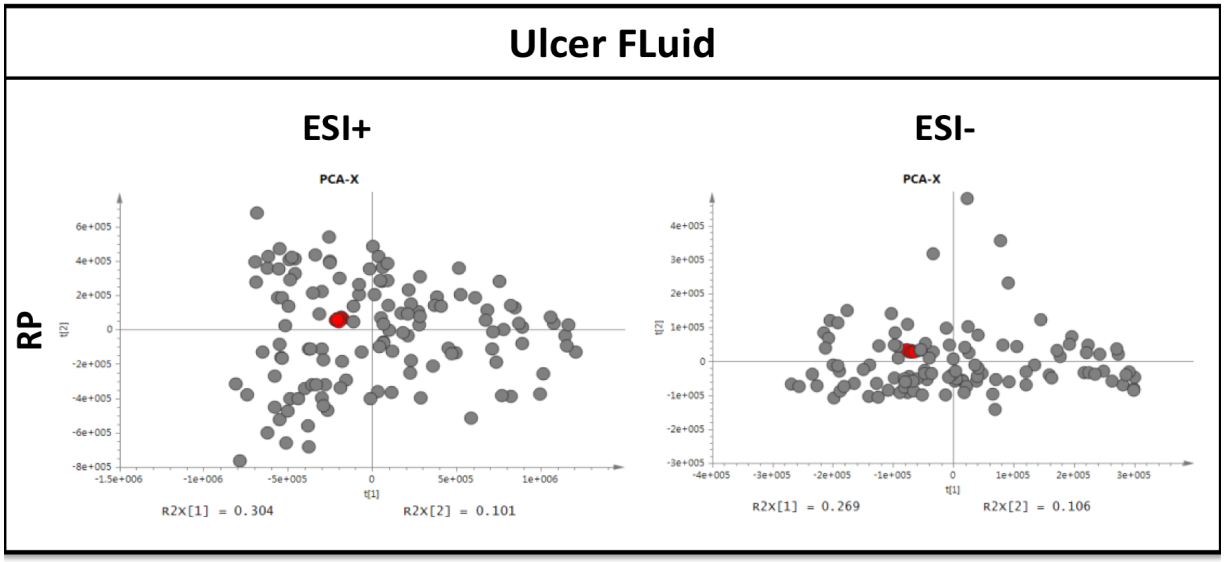


Figure 6.1: PCA scores plot with each spectra represented as a point. Spectra in red represent 'QC' samples.

Figure 6.1 is a scores plot for RP-UPLC-MS of all the samples in the CVU fluid cohort in the positive and negative electrospray modes. A grey dot is representative of a spectrum from a given sample from a patient at a given time point. Red dots represent summated QC samples. The clustering of QC samples infers that the variation between spectra is due to biological variation as opposed to variation in analytical conditions.

6.3.2 Analysis of Spectra Using Clinical Correlates

Having determined the analytical validity of this approach it was then sought to identify the metabolic phenotype of samples stratified by clinical variables described in the previous chapter namely:

1. Healing status at week 20.
2. Initial ulcer size at week 0.
3. Ulcer age at week 0.

4. Healing rate between week 0 and week 4.

OPLS-DA was utilised to optimally extract potential biomarkers responsible for variation within the dataset between wounds that healed within the study timeframe and those that did not (variable 1). OPLS models were generated to demonstrate differences for factors related to ulcer age, ulcer healing rate and ulcer size (at week 0 and throughout the study). CV-ANOVA is a tool for assessing the reliability of OPLS models and was undertaken here to provide a significance level of the models evaluated. For the purposes of these results a P-value of less than 0.05 is taken to represent significance.

Table 6.1 summarises all the model characteristics from all the permutations of experiment and analyses (including lipid profiling MS in both electrospray modes and NMR). The Q2 value demonstrates the variation in the model when correlated with clinical factor, the higher the percentage, the more predictive the model is.

As demonstrated by the table below, positive Q2 values were demonstrated in 8/12 model permutations and statistical significance was seen in 7/8 models. In broad terms this infers that the clinical variation between given samples accounts for different metabolic phenotypes demonstrated in OPLS modelling.

	Healers (n=60) v. Non-healers (n=66)(week 20)	**Ulcer Size (week 0) (n=126)**	**Ulcer age / Chronicity (at week 0 (n=126)**	**Healing Rate (Week 4 / Week 0) (n=126)**
	OPLS-DA	**OPLS-Regression**	**OPLS-Regression**	**OPLS-Regression**
Ulcer Fluid Lipid Profiling Positive (UPLC-MS ESI+ve)	Q_2=0	Q_2=8.8% P value = 0.002	Q_2=11.9% P value= 0.003	Q_2=23% P value = 1.9e-6
Ulcer Fluid Lipid Profiling Negative (UPLC-MS ESI-ve)	Q_2 = 33.4% P value = 5.9e-7	Q_2 = 5% NS	Q_2 = 35.6% P value = 7.2e-8	Q_2 = 38.3% P value = 1.5e-6
Ulcer Fluid NMR	Q_2=0	Q_2<0	Q_2<0	Q_2 = 41% P value = 3.7e-7

Table 6.1: Summated Q2 scores and P values for all permutation of experiments carried out using ulcer fluid as a substrate.

6.3.3 Individual Scores Plots Stratified by Clinical Variable

The following charts illustrate the examples of the scores and cross validation scores plots obtained in the process of CVU fluid analysis using OPLS and OPLS-DA multivariate statistical analysis.

Figure 6.2 refers to the primary outcome of complete wound healing within the 20 week period of the clinical study (RP-UPLC-MS ESI-). As alluded to above, a strong Q2 value of 33.4% with statistical significance was demonstrated in the model. This is

echoed by separation of the samples from patients who healed and ultimately did not heal in the plot.

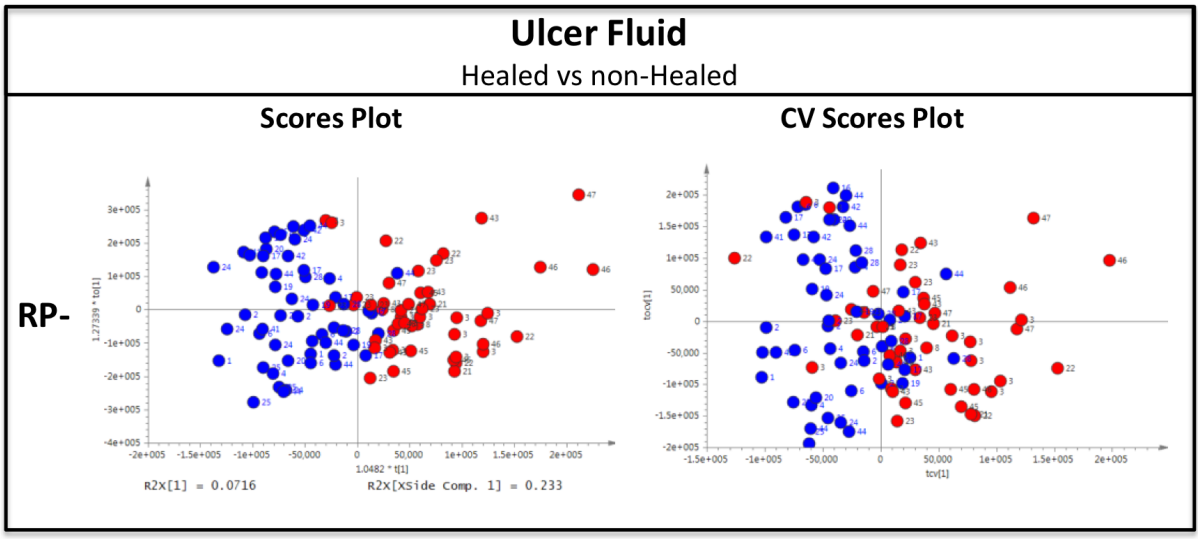


Figure 6.2: RP-UPLC-MS ESI-ve, all analysed ulcer fluid samples. Red represents samples from non-healers at 20 weeks and blue represents samples from patients healed at 20 weeks.

Figure 6.3 demonstrates a cross validated OPLS analysis of all ulcer fluid samples taken from the study cross referenced against the healing rate calculated at week 4. It is must be borne in mind that the points on the chart refer to an individual sample from a patient at a given time point. As demonstrated in the figure there is a separation of samples based on a calculated early healing rate which demonstrates a Q value of 23% and 38.3% for the lipid positive and lipid negative modes both achieving statistical significance. The NMR cross validation OPLS analysis was the only permutation using NMR that demonstrated a positive Q2 value of 41% with strong statistical significance.

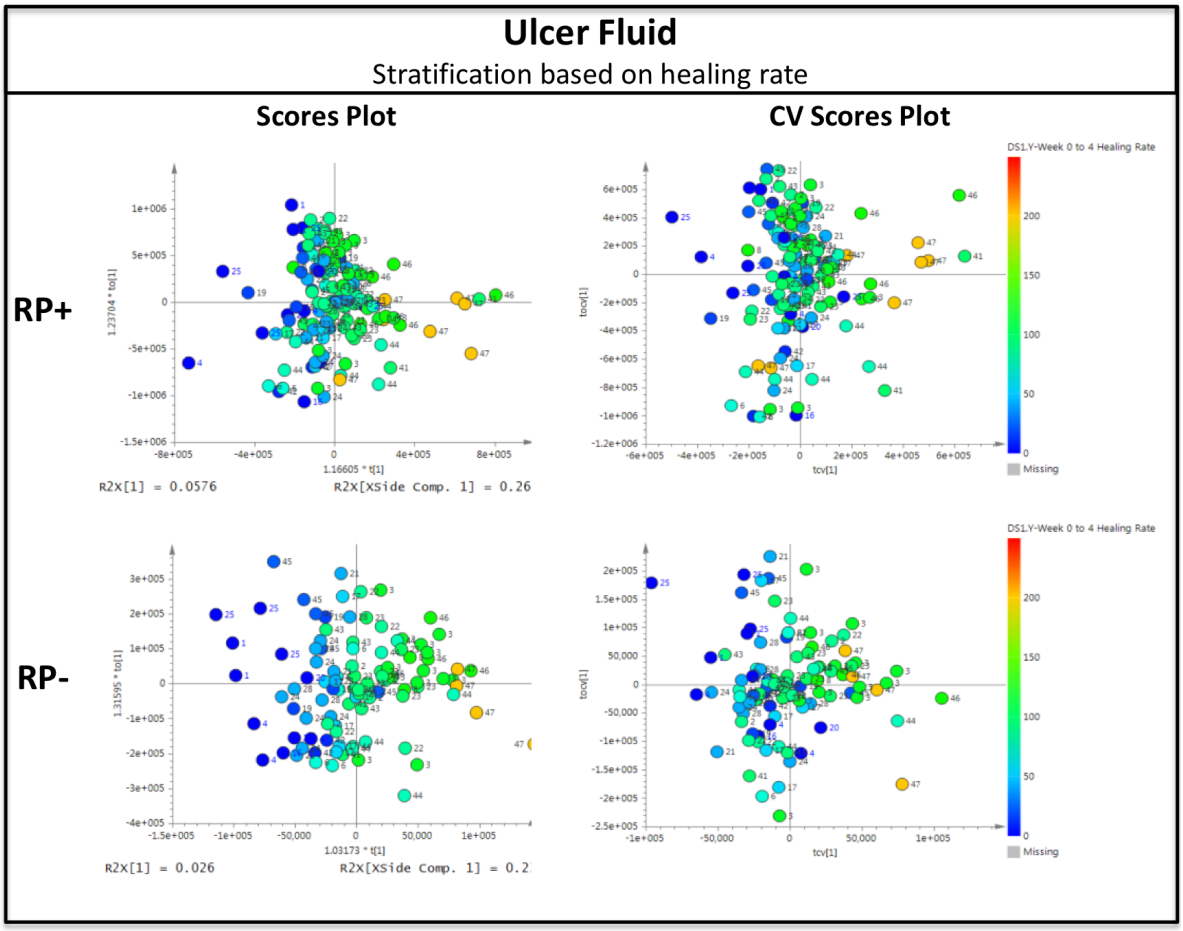


Figure 6.3: Scores plots and Cross-validated (CV) scores plots of OPLS analysis, demonstrating differences between the ulcer fluid profiles related to healing rate obtained from the lipid RP-UPLC-MS ESI+ and ESI- analysis. The colour bar indicates the healing rate.

Figure 6.4 demonstrates a visualisation of a scores plot and cross validated scores plot of OPLS of all ulcer fluid samples based on ulcer size at the commencement of the study (week 0). The model has weak prediction based on the clinical correlate based on Q2 of 8.8% (Lipid ESI positive) and 5% (Lipid ESI negative - not shown in figure). Only the model generated for the Lipid ESI positive achieved statistical significance (P value 0.002).

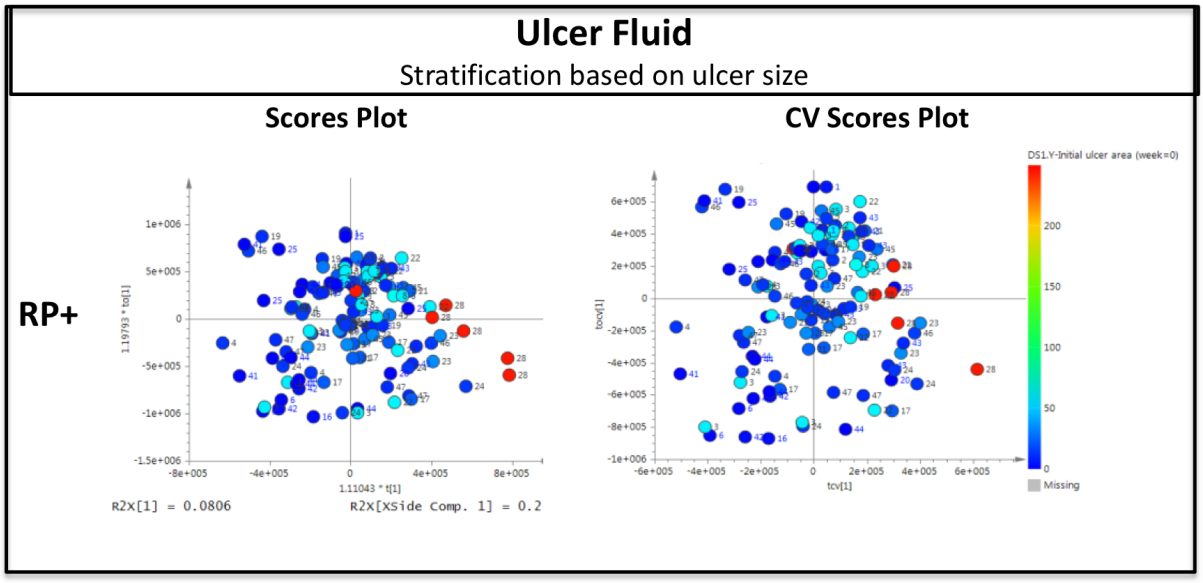


Figure 6.4: Scores plots and Cross-validated (CV) scores plots of OPLS analysis, demonstrating differences between the ulcer fluid profiles related to ulcer size obtained from the lipid RP-UPLC-MS ESI+ analysis. The colour bar indicates the ulcer size.

Figure 6.5 demonstrates an OPLS scores plot and a cross validated OPLS scores plot for the variable of ulcer age at the commencement of the study (week 0). Again all samples were cross referenced to the clinical subject they originated from. The figure demonstrates separation of the samples in the lipid profiling ESI positive and ESI negative modes. The lipid profiling ESI positive model demonstrated a weaker prediction in such that the Q2 was 8.8% (p value 0.003) whereas in the positive mode, the values were stronger with a Q2 of 35.6% (p value 0.0000000072).

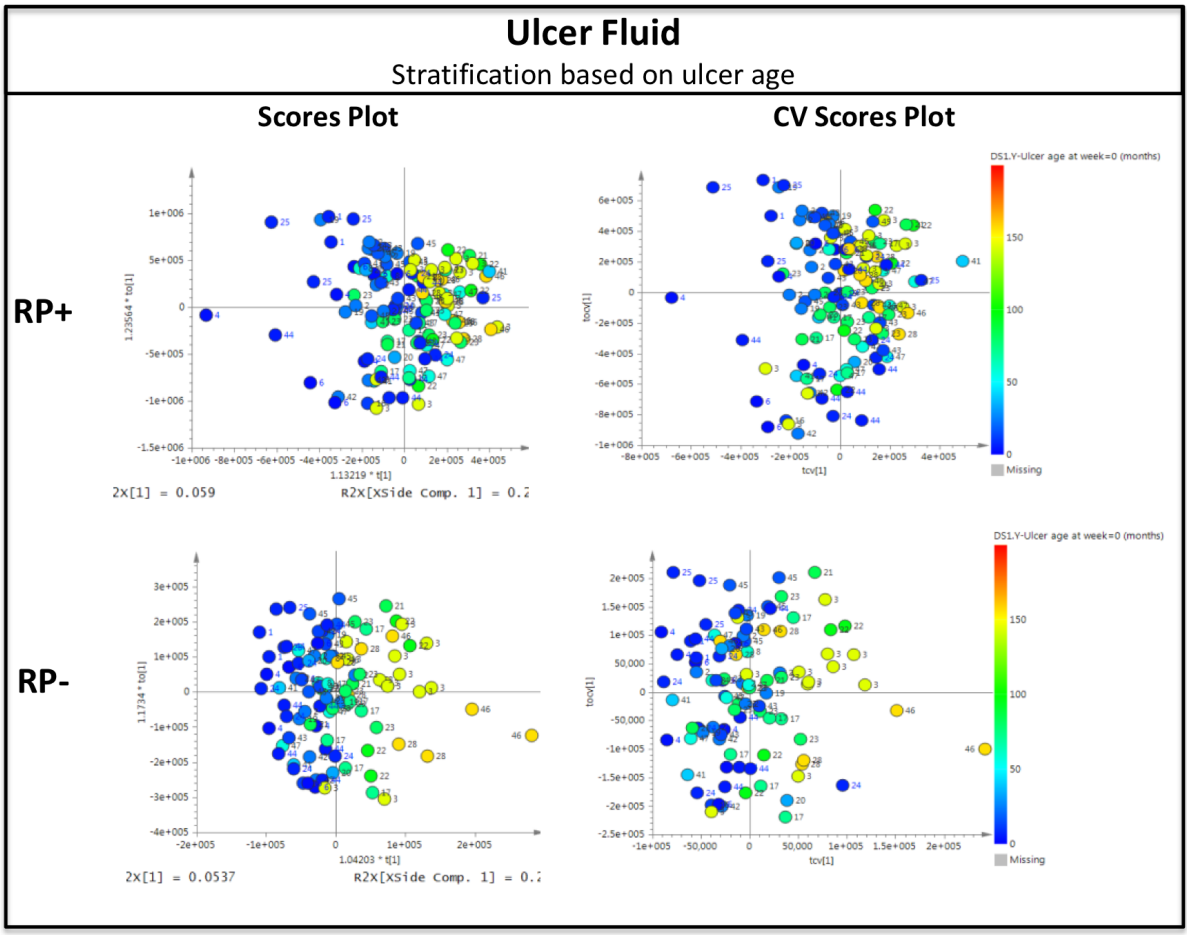


Figure 6.5: Scores plots and Cross-validated (CV) scores plots of OPLS analysis, demonstrating differences between the ulcer fluid profiles related to ulcer age obtained from the lipid RP-UPLC-MS ESI+ and ESI- analysis. The colour bar indicates the ulcer age.

NMR OPLS models did not show variation that could be predicted by the clinical variables of ulcer size nor ulcer age at the outset of the study.

6.3.4 Candidate Biomarkers

Loadings plots were generated from the acquired data sets to identify the metabolite features most likely to explain separation between groups. Metabolite features were inputted into Scripps Center for Metabolomics website (metlin.scripps.edu) to generate metabolites of interest. Table 6.2 identifies potential biomarkers correlated with ulcer healing or lack of healing by the end of the study period and those associated with ulcers of a greater chronicity.

POSITIVE - Healing status	NEGATIVE - Healing status	POSITIVE - Ulcer chronicity
L-Carnitine	TG(16:0/18:1/18:3)	LPC(16:0)
TG(16:0/18:1/18:1)	SM(d18:1/24:1)	LPC(18:2)
PC(16:0/18:1)	PC(18:1/18:2)	PC(16:0/18:2)
PC(16:0/18:2)	PC(18:1/18:1)	
PC(16:0/20:4)	PC(18:0/18:1)	
PC(18:0/18:2)		
PC(18:0/20:4)		

Table 6.2: List of metabolite species identified as being potential responsible for differences when variable clinical factors were applied to modelling.

6.4 Discussion

A novel approach to metabolic profiling in chronic venous ulcer fluid samples has been successfully applied as demonstrated in this chapter. Building from preliminary work described in the earlier portion of this thesis, sterile filter paper discs impregnated with chronic venous ulcer fluid from 28 patients were stratified according to various clinical outcomes including healing status by the completion of the study, initial ulcer size, ulcer chronicity and a healing rate based on planimetric estimates of size change between week 0 and week 4. A quality control approach was utilised and has demonstrated system and analytical stability in this approach.

This chapter demonstrates the novel utilisation of metabolic profiling in the use of CVU fluid and correlated with clinical characteristics although the literature demonstrates applications in acute wounds in mice [191] and human chronic pressure ulcer wounds[192, 193].

The majority of positive findings (predictive models with statistical significance) were demonstrated in lipid profiling mass spectrometry although a predictive model was identified using nuclear magnetic resonance was identified in the context of wound healing rate. Inherently this most likely reflects the reduced ‘resolution’ of NMR compared to MS in terms of detecting metabolites.

Conversely it is promising that MS based metabolic profiling analysis has generated models that are predicted by a clinical variable with statistical significance. A relationship exists based on wound healing status, initial ulcer size, ulcer chronicity

and ulcer healing rate at 4 weeks. This suggests with the more sensitive platform of MS that within the local metabolome of chronic venous ulcers, there may be phenotype variation ascribed to characteristics that classically predicted positive venous ulcer outcome.

Certain lipid metabolite species have been associated with overall healing or absence of healing at week 20 (completion of follow up in the study). These may not necessarily be intermediates or end products of cellular pathways directly implicated in wound healing processes without further evaluation. However; at this point, what may be inferred from the data is a potential fingerprint that may be sought from ulcer specimens to determine the probability of successful ulcer healing. This would require further assessment using targeted metabolic profiling techniques in a larger cohort.

Chapter 7: Global Metabolic Profiling of Serum from Chronic Venous Ulcer Participants

7.1 Introduction

The second tranche of metabolic profiling results comes from the analysis of serum from participants with chronic venous ulceration of the lower limb. Metabolic profiling of samples of systemic biofluids is well established process[134, 135, 138, 140, 141].

In this chapter, it is sought to determine whether changes in metabolic phenotype can be ascertained from a variation in clinical factors that can predict venous ulcer outcome. The prime motivation for the work in this chapter is to determine whether metabolic phenotype changes can be detected in systemic biofluds as opposed to the local ulcer environment.

This is undertaken in a manner similar to the previous chapter and will be fully described.

7.2 Methods

7.2.1 Clinical Sample Collection

The protocol for the clinical study has been described in detail in a previous chapter but in summary, samples of urine and serum were collected routinely at all clinical assessments where possible according to the participant's ability to provide a sample.

7.2.2 Serum Collection

Blood sampling was performed using a 21 gauge sterile needle and 10ml syringe from a superficial vein in the right antecubital fossa without the use of alcohol based preparation. Blood was transferred into two 13*100 mm (5ml) plastic serum tubes (Beckton Dickson and Company, New Jersey, USA), allowed to stand for 30 minutes to allow clotting at room temperature, centrifuged at 2500 RCF for 10 minutes and the serum fraction was used to generate a minimum of three 0.4ml aliquots of serum which were immediately placed on dry ice and transferred to a 80^{o}C freezer.

7.2.3 NMR Metabolic Profiling of Serum

Serum analysis was carried out exactly according to the procedures outlined in Chapter 3 (3.2.5.3 NMR Metabolic Profiling of Serum).

7.2.4 RP-UPLC-MS Lipid Profiling of Serum

Serum RP-UPLC-MS analysis was carried out exactly according to the procedures outlined in Chapter 3 (3.2.5.4 MS Metabolic Profiling of Serum).

7.2.5 HILIC-UPLC-MS profiling of Serum

HILIC analysis was undertaken to provide greater breadth in metabolite feature capture given the wide potential distribution of metabolites in serum.

For HILIC-UPLC-MS analysis neat urine samples were vortexed and centrifuged without any dilution and aliquoted into 96 well-plates. The HILIC-UPLC-MS method used for the analysis of the urine samples was the same as in the other biofluids.

Metabolic profiling was performed on an Acquity UPLC system (Waters Ltd. Elstree, U.K.) coupled to a XEVO G2 Q-TOF mass spectrometer (Waters MS Technologies, Ltd., Manchester, U.K.). Chromatography was performed using an Acquity BEH HILIC (1.7 µm, 2.1 × 100 mm) column (Waters Corporation, Milford, U.S.A.) kept at 40 °C. UPLC separation was conducted using an Acquity UPLC System (Waters Corp, USA). Column temperature was set at 40 °C. Mobile phase A consisted of acetonitrile (ACN)/water (95:5) and mobile phase B ACN/water (50:50). In both solutions ammonium acetate was diluted to 10 mM and formic acid to 0.1%. The elution gradient was set as follows: 99% A (0.0–2.0 min; 0.4 mL/min), 99–45% A (2.0–8.0 min; 0.4 mL/min), 45–1% A (8.0–9.0 min; 0.4 mL/min), 1% A (9.0–9.1 min; 0.4–0.6 mL/min), 1% A (9.1–11.1 min; 0.6 mL/min), 1–99% A (11.1–11.2 min; 0.65 mL/min), 99% A (11.1–11.7 min; 0.65-0.9 mL/min), 99% A (11.7–17.5 min; 0.9 mL/min), 99% A (17.5–17.8 min; 0.9–0.4 mL/min), 99% A (17.8–20.0 min; 0.4 mL/min).

Mass spectrometry was performed using electrospray in both positive and negative ESI ionization modes. The capillary voltage was 1.5 kV (ESI+ and ESI−), cone voltage was 30 V, desolvation temperature was 600 °C, and source temperature was 120 °C. The cone gas flow rate was 50 L/h, and desolvation gas flow rate was 1,000 L/h. The MS was operated in sensitivity mode with a scan time of 0.2 s. For mass accuracy, a LockSpray interface was used with a 9 µg/L leucine enkephalin (555.2645 amu) solution (50/50 ACN/H2O with 0.1% v/v formic acid) at 10 µL/min was used as the lock mass. Data were collected in centroid mode with a scan range of 50−1200 m/z, with lockmass scans collected every 30 s and averaged over 4 scans to perform mass correction.

7.2.6 Data Processing and Statistical Analysis

NMR and MS Data importation, processing and statistical analysis have been previously described in Chapter 3 of this thesis and in this set of experiments were carried out exactly as outlined (Section 2.2.6.4).

7.3 Results

In a similar style to the chapter describing ulcer fluid analysis, the metabolic profiling results of serum and urine will be outlined with reference to quality control procedures, statistical analysis and indication of biological molecules that may be responsible for variation between groups.

7.3.1 Quality Control Assessment

The role of QC assessment in these experiments was to assess the reproducibility of the NMR and MS platforms utilised in the throughput analysis of human serum. Serum is a commonly analysed biofluid in metabolic profiling studies and the QC approach has been commonly described in the literature [190, 194].

In the figure 7.1, serum QC scores plots for mass spectrometry experiments are presented with highlighted QC plots that represent summated samples from all participants in the study and analysed in the main batches at regular intervals. The relative clustering of QC samples in all figures suggests reproducibility and stability of the analytical systems and thus allows further statistical analysis of the data to be made. This result infers that any variation in the samples is because of variation in participants as opposed to arising from instability in the analytical system.

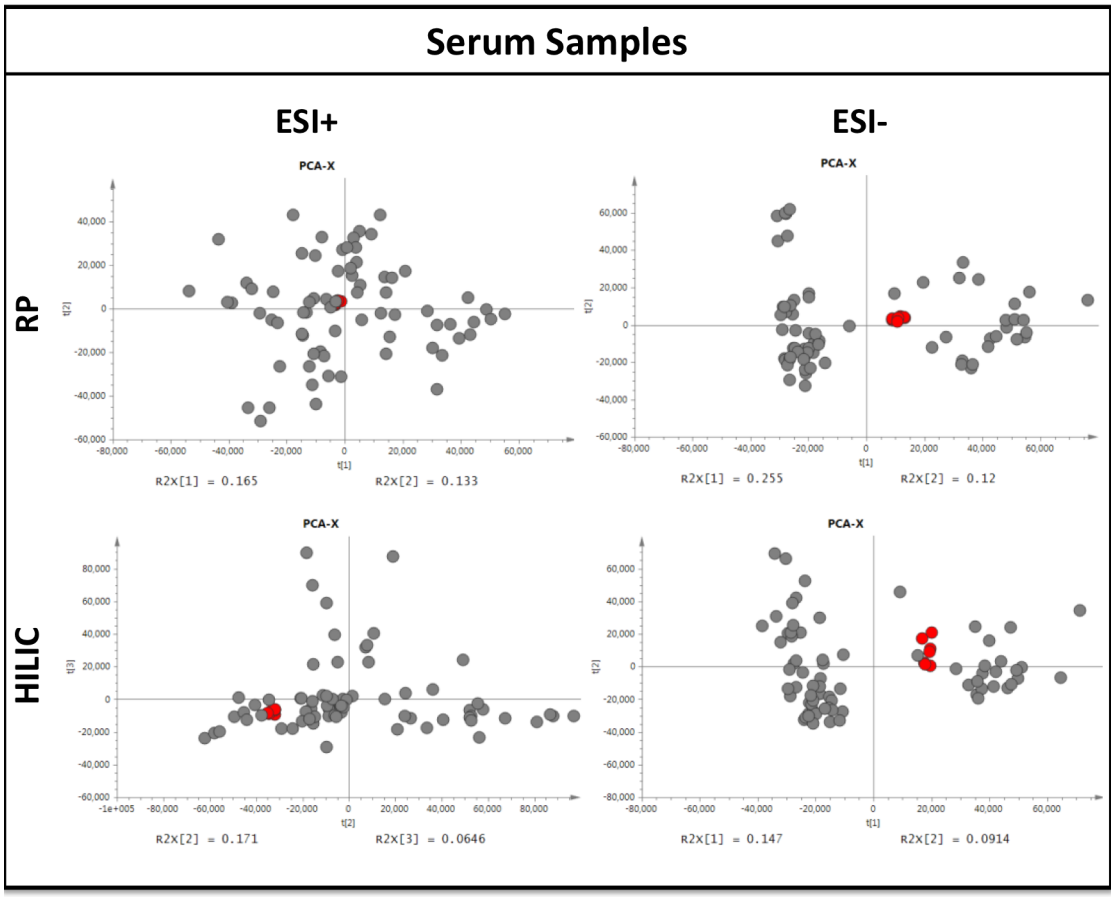


Figure 7.1: PCA scores plot for all serum samples, QC samples highlighted in red. RP-UPLC-MS (lipid profiling) and HILIC in both ESI+ and ESI- modes.

7.3.2 Metabolic Profiling Results and Modelling Outcomes

Table 7.1 outlines the technical and analytical permutations used to examine the serum samples from the study cohort. OPLS-DA and OPLS regression are utilised to ascertain if clinical variables used to define groups can in turn show variation as demonstrated by a positive Q2 value. Statistical significance of the model is represented with a P value < 0.05.

The clinical factors used to examine data were

1. Healing status at week 20.
2. Initial ulcer size at week 0.
3. Ulcer age at week 0.
4. Healing rate between week 0 and week 4.

13/16 models demonstrate strongly predictive models with strong statistical significance including all permutations of analysis with serum lipid profiling in the negative mode, non-healers versus healers in the lipid positive mode and all permutations for HILIC in the positive and negative mode. The remaining serum lipid positive permutations either has a weakly positive model with non-significant ANOVA results or a model that was not predictive.

These data broadly suggest that it is possible to separate metabolic profiles from various groups based on clinical stratification relating to healing status, ulcer size, chronicity and healing rate based on biofluid obtained from human serum.

	Non-healers v Healers **N = 45 v 26**	**Ulcer size week 0** **N=71**	**Ulcer chronicity (age at week 0)** **N=71**	**Healing rate week 4** **n=71**
	OPLS-DA	**OPLS regression**	**OPLS regression**	**OPLS regression**
Serum Lipid Profiling Positive (ESI+)	Q_2= 42.6% P = 0.002	Q_2=3.8% P >0.05	Q_2= 0.06 P >0.05	Q_2=0
Serum Lipid Profiling Negative (ESI-)	Q_2= 31.4% P = 0.0000005	Q_2= 25% P = 0.0007	Q_2= 35.5% P = 0.00000036	Q_2= 26.4% P = 0.0025
Serum HILIC ESI+	Q_2= 26.4% P = 0.0004	Q_2= 25% P = 0.0007	Q_2= 36.5% P = 0.00000039	Q_2=26.4% P = 0.000000169
Serum HILIC ESI-	Q_2=48.4% P= 0.000000009	Q_2=46% P= 0.00000007	Q_2=37% P= 0.000000000029	Q_2=43% P = 0.000000169

Table 7.1: Global summation of all serum profiling modalities and permutations assessed with modelling.

Figures 7.2, 7.3, 7.3 and 7.5 demonstrates scores and cross validated scores plots with each point representing a sample from a given patient with varying colours determined by clinical variables.

Figure 7.2 stratifies samples based upon the healing status of the wound by completion of the study. Figure 7.3 is stratified based upon healing rate in the first four weeks of the study. Figure 7.4 is based upon ulcer size. Figure 7.5 is based upon ulcer chronicity reflecting the ulcer age in months at the commencement of the study. The majority of samples demonstrate a reasonable separation from each other confirming that the groups can be sufficiently discerned.

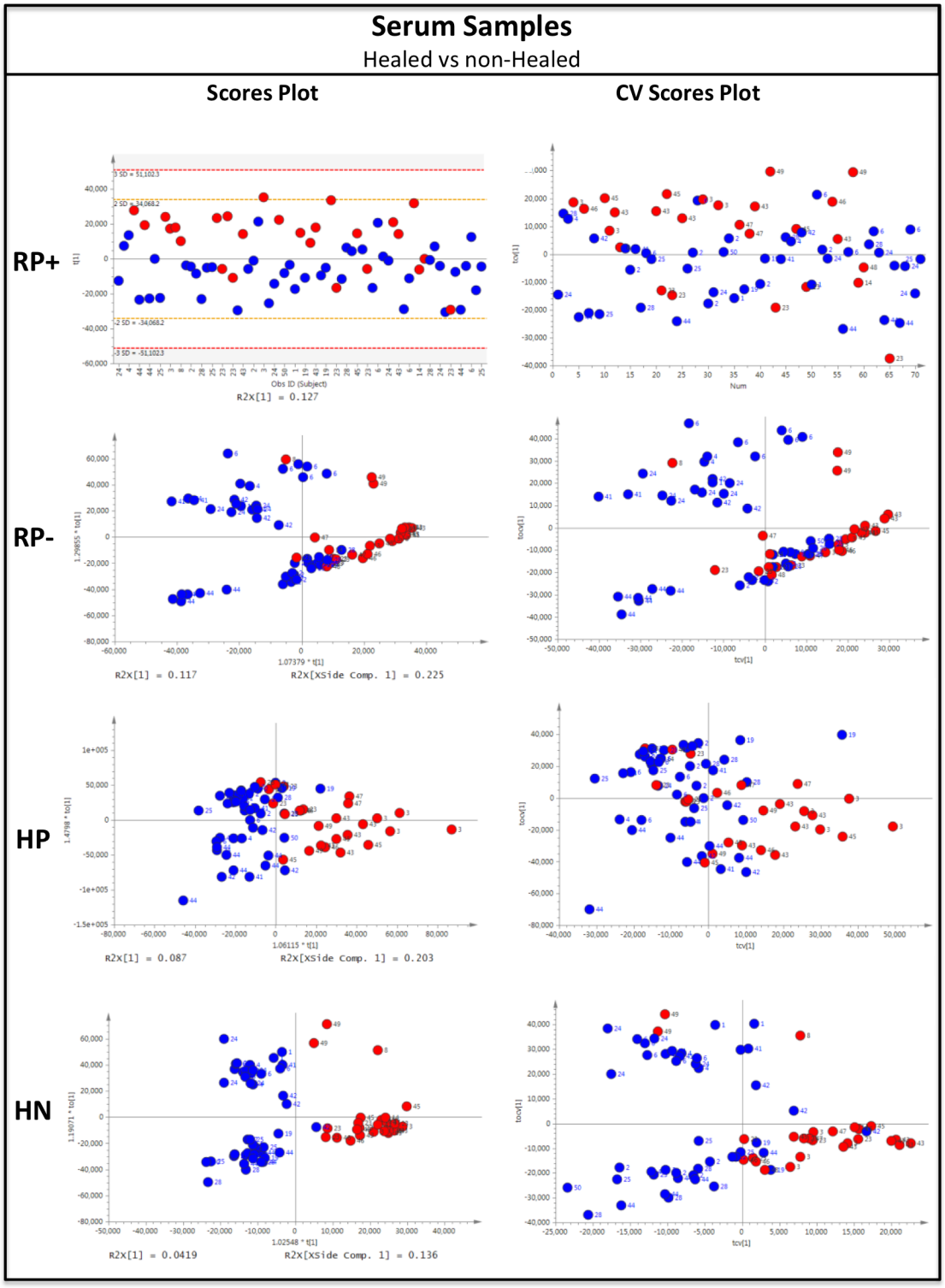


Figure 7.2: Scores plots and Cross-validated (CV) scores plots of OPLS-DA analysis, demonstrating differences between the serum profiles of healed (blue) and non-healed (red) patients obtained from both the lipid RP- and HILIC-UPLC-MS ESI+ and ESI- analysis.

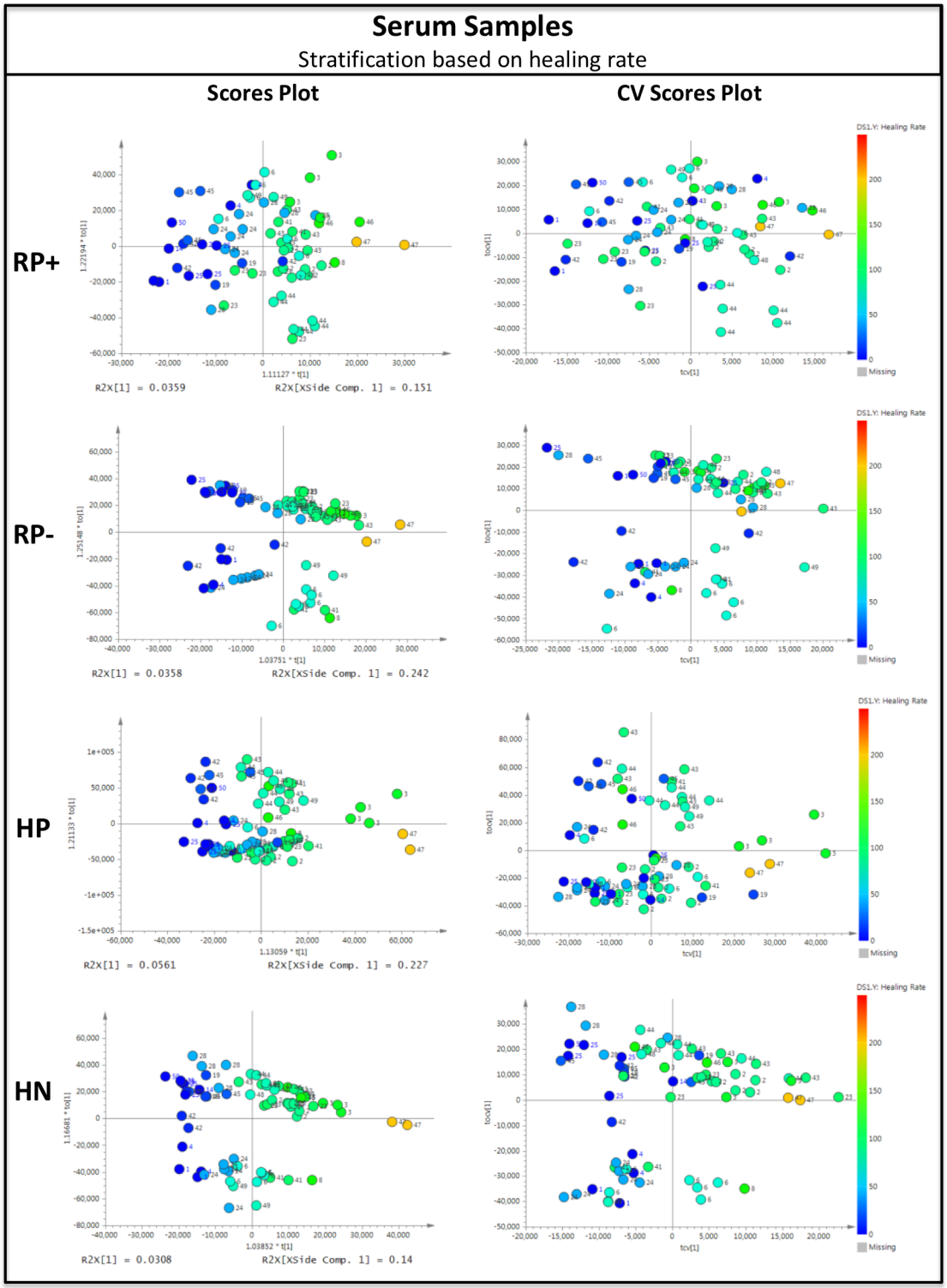


Figure 7.3: Scores plots and Cross-validated (CV) scores plots of OPLS analysis, demonstrating differences between the serum profiles related to healing rate obtained from both the lipid RP- and HILIC-UPLC-MS ESI+ and ESI- analysis. The colour bar indicates the healing rate - Colours towards red denote faster healers and towards blue are slower healers.

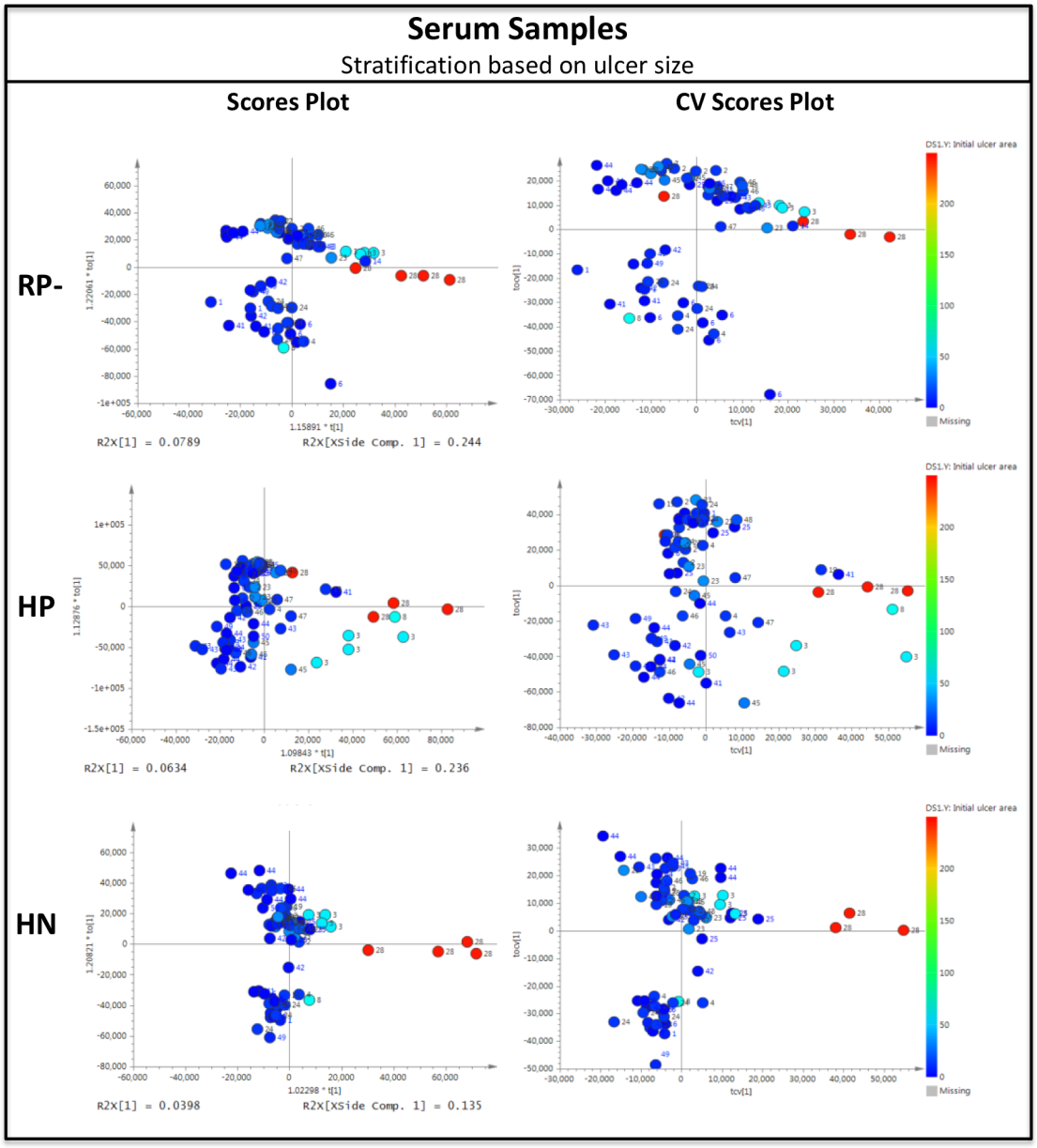


Figure 7.4: Scores plots and Cross-validated (CV) scores plots of OPLS analysis, demonstrating differences between the serum profiles related to ulcer size obtained from all analysis and electrospray ionization polarity modes from both the lipid RP- and HILIC-UPLC-MS ESI+ and ESI- analysis. The colour bar indicates the ulcer size with larger ulcers depicted as red and smaller ulcers depicted as blue on the scale.

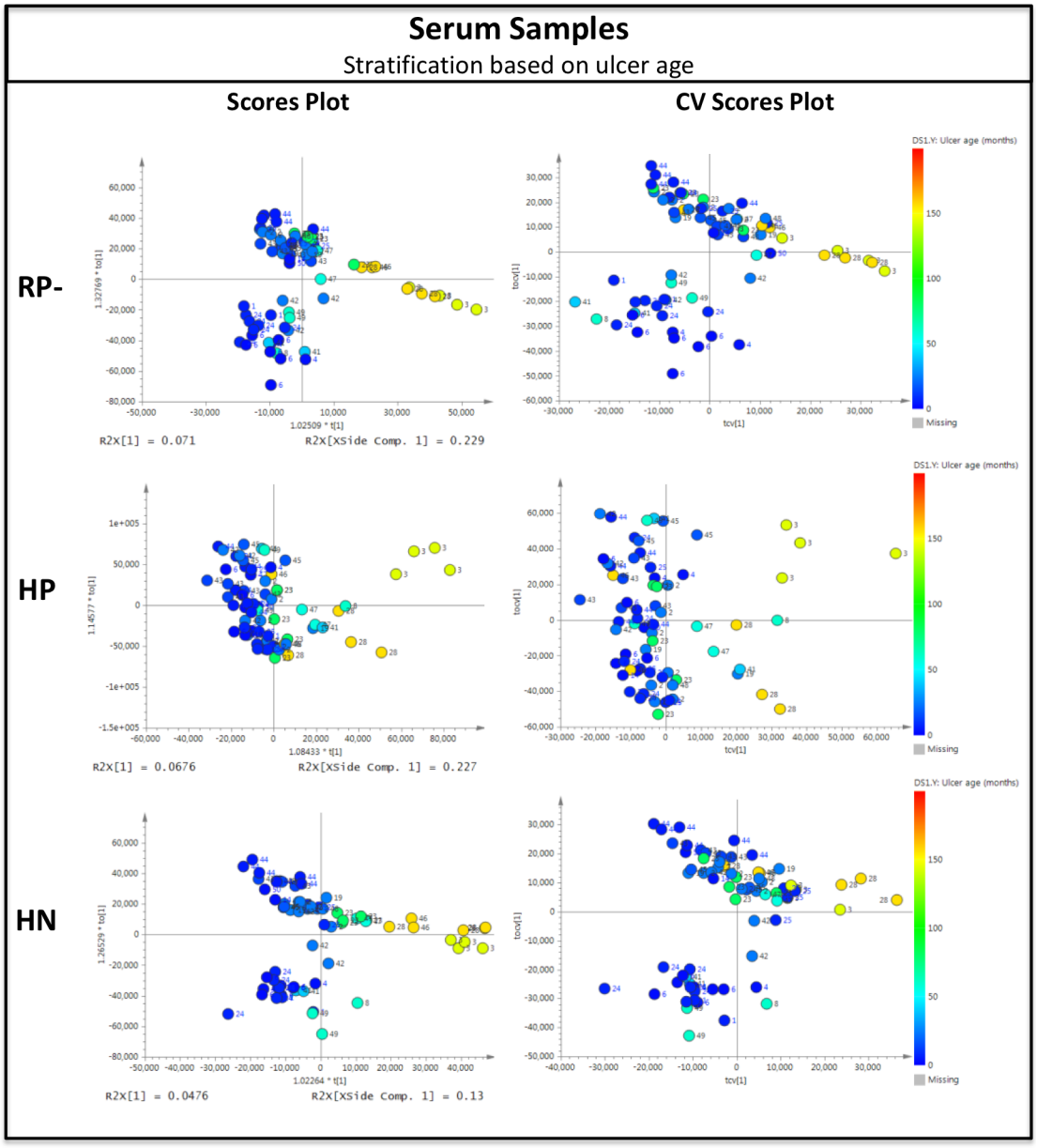


Figure 7.5: Scores plots and Cross-validated (CV) scores plots of OPLS analysis, demonstrating differences between the serum profiles related to ulcer age obtained from all analysis and electrospray ionization polarity modes from both the lipid RP- and HILIC-UPLC-MS ESI+ and ESI- analysis. The colour bar indicates the ulcer age in months with 'older' ulcers trending towards red points and dark blue representing 'younger' ulcers.

7.3.2.1 Candidate Biomarkers

Table 7.2 below outlines the metabolite species identified, the metabolite features most responsible for the variation between clinically defined groups were extracted from the OPLS loadings plot. These data were then transposed to the Scripps metabolite database (www.scripps.edu) to determine the designations.

Positive – Healing Status	**Negative – Healing Status**	**Positive – Ulcer Chronicity**
L-Carnitine	TG(16:0/18.1/18:3)	LPC(16:0)
TG(16:0/18.1/18:1)	SM(d18:1/24.1)	LPC(18:2)
PC(16:0/18:1)	PC(18:1/18:2)	PC(16:0/18:2)
PC(16:/18:2)	PC(18:1/18:1)	
PC(16/20.4)	PC(18:0/18:1)	
PC(18:0/18:2)		
PC(18:0/20:4)		

Table 7.2: List of metabolite species identified as being potential responsible for differences when variable clinical factors were applied to modelling for serum samples.

7.4 Discussion

This chapter has outlined the novel application of a standardised and validated metabolic profiling workflow applied to human serum from 28 participants with chronic venous ulceration. Initially, work was directed at determining that the analytical workflow was free of undue variation secondary to changes in ambient conditions or contamination from sample preparation processes which allows the analysis of samples against clinical variables to occur.

28 participants gave rise to 71 individual samples of serum at various assessments at given time points throughout the study. Of interest, 13/16 models demonstrate a positive relationship with statistical significance achieved. This suggests that throughout the journey it is certainly possible to ascribe separation in the metabolic phenotype to variation in the clinical factors.

The models that did not achieve stronger predictive values with statistical significance all emanated from the serum lipid positive profiling set of analyses namely for the factors: ulcer size, ulcer chronicity and healing rate at week 4. This may well reflect that the number of samples was too small for a regression technique to demonstrate a difference with a continuous variable as opposed to the binary question of healed or not healed which was asked using an OPLS-DA model. Nonetheless, for all the other permutations of mass spectrometry analysis, it was clearly seen that predictive models with statistical significance can be achieved with all clinical correlates.

This therefore leads to the interesting observation that serum samples can in most cases been seen to demonstrate a differing metabolic phenotype in participants with differences in clinical predictive factors relating to their chronic venous ulcer. This may be regarded as a systemic aberration relating to the local biology of the ulcer or possibly a systemic factor that perhaps relates in a way to the participant's ability to heal.

A caveat to the interpretation of the data is firstly that metabolic profiling studies can reach participant levels in the thousands and that this study population is on the smaller end of a clinical recruitment spectrum. It must also be considered that samples per say belong to a participant and are analysed as such rather than considering the participant's progression through a clinical study except for the healing rate subset.

Although metabolite features for various lipid species have been identified as potential biomarkers, further work will be required to examine the metabolite features that allow for differences between clinical subgroups and perhaps to suggest an explanation for the results identified. A more simplistic interpretation of the data achieved may be that a metabolic profile may predict how a participant's ulcer may progress over time and would again be the source of future study. The fact that serum is a well utilised biofluid with relative ease of standardised collection would certainly make further study a relatively easy future source of study.

Chapter 8: Global Metabolic Profiling of Urine from Chronic Venous Ulcer Participants

8.1 Introduction

The final group of clinical biofluid samples from the cohort recruited for the main study of this thesis is urine. Like serum in the previous chapter, urine is a very common biofluid utilised in global metabolic profiling studies, reflecting the ease of non-invasive sampling.

Urine metabolic profiling methodology and techniques have been extensively described in the literature with validated assays and quality control techniques[134, 138].

Using the literature and departmental advice as a guide, a global approach utilising lipid profiling and HILIC mass spectrometry in addition to NMR was utilised in this chapter.

As per the previous chapters, the methodology will be outlined with a description of quality control and modelling outcomes.

The hypothesis for this chapter is that global urine metabolic profiles will show a evidence of a differing chemical phenotype when analysing the dataset according to clinical stratification.

8.2 Methods

8.2.1 Clinical Sample Collection

The protocol for the clinical study has been described in detail in a previous chapter but in summary, samples of urine and serum were collected routinely at all clinical assessments where possible according to the participant's ability to provide a sample.

8.2.2 Urine Collection

Patients were asked to provide a 50ml mid-stream urine sample into a 50ml Falcon tube (Beckton Dickson and Company, New Jersey, USA). Three 1ml aliquots of urine were prepared, immediately placed on dry ice and as soon as practicable placed in a -80°C freezer.

8.2.3 NMR Profiling of Urine

This has been fully described in Chapter 3 of this thesis (Section 3.2.5.1 NMR Profiling of Urine).

8.2.4 RP-UPLC-MS Profiling of Urine

This has been fully described in Chapter 3 of this thesis (Section 3.2.5.2 MS Profiling of Urine).

8.2.5 HILIC-UPLC-MS Profiling of Urine

This further method of MS analysis was utilised to provide further metabolite coverage. For HILIC-UPLC-MS analysis neat urine samples were vortexed and centrifuged without any dilution and aliquoted into 96 well-plates. The HILIC-UPLC-MS method used for the analysis of the urine samples was the same as in the other biofluid such as serum as described earlier.

Metabolic profiling was performed on an Acquity UPLC system (Waters Ltd. Elstree, U.K.) coupled to a XEVO G2 Q-TOF mass spectrometer (Waters MS Technologies, Ltd., Manchester, U.K.). Chromatography was performed using an Acquity BEH HILIC (1.7µm, 2.1 × 100 mm) column (Waters Corporation, Milford, U.S.A.) kept at 40 °C. UPLC separation was conducted using an Acquity UPLC System (Waters Corp, USA). Column temperature was set at 40°C. Mobile phase A consisted of acetonitrile (ACN)/water (95:5) and mobile phase B ACN/water (50:50). In both solutions ammonium acetate was diluted to 10mM and formic acid to 0.1%. The elution gradient was set as follows: 99% A (0.0–2.0 min; 0.4 mL/min), 99–45% A (2.0–8.0 min; 0.4 mL/min), 45–1% A (8.0–9.0 min; 0.4 mL/min), 1% A (9.0–9.1 min; 0.4–0.6 mL/min), 1% A (9.1–11.1 min; 0.6 mL/min), 1–99% A (11.1–11.2 min; 0.65 mL/min), 99% A (11.1–11.7 min; 0.65-0.9 mL/min), 99% A (11.7–17.5 min; 0.9 mL/min), 99% A (17.5–17.8 min; 0.9–0.4 mL/min), 99% A (17.8–20.0 min; 0.4 mL/min).

Mass spectrometry was performed using electrospray in both positive and negative ESI ionization modes. The capillary voltage was 1.5kV (ESI+ and ESI–), cone voltage

was 30 V, desolvation temperature was 600°C, and source temperature was 120 °C. The cone gas flow rate was 50 L/h, and desolvation gas flow rate was 1,000 L/h. The MS was operated in sensitivity mode with a scan time of 0.2 s. For mass accuracy, a LockSpray interface was used with a 9 µg/L leucine enkephalin (555.2645 amu) solution (50/50 ACN/H2O with 0.1% v/v formic acid) at 10 µL/min was used as the lock mass. Data were collected in centroid mode with a scan range of 50–1200 m/z, with lockmass scans collected every 30 s and averaged over 4 scans to perform mass correction.

8.2.6 Data Processing and Statistical Analysis

NMR and MS data importation and statistical analysis have been described in detail in Chapter 3 of this thesis and the same procedures were followed exactly (Section 2.2.6.4)

8.3 Results

8.3.1 Quality Control Assessment

As standard practise with metabolic profiling studies, a QC assessment was undertaken to ensure that any variation in profiles was not a function of an analytical drift in the workflow related to change in environmental conditions.

As demonstrated in figure 8.1, a PCA scores plot for each of permutations of analytical mode is displayed. Importantly, a clustering of summated QC samples containing urine from the whole cohort is demonstrated. This suggests in variation in the dataset is a function of biological variation as opposed to analytical instability and allows full interpretation of the results.

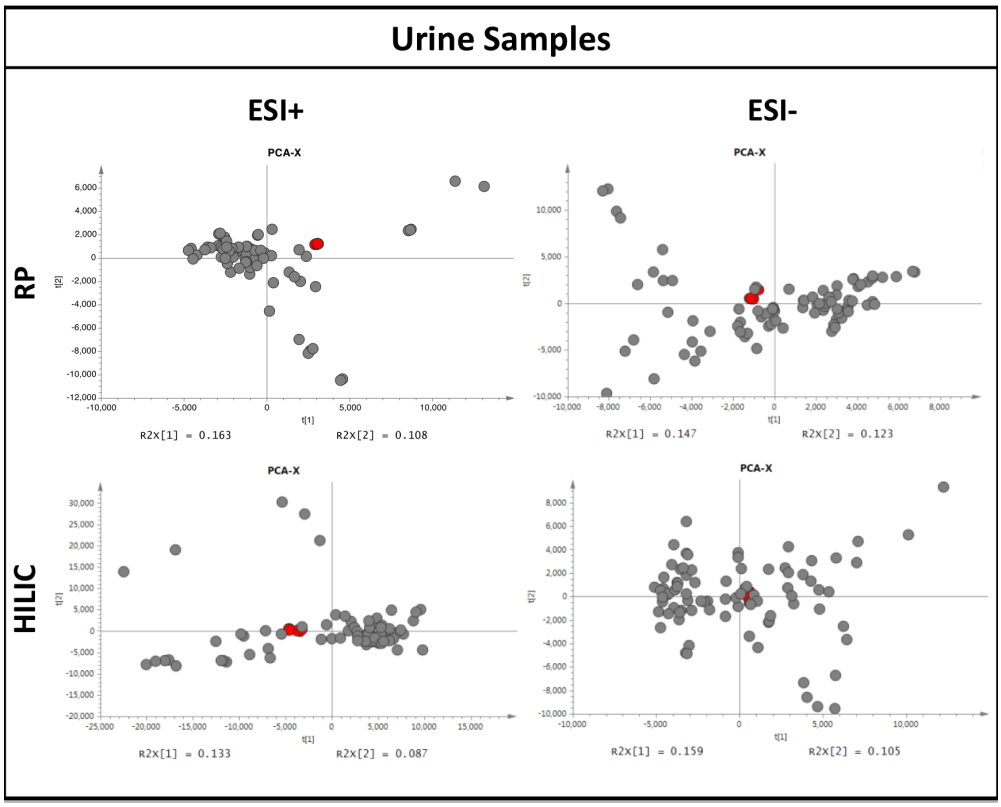


Figure 8.1: PCA scores plot for all urine samples. QC samples designated in red and plots refer to RP-UPLC-MS and HILIC ESI+ and ESI- modes.

8.3.2 Metabolic Profiling Results and Modelling Outcomes

Table 8.1 demonstrates a summated modelling outcome for all permutations of analysis for MS experiments. All sixteen permutations demonstrated strong Q2 values with strong statistical significance. The primary interpretation is that when biological stratification is applied to the data set that separation of the groups can be seen.

This broadly demonstrates that differences in the metabolic profile in subsets of samples of human urine from participants with CVU can be identifies and are the basis of further study.

	Non-Healers v Healers N= 53 v 31 **OPLS-DA**	**Ulcer Size Week 0 N=84** **OPLS Regression**	**Ulcer Chronicity (Age at Week 0) N=84** **OPLS Regression**	**Healing Rate at Week 4 N=84** **OPLS Regression**
Urine RP ESI+	Q2=48.3% P value =1.8e-9	Q2=55.5% P value = 7.58e-12	Q2=50.8% P value = 2.94e-12	Q2=43% P value = 4.68e-6
Urine RP ESI-	Q2=47.4% P value = 9.03e-8	Q2=46.7% P value = 5.59e-7	Q2=47.7% P value = 2.7e-9	Q2=35.3% P value = 5.16e-7
Urine HILIC ESI+	Q2=46.2% P value = 7.9e-9	Q2=80.2% P value = 2.36e-23	Q2=61.3% P value = 8.6e-13	Q2=41.7% P value = 1.44e-7
Urine HILIC ESI-	Q2=44.4% P value = 2.5e-8	Q2=59.2% P value = 5.8e-12	Q2=42.9% P value = 6.6e-7	Q2=34.7% P value = 5.8e-5

Table 8.1: All MS methods correlated against clinical permutations with Q and P values.

Figure 8.2 below demonstrates scores and cross validated scores plots for all urine samples when referring to the final outcome of healing for the specific participants. As demonstrated in the table above, all Q2 values are strongly positive with statistical significance. In addition, it can be demonstrated that for the outcome of ultimate healing, samples of urine do on the whole demonstrate a clear separation suggesting an altered chemical phenotype in mass spectrometry analysis of urine samples.

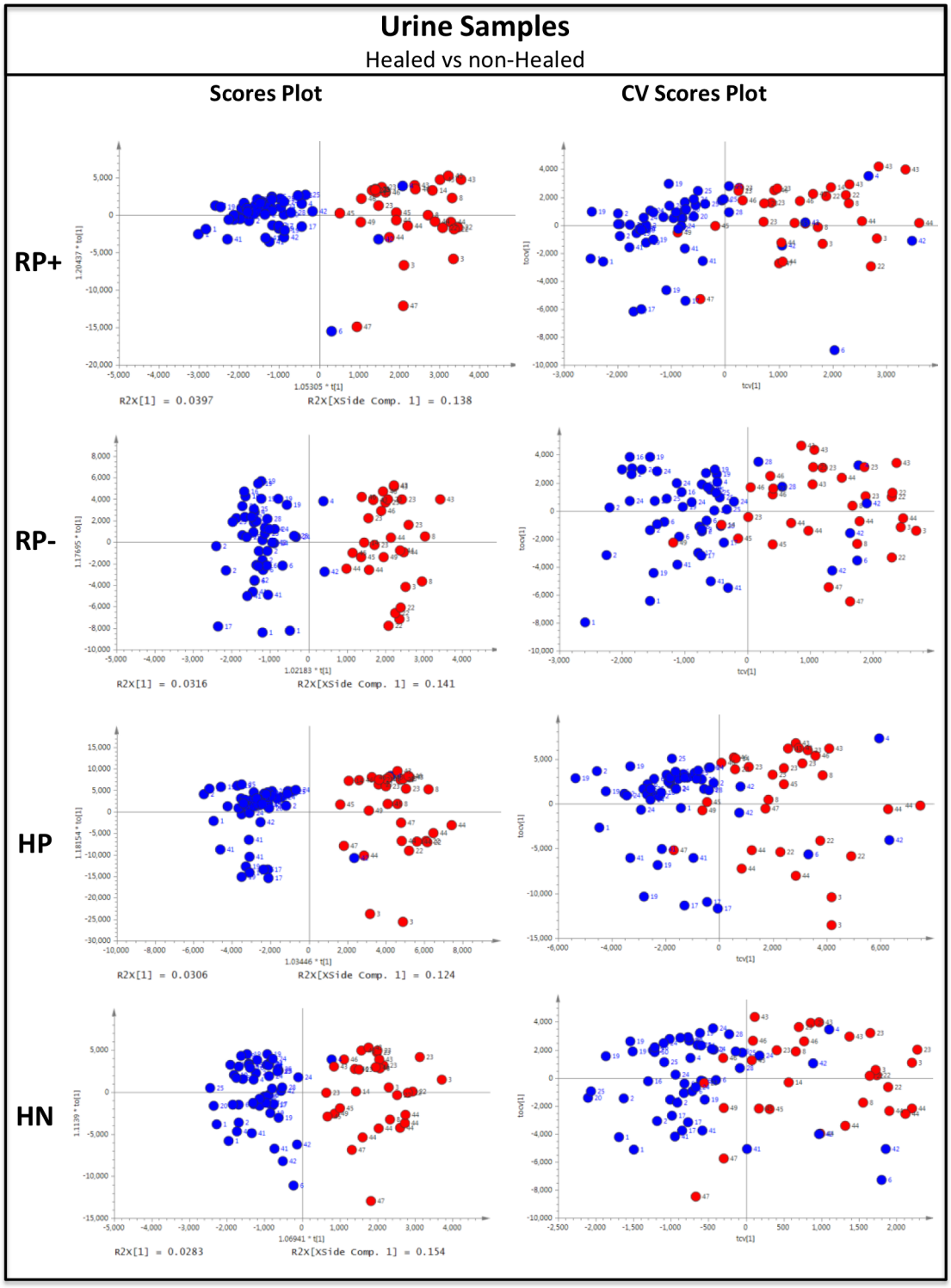


Figure 8.2: Scores plots and Cross-validated (CV) scores plots of OPLS-DA analysis, demonstrating differences between the urine profiles of healed (H) and non-healed (NH) patients from both the lipid RP- and HILIC-UPLC-MS ESI+ and ESI- analysis – Blue points represent participants who healed and red points represent non-healers.

Figure 8.3 below demonstrates scores and cross validated scores plots for all samples categorised as per the participants calculated healing rate at week 4. Again, a strong relationship with statistical significance was identified and the plots do identify a trend towards samples clustering towards healing rate suggesting a differential metabolic phenotype becoming evident.

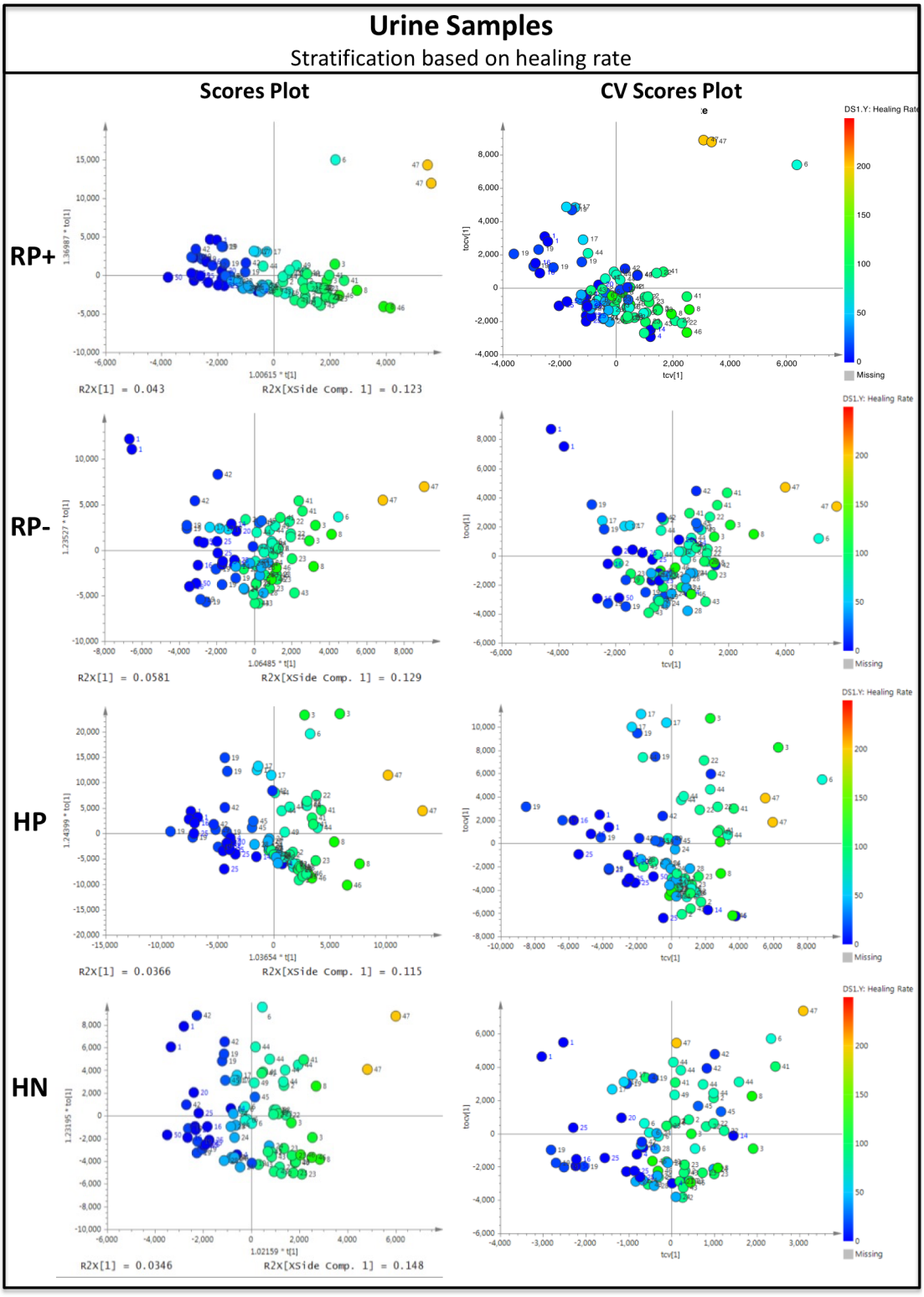


Figure 8.3: Scores plots and Cross-validated (CV) scores plots of OPLS analysis, demonstrating differences between the urine profiles related to healing rate obtained from both the lipid RP- and HILIC-UPLC-MS ESI+ and ESI- analysis. The colour bar indicates the healing rate with blue points represent faster healers and yellow to red representing slower healers.

Figure 8.4 below demonstrates scores plots and cross validated scores plots generated from MS data for all samples relating to initial ulcer size at the outset of the study for a given patient. Although the modelling suggests a strong relationship with statistical significance it can be seen that most ulcers were categorised as less then $50cm^2$ although there does appear to be clustering according to ulcer size, more evident in the fact that smaller ulcers do tend to cluster more which may reflect the population recruited to the study.

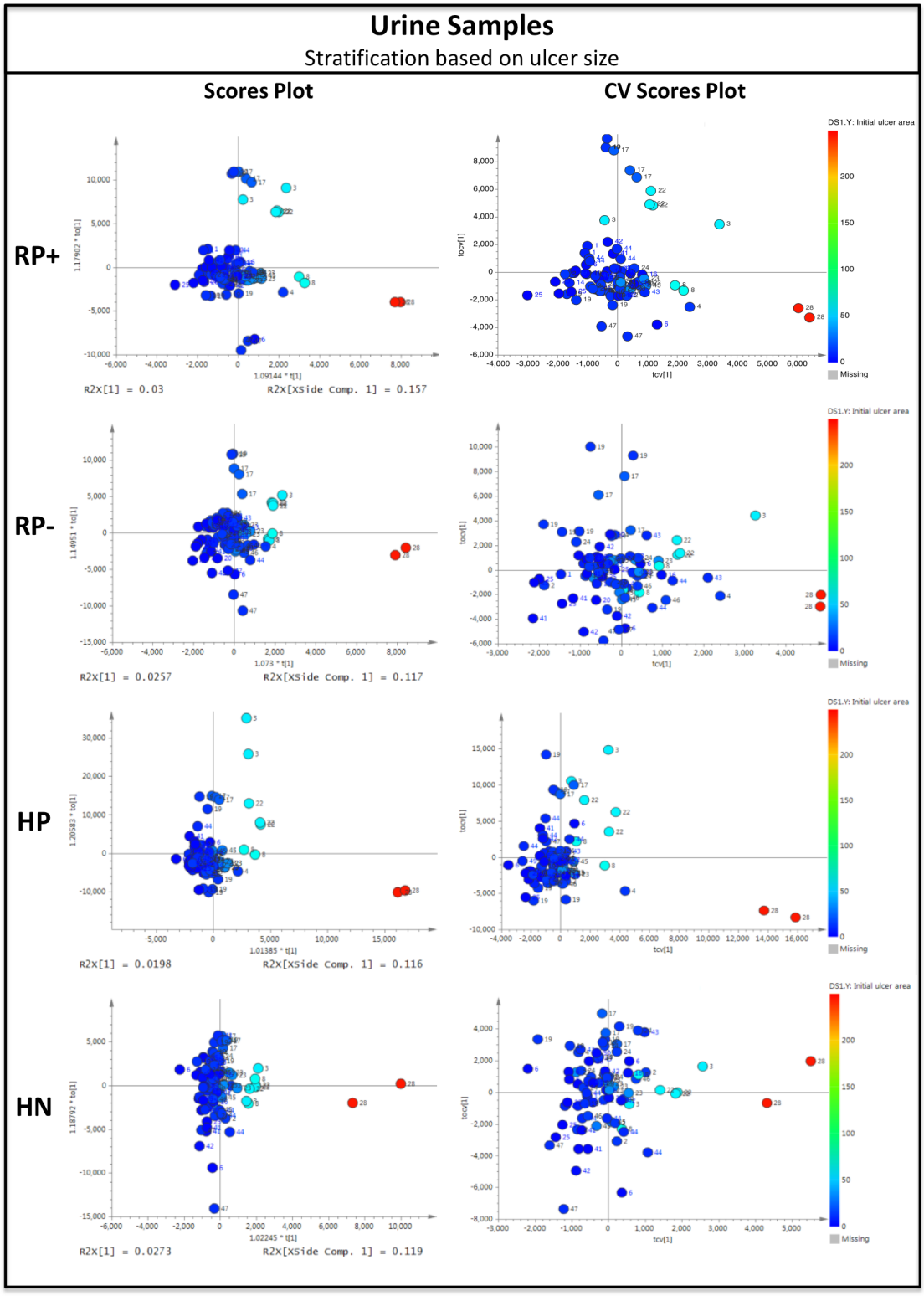


Figure 8.4: Scores plots and Cross-validated (CV) scores plots of OPLS analysis, demonstrating differences between the urine profiles related to ulcer size obtained from both the lipid RP- and HILIC-UPLC-MS ESI+ and ESI- analysis. The colour bar indicates the ulcer size with smaller ulcers as dark blue points (<5cm^2) with larger ulcers trending towards red points.

The final figure (8.5) for MS urine analysis demonstrates scores and cross validated scores plots for all samples undergoing MS analysis with reference to the chronicity of the ulcer i.e. the age of the ulcer in months at the outset of the study (week 0). As demonstrated in the table, the models all demonstrate a strong positive relationship to the variable with statistical significance achieved. There is clustering of samples according to initial ulcer age with the most clustering taking place with ulcers of a younger age at the onset of the study.

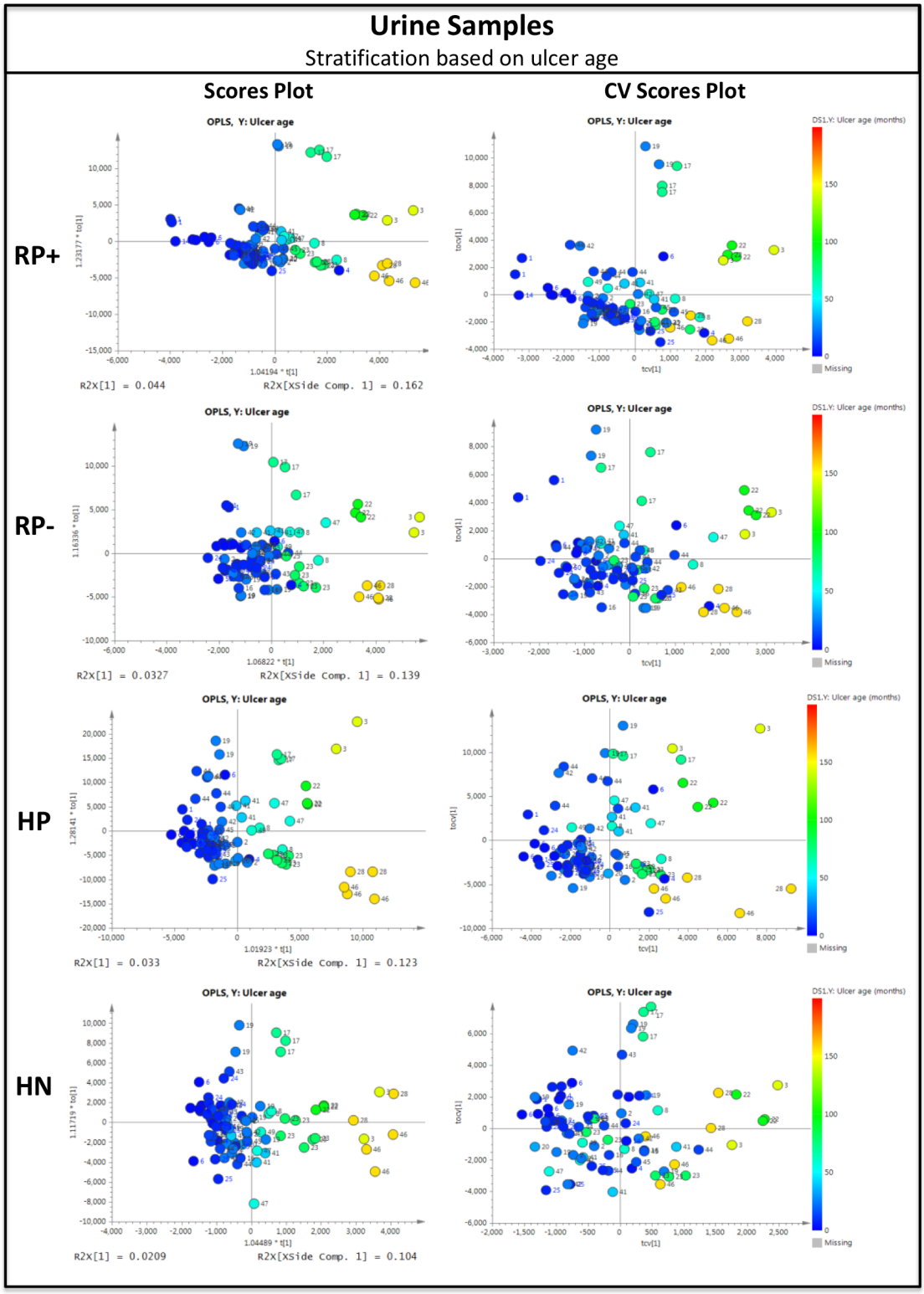


Figure 8.5: Scores plots and Cross-validated (CV) scores plots of OPLS analysis, demonstrating differences between the urine profiles related to ulcer age obtained from both the lipid RP- and HILIC-UPLC-MS ESI+ and ESI- analysis. The colour bar indicates the ulcer age in months with blue representing 'younger' and red representing 'older' ulcers as measured in months.

8.3.3 Candidate Biomarkers

In a similar manner to the previous two chapters, OPLS loadings plots were examined to determine the metabolite features responsible for variation between clinically defined groups which in turn identification was achieved by cross-referencing the Scripps database of metabolites (www.scripps.edu). The identified candidate biomarkers are listed in table 8.2.

Positive – Healing Status	**Negative – Healing Status**
Penicilloic acid	1-Isothiocyanato-4- methylpentane
2,6 dimethylheptanoyl carnitine	Tramadol
p-cresyl sulphate	N-oxide gliclazide
1,3 Dimethyluric acid	erythromycin

Table 8.2: List of metabolite species identified as being potential responsible for differences when variable clinical factors were applied to modelling for urine samples.

8.4 Discussion

In this chapter, it has been reaffirmed as per previous work in the literature that the metabolic profiling of urine is feasible. It has been demonstrated that the collection and analysis from patients with chronic venous ulceration is feasible and that quality control protocols have generated a data set free of contamination from preparative processes or analytical drift secondary to variation in ambient conditions.

Interestingly, to a greater extent than samples of serum, urine samples taken from CVU participants throughout their journey through the clinical study appear to clearly separate with positive models demonstrating statistical significance when correlated with clinical correlates known to predict outcome in CVU. The urine samples from the 28 participants in the study correlate well with healing outcome at the end of the study, ulcer chronicity, ulcer age and ulcer healing rate at week 4.

Based on separations of urine samples evident on the scores and cross validated scores plots it may be possible to infer that a differential metabolic phenotype exists between groups of participants in the study.

Although a differential metabolic phenotype appears to discern different clinical prognostic groups, it cannot be said at this point that the systemic features in urine represent exactly perturbations the ulcer microenvironment but may be related to inherent variation between subjects. By standardisation of the clinical collection of urine restricting samples to a mid-stream urinary sample taken in the morning with rapid freezing on dry ice prior to definitive deep freeze storage, it has been sought to

minimise variation. However, clinical variation does exist between patients on the basis of factors such as age, gender, medication usage and pre-existing illness. It must also be taken into account that patients with CVU are usually by definition elderly with a consequent increase in co-morbid conditions. Furthermore, participant samples at multiple time points were analysed together which may have introduced a bias into the system.

However, taking the data obtained with the caveats and considering the nature of the sample size with the implication of altered metabolic phenotype based on clinical prognostic factors, it may be feasible to suggest that this work may be the basis of further study to validate a metabolic fingerprint that may predict clinical outcome in patients with CVU.

Chapter 9: Final Discussion

9.1 Rationale for This Body of Research

Chronic venous ulceration remains a common condition that is expensive to treat (a manifestation of nursing time), it poses significant adverse effects in terms of quality of life on patients and carers and although it is reasonably well accepted that elevated venous hypertension is the underlying cause of venous ulceration, it is unclear how sustained elevations of venous pressure translate into a syndrome of tissue fragility and loss. It can sometimes be unclear how to accurately predict response to treatment in patients with chronic venous ulceration apart from utilising clinical correlates. It is also unclear how patients with for example uncomplicated superficial venous disease progress into more complicated forms of venous disease typified by CEAP clinical stages C4-6.

Multiple avenues of hypothesis directed research have outlined broad theories to explain the genesis of chronic venous ulceration namely the fibrin cuff theory and the white cell/cytokine tape hypotheses.

Metabolic profiling has rapidly evolved over the course of the last twenty years driven by improvements in throughput, analytical platform performance and computer processing have driven large scale studies examining a large range of human and animal tissues and biofluids. One of the advantages in a modern data driven environment with rapid throughput is the premise of a hypothesis gathering approach which has been partially been utilised in the studies of chronic venous ulceration. It

has the potential to reveal hitherto unrecognised pathways and connections in venous ulcer biology.

Finally, in addition to work on freshly frozen varicose vein specimens in this thesis, there has been an effort to sample the venous ulcer microenvironment. Although multiple methods such has biopsy, occlusive dressings, direct elution from dressings and filter paper and this thesis had in part agreed with the findings of Tarlton [108] in such that filter paper elution is feasible and furthermore that metabolic profiling studies are possible with biofluids eluted onto filter paper. Although ulcer fluid has been a preferred and validated sampling method in past studies [65] [170], the ease of filter disc placement has been noted in this thesis.

There is also the interesting view that local wound changes may give rise to variation in systemic biofluid compartments and in this thesis, samples from urine and serum have confirmed this.

9.2 Final Conclusions

9.2.1 Clinical Work and Sampling

The initial work in the thesis examined the feasibility of a metabolic profiling approach to venous ulcer fluid samples. The methods of occlusive dressing and filter disc placement were undertaken. In general, sampling using the filter discs was a more effective process.

Filter disc weights post clinical sampling reflected how much fluid had been absorbed and although this was corrected for in the analysis there is a chance that larger ulcers may produce more fluid and have the potential to skew results.

Urine and serum sampling was in many respects and easier biofluid to standardise in terms of collection and analysis. However, it was difficult to obtain a sample at every assessment usually because of impossibility in sampling or being unable to produce a urine sample. This is perhaps to be expected in a patient group who are typically elderly.

9.2.2 Metabolic Profiling in Varicose Vein Samples

Varicose vein demonstrate tissue demonstrates a different metabolic phenotype to normal vein and this thesis has reaffirmed previous work in this area. It is interesting that in the systemic biofluid compartments, prominent varicose vein metabolites can be detected. However, no change between clinical stage of venous disease and systemic or vein features can be detected. This may reflect the fact that the CEAP

classification clinical stage is dependent on skin changes which are mediated locally within the skin secondary to the haemodynamic manifestations of abnormal venous circulation. Nonetheless, the identification of implicated biomarkers in systemic compartments warrants further attention.

9.2.3 Metabolic Profiling in Chronic Venous Ulcer Fluid

Particularly in the lipid profiling negative mode, models based on clinical correlates do appear to positively explain the separation of groups in some cases with statistical significance. The lack of clear cut separation in permutations may either reflect a lack of profile variation in ulcer fluid samples with clinical factors applied or it may reflect the difficulty and heterogeneity of sampling the ulcer microenvironment.

9.2.4 Metabolic Profiling of Serum and Urine

It is interesting that multiple models from various metabolic profiling models demonstrated positive values with statistical significance. The implication is that this may represent an altered metabolic phenotype discernible within a systemic biofluid compartment detectable at site remote to the disease process which in this case represents a venous ulcer. If a metabolite feature or set of metabolite features could be extracted from the dataset, then a biomarker predictive of CVU outcome could be screened for from an easily accessible sample (serum or urine).

9.3 Critique of the Research

The studies carried out were undertaken in established vascular surgery departments and although a relatively small population of 28 patients was recruited for the study in terms of metabolic profiling, this is on par with comparable studies of CVU. Nonetheless, recruitment of patients who are typically frail and elderly is a difficult process and limits numbers especially given the requirement for repeat assessments. Recruitment of patients with superficial disease for the CEAP study was much more successful given the younger, more mobile patients only requiring a single assessment.

To reduce variability, all samples and assessments were collected by the author of this thesis as was the recruitment of participants. Sample storage preparation in the laboratory was mostly performed by the author of this thesis under supervision of a post-doctoral researcher again to reduce variability.

The filter disc technique has been described previously in CVU biological research but it remains unclear as to what ulcer fluid represents within the biological concept of wounds and wound healing in general. It would have been useful to correlate wound fluid results with validation against the biopsy of ulcer wound edges which are thought to be most active biological portion of the wound. However, this was unfeasible in the main study of the thesis given the numbers of patients recruited and the assessment periods.

For all studies, the inclusion and exclusion criteria were kept sufficiently broad to maximise patient recruitment but this ultimately meant that heterogeneity was introduced into the process by virtue of factors such as ulcer size, chronicity, comorbid conditions and medication history.

9.4 Future Studies

9.4.1 Validation of Ulcer Differential Metabolic Phenotypes

With urine and to a lesser extent serum and fluid eluted onto filter discs, differential metabolic phenotypes can be seen. To assess if the metabolic signatures can predict outcome, namely ulcer healing after a defined period with optional treatment, it would be useful to sample participants at the start of treatment with interval clinical follow up.

9.4.2 Ulcer Biopsy

This would go some way in answering the question as to the relevance of ulcer fluid sampling to compare the metabolite features with imaging based MS from fluid against the metabolite features of biopsy of the ulcer edge which is thought to be the most active area of the wound when it comes to wound healing and the failure of wound healing to progress.

Appendix

MS Metabolic Profiling Conditions For Urine

Column	Acquity® UPLC BEH HILIC 2.1 x 100 mm, 1.7 μm			
Column temperature	40 °C			
Mobile phase A	ACN:H_2O (95:5%), 10mM Ammonium Acetate, 0.1 % Formic Acid			
Mobile Phase B	ACN:H_2O (50:50%), 10mM Ammonium Acetate, 0.1 % Formic Acid			
Injection Volume	2.5 μL in ESI+ and 3 μL in ESI- 5µL loop, partial loop setting, with 4µL needle overfill factor			
	Gradient			
Time (min)	**Flow (ml/min)**	**A (%)**	**B (%)**	**Curve**
Initial	0.4	99	1	Initial
2.0	0.4	99	1	6
8.0	0.4	45	55	6
9.0	0.6	1	99	6
9.1	0.6	1	99	6
11.0	0.6	1	99	6
11.1	0.6	99	1	6
19.0	0.6	99	1	6
19.1	0.4	99	1	6
23.0	0.4	99	1	6

Table A1: Conditions of the HILIC-UPLC-MS metabolic profiling methodology

Column	Acquity® UPLC HSS T3 2.1 x 100 mm, 1.7 μm			
Column temperature	40 °C			
Mobile phase A	H_2O, 0.1 % Formic Acid			
Mobile Phase B	ACN, 0.1 % Formic Acid			
Injection Volume	2.5 μL in ESI+ and 3 μL in ESI- 5µL loop, partial loop setting, with 4µL needle overfill factor			
	Gradient			
Time (min)	**Flow (ml/min)**	**A (%)**	**B (%)**	**Curve**
Initial	0.5	99	1	Initial
1.0	0.5	99	1	6
3.0	0.5	85	15	6
6.0	0.5	50	50	6
9.0	0.5	5	95	6
10.0	0.5	5	95	6
10.1	0.5	99	1	6
12.0	0.5	99	1	6

Table A2: Conditions of the RP-UPLC-MS metabolic profiling methodology

Acquisition Mode	MS High Resolution mode, centroid mode
Ionisation Mode	ESI positive and negative
Capillary Voltage	1.5 KV (positive) 2.0 KV (negative)
Sampling Cone Voltage	40 V
Desolvation Temperature	400 °C
Desolvation Gas Flow	800 L/Hr
Source Temperature	120 °C
Acquisition Range	50 – 1200 m/z
Scan Time	0.1 sec

Table A3: Mass spectrometry parameters for the HILIC-UPLC-MS metabolic profiling.

Acquisition Mode	MS Sensitivity mode, centroid mode
Ionisation Mode	ESI positive and negative
Capillary Voltage	3.0 KV (positive) 1.5 KV (negative)
Sampling Cone Voltage	30 V
Desolvation Temperature	600 °C
Desolvation Gas Flow	1000 L/Hr
Source Temperature	120 °C
Acquisition Range	50 – 1200 m/z
Scan Time	0.15 sec

Table A4: Mass spectrometry parameters for the RP-UPLC-MS metabolic profiling.

MS Metabolic Profiling Conditions for Serum

Column	Acquity® UPLC CSH™ C_{18}, 2.1 x 100 mm, 1.7 μm			
Column temperature	55 °C			
Mobile phase A	ACN:H_2O (60:40%), 10mM Ammonium Formate, 0.1 % Formic Acid			
Mobile Phase B	IPA:ACN (90:10%), 10mM Ammonium Formate, 0.1 % Formic Acid			
Injection Volume	3 μL in ESI+ and 10 μL in ESI- 10µL loop, partial loop setting, with 4µL needle overfill factor			
	Gradient			
Time (min)	**Flow (ml/min)**	**A (%)**	**B (%)**	**Curve**
Initial	0.4	60	40	Initial
2.0	0.4	57	43	6
2.1	0.4	50	50	1
12.0	0.4	46	54	6
12.1	0.4	30	70	1
18.0	0.4	1	99	6
18.1	0.4	60	40	6
20.0	0.4	60	40	1

Acquisition Mode	MS V mode, centroid mode
Ionisation Mode	ESI positive and negative
Capillary Voltage	2.5 KV (positive) 2.5 KV (negative)
Sampling Cone Voltage	30 V
Desolvation Temperature	400 °C
Desolvation Gas	800 L/Hr
Source Temperature	120 °C
Acquisition Range	50 – 1200 m/z
Scan Time	0.2 sec

Table A5: MS Metabolic Profiling Conditions for Serum.

Patient Information Sheet – Imperial Site Version

Imperial College
London

Participant Information Sheet

Academic Section of Vascular Surgery, Imperial College London

Vascular Unit, Imperial College NHS Trust

You will be given a copy of this information sheet to keep

CHIEF INVESTIGATOR – PROFESSOR ALUN DAVIES

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Study Title: Clinical Study to Identify Biological Markers to Predict Wound Healing in Patients with Chronic Venous Ulcers

Introduction

We are setting up a clinical study to further our knowledge about leg ulcer healing. We would like to invite you to take part in this study. Before you decide to take part, it is important for you to understand why we are doing this study and taking samples. It is also important for you to understand what tests we will be doing with samples that we collect.

Please take your time in reading this information sheet carefully and feel free to discuss it with your friends, family and your own doctor. Please feel free to ask us anything that you do not understand or if you would like further information.

Once you have all the information you need you should be ready to decide whether you would like to take part in this study.

Thank you for reading this sheet.

What is the purpose of the study?

The aim of this study is to investigate the reasons why chronic venous ulcers in the leg develop. This may help in the future in determining which patients need extra attention to help their ulcers heal and may also provide ideas in how we can treat patients with chronic venous ulceration better.

We would also like to investigate whether a special dressing helps in treating ulcers that are slow to heal and we would like to investigate its effects on the ulcer.

What samples will be collected?

The samples taken will include

- Blood (two teaspoons worth or 10ml) from a vein near the elbow
- Urine (at least 50mls or three tablespoons)
- Fluid from your ulcer (We predict that this will be at most 2mls or small teaspoon)

What will happen to my samples?

Once the samples have been taken, they will be prepared for storage in the freezer at Imperial College. We will then perform special tests on all the samples at once that will examine for compounds associated with the healing of wounds and also a newer form of testing for the by-products produced by cells (metabolites).

Because we are taking samples over time, we would be able to chart the progress of the healing wound over time in order to better understand the process of healing.

We would also ask you some questions about your ulcer, your general health, medications and your diet to better interpret our results. We will also perform an examination of the ulcer, perform a general examination and we would also like to take special photographs and tracings of the ulcer in order to measure the rate of healing over time with some special computer programmes.

Why have I been invited?

You have been invited because you have been referred to the vascular unit at Imperial NHS trust for management of your venous ulcer. We believe because of this that you would be suitable for participation in this study because you are currently undergoing or about to undergo treatment with compression bandaging for the ulcer or ulcers affecting your leg.

What will happen to me if I take part?

If you agree to take part, we would ask for a urine sample and permission to take a blood sample. These samples will be frozen until they are tested.

During the study; your ulcer shall be treated as appropriate with compression bandaging which is the preferred option in the United Kingdom for the treatment of this condition. As part of your routine care, you will also have the veins in your leg scanned (duplex ultrasound, a test that measures the size of veins and the flow of blood within them), the blood pressure in your legs measured (ankle brachial pressure index) and some routine blood tests shall be taken.

We would also like to take samples of leg ulcer fluid. This will involve the wound dressing being removed and the placement of filter paper over the wound and a special plastic dressing. After ninety minutes, we would pop the dressing with a needle and suck out any fluid with a syringe (this will not hurt as the needle will not pierce the skin) and remove the filter paper. We would prepare the soaked paper and the fluid for storage until they are tested.

We will also take photographs of the ulcer in order to chart its progress over time. The pictures will be analysed by computer to calculate the size of your ulcer.

We would like to see you six times over a twenty week period in order to take samples from you. If at twenty weeks, your ulcer is not healing with the normal treatment. We would offer you a special dressing in order to see if it helps and we would like to see you for a further five appointments over eight weeks.

The dressing is called Urgostart® and is manufactured by Laborotoires Urgo, a French pharmaceutical company who are funding this study.

The Urgostart® dressing would be used in conjunction with your standard compression dressing. It takes the form of a foam-dressing pad with some substances involved with normal wound healing. In more detail, it is thought that the dressing may stop the inappropriate breakdown of proteins and also help new skin be laid down. The dressing is fully approved by the regulatory authorities in the United Kingdom for use in patients with venous ulcers.

Scheme of Appointments

This table shows what will happen at each time you come to the unit for the study.

First Meeting	Introductions, discussion and consent
Initial Assessment – Week 0	Preliminary evaluation – Interview about medical problems, physical examination. Blood, urine and ulcer fluid sampling. Wound dressing.
Week 1,2, 4, 8 and 12	Clinical review Blood, urine and wound ulcer fluid sampling
Week 20	Clinical review Blood, urine and wound ulcer fluid sampling If wound has not healed, apply Urgostart® dressing to wound dressing. If wound has healed – end of study.
Week 21, 22, 23 and 25	Clinical review Blood, urine and wound ulcer fluid sampling Continue dressing with Urgostart®
Week 28	Clinical review Blood, urine and wound ulcer fluid sampling Completion of Urgostart® dressing End of study

Do I have to take part?

Not at all, it is entirely your choice to take part in this study. If you do decide to take part, you will receive a copy of this sheet and your consent form. You are free to change your mind at any point. This project will not affect your standard care in any way.

What are the possible advantages/disadvantages and risks of taking part?

In the initial part of the study you are not expected to gain any personal benefit in terms of improved health, fitness or personal well-being associated from taking part in this study. You may benefit from the special dressing that we would use if the ulcer is not healed at twenty weeks.

There will be a degree of inconvenience in attending the extra clinics, but we are able to reimburse your expenses in getting to these appointments.

There would also be some time where you have to sit in a chair for ninety minutes in order to allow the wound ulcer fluid to be taken and there will be some slight discomfort when a blood sample is taken.

What happens when the research study stops?

You will normally be followed up in the outpatient clinic as clinically required. We would like to keep the tissue, blood, urine or fluid for use in future studies if you agree. Any other research done with the samples will have received approval from a research ethics committee. If an external researcher wishes to use the samples for research purposes, we will submit a substantial amendment in the ethics through research ethics committee for approval before initiating any such proposal.

Will my taking part in this study be kept confidential?

Your confidentiality is of the utmost importance to us. All information that we gain from a consultation with you, sample collection and reviewing your notes shall be kept confidential. We will keep a copy of your consent form, clinical details relevant to the study and some personally identifiable information on secured Imperial College computers or premises. However, access to your personally identifiable information will be strictly limited to a small group of authorised people in this study.

Your samples will be analysed in Imperial College's South Kensington campus and it will be impossible to identify you from them without reference to our secured records.

The reason we require clinical information (such as medical problems and medications taken) and personally identifying information (such as your age and gender) is because it helps us to interpret the complex tests of metabolism.

Imperial College London are the research sponsor and in addition to research regulatory authorities may have access to your notes and research documentation in order to make sure the research is being conducted correctly.

Will my samples or medical records be released to external researchers?

Your samples of blood, urine and wound ulcer fluid shall be kept in secure area at Imperial College's South Kensington campus and your clinical records shall be remain as they normally would with <insert NHS Trust>. Any clinical information that we record as part of the study will be kept securely in our department in the Imperial College, Charing Cross Campus under lock and key. Sometimes, researchers from other institutions may wish to use your clinical data or samples to see if they could answer scientific questions in a different manner. If this is the case, they would have to approach the Chief Investigator, Professor Davies. If Professor Davies felt that a valid scientific question could be answered, we would then have to discuss the process with a NHS Research Ethics Committee to gain their approval for the use of your anonymised samples and data.

Will my own doctor be informed?

We would like to inform your general practitioner about your participation in the study having taken your permission first. This is important as your own doctor plays an important role in coordinating all of your healthcare needs.

What will happen to the results of the research study?

We estimate that the analysis of the tissues removed will take us about 12-24 months. We will analyse the specimens in batches and compare the results to the medical information we have collected. At the end of the analysis, the results will be an aggregate of all the samples collected and thus it would be impossible to provide individual results.

The research will form the basis of Mr Velineni's research degree and shall be presented at scientific meetings. It will also be submitted for publication in scientific journals. We would also publicise our findings within the hospital. If you would like, we could send you a summary of the results once we have completed our work.

Who is organising and funding the research?

The project is organized by Imperial College. Funding for this study has been received from Laborotoires Urgo in the form of an unrestricted academic grant, which means that the research team at Imperial College has full independence in the design and conduct of the study. No member of the research team is being paid to individually recruit patients.

Who has reviewed the study?

This research study has been reviewed by the NRES Committee East of England – Cambridge South. This is a committee of people separate from your doctor, whose primary concerns are the safety, rights and welfare of patients on this study. The research ethics committee has reviewed and approved all written materials about this study including this information sheet.

What if something goes wrong?

Imperial College London holds insurance policies which apply to this study. If you experience serious and enduring harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator (Insert name and contact details). The normal National Health Service complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial AHSC Joint Research Compliance Office

Contact Details

Imperial AHSC Joint Research Compliance Office

510A, 5th Floor Lab Block

Charing Cross Hospital

Fulham Palace Road

London

W6 8RF

0203 311 0203

Imperial College Healthcare NHS Trust Patient Advice and Liason Service

Consent Form for Study – Imperial Site Version

Chronic Venous Ulcer Healing Consent Form Version 6 04/10/2013

REC Ref: 13/EE/0137

STUDY NUMBER……

One copy for clinical record, one copy for study file, one copy to patient

Imperial College
London

Consent Form

Study Title: Clinical Study to Identify Biological Markers to Predict Wound Healing in Patients with Chronic Venous Ulcers

Principal Investigator: Professor Alun H Davies

1. I confirm that I have read and understood the subject information sheet "Chronic Venous Ulcer Healing Information Sheet Version 6" dated 07/06/2013 and have had the opportunity to ask questions which have been answered fully.

Please initial ☐

2. I understand that my participation in this study is entirely voluntary and I am free to withdraw without giving any reason without my medical care or legal rights being affected.

Please initial ☐

3. I understand that sections of any of my medical records may be looked at by responsible individuals from Imperial College London or from regulatory authorities where it is relevant to taking part in this research study. I give permission for these individuals to access my records that are relevant to this research. I understand that some potentially identifying details may be kept on Imperial College computers or premises.

Please initial ☐

4. I give my permission for my blood and urine to be taken, stored and analysed for the purposes of this study.

Please initial ☐

5. I give permission for samples of leg ulcer fluid to be taken, stored and then analysed for the purposes of this study.

Please initial ☐

Chronic Venous Ulcer Healing Consent Form Version 6 04/10/2013

REC Ref: 13/EE/0137

STUDY NUMBER.....

One copy for clinical record, one copy for study file, one copy to patient

6. I give permission for anonymised photographs of my ulcer to be taken and digital analysis to be performed in order to calculate its healing. This is for purposes of the study.

7. I agree for my samples to be used in future ethically approved research.

Please initial ☐

8. If my ulcer has not healed after twenty weeks. I give permission for the use of an Urgostart® Dressing.

Please initial ☐

9. I give permission for my general practitioner to be informed about my participation in the study.

Please initial ☐

10. I agree to take part in the above study.

Please initial ☐

Patient	**Person Taking Consent**
Signature:	Signature:
Print Name:	Status:
Date:	Print Name:
	Date:

Confirmation of Ethical Approval for Main Study

NHS
Health Research Authority

NRES Committee East of England - Cambridge South
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Telephone: 0115 8839311
Facsimile: 0115 8839294

04 July 2013

Professor Alun H Davies
Professor of Vascular Surgery
Imperial College London
4 East Charing Cross Hospital, Fulham Palace Road
London
W6 8RF

Dear Professor Davies

Study title:	**Clinical Study to identify biological markers to predict wound healing in patients with chronic venous ulcers**
REC reference:	**13/EE/0137**
IRAS project ID:	**120922**

Thank you for your letter of 19 June 2013, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Ms Trish Wheat, nrescommittee.eastofengland-cambridgesouth@nhs,net

Confirmation of ethical opinion

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

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

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

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

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

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

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

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

Approved documents

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

Document	*Version*	*Date*
Advertisement	1	07 March 2013
Covering Letter		22 April 2013
Evidence of insurance or indemnity		30 July 2012
Investigator CV	Professor Alun Davies	
Investigator CV	Mr Rahul Velineni	22 April 2013
Letter from Sponsor		17 April 2013
Other: GP Letter	3	07 June 2013
Participant Consent Form	5	07 June 2013
Participant Information Sheet	6	07 June 2013
Protocol	5	07 June 2013
REC application	120922/440783/1/728	22 April 2013
Response to Request for Further Information		19 June 2013

Statement of compliance

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

After ethical review

Reporting requirements

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

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

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

Feedback

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

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

13/EE/0137	**Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

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

Yours sincerely

Dr Leslie Gelling
Chair

Email:nrescommittee.eastofengland-cambridgesouth@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Miss Becky Ward*

Clinical Report Form for Main Venous Ulcer Study

MOJITO CRF

Date of assessment / /

Patient Initials ☐ ☐ ☐

Study Number ☐ ☐

Version 1 14/11/2013

Initial Assessment

Eligibility Criteria

Inclusion Criteria	Yes	No
1. >18 years		
2. Chronic venous ulceration - Defined as wound of greater than four weeks in duration between the foot and the ankle		
3. APBI > 0.85		
4. Colour venous duplex evidence of CVI with either reflux, obstruction or both.		
Exclusion Criteria	**Yes**	**No**
1. History of any malignant disease in the affected limb.		
2. Acute infection in the limb <4 weeks		
3. History of connective tissue disease		
4. Use of current immunosupressive treatment (steroids, anti-cancer, biologicals)		

Is the patient eligible for the MOJITO study?

Yes ☐ No ☐

Consent form Chronic Venous Ulcer Healing Consent Form Version 6 04/10/2013 signed?

Yes ☐ No ☐

Study Identifying Number = ☐ ☐

Site = London / Cambridge (delete as appropriate)

MOJITO CRF

Date of assessment	/ /
Patient Initials	☐ ☐ ☐
Study Number	☐ ☐

Version 1 14/11/2013

WEEK 0

Date of Birth (dd/mm/yyyy)	
Gender	☐ Male ☐ Female

Ulcer and Venous Baseline

Month and Year of Ulcer Appearance	
Study leg	☐ Right ☐ Left
Ulcer location	☐ Medial ☐ Lateral ☐ Circumferential
VCSS	Score =
CEAP	Score =
Previous DVT?	YES / NO Details:
Previous venous ulcer	YES / NO Date (month / year) = ☐ Right ☐ Left Treatment:
Previous venous interventions	
Pentoxyfyline Use?	YES / NO
Ulcer Primary Dressing	
Compression	☐ Three layer ☐ Four layer ☐ European short stretch ☐ other ☐ none
ABPI	

MOJITO CRF

Date of assessment / /

Patient Initials ☐ ☐ ☐

Study Number ☐ ☐

Version 1 14/11/2013

Diabetes	YES / NO Type Treatment
Medical comorbidity	Cardiac: Hypertension: Stroke: Other:
Medications	Antiplatelet YES/NO Details: Anticoagulant YES/NO Details: Statin YES/NO Details: Others:

Urine sample obtained	☐
Ulcer digital photograph	☐
Transparent tracing grid	☐
Approximate area	cm^2
Clean ulcer and apply three filter disks (note if less than 3)	☐ If < 3, how many filter disks?
Application of occlusive dressing	☐
Venous blood sample obtained	☐
Time dressing placed	24h clock
Time of aspiration	YES / NO Details:
Volume aspirated and appearance	ml
Labelling complete	☐
Wound redressed	☐
Comments	

MOJITO CRF

Date of assessment	/ /
Patient Initials	☐ ☐ ☐
Study Number	☐ ☐

Version 1 14/11/2013

WEEK

Patient happy to continue	☐
Urine sample obtained	☐
Ulcer digital photograph	☐
Transparent tracing grid	☐
Approximate area	cm^2
Clean ulcer and apply three filter disks (note if less than 3)	☐ If < 3, how many filter disks?
Application of occlusive dressing	☐
Venous blood sample obtained	☐
Time dressing placed	24h clock
Time of aspiration	YES / NO Details:
Volume aspirated and appearance	ml
Labelling complete	☐
Wound redressed	☐
Comments	

Published Abstracts Arising From This Work

Metabolic profiling in chronic venous ulceration of the lower limb - a new approach to an old problem?

R. Velineni, K. Spagou, M. S. Gohel, E. Holmes, A. H. Davies

Imperial College London

Introduction: Chronic venous ulceration (CVU) of the lower limb remains a challenge yet the precise pathophysiological translation from sustained venous hypertension to tissue loss and failure of healing remains unclear. Metabolic profiling of the end-product of cellular processes has emerged as a viable tool in systems biology driven by the development of multivariate statistical models. We seek to determine the utility of these techniques in the understanding of CVU.

Methods: With ethical approval (13/EM/0011) and after informed consent. Ulcer fluid was sampled from ten patients with chronic venous ulceration by the placement of sterile absorbent filter disks and the direct sampling of fluid beneath an occlusive dressing. Aqueous and organic metabolites were extracted using a bilayer extraction protocol. Aqueous extracts from filter disks underwent proton nuclear magnetic resonance (^{1}H NMR) and organic extracts from ulcer fluid and filter disks underwent reverse phase-ultra performance liquid chromatography - mass spectrometry (RP-UPLC-MS). Blank solvents and filter disks were used as controls.

Results: Fluid collection was 100% successful with filter disks and 90% successful with dressings. Ulcer area determined fluid volume. RP-UPLC-MS using filter disks and ulcer fluid demonstrated clear separation of common lipid classes including lysophospholipids, sphingomyelins, phospholipids, fatty acyls and cholesterol esters. ^{1}H NMR using filter disks demonstrated multiple metabolites including choline, lactate, succinate, creatine and succinate. Principal component analysis from repeat samples confirmed reproducibility.

Discussion: This pilot study has demonstrated the feasibility of metabolic profiling in bio-fluid samples from patients with CVU.

Take-home message: The biology of venous ulceration is complex and incompletely understood. We have demonstrated for the first time that metabolic profiling is possible in venous ulcer samples and a systems biology approach may shed some new light on a poorly understood subject.

SARS Annual Meeting 2014 – University of Cambridge (BJS 2014 S4 1-73)

Metabolic profiling of patients with differing severity of venous disease

K. Spagou, R. Velineni, J. W. Penn, A. Dona, E. Holmes, A. H. Davies

Academic Section of Vascular Surgery, Imperial College London

Introduction: Superficial lower limb venous disease lies within a spectrum of clinical severity (CEAP1-6) although the precise biological mechanisms underlying linical up-migration remain uncertain. Previous work has demonstrated distinctive metabolic profile changes between normal and varicose vein tissue. This study looks to see if there is a difference between the metabolic profile of patients presenting with varying clinical severity of venous disease.

Method: Patients with defined venous disease attending for treatment were recruited. Urine, serum and vein tissue were analysed using mass spectrometry and proton nuclear magnetic resonance. Data analysed within a multivariate statistical analysis environment. Data was correlated with clinical data into two groups 1. No venous skin damage (C1-3) and 2. Venous skin damage present (C4-6).

Result: 44 participants were recruited (group 1 – 20 subjects, group 2 – 24 subjects). Sphingomyeline, phosphatidylcholine and phosophorylethanolamine were found to be elevated in vein tissue and for the first time identified in serum in both groups whilst taurine was present in urine. However multivariate models demonstrated no significant difference between the metabolic profile of the two groups in vein tissue, urine or serum.

Conclusion: Previously implicated compounds that have been detected in tissue have now been identified in the serum and urine of patients with varicose veins which may further support a systemic component in patients presenting with venous disease. However, in this study there was no difference in the metabolic profile dependent on the severity of the venous disease as judged by skin changes.

Take-home message: 1. There may be a systemic component to venous disease. 2. Skin manifestations of venous disease may be determined by localised tissue interactions.

SARS Annual Meeting 2015 – University of Durham (BJS 2015 102 S5 3-52)

Metabolic profiling in chronic venous ulceration of the lower limb - a potential tool for discovering biomarkers of prognosis?

R. Velineni, K. Spagou, M. S. Gohel, A. H. Davies

Imperial College London

Introduction: Despite the enormous clinical challenge of chronic venous ulceration (CVU) of the leg, the pathophysiological pathways from sustained venous hypertension to tissue loss and failure of healing remain unclear. Metabolic profiling of the end-products of cellular processes has emerged as a viable tool in systems biology driven by multivariate statistical models. We sought to apply these methods to CVU.

Method: Ulcer fluid was obtained from multiple patients with CVU by the placement of filter discs on the wound (ethical approval 13/EE/0137). Ulcer fluid was extracted from filter disks for metabolic profiling analysis. The aqueous extracts were analysed by proton nuclear magnetic resonance (1H-NMR) and the organic extracts by reversed-phase ultra performance liquid chromatography coupled to mass spectrometry (RP-UPLC-MS). Pooled samples, blank solvents and blank discs analysed for quality control assessment. Processed data underwent multivariate statistical analyses (orthogonal partial least squares discriminant analysis & partial least squares regression) with stratification for clinical data including ulcer size, ulcer age, healing status and healing rate.

Result: Samples were obtained from 28 patients with CVU (18 male, 10 female, median age 70.5 years) with median ulcer duration of 24.5 months. Multivariate statistical analysis revealed significant differences in the lipid species of the ulcer fluid microenvironment associated to the healing status, the chronicity of the wound and the healing rate.

Conclusion: Metabolic profiles in ulcer fluid from CVU appear to vary depending on key clinical outcomes, including healing status. Identification of biomarkers of healing may be feasible using a metabolic profiling approach.

Take-home message: Biomarkers predictive of venous ulcer healing may lead to more effective prognostication in a patient with venous ulceration or direct future study towards novel therapeutic targets.

SARS Annual Meeting 2016 – Royal College of Surgeons, London (BJS 2016 103 S3 p50-530

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