Tony J. Parker, PhD

Submitted for publication June 23, 2013.

*Correspondence: Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Avenue, Kelvin Grove, Brisbane, 4059 Queensland, Australia (e-mail: a.parker@qut.edu.au)

Abbreviations and Acronyms

CVI = chronic venous insufficiency

ECM = extracellular matrix

FIII = factor III

FVII = factor VII

FVIIa = activated Factor VII

FXIIIa = activated Factor XIII

FN = fibronectin

FpA/B = fibrinopeptide A/B

GP = glycoprotein

ICAM-1 = intercellular adhesion molecule-1

IGF = insulin like growth factor

kDa = kilodalton

MMP, matrix metalloprotease

MW = molecular weight

PAI-1/2 = plasminogen activator inhibitor-1/2 (continued)

Provisional Matrix Deposition in Hemostasis and Venous Insufficiency: Tissue Preconditioning for Nonhealing Venous Ulcers

Tony J. Parker,^{1,*} James A. Broadbent,¹ Jacqui A. McGovern,¹ Daniel A. Broszczak,¹ Christina N. Parker,² and Zee Upton¹

¹Tissue Repair and Regeneration Program, Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Queensland, Australia.

²Institute of Health and Biomedical Innovation, School of Nursing, Queensland University of Technology, Brisbane, Queensland, Australia.

Significance: Chronic wounds represent a major burden on global healthcare systems and reduce the quality of life of those affected. Significant advances have been made in our understanding of the biochemistry of wound healing progression. However, knowledge regarding the specific molecular processes influencing chronic wound formation and persistence remains limited.

Recent Advances: Generally, healing of acute wounds begins with hemostasis and the deposition of a plasma-derived provisional matrix into the wound. The deposition of plasma matrix proteins is known to occur around the microvasculature of the lower limb as a result of venous insufficiency. This appears to alter limb cutaneous tissue physiology and consequently drives the tissue into a 'pre-conditioned' state that negatively influences the response to wounding.

Critical Issues: Processes, such as oxygen and nutrient suppression, edema, inflammatory cell trapping/extravasation, diffuse inflammation, and tissue necrosis are thought to contribute to the advent of a chronic wound. Healing of the wound then becomes difficult in the context of an internally injured limb. Thus, interventions and therapies for promoting healing of the limb is a growing area of interest. For venous ulcers, treatment using compression bandaging encourages venous return and improves healing processes within the limb, critically however, once treatment concludes ulcers often reoccur.

Future Directions: Improved understanding of the composition and role of pericapillary matrix deposits in facilitating internal limb injury and subsequent development of chronic wounds will be critical for informing and enhancing current best practice therapies and preventative action in the wound care field.

SCOPE AND SIGNIFICANCE

THIS REVIEW BEGINS with an exploration of the roles of specific plasma-derived matrix proteins during the earliest stages of acute wound repair, hemostasis. The focus then shifts to chronic wounds where we introduce the concept of 'tissue preconditioning', a phase of wounding before hemostasis. Tissue precon-

ditioning is characterized by the extravascular deposition of plasma matrix proteins and is associated with symptoms of venous disease. Finally, we discuss the relationship between chronic wound management and plasma matrix protein deposition and we suggest that a factor in wound perpetuation may lie in the health of the limb before macroscopic injury.

TRANSLATIONAL RELEVANCE

The study of the underlying molecular and matrix biology of venous insufficiency and the associated development of chronic venous ulcers could offer insight into appropriate treatment selection in the clinic and general health practice. In particular, this review highlights the need for further investigations into how plasma matrix protein deposition alters lower limb tissue physiology. Such studies will provide a clearer understanding of the molecular and broader physiological effects of properly implemented compression therapy and targeted exercise regimes for improved blood flow in affected limbs.

CLINICAL RELEVANCE

This review highlights the importance of understanding the underlying pathophysiology of internally injured limbs before the onset of chronic wounds. Identification of at risk patients may encourage preventative interventions. In addition, increased understanding of the molecular and matrix biology of hemostasis and abnormal hemostatic events, described herein as tissue preconditioning, could provide a more complete picture of why chronic wounds form, become difficult to heal with standard treatments, and frequently reoccur after healing if compression therapy ceases.

INTRODUCTION

The prevalence of chronic wounds is high with ~1–3% of the global population affected by a chronic leg ulcer and this prevalence is known to increase with age.^{1,2} Of all leg ulcer cases, 70% are diagnosed with venous insufficiency as the underlying etiology.³ These chronic wounds cost on average 3% of total health expenditure in developed nations⁴ and

impact these economies further via indirect costs associated with lost productivity of both patients and carers. Despite advances in treatments, chronic wounds can persist for months or years before healing occurs.⁵

Under normal healthy conditions, an acute wound will heal within weeks, depending on the extent of damage, and result in the formation of scar tissue with similar tensile strength and functionality as the original unwounded tissue. The process of healing a wound can be best understood as a cascade of overlapping events, where injury sets into motion a coordinated series of physiological responses that results in tissue repair. These overlapping phases include: hemostasis; inflammation; migration and proliferation; and tissue remodeling or resolution (Table 1 and Fig. 1a). These events will not be described in length here, since they have been comprehensively reviewed elsewhere.^{6–13} Briefly however, a prolonged inflammatory phase is associated with a pathological state and often results in a 14 In particular, chronic venous ulcers remain within a sustained flux of inflammation and repair, with excessive host tissue damage, degradation and leukocyte infiltration delaying healing (Fig. 1b). Importantly, there is extensive disruption to the underlying extracellular matrix (ECM) of the skin, which can further perturb the wound healing process. The ECM is not simply a structural scaffold, it provides spatial cues for cells and is intimately involved in the regulation of cellular functions, such as migration, proliferation and differentiation. Therefore, its deposition is not random following wounding but rather, coordinated to initiate specific cellular responses.

Importantly, this review will first focus on connective tissue deposition

Abbreviations and Acronyms (*continued*)

PKA = protein Kinase A

PVD = peripheral vascular disease

RGD = arginine, glycine, aspartic acid

TGF- β 1 = transforming growth factor-beta 1

TGF- β RII = type II transforming growth factor beta receptors

TF = tissue Factor

uPA = urokinase plasminogen activator

uPAR = urokinase plasminogen activator receptor

VN = vitronectin

vWF = von Willebrand factor

Table 1. *The phases of wound healing*

<i>Phase</i>	<i>Action</i>	<i>Biological Effects</i>
1. Hemostasis	Clot formation and platelet aggregation	Impede blood loss and release of growth factors/chemical stimuli. ²⁵
2. Inflammation	Arrival of neutrophils	Neutrophils are attracted by chemical stimuli released during hemostasis necrotic material is consumed. ²⁶
	Monocyte arrival and differentiation to macrophage	Release of growth factors/chemical stimuli. ²⁸
3. Proliferation	Fibroblasts arrive	Fibroblasts are attracted by macrophage-released chemical stimuli, collagen is produced and growth factors/chemical stimuli are released. ^{24,27}
	Fibroblast-myofibroblast transformation	Myofibroblasts align with new ECM to contract the wound site. ²⁴
	Keratinocyte migration across the ECM	Keratinocytes are attracted by chemical stimuli released by fibroblasts, ²⁷ leading to creation of an epithelial monolayer covering the wound area. ²³
	Release of MMPs from fibroblasts and keratinocytes	Basement membrane digestion. ²⁸
	New blood vessel formation	Capillary sprouts from pre-existing blood vessels vascularise wound area. ²⁶
4. Remodelling	Vascularised wound area	Elimination of hypoxic environment, leading to reduction of vascularisation. ²²
	Collagen remodelling	Complete wound contraction and increased strength of the wound. ²⁴

ECM, extracellular matrix; MMP, matrix metalloprotease.

during the earliest phase in the healing cascade, hemostasis, and its role in the orchestration of inflammation, subsequent re-epithelialization and tissue repair. In addition, this review will examine the 'fibrin cuff' theory, originally proposed by Browse and Burnand,¹⁵ in the context of more recent evidence regarding the roles of the plasma derived connective tissue proteins common to both classical hemostasis and the fibrin cuff theory. In particular, we will examine their role in 'preconditioning' of the lower limb tissue before development of chronic venous ulcers (Fig. 1b). In this context, we will also examine the effects of compression therapy on the deposition and organization of these proteins as a possible factor in the documented success of compression in facilitating wound healing (Fig. 1c).

DISCUSSION

Hemostasis

Hemostasis is initiated immediately after a traumatic wound event, during which, bleeding into the wound bed from the damaged vasculature provides an opportunity for the deposition of the polymeric plasma proteins fibrinogen, fibronectin (FN) and vitronectin (VN) into a provisional matrix.¹⁶ While many other molecular components are also included in the provisional matrix, this review will focus on the central role of these three

connective tissue proteins in regulating both acute wound healing and their potential involvement in extravascular tissue 'preconditioning' for non-healing venous ulcers.

Fibrinogen. Fibrinogen is a 340 kilodalton (kDa) soluble glycoprotein (GP) which is produced and released into the circulation by hepatocytes. It consists of a pair of three independently encoded polypeptides known as α ₂, β ₂, and γ connected by 29 disulfide bonds (a recent review of the molecular structure and deposition of fibrin(ogen) can be found in¹⁷).

The fibrinogen deposition process is part of the cascade of events known as hemostasis, which is facilitated by two cooperating systems involving platelet attachment and activation and the coagulation cascade. Platelet activation is initiated by the interaction of plasma derived von Willebrand factor (vWF) with type I and III fibrillar collagens, at the site of injury, where it mediates the capture and tethering of platelets via binding with GP Ib α on the platelet surface. Subsequent adhesion stabilization, aggregation and platelet activation is facilitated by the interactions of GP VI and various platelet surface integrins with the perivascular ECM (see¹⁸ for a detailed review). Concomitantly, the coagulation cascade is initiated by complex formation between the perivascular, transmembrane GP Tissue Factor (TF), otherwise known as

Figure 1. The phases of wound healing in: **(a)** the acute wounding of a healthy individual; and **(b)** the commonly described disrupted phases of wound healing in the chronic condition. We propose that extravascular plasma-derived matrix deposition (a type of hemostasis) and edema associated with chronic venous insufficiency (CVI) results in persistent and diffuse inflammation, which along with other factors 'preconditions' the lower limb cutaneous tissues before the apparent injury event (grey dashed line). Cellular proliferation and tissue remodeling/resolution fail under high levels of sustained inflammation and poor circulation. **(c)** The phases of wound healing in the chronic condition with the application of compression therapy (arrow) results in the return of venous clearance; subsequent reduction of inflammation and disrupted cellular proliferation; and improved tissue remodeling/resolution phase of healing. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

Factor III (FIII) and the circulating zymogen, Factor VII (FVII). The TF/activated FVII complex then proteolytically activates Factor X directly; or indirectly by serial proteolytic activation of Factors VIII, XII, XI, and IX, which subsequently also leads to activation of Factor X.¹⁹ These two pathways

lead to the conversion of the circulating zymogen prothrombin to thrombin, which then mediates calcium dependent production of insoluble fibrin from fibrinogen that has adsorbed to sub-endothelial matrix proteins in the injured vessel wall. The fibrin monomer ($\alpha\beta\gamma$)₂ is formed by the cleavage and release of fibrinopeptides A (FpA) and B (FpB) from the N-terminal of the fibrinogen α and β chains; thus, exposing polymerization sites, which result in fibrin fibril formation.²⁰ The extending fibrin polymers are covalently cross-linked by activated Factor XIII (FXIIIa), significantly contributing to the stabilization of the developing fibrin clot (Fig. 2). The detailed molecular mechanisms of the role that FXIIIa plays in this process have recently been reviewed.²¹ Interestingly, recent evidence suggests that the release kinetics of fibrinopeptides is different between soluble and surface bound fibrinogen, with FpB being released at a higher rate than FpA from the surface bound configuration.²⁰ This may have important functional consequences *in vivo* because the FpB is a known chemoattractant for neutrophils and fibroblasts *in vitro*.²² Moreover, the structure of the fibrin network is dependent on local conditions, including the concentration of fibrinogen, calcium, and thrombin, and the flow rate of blood in the injured vessels. Of relevance to this review, fibrin fiber alignment and diameter after deposition are dependent on blood flow rate, where fibers are aligned with the direction of flow and have a larger diameter than when there is no blood flow,^{23,24} in the perivascular matrix, for example. The complete formation of the fibrin clot represents one of the initial connective tissue deposition events in wound healing (Fig. 3a). To the best of our knowledge there is little evidence describing any potential differences in the architecture of fibrin deposition or other matrix proteins between typical acute wounds and mild traumas, which will ultimately develop into a chronic wound.

Fibronectin. FN is a multifunctional GP present in plasma and tissue ECM and typically exists as a homo-dimer of two 250 kDa disulfide linked subunits. However, the molecular weight (MW) of FN can vary depending on alternative splice variations.²⁵ Plasma derived FN typically has a lower MW than cellular FN, is largely synthesized by hepatocytes in the liver and is present in plasma at $\sim 300 \mu\text{g/mL}$.²⁶ A number of different cell surface adhesion molecules known as integrins are capable of binding to FN, including the $\alpha\text{IIb}\beta_3$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ integrins.^{27–30} FN is also capable of binding a number of other ECM components, including type I

Figure 2. The coagulation cascade. A summary of the classical coagulation cascade in which fibrin(ogen) is deposited and cross-linked into a fibrin clot along with other plasma derived connective tissue proteins, such as fibronectin and vitronectin (not shown). Although the coagulation cascade is well-characterized, the complete composition of fibrin clots and the functional and mechanistic response of surrounding tissue to these deposits during acute and abnormal wound healing remains only partially understood. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

and type IV collagen, fibrin, heparin and heparan/chondroitin/dermatan sulfate proteoglycans.^{28,31} Plasma derived FN is known to be cross-linked into the fibrin provisional matrix by FXIIIa, where it serves as a basement membrane for cutaneous

re-epithelialization.^{16,32,33} Clark *et al.*¹⁶ demonstrated in a guinea pig excisional wound model that during re-epithelialization, epidermal cells migrated from the wound edge dissecting the deposited provisional matrix. This resulted in a thick

Figure 3. Models of matrix deposition in acute and chronic dermal injury. **(a)** Epidermal growth after matrix deposition during hemostasis, **(b)** pericapillary matrix deposition and 'tissue preconditioning' preceding development of a nonhealing chronic wound during venous insufficiency. Note distended capillaries (relative to the acute tissue), plasma leakage (causing edema), and fibrin cuffs (arrows) diffuse presence of inflammatory cells and injured but intact epidermis. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

(compared to normal basement membrane) irregular bed of fibrin and FN beneath the migrating epithelial front, and a fibrin (and presumably FN and VN) eschar above.¹⁶ Thin fibrils of both fibrin and FN also extended into the dermis, although it was not clear if dermal fibroblasts played a role in the deposition of these perpendicular fibrils as opposed to deposition during the hemostatic process. The study also demonstrated that the perpendicular basilar provisional fibrin and FN deposit disappeared once epithelial migration had ceased and epithelial differentiation and maturation had begun. Fibrin and FN remained associated with the dermis but in a more reticular pattern compared to the original perpendicular orientation observed during re-epithelialization (Fig. 3a).¹⁶ Thus, provisional matrix deposition and remodeling during the wound healing process is highly coordinated, yet still incompletely understood. Static *in vitro* experiments have also shown that FN matrix assembly is mediated by platelets adhered to fibrin and FN but not by platelets adhered to fibrinogen, vWF or VN. The negative effect on FN formation by platelets adhered to fibrinogen is facilitated by the binding interaction of the $\alpha\text{IIb}\beta_3$ integrin with the C-termini of the γ chain of fibrinogen. Polymerization and cross-linking of fibrin alters the accessibility of the integrin for its binding site and consequently the ability of platelets to modulate FN deposition.³⁴ Interestingly, $\alpha_6\beta_1$ integrin mediated platelet adhesion to laminin-111 also induces FN matrix assembly but only under static or stagnant blood flow conditions.³⁵ This may have implications for the structural morphology of fibrin/FN matrix deposition into the extravascular tissue, as has been associated with venous insufficiency.¹⁵

Vitronectin. VN is a 75 kDa multifunctional GP localized to the ECM and plasma. VN is involved in a wide variety of biological activities, including promotion of cell adhesion, spreading, proliferation and migration. It is also involved in immune defense through interactions with complement complexes, and hemostasis through interactions with fibrin. Plasma VN is largely synthesized in the liver and secreted into the circulation in a nonadhesive, monomeric 'native' conformation.³⁶ Native VN is structurally altered through interactions with a number of plasma proteins to form 'denatured' VN. Denatured VN can form multimers after endogenous cleavage of the 75 kDa native protein into 10 and 65 kDa fragments, linked by a disulfide bond (Cys²⁷⁴-Cys⁴⁵³).³⁷ VN has been shown to be incorporated into the provisional fibrin matrix af-

ter injury,³⁸ where it contributes to the stabilization of the newly formed clot against premature fibrinolysis (Fig. 3a).³⁹ This is achieved, in part, through the binding and latency stabilization of, plasminogen activator inhibitor-1 (PAI-1) with the N-terminal of the somatomedin B domain of VN,^{40,41} where PAI-1 is able to inhibit plasmin formation and therefore, early clot fibrinolysis (Fig. 3a).³⁹ The subsequent release of Protein Kinase A (PKA) from activated platelets can phosphorylate fibrin bound VN; thus, reducing the affinity of PAI-1 for VN.⁴² The release of PAI-1 via this mechanism is thought to reduce its half life; thus, fibrinolysis is allowed to progress at specific loci in the clot. Moreover, interaction of VN bound PAI-1 with urokinase plasminogen activator (uPA) or tissue plasminogen activator (tPA) results in the release of PAI-1 from VN, facilitating $\alpha_v\beta_3$ integrin and uPA receptor (uPAR) mediated cellular migration into the provisional matrix.^{43,44} While PAI-1 competitively disrupts uPAR and integrin binding of VN, other evidence suggests that PAI-1 can induce cell detachment from VN by interacting with active uPA bound to uPAR in a VN-independent process. In addition, this process is found to result in the inactivation and internalization of uPAR-uPA- α_v integrin complexes.⁴⁵ Furthermore, VN interacts with $\alpha\text{IIb}\beta_3$ integrins on the surface of platelets, thereby facilitating platelet aggregation and activation in the developing fibrin meshwork, which also serves to provide additional stabilization of the clot after injury.^{46,47} Cell attachment to VN occurs through integrin binding via the arginine, glycine, aspartic acid (RGD) motif and/or via VN-uPAR-uPA $\pm \alpha_v$ integrin complex formation.⁴⁸

Interestingly, in a murine model of glomerulonephritis, induced by nephrotoxic injury to glomerular capillary basement membranes, Mesnard *et al.*⁴⁹ demonstrated that plasma derived VN facilitates intra-glomerular localization of PAI-1, which results in reduced fibrinolysis of pericapillary fibrin deposits. The authors also showed that the VN deposition around the injured capillary bed resulted in increased macrophage extravasation early in the disease progression and eventual glomerular capillary occlusion.⁴⁹ The deposition of extra-capillary fibrin and VN presented in this study bears striking resemblance to the deposition of fibrin around capillary beds of patients suffering venous insufficiency, the so called 'fibrin cuff' theory (Fig. 3b).^{15,50} In addition, multimeric VN has recently been shown to induce vascular permeability *in vitro* by causing the internalization of vascular endothelial (VE)-cadherin in human vascular endothelial cells via engagement of $\alpha_v\beta_3$

integrins and induction of Src kinase signaling.⁵¹ In this study, the authors also demonstrated that VN null mice exhibited impaired vascular permeability compared to wild type controls in a hind limb ischemia model. Moreover, the ischemia appeared to increase both the local deposition and multimerization of extravascular VN which was required for facilitation of extravasation of macrophages to the area of injury.⁵¹ Thus, the deposition and conformation of VN in the wound environment plays an important role in the initialization of the inflammatory response to injury and removal of the provisional matrix via regulation of fibrinolysis.

Importantly, FN and VN are known to interact with a variety of growth factors and cytokines, including members of the insulin like growth factor (IGF) family,^{52–54} transforming growth factor-beta 1 (TGF- β_1)⁵⁵ and hepatocyte growth factor.⁵⁶ Each of these growth factors has been shown to have important effects on skin cell function.^{52,57–59} Indeed, VN/IGF complexes have been developed for use as a potential wound healing therapeutic.^{60,61}

In summary, these three plasma matrix proteins not only interact, multimerize and function to form a stabilized provisional matrix to prevent excessive blood loss, but also embed with them the molecular elements required to regulate inflammation, cellular migration, proliferation, differentiation and tissue remodeling. This is achieved either directly by interacting with cell surface receptors, such as integrins, or indirectly by acting as carrier proteins for growth factors or inhibitors, which regulate cellular function.

Tissue preconditioning: an undefined prerequisite for venous leg ulceration

Venous disease and chronic venous insufficiency. Venous disease is caused by inadequacies in the superficial or deep venous system of the legs where venous return to the heart is impaired by reflux, due to obstruction or failure of the calf muscle pump, resulting in an accumulation of blood in the legs.^{62,63} Half the adult population are estimated to have venous disease in the lower limbs with the prevalence ranging from 40–50% in men and 50–55% in women.³ The normal venous system may be affected by deep vein thrombosis or history of injury to legs, such as fractures or minor trauma, which can result in permanent damage to valves in the superficial venous system.⁶⁴ Furthermore, occlusion of the venous system may occur due to obesity or pregnancy where increased pressure on abdominal veins reduces venous outflow from the legs.⁶⁴ Any damage to the venous system that increases fluid in the legs and elevates venous

pressure in the veins of the lower limbs, is known as venous hypertension.⁶⁵ Symptoms of venous disease may include: varicose veins, edema and venous skin changes, such as lipodermatosclerosis and/or hemosiderin staining.⁶⁶ Lipodermatosclerosis is a painful induration which, if severe, gives the lower leg an inverted shape with enlargement of the calf and narrowing at the ankle.⁶⁷ Hemosiderin pigmentation is characterized by a brown coloring of the leg due to iron, derived from hemoglobin, being deposited in extravascular tissues of the leg (Fig. 4).⁶⁴

Chronic Venous Insufficiency (CVI) is an extreme form of venous disease⁶³ leading to extravasation of red blood cells and large protein molecules that leak out from capillaries resulting in edematous skin. This results in impaired oxygen diffusion,⁶³ and damage to the lymphatic system.⁶⁵ The prevalence of CVI ranges from 2–7% in men and 3–7% in women,³ although another study reported men to have a higher incidence than women.⁶⁸ The influence of androgens on the expression and activity of matrix metalloproteases (MMPs) and therefore, collagen and FN matrix turnover in affected limbs may underpin the observed gender differences.⁶⁹ Furthermore, prevalence data may be underestimated due to differing definitions that exist in relation to venous disease and CVI, as well as asymptomatic disease.³ Other symptoms may include mild to severe swelling, aching legs, leg heaviness, and skin changes.⁷⁰ In the most severe cases, patients experience venous leg ulceration where the skin on the lower limbs breaks down to form a perpetuating wound. These wounds were shown to affect 3.6% of patients with CVI in a study of varicose veins and CVI in a Brazilian population and are estimated to have a global incidence of 1% in the adult population.^{66,71} These wounds lead to long-term poor health, pain, immobility, psychological and financial hardship,⁷² and consume a significant proportion of health care expenditure in developed nations. Despite their high incidence and cost to patients and society, the processes involved in the transition from healthy tissue to chronic dermal ulceration remains under-characterized.⁷³

Tissue preconditioning. While there is certainly a link between valve malfunction, venous disease and chronic venous leg ulcers, not all CVI patients have venous leg ulcers or will necessarily develop leg ulcers.⁷⁴ Instead there is a mixed population of CVI patients with and without venous ulcers. As such, those patients who go on to develop these ulcers must experience unique or specific events

Figure 4. Extent of injured tissue in acute wounds and edematous limbs as a result of venous insufficiency. In acute wounds localized hemostasis and inflammation are resolved due to adequate vascular hemodynamics and from the relatively uninjured/healthy surrounding tissues. In venous insufficiency, wound healing is difficult in the context of diffuse internal vascular injury which is undergoing a perpetual hemostatic and inflammatory response. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

which predispose them to dermal ulceration. This also suggests that only through a process of specific or severe tissue disruption, or biochemical perturbation, does CVI ultimately cause venous leg ulceration. These events are a discrete but undefined aspect of venous disease that conditions the tissues in the lower limb making them prone to ulceration. For differentiation from general venous-related pathogenesis, we have referred to this active and causative aspect of venous disease as tissue preconditioning.

The concept of tissue preconditioning is not novel. Many investigators have been searching for the causative agent or event that ultimately triggers the presentation of a chronic leg ulcer after the onset of venous disease. Burnand *et al.* observed changes in matrix deposition in the tissues of leg ulcer patients in 1982 and proposed one of the first theories for the cause of chronic venous leg ulcers.⁷⁵ Since Burnand *et al.*,⁷⁵ proposed the so called 'fibrin cuff' theory, others have proposed the so called 'white cell trap' theory and have investigated the role of: inflammation; the ECM; nerves; growth factors; and proteases in the development of chronic leg ulcers. A particularly interesting hypothesis proposed by Simka⁷⁶ suggests that epidermal degeneration and initial ulcer formation results from T lymphocyte attack (Fig. 3b). The hypothesized mechanism involves the combined interplay between extravasated macrophages,

which are engaged in the elimination of erythrocyte remnants as a result of increased vascular permeability, excessive ferric ion deposition (from hemoglobin), T Lymphocytes, epidermal keratinocytes and cytokine cascades.⁷⁶ While a detailed description of this hypothesis is beyond the scope of this review, it fits very well into the concept of tissue preconditioning along with the fibrin cuff and other theorized mechanisms of ulcer causation and perpetuation. Indeed, readers are directed to detailed reviews of these investigations.^{73,76–78} These discuss a number of mechanical, cellular and extracellular changes that have been observed to take place between the failure of the venous circulation in the lower limbs and the onset of chronic venous leg ulcers,⁷³ any or a combination of which may comprise tissue preconditioning. Although well described, the causal relationship of these tissue changes with the development of venous leg ulcers remains contentious.⁷³

Fibrin cuffs as a tissue preconditioning candidate. One of the highly cited changes in the lower limb tissues of patients with venous insufficiency, and a clear perturbation in matrix deposition, is the presence of fibrin cuffs (Fig. 3b). These perivascular structures form a sheath around the dermal capillaries and were first observed by Burnand *et al.* in the hypertensive canine hind limb.⁷⁹ The authors went on to detect these

structures in the tissues of leg ulcer patients with lipodermatosclerosis and implicated them as causative in the genesis of lipodermatosclerosis and venous ulceration.⁷⁵ Since this time fibrin cuffs have been observed even in the capillary beds of nonulcerated but venous compromised patients, and were found to contain fibrin, laminin, FN, tenascin, collagen and trapped leukocytes in ordered structures.⁸⁰ A direct link between these structures and the occurrence of chronic venous leg ulcers has been limited by a number of factors, such as their persistence with treatment and wound healing, which suggested a lack of functional association with ulcer biology.⁸¹ However, this observation was partially refuted when it was shown that fibrin cuffs can be resolved with compression therapy.⁸⁰ These structures are also observed in other chronic ulcerative diseases.⁸² In this regard, however, the inability of the affected capillaries to supply oxygen to the surrounding tissues was found to be unique to venous insufficiency,⁸³ although this remains contentious.⁸⁴ While the role of fibrin cuffs in oxygen supply remains debatable, these matrix-rich deposits may impart other functional consequences in the wound environment.

Fibrin(ogen) as an inflammatory mediator in chronic wounds. The role of fibrinogen in the extra-vascular environment is well understood in the context of hemostasis. In this role the cleavage of fibrinogen into fibrin, and the subsequent polymerization of these products, is critical to the clotting process and the provision of a transient matrix comprising fibrin, FN, and VN. This matrix is tightly regulated in space and time by fibrinogenic and fibrinolytic mediators (Fig. 3a). In instances where the degradation of the fibrin is compromised, as observed in mice with a deficiency in plasminogen (a fibrinolytic enzyme), there is sustained fibrin deposition in tissues leading to a myriad of consequences, including delayed wound healing.⁸⁵ Reciprocal models, including the FN null mouse demonstrate generally reduced inflammation, lower levels of inflammatory cytokines and reduced macrophage and neutrophil infiltration.⁸⁶ With regard to chronic venous leg ulcers, there is clear evidence for the extensive extravascular deposition of fibrin(ogen) in ulcer tissues and affected limbs. These limbs demonstrate signs of elevated inflammatory cell infiltrates^{87,88} known to be induced by fibrin(ogen) derived peptides.^{22,89,90} Once there, these cells are proposed to increase local concentrations of proteases, such as MMPs and reactive oxygen species in the local environment⁹¹ causing oxidative stress, leading to the damage of vessel

walls and thereby facilitating the release of yet further fibrinogen into the extracellular space (Fig. 3b). Investigators have also observed increased intercellular adhesion molecule-1 (ICAM-1) levels in chronic wound tissues,⁹² suggesting an increased capacity for leukocyte-endothelial adhesion. Critically, fibrinogen has also been demonstrated to bind ICAM-1⁹³ and $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrins⁹⁴⁻⁹⁶ and therefore, may play a critical role in the recruitment of leukocytes to the wound environment through the bridging of the endothelium and leukocyte surfaces, although this role has not been specifically investigated in chronic wounds.

Clearly fibrin(ogen) function in the context of chronic venous ulcers remains under-characterized. The roles for this matrix protein in the induction and mediation of inflammatory functions have been extensively reviewed previously.⁸⁶ Critically, this review demonstrates the diverse mechanisms by which fibrin(ogen) both drives and mediates inflammation, and suggests roles for extravascular fibrin(ogen) within chronic venous ulcers far beyond that of a physical barrier to oxygen and nutrient diffusion.

Fibrin and fibrin cuffs in acute and chronic wounds. Wound healing is generally considered as four overlapping phases comprising hemostasis, inflammation, proliferation, and remodeling. The deposition and degradation of ECM is critical to all phases of this process; however, this model assumes that the affected tissue is healthy before physical trauma. The concept of tissue preconditioning suggests that tissues affected by discrete aspects of venous disease are critically injured before trauma or epidermal disintegration. In this regard, the preconditioning of tissues could be considered as a prehemostatic phase of the wound healing process where tissues are compromised and primed for inflammation rather than classical hemostasis and prohealing ability (Fig. 3b).

In terms of gross matrix deposition in acute and chronic wounds, fibrin, laminin, FN, tenascin, and collagen are all highly abundant in discrete prehemostatic chronic wound tissues,⁸⁰ whereas they are absent from the pericapillary space of healthy tissues⁷⁵ and have not been reported as being specifically associated with the pericapillary microenvironment in acute wounds. Not only are these ECM proteins enriched in the prehemostatic tissues of chronic wounds, but the ability to digest and reabsorb these deposits has been shown to be reduced as compared to healthy controls.¹⁵ To the best of our knowledge, no study has yet specifically examined the potential deposition of VN into

pericapillary fibrin deposits, despite the fact that VN incorporation into fibrin clots has been described previously.⁹⁷ This is especially striking, given the conspicuous deposition of PAI-1 in the capillary beds of patients with CVI⁹⁸ and that VN is known to mediate incorporation and thus, the biological activity of PAI-1^{38,41} in the fibrin matrix, thereby regulating fibrinolysis and subsequent fibroblast migration into the clot provisional matrix.^{38,99} Similarly, but less well studied, is the potential role of PAI-2, which also regulates fibrinolysis, is produced by macrophages and is cross linked to fibrin.¹⁰⁰ A preliminary study indicated that PAI-2 tissue concentration was higher at the edges compared to the centre or surrounding skin of healing wounds and lower in chronic venous wounds at the wound edges and in the centre of the wound particularly.¹⁰¹ It is not yet clear if PAI-2 is incorporated into fibrin cuffs or if it plays a significant role in the pathogenesis of venous insufficiency and chronic wounds.

Finally, fibrin(ogen) is known to be incorporated into the clot matrix several days after wounding, due to clot bound thrombin (possibly through binding to VN or fibrin); thus, it is possible that this contributes to the persistence of fibrin deposition associated with venous insufficiency.¹⁰² Moreover, as eluded to above, extravascular fibrin fiber deposition may not be subjected to the same rate of blood flow as in the vasculature itself, thereby leading to a relatively random arrangement of the fibers compared to normal wounding where there is often a flow of blood through damaged vasculature and into the newly formed wound bed suggesting the fibers may be more aligned.^{23,24}

In summary, the presence and function of fibrin(ogen) and other plasma derived matrix proteins are compromised in both space and time, appearing outside of their native setting, before vascular trauma and persisting when not required. These observations represent major points of difference in matrix deposition between chronic and acute wounds. It remains unclear, however, as to the functional implication of such disparity between chronic and acute wounds. In addition to the dysregulation of connective tissue deposition afforded by fibrin cuff formation and associated proteins, other observations have brought to light further differences in connective tissue deposition between acute and chronic wounds.

Hypoxia and its effect on matrix deposition as a tissue preconditioning candidate. Local and transient hypoxia is inevitable and crucial to wound healing through the stimulation of angiogenesis

which enables wound reperfusion.¹⁰³ In an acute wound environment, this initial hypoxia is thought to stimulate the progression of cell proliferation, migration and cellular differentiation via the induction of cytokines and growth factors, which stimulate intracellular signaling pathways.¹⁰³ Physiologically, the central region of the wound environment is the most hypoxic, with an increase in the oxygen gradient toward the uninjured tissue at the periphery.¹⁰⁴ It is this oxygen gradient from wounded to normal tissue that is thought to drive the wound healing cascade and thus, promotes the diffusion of oxygen to the hypoxic tissue.¹⁰³ This elevated diffusion then provides the requisite oxygen needed to continue the wound healing process. Critically, tissue hypoxia in conjunction with ischemia is also often viewed as a key characteristic of chronic wounds and is related to the inability of such wounds to heal.¹⁰⁵ In a chronic wound environment, hypoxia becomes extreme and therefore, if hypoxia alone was sufficient to support healing, all ischemic wounds would heal rapidly; however, clinical observations indicate the exact opposite. Moreover, several animal studies have demonstrated that chronic hypoxia results in decreased granulation tissue production and delayed onset of re-epithelialization.^{106,107}

In 1985 Clyne *et al.* reported that the diffusion of oxygen in lipodermatosclerotic or ulcerated gaiter area skin was lower than in the skin of subjects without significant disease.¹⁰⁸ Since it had also been proposed that pericapillary fibrin deposits might be a potential causative factor in limiting oxygen diffusion, thus contributing to tissue necrosis and ulceration,¹⁵ Stacey *et al.* conducted a critical study, which showed that such deposits preceded observable lipodermatosclerotic skin alterations and importantly tissue hypoxia.⁵⁰ The authors also found a moderate but significant inverse correlation between the extent of fibrin deposition and transcutaneous oxygen measurements, suggesting that pericapillary fibrin might indeed be involved in the development of hypoxia in these limbs. Although they also found that the extent of pericapillary FN deposits were higher in biopsies taken from gaiter area skin of the lower leg, compared to biopsies of thigh tissue, this was not correlated with levels of transcutaneous oxygen or fibrin deposition. This would seem to suggest that FN is not always associated with pericapillary fibrin deposits; however, others have found that FN is commonly associated with fibrin cuffs, although most of these studies utilized biopsies from venous ulcers or ulcer margins.⁸⁰ A crucial step in the normal wound healing process is the migration

of fibroblasts from surrounding healthy tissue into the wound area and provisional matrix through the expression of integrins, which are able to interact with fibrin, FN and VN.^{109,110}

Mogford *et al.* reported that fibroblasts grown in a chronically hypoxic environment expressed lower levels of type I and type II TGF- β_1 receptors (TGF- β RI & II).¹¹¹ This supported previous work by others which demonstrated that primary dermal fibroblasts exposed to *acute* hypoxic conditions (< 1 week) *in vitro*, results in cellular proliferation, TGF- β_1 expression and type I collagen production. However, exposure to *prolonged* culture in hypoxic conditions (> 1 week), thus, mimicking an ischemic dermal environment, did not support these processes.¹¹² Similarly, other studies using dermal fibroblasts have reported up-regulated expression of pro- $\alpha 1$ (I)¹¹³ and (III) collagens^{114,115} after acute exposure to hypoxic conditions *in vitro*, followed by decreased expression in response to prolonged hypoxia.^{114,115}

Under hypoxic conditions dermal fibroblast-produced collagen cannot be synthesized or cross-linked effectively, since the enzymes required for collagen polymerization and crosslinking, prolyl hydroxylase, lysyl hydroxylase, and lysyl oxidase, all require molecular oxygen as a cofactor to perform their biological function in the collagen synthesis and maturation pathway.^{116–118} More specifically, prolyl hydroxylase is required to convert proline residues to hydroxyproline, which allows the procollagen peptide chains to assume their triple helix configuration.¹¹⁹ Thus, in the absence of sufficient oxygen, collagen cannot be correctly formed, and since collagen deposition provides an essential matrix for angiogenesis and cellular migration this negatively effects wound healing.¹⁰⁵ In addition, the enzyme lysyl oxidase, is also involved in the cross-linking of the ECM protein elastin.¹¹⁷ Elastin is a protein which possesses elastic-like qualities and imbues tissues with the ability to retain their shape after stretching.¹²⁰ Berk *et al.*, reported that rat lung-isolated fibroblasts produced up to 63% less elastin when grown under chronically hypoxic conditions than under normoxic conditions.¹²¹ Therefore, it is clear that synthesis and stabilization of these ECM proteins requires oxygen and a prolonged lack of oxygen in the chronic wound environment ultimately leads to production of a dysfunctional ECM.

In a study of paired biopsies from injured and noninjured skin obtained from lower limbs in patients with chronic ischemia, secondary to peripheral vascular disease (PVD), Dalton *et al.* also proposed that abnormalities in uninjured skin

might predispose the tissue to breakdown.¹²² The authors reported increased collagen synthesis in ischemic tissue compared to nonischemic tissue, yet the total collagen in both tissue types was equivalent.¹²² In a subsequent study, Dalton *et al.*¹²³ reported increased yet defective angiogenesis in noninjured ischemic tissue compared with nonischemic tissue. Furthermore, the authors reported an increase in TGF- β_1 , TGF- β RI and TGF- β RII expression in ischemic limb fibroblasts, which coincided with an increase in Smad 2/3 phosphorylation and type I collagen synthesis.¹²³ In contrast, Kim *et al.*¹²⁴ analyzed fibroblasts taken from chronic venous ulcer biopsies and compared them to patient matched upper thigh biopsy fibroblasts. They reported that there was a decreased expression of TGF- β RII in chronic wound fibroblasts, resulting in decreased phosphorylation of Smad 2/3 and mitogen-activated protein kinase. The authors concluded that chronic venous wound fibroblasts are less responsive to TGF- β_1 .¹²⁴ In a follow-up study, the authors found that the TGF- β_1 inducible, β ig-h3 gene and protein exhibited decreased expression in fibroblasts and tissue sections obtained from chronic wound biopsies. β ig-h3 is thought to be involved in cell adhesion, migration and proliferation and has recently been found to be involved in platelet activation and thrombus formation, highlighting its potential role in facilitating wound healing.¹²⁵ It is clear that further studies need to be conducted to determine if fibroblasts from preconditioned tissues from various vascular etiologies differ in their ability to respond to key wound healing signals. In each of the above mentioned studies, the response of dermal fibroblasts to tissue hypoxia appears to be different. While this is seemingly counterintuitive, the causative modality of the tissue hypoxia is quite different. Hypoxia in PVD is due to occlusive processes, which prevent adequate capillary perfusion, whereas in venous insufficiency occlusion, and therefore, oxygen deprivation (according to 'fibrin cuff theory'), occurs between the microcirculation and the extravascular tissue. Thus, it is possible that fibroblasts derived from these different etiologies may be primed to respond differently to tissue hypoxia due to other differences in the extravascular microenvironment rather than the lack of oxygen *per se*.

Translations/interventions

Compression therapy. A systematic review of all randomized controlled trials of the clinical effectiveness of compression bandage or stocking systems in the treatment of venous leg ulceration

concluded that compression increased healing rates compared with no compression. In addition, multicomponent systems were more effective than single-component systems, especially when the multicomponent systems contained an elastic bandage (Fig. 5a, b).¹²⁶ O'Brien *et al.* obtained a 54% healing rate at 3 months in patients using a four layer compression system, in comparison to 34% healed in a control group receiving 'usual care', where only 5 of 100 participants received some type of compression.¹²⁷

In a standing individual, the venous pressure, which equals the weight of the blood column between the foot and right atrium, is about 80–100 mm Hg. During walking however, the blood flow is accelerated by the combined action of the calf muscle pump and the foot pump, which in patients with competent valves, decreases the volume of venous blood in the foot and reduces venous pressure to about 10–20 mm Hg.¹²⁸ Incompetent veins will cause blood to oscillate up and down in venous segments lacking functional valves, resulting in retrograde (backward) flow in the veins of the lower leg (venous reflux) leading to a reduction in venous pressure during walking (ambula-

tory venous hypertension).¹²⁹ For ambulant patients with venous insufficiency, high levels of compression (*e.g.*, 40–50 mm Hg) are required to produce beneficial hemodynamic effects.¹²⁹

Venous hypertension in patients with CVI is the trigger for functional alterations about the micro-circulation although, as this review discusses, these alterations are complex and only partially understood.¹²⁸ The use of high compression therapy has long been identified as the cornerstone of management of venous leg ulcers.¹²⁹ The aim of graduated compression is to provide greater pressure at the ankle (~ 40 mm Hg) which is required to reverse venous hypertension and reduce blood pressure in the superficial venous system.^{130,131} Using the law of Laplace, a garment that is applied at constant tension will produce graduated compression, provided that it is shaped to fit as accurately at the ankle as at the calf.¹³² At the tissue level the edema or accumulation of extravascular fluid occurs as a result of complex interactions involving the permeability of capillary walls and hydrostatic and oncotic pressure gradients that exist between the blood vessels and surrounding tissue.¹²⁸ Venous hypertension results in elevated

Figure 5. Compression therapy, calf muscle activation and Leg elevation. (a) Multilayer and (b) single layer (stockings) compression treatments. (c–e) Exercise of calf muscle pump (heel raises), note contracted gastrocnemius muscles in (d). (f) Leg elevation above the level of the heart. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

hydrostatic pressure within the capillaries and contributes to distension, increased vascular permeability and an increased net flow of fluid extravascularly. Since the increase in vascular permeability also allows the passage of plasma proteins, including fibrinogen, FN, and VN, this reduces the colloid osmotic pressure (oncotic pressure) of the plasma, further exacerbating net fluid loss from the vasculature. Since fibrinogen, FN and potentially VN are polymerized into an extravascular provisional matrix it is possible that the diffusion coefficient of the proteins out of the vasculature is maintained. However, this is yet to be demonstrated. Starling's equation suggests that the application of external compression will counteract the loss of capillary fluid by increasing the interstitial fluid hydrostatic pressure.¹²⁸ This in turn reduces the elevated net filtration pressure within the capillaries and reinforces fluid reabsorption into the veins and lymph vessels, increasing orthograde (towards the heart) blood flow and reduction in venous reflux.¹²⁸ The acceleration of blood flow in the microcirculation, facilitated by compression, has been noted to favor white cell detachment from the endothelium and prevent further adhesion.⁹¹ Taken together, this presumably allows fibrinolytic processes and inflammatory cells within the extravascular tissue to remove protein deposits without them being constantly replaced through plasma outflow; thus, resolving one of the key inflammatory stimuli. Observations consistent with this hypothesis have been made previously,⁸⁰ and intermittent pneumatic compression therapy has been demonstrated to stimulate fibrinolytic activity systemically.¹³³ However, more in depth analysis of the effects of hemodynamics on the molecular processes at work as a result of compression therapy are required. Taradaj *et al.* concluded that the hemodynamic effect (improvement of arterial microcirculation inside the venous leg ulcer) is one of the most significant biophysical effects of healing after clinically efficient compression therapy.¹³⁴ Although under-characterized, these observations provide a link between the application of compression bandaging and the molecular perturbations to matrix deposition found associated with venous insufficiency and chronic venous leg ulcers.

Exercise. The deep veins in the lower extremities are surrounded by calf muscle that has a function in assisting venous blood return.¹³⁵ When the calf muscle pump is relaxed, blood pools in the veins and when the calf muscle contracts the pumping action propels blood back to the heart.¹³⁵

Thus, heel-toe walking produces optimal calf muscle pump function.¹³⁵ Moreover, ankle range of motion has been significantly correlated with severity of venous insufficiency^{136,137} and this may be related to the observed impaired calf muscle function in patients with venous disease.¹³⁸ Importantly, a single-arm pilot study and two small randomized control trials indicated that exercises that increase calf muscle strength and mobility also improve calf muscle function.^{139–141} Such exercises include heel raises and active plantar flexion using resistance (Fig. 5c–e). Additional research is required to determine the effects of calf muscle exercise on: hemodynamics within pre-conditioned limbs; the effect on extravascular matrix deposition and/or fibrinolysis; and wound healing. This would seem particularly important given the cost effectiveness of exercise for prevention over treatment for healing.

Elevation. Limb elevation is frequently advocated in the treatment of venous leg ulcers and the Australian and New Zealand Clinical Practice Guidelines for Prevention and Management of Venous Leg Ulcers recommend the elevation of legs, ideally above the level of the heart, to promote changes in microcirculation and decrease lower limb edema (Fig. 5f).¹³⁵ Barnes *et al.* concluded that continuous elevation (24 h) led to changes in skin microcirculation.¹⁴² Karaca and Nilsson showed that prolonged bed rest raised the level of leg vein wall fibrinolytic activity to a level similar to that of the arm in hemiplegic patients.¹⁴³ This critical investigation again demonstrates the potential importance of restoring fibrinolytic activity to physiological levels through clinical interventions aimed at restoring normalized hemodynamics in the lower limb.

Nutrition. Tissue repair requires adequate nutrition in the form of protein, calories, vitamins and minerals.¹⁴⁴ There has been very little research into the effect of vitamins and minerals in wound healing although it has generally been reported that levels of vitamin A, vitamin D, iron, zinc, and selenium levels are often lower in chronic wound patients; therefore, it has been deemed that they may have a role in wound healing and may also influence the nonhealing of wounds. However, many of the studies in relation to these factors were performed long ago or were conducted using animal models.¹⁴⁵ One investigation by Hunt indicated that vitamin C (Ascorbic acid) had great importance in wound healing whereby collagen formation was impaired during wound healing in

guinea pigs that were partially deficient in vitamin C.¹⁴⁶ In this regard, vitamin C is a known cofactor for collagen synthesis, with a deficiency in vitamin C resulting in the breakdown of connective tissue in and around the walls of blood vessels, thereby resulting in poor healing of wounds.¹⁴⁷ A systematic literature review found that many leg ulcer patients have low levels of vitamin A, vitamin C, zinc, and carotenes and there is some evidence that zinc may have a beneficial effect on healing of venous ulcers in people with low baseline serum zinc levels.^{148,149}

CONCLUSION

The deposition of plasma derived matrix proteins in both acute wounds and CVI exhibit similarities in terms of the specific protein species deposited and their effects on surrounding tissue. In acute wounding, plasma-derived provisional matrix deposition during hemostasis is localized about the wounded area and is a consequence of a traumatic injury (Fig. 4). However, for chronic venous ulcers the plasma derived matrix deposition typically occurs as a consequence of CVI (effectively internal bleeding), is diffuse and formed before the formation of an open wound (Fig. 1b). The 'preconditioning' of the extravascular tissue with a clot-like matrix and excessive fluid is associated with influx and persistence of inflammatory cells (Figs. 3b and 4). Therapies which address CVI, such as compression therapy and/or limb elevation, positively influence hemodynamics in affected limbs resulting in reduction of plasma leakage into the extravascular tissues. This reduces the edema and seems to allow for the removal of aberrant matrix deposits, possibly by persistent immune phagocytes. The subsequent resolution of inflammation seems to be a prerequisite for allowing wound healing progression (Fig. 1c). Detail of the molecular factors and events involved in 'tissue preconditioning' during CVI remain poorly understood compared to those involved in 'normal' hemostasis, despite significant apparent similarities between these processes. Similarly, the potential role of epigenetics in modulating processes, such as the inflammatory response to CVI and resolution after treatment is an area of increasing research opportunity. Finally,

TAKE-HOME MESSAGES

Basic science

- Plasma derived proteins, such as fibrin(ogen), FN, and VN are deposited into wounds after an acute wounding event, where they form a provisional matrix to both inhibit blood loss and enable the initiation of tissue repair.
- These same proteins are deposited in pericapillary tissue as a consequence of venous insufficiency and may play a significant role in mediating tissue hypoxia and inflammatory processes, thereby 'preconditioning' the tissue for the development of a nonhealing venous ulcer.
- More extensive characterization of the molecular composition of these deposits and the effects they have on tissue pathology will be an important area of future research.

Clinical science

- Chronic nonhealing wounds potentially develop as a result of internal tissue injury which can essentially be characterized as internal bleeding followed by inflammatory processes, which are an attempt to 'heal' the injured tissue. However, the inflammatory response is perpetuated by persistent plasma leakage and associated matrix protein deposition as a consequence of the underlying venous insufficiency.
- Compression therapy has been shown to improve tissue hemodynamics, thereby reducing edema by reinforcing fluid reabsorption into the capillaries and lymphatic system. This presumably reduces aberrant matrix protein deposition, allowing endemic macrophages and neutrophils to break down and remove the inflammatory material.

Relevance to clinical care

- Compression therapies may be further optimized through a better understanding of the role of matrix deposition in the 'preconditioning' of lower limb tissues for development of a nonhealing wound.
- Exercises aimed at strengthening and improving calf muscle function may reduce pericapillary matrix protein deposition through improved hemodynamics.
- Leg elevation reduces edema and likely reduces the effects of tissue preconditioning of lower limb by similar mechanisms as compression and exercise.

research into the effects of compression therapies, exercise and nutrition on wound matrix biology will add to our understanding of how to effectively heal chronic wounds.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Natalie Pecheniuk for her useful discussions regarding the coagulation cascade and Ms. Jaz Lyons-Reid for her assistance with proof reading early drafts of the manuscript. The authors would also like to acknowledge the salary (J.A.B.), scholarships (D.A.B. and C.N.P.), and research support (T.J.P., J.A.B., D.A.B., C.N.P., and Z.U.) provided by the Wound Management Innovation Cooperative Research Centre.

AUTHOR DISCLOSURE AND GHOSTWRITING

Z.U. is a named inventor on patents related to VN/IGF complexes noted in this review. These complexes are licensed to Tissue Therapies Ltd., a company spun out of QUT to commercialize this technology. T.J.P. and Z.U. own shares in Tissue Therapies and ZU also provides consulting services to Tissue Therapies.

The content of this article was expressly written by the authors listed. No ghostwriters were used to write this article.

ABOUT THE AUTHORS

Dr. Tony Parker is a Lecturer in Anatomy and Physiology in the Faculty of Health, Queensland University of Technology (QUT) and research team leader of the Systems Biology Group within the Tissue Repair and Regeneration (TRR) Program at the Institute of Health and Biomedical Innovation

(IHBI), QUT. His research expertise includes tissue injury with an emphasis on matrix biology and biomarker discovery for diagnostic development. **Dr. James Broadbent** is a Research Associate within the TRR Program with expertise in proteomics and chronic wound inflammation. **Dr. Jacqui McGovern** is a Research Associate within the TRR Program with expertise in cutaneous tissue hypoxia and epidermogenesis. **Daniel Broszczak** is a senior PhD candidate within the TRR Program with expertise in the molecular composition of chronic wound fluid. **Christina Parker** is a registered nurse and senior PhD candidate in the School of Nursing, QUT with expertise in wound risk factor analysis. **Professor Zee Upton** is the Assistant Dean (Research) in the Faculty of Health at QUT, a biochemist, an inventor, and tissue engineer, recognized internationally for her research in growth factors, ECM proteins and wound healing. She is the founder of the TRR Program, an interdisciplinary consortium of 50+ researchers.

REFERENCES

- Briggs M and Closs S: The prevalence of leg ulceration: a review of the literature. *EWMA J* 2003; **3**: 14.
- Margolis DJ, Bilker W, Santanna J, and Baumgarten M: Venous leg ulcer: incidence and prevalence in the elderly. *J Am Acad Dermatol* 2002; **46**: 381.
- Abbade LP and Lastória S: Venous ulcer: epidemiology, physiopathology, diagnosis and treatment. *Int J Dermatol* 2005; **44**: 449.
- Posnett J and Franks PJ: The burden of chronic wounds in the UK. *Nurs Times* 2008; **104**: 44.
- Ebbeskog B, Skonda E, Lindholm C, Grauers M, and Ohman S: A follow-up study of leg ulcer patients in south Stockholm. *J Wound Care* 1999; **8**: 170.
- Reinke JM and Sorg H: Wound repair and regeneration. *Eur Surg Res* 2012; **49**: 35.
- Gordillo GM and Sen CK: Revisiting the essential role of oxygen in wound healing. *Am J Surg* 2003; **186**: 259.
- Li J, Chen J, and Kirsner R: Pathophysiology of acute wound healing. *Clin Dermatol* 2007; **25**: 9.
- Martin P: Wound healing—aiming for perfect skin regeneration. *Science* 1997; **276**: 75.
- Martin P and Leibovich SJ: Inflammatory cells during wound repair: the good, the bad and the ugly. *Trends Cell Biol* 2005; **15**: 599.
- Werner S, Krieg T, and Smola H: Keratinocyte-fibroblast interactions in wound healing. *J Invest Dermatol* 2007; **127**: 998.
- Wilgus TA: Immune cells in the healing skin wound: influential players at each stage of repair. *Pharmacol Res* 2008; **58**: 112.
- Yager DR and Nwomeh BC: The proteolytic environment of chronic wounds. *Wound Repair Regen* 1999; **7**: 433.
- Menke NB, Ward KR, Witten TM, Bonchev DG, and Diegelmann RF: Impaired wound healing. *Clin Dermatol* 2007; **25**: 19.
- Browse NL and Burnand KG: The cause of venous ulceration. *Lancet* 1982; **2**: 243.
- Clark RA, Lanigan JM, DellaPelle P, Manseau E, Dvorak HF, and Colvin RB: Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound reepithelialization. *J Invest Dermatol* 1982; **79**: 264.
- Weisel JW and Litvinov RI: Mechanisms of fibrin polymerization and clinical implications. *Blood* 2013; **121**: 1712.
- Ruggeri ZM: The role of von Willebrand factor in thrombus formation. *Thromb Res* 2007; **120 Suppl 1**: S5.
- Lippi G, Favaloro EJ, Franchini M, and Guidi GC: Milestones and perspectives in coagulation and hemostasis. *Semin Thromb Hemost* 2009; **35**: 9.
- Riedel T, Suttner J, Brynda E, Houska M, Medved L, and Dyr JE: Fibrinopeptides A and B release in the process of surface fibrin formation. *Blood* 2011; **117**: 1700.
- Richardson VR, Cordell P, Standeven KF, and Carter AM: Substrates of Factor XIII-A: roles in thrombosis and wound healing. *Clin Sci* 2013; **124**: 123.
- Skogen WF, Senior RM, Griffin GL, and Wilner GD: Fibrinogen-derived peptide B beta 1–42 is a multidomained neutrophil chemoattractant. *Blood* 1988; **71**: 1475.
- Gersh KC, Edmondson KE, and Weisel JW: Flow rate and fibrin fiber alignment. *J Thromb Haemost* 2010; **8**: 2826.
- Campbell RA, Aleman M, Gray LD, Falvo MR, and Wolberg AS: Flow profoundly influences fibrin network structure: implications for fibrin formation and clot stability in haemostasis. *Thromb Haemost* 2010; **104**: 1281.
- Tamkun JW, Schwarzbauer JE, and Hynes RO: A single rat fibronectin gene generates three different mRNAs by alternative splicing of a complex exon. *Proc Natl Acad Sci U S A* 1984; **81**: 5140.
- Yamada KM and Kennedy DW: Fibroblast cellular and plasma fibronectins are similar but not identical. *J Cell Biol* 1979; **80**: 492.
- Pankov R and Yamada KM: Fibronectin at a glance. *J Cell Sci* 2002; **115**: 3861.

28. Ruoslahti E: Fibronectin and its receptors. *Ann Rev Biochem* 1988; **57**: 375.
29. Gardner JM and Hynes RO: Interaction of fibronectin with its receptor on platelets. *Cell* 1985; **42**: 439.
30. Plow EF, McEver RP, Collier BS, Woods VL, Jr., Marguerie GA, and Ginsberg MH: Related binding mechanisms for fibrinogen, fibronectin, von Willebrand factor, and thrombospondin on thrombin-stimulated human platelets. *Blood* 1985; **66**: 724.
31. Romberger DJ: Fibronectin. *Int J Biochem Cell Biol* 1997; **29**: 939.
32. Mosher DF: Cross-linking of cold-insoluble globulin by fibrin-stabilizing factor. *J Biol Chem* 1975; **250**: 6614.
33. Mosher DF: Action of fibrin-stabilizing factor on cold-insoluble globulin and alpha2-macroglobulin in clotting plasma. *J Biol Chem* 1976; **251**: 1639.
34. Cho J, Degen JL, Collier BS, and Mosher DF: Fibrin but not adsorbed fibrinogen supports fibronectin assembly by spread platelets. Effects of the interaction of alphaIIb beta3 with the C terminus of the fibrinogen gamma-chain. *J Biol Chem* 2005; **280**: 35490.
35. Cho J and Mosher DF: Characterization of fibronectin assembly by platelets adherent to adsorbed laminin-111. *J Thromb Haemost* 2006; **4**: 943.
36. Seiffert D and Smith JW: The cell adhesion domain in plasma vitronectin is cryptic. *J Biol Chem* 1997; **272**: 13705.
37. Schwartz I, Seger D, and Shaltiel S: Vitronectin. *Int J Biochem Cell Biol* 1999; **31**: 539.
38. Podor TJ, Peterson CB, Lawrence DA, Stefansson S, Shaughnessy SG, Foulon DM, Butcher M, and Weitz JI: Type 1 plasminogen activator inhibitor binds to fibrin via vitronectin. *J Biol Chem* 2000; **275**: 19788.
39. Zhou A, Huntington JA, Pannu NS, Carrell RW, and Read RJ: How vitronectin binds PAI-1 to modulate fibrinolysis and cell migration. *Nat Struct Biol* 2003; **10**: 541.
40. Seiffert D, Ciambone G, Wagner NV, Binder BR, and Loskutoff DJ: The somatomedin B domain of vitronectin. Structural requirements for the binding and stabilization of active type 1 plasminogen activator inhibitor. *J Biol Chem* 1994; **269**: 2659.
41. Trelle MB, Hirschberg D, Jansson A, Ploug M, Roepstorff P, Andreassen PA, and Jorgensen TJ: Hydrogen/Deuterium exchange mass spectrometry reveals specific changes in the local flexibility of plasminogen activator inhibitor 1 upon binding to the somatomedin B domain of vitronectin. *Biochemistry* 2012; **51**: 8256.
42. Morgenstern E, Gnad U, Preissner KT, Dierichs R, Belleli A, Chestukhin A, Schwartz I, and Shaltiel S: Localization of protein kinase A and vitronectin in resting platelets and their translocation onto fibrin fibers during clot formation. *Eur J Cell Biol* 2001; **80**: 87.
43. Stefansson S and Lawrence DA: The serpin PAI-1 inhibits cell migration by blocking integrin alpha V beta 3 binding to vitronectin. *Nature* 1996; **383**: 441.
44. Leavesley DI, Ferguson GD, Wayner EA, and Cheres DA: Requirement of the integrin beta 3 subunit for carcinoma cell spreading or migration on vitronectin and fibrinogen. *J Cell Biol* 1992; **117**: 1101.
45. Czekay RP, Aertgeerts K, Curriden SA, and Loskutoff DJ: Plasminogen activator inhibitor-1 detaches cells from extracellular matrices by inactivating integrins. *J Cell Biol* 2003; **160**: 781.
46. Chavakis T, Boeckel N, Santoso S, Voss R, Isordia-Salas I, Pixley RA, Morgenstern E, Colman RW, and Preissner KT: Inhibition of platelet adhesion and aggregation by a defined region (Gly-486-Lys-502) of high molecular weight kininogen. *J Biol Chem* 2002; **277**: 23157.
47. Wu YP, Bloemendal HJ, Voest EE, Logtenberg T, de Groot PG, Gebbink MF, and de Boer HC: Fibrin-incorporated vitronectin is involved in platelet adhesion and thrombus formation through homotypic interactions with platelet-associated vitronectin. *Blood* 2004; **104**: 1034.
48. Kjoller L: The urokinase plasminogen activator receptor in the regulation of the actin cytoskeleton and cell motility. *Biol Chem* 2002; **383**: 5.
49. Mesnard L, Rafat C, Vandermeersch S, Hertig A, Cathelin D, Xu-Dubois YC, Jouanneau C, Keller AC, Ribeil JA, Leite-de-Moraes MC, and Rondeau E: Vitronectin dictates intraglomerular fibrinolysis in immune-mediated glomerulonephritis. *Faseb J* 2011; **25**: 3543.
50. Stacey MC, Burnand KG, Bhogal BS, and Black MM: Pericapillary fibrin deposits and skin hypoxia precede the changes of lipodermatosclerosis in limbs at increased risk of developing a venous ulcer. *Cardiovasc Surg* 2000; **8**: 372.
51. Li R, Ren M, Chen N, Luo M, Zhang Z, and Wu J: Vitronectin increases vascular permeability by promoting VE-cadherin internalization at cell junctions. *PLoS One* 2012; **7**: e37195.
52. Kricker JA, Towne CL, Firth SM, Herington AC, and Upton Z: Structural and functional evidence for the interaction of insulin-like growth factors (IGFs) and IGF binding proteins with vitronectin. *Endocrinology* 2003; **144**: 2807.
53. Upton Z, Webb H, Hale K, Yandell CA, McMurtry JP, Francis GL, and Ballard FJ: Identification of vitronectin as a novel insulin-like growth factor-II binding protein. *Endocrinology* 1999; **140**: 2928.
54. Gui Y and Murphy LJ: Insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3) binds to fibronectin (FN): demonstration of IGF-I/IGFBP-3/fn ternary complexes in human plasma. *J Clin Endocrinol Metab* 2001; **86**: 2104.
55. Schoppet M, Chavakis T, Al-Fakhri N, Kanse SM, and Preissner KT: Molecular interactions and functional interference between vitronectin and transforming growth factor-beta. *Lab Invest* 2002; **82**: 37.
56. Rahman S, Patel Y, Murray J, Patel KV, Sumathipala R, Sobel M, and Wijelath ES: Novel hepatocyte growth factor (HGF) binding domains on fibronectin and vitronectin coordinate a distinct and amplified Met-integrin induced signaling pathway in endothelial cells. *BMC Cell Biol* 2005; **6**: 8.
57. Hollier B, Harkin DG, Leavesley D, and Upton Z: Responses of keratinocytes to substrate-bound vitronectin: growth factor complexes. *Exp Cell Res* 2005; **305**: 221.
58. Hyde C, Hollier B, Anderson A, Harkin D, and Upton Z: Insulin-like growth factors (IGF) and IGF-binding proteins bound to vitronectin enhance keratinocyte protein synthesis and migration. *J Invest Dermatol* 2004; **122**: 1198.
59. Beanes SR, Dang C, Soo C, and Ting K: Skin repair and scar formation: the central role of TGF-beta. *Expert Rev Mol Med* 2003; **5**: 1.
60. Upton Z, Wallace HJ, Shooter GK, van Lonkhuyzen DR, Yeoh-Ellerton S, Rayment EA, Fleming JM, Broszczak D, Queen D, Sibbald RG, Leavesley DI, and Stacey MC: Human pilot studies reveal the potential of a vitronectin: growth factor complex as a treatment for chronic wounds. *Int Wound J* 2011; **8**: 522.
61. Upton Z, Cuttle L, Noble A, Kempf M, Topping G, Malda J, Xie Y, Mill J, Harkin DG, Kravchuk O, Leavesley DI, and Kimble RM: Vitronectin: growth factor complexes hold potential as a wound therapy approach. *J Invest Dermatol* 2008; **128**: 1535.
62. O'Meara S, Cullum NA, and Nelson EA: Compression for venous leg ulcers. *Cochrane Database Syst Rev* 2009; **21**: CD000265.
63. Coghlan D: Chronic venous insufficiency. *ASUM Ultrasound Bulletin* 2004; **7**: 14.
64. Casey G: Chronic wound healing: Leg ulcers. *Kai Tiaki Nurs N Z* 2011; **17**: 24.
65. Kerstein MD: The non-healing leg ulcer: peripheral vascular disease, chronic venous insufficiency, and ischemic vasculitis. *Ostomy Wound Manage* 1996; **42**: 19S.
66. Fowkes FGR, Evans CJ, and Lee AJ: Prevalence and risk factors of chronic venous insufficiency. *Angiology* 2001; **52**: S5.
67. Beers MH, Porter RS, Jones TV, Kaplan JL, and Berkwitz M (eds): *The Merck Manual of Diagnosis and Therapy*. New Jersey: Merck Research Laboratories, 2006.
68. Scott TE, LaMorte WW, Gorin DR, and Menzoian JO: Risk factors for chronic venous insufficiency: a dual case-control study. *J Vasc Surg* 1995; **22**: 622.
69. Gilliver SC, Ruckshanthi JP, Atkinson SJ, and Ashcroft GS: Androgens influence expression of matrix proteins and proteolytic factors during cutaneous wound healing. *Lab Invest* 2007; **87**: 871.
70. Black SB: Venous stasis ulcers: a review. *Ostomy Wound Manage* 1995; **41**: 20.

71. Callam MJ: Epidemiology of varicose veins. *Br J Surg* 1994; **81**: 167.
72. Walshe C: Living with a venous leg ulcer: a descriptive study of patients' experiences. *J Adv Nurs* 1995; **22**: 1092.
73. Liu YC, Margolis DJ, and Isseroff RR: Does inflammation have a role in the pathogenesis of venous ulcers? A critical review of the evidence. *J Invest Dermatol* 2011; **131**: 818.
74. Maffei FH, Magaldi C, Pinho SZ, Lastoria S, Pinho W, Yoshida WB, and Rollo HA: Varicose veins and chronic venous insufficiency in Brazil: prevalence among 1755 inhabitants of a country town. *Int J Epidemiol* 1986; **15**: 210.
75. Burnand KG, Whimster I, Naidoo A, and Browse NL: Pericapillary fibrin in the ulcer-bearing skin of the leg: the cause of lipodermatosclerosis and venous ulceration. *Br Med J* 1982; **285**: 1071.
76. Simka M: Cellular and molecular mechanisms of venous leg ulcers development—the "puzzle" theory. *Int Angiol* 2010; **29**: 1.
77. Agren MS and Werthen M: The extracellular matrix in wound healing: a closer look at therapeutics for chronic wounds. *Int J Low Extrem Wounds* 2007; **6**: 82.
78. Coleridge Smith P: The causes of skin damage and leg ulceration in chronic venous disease. *Int J Low Extrem Wounds* 2006; **5**: 160.
79. Burnand KG, Clemenson G, Whimster I, Gaunt J, and Browse NL: The effect of sustained venous hypertension on the skin capillaries of the canine hind limb. *Br J Surg* 1982; **69**: 41.
80. Herrick SE, Sloan P, McGurk M, Freak L, McCollum CN, and Ferguson MW: Sequential changes in histologic pattern and extracellular matrix deposition during the healing of chronic venous ulcers. *Am J Pathol* 1992; **141**: 1085.
81. Falanga V, Kirsner R, Katz MH, Gould E, Eaglstein WH, and McFalls S: Pericapillary fibrin cuffs in venous ulceration. Persistence with treatment and during ulcer healing. *J Dermatol Surg Oncol* 1992; **18**: 409.
82. Joshi A and Sloan P: Role of "fibrin" cuffs in chronic nonspecific oral ulceration. *Wound Repair Regen* 2004; **12**: 18.
83. Neumann HA, van den Broek MJ, Boersma IH, and Veraart JC: Transcutaneous oxygen tension in patients with and without pericapillary fibrin cuffs in chronic venous insufficiency, porphyria cutanea tarda and non-venous leg ulcers. *Vasa* 1996; **25**: 127.
84. Gschwandtner ME and Ehringer H: Microcirculation in chronic venous insufficiency. *Vasc Med* 2001; **6**: 169.
85. Kjoller L, Engelholm LH, Hoyer-Hansen M, Dano K, Bugge TH, and Behrendt N: uPARAP/endo180 directs lysosomal delivery and degradation of collagen IV. *Exp Cell Res* 2004; **293**: 106.
86. Davalos D and Akassoglou K: Fibrinogen as a key regulator of inflammation in disease. *Semin Immunopathol* 2012; **34**: 43.
87. Thomas PR, Nash GB, and Dormandy JA: White cell accumulation in dependent legs of patients with venous hypertension: a possible mechanism for trophic changes in the skin. *Br Med J* 1988; **296**: 1693.
88. Takase S, Schmid-Schonbein G, and Bergan JJ: Leukocyte activation in patients with venous insufficiency. *J Vasc Surg* 1999; **30**: 148.
89. Richardson DL, Pepper DS, and Kay AB: Chemotaxis for human monocytes by fibrinogen-derived peptides. *Br J Haematol* 1976; **32**: 507.
90. Gross TJ, Leavell KJ, and Peterson MW: CD11b/CD18 mediates the neutrophil chemotactic activity of fibrin degradation product D domain. *Thromb Haemost* 1997; **77**: 894.
91. Abu-Own A, Shami SK, Chittenden SJ, Farrah J, Scurr JH, and Coleridge Smith PD: Microangiopathy of the skin and the effect of leg compression in patients with chronic venous insufficiency. *J Vasc Surg* 1994; **19**: 1074.
92. Peschen M, Lahaye T, Hennig B, Weyl A, Simon JC, and Vanscheidt W: Expression of the adhesion molecules ICAM-1, VCAM-1, LFA-1 and VLA-4 in the skin is modulated in progressing stages of chronic venous insufficiency. *Acta Derm Venereol* 1999; **79**: 27.
93. Duperray A, Languino LR, Plescia J, McDowall A, Hogg N, Craig AG, Berendt AR, and Altieri DC: Molecular identification of a novel fibrinogen binding site on the first domain of ICAM-1 regulating leukocyte-endothelium bridging. *J Biol Chem* 1997; **272**: 435.
94. Ugarova TP, Lishko VK, Podolnikova NP, Okumura N, Merkulov SM, Yakubenko VP, Yee VC, Lord ST, and Haas TA: Sequence gamma 377–395(P2), but not gamma 190–202(P1), is the binding site for the alpha M1-domain of integrin alpha M beta 2 in the gamma C-domain of fibrinogen. *Biochemistry* 2003; **42**: 9365.
95. Ugarova TP, Solovjov DA, Zhang L, Loukinov DI, Yee VC, Medved LV, and Plow EF: Identification of a novel recognition sequence for integrin alphaM beta2 within the gamma-chain of fibrinogen. *J Biol Chem* 1998; **273**: 22519.
96. Ugarova TP and Yakubenko VP: Recognition of fibrinogen by leukocyte integrins. *Ann N Y Acad Sci* 2001; **936**: 368.
97. Podor TJ, Campbell S, Chindemi P, Foulon DM, Farrell DH, Walton PD, Weitz JI, and Peterson CB: Incorporation of vitronectin into fibrin clots. Evidence for a binding interaction between vitronectin and gamma A/gamma' fibrinogen. *J Biol Chem* 2002; **277**: 7520.
98. Kolbach DN, Hamulyak K, Prins MH, Neumann HA, and Cleutjens JP: Severity of venous insufficiency is related to the density of microvascular deposition of PAI-1, uPA and von Willebrand factor. *Vasa* 2004; **33**: 19.
99. Preissner KT: Specific binding of plasminogen to vitronectin. Evidence for a modulatory role of vitronectin on fibrin(ogen)-induced plasmin formation by tissue plasminogen activator. *Biochem Biophys Res Commun* 1990; **168**: 966.
100. Ritchie H, Robbie LA, Kinghorn S, Exley R, and Booth NA: Monocyte plasminogen activator inhibitor 2 (PAI-2) inhibits u-PA-mediated fibrin clot lysis and is cross-linked to fibrin. *Thromb Haemost* 1999; **81**: 96.
101. Stacey MC and Mata SD: Lower levels of PAI-2 may contribute to impaired healing in venous ulcers—a preliminary study. *Cardiovasc Surg* 2000; **8**: 381.
102. Pan CP, Shi Y, Amin K, Greenberg CS, Haroon Z, and Faris GW: Wound healing monitoring using near infrared fluorescent fibrinogen. *Biomed Opt Express* 2010; **1**: 285.
103. Bishop A: Role of oxygen in wound healing. *J Wound Care* 2008; **17**: 399.
104. Remensnyder JP and Majno G: Oxygen gradients in healing wounds. *Am J Pathol* 1968; **52**: 301.
105. Sen CK: Wound healing essentials: let there be oxygen. *Wound Repair Regen* 2009; **17**: 1.
106. Mustoe TA, Tae Ahn S, Tarpley JE, and Pierce GF: Role of hypoxia in growth factor responses: differential effects of basic fibroblast growth factor and platelet-derived growth factor in an ischemic wound model. *Wound Repair Regen* 1994; **2**: 277.
107. Zhao LL, Davidson JD, Wee SC, Roth SI, and Mustoe TA: Effect of hyperbaric oxygen and growth factors on rabbit ear ischemic ulcers. *Arch Surg* 1994; **129**: 1043.
108. Clyne CA, Ramsden WH, Chant AD, and Webster JH: Oxygen tension on the skin of the gaiter area of limbs with venous disease. *Br J Surg* 1985; **72**: 644.
109. Greiling D and Clark RA: Fibronectin provides a conduit for fibroblast transmigration from collagenous stroma into fibrin clot provisional matrix. *J Cell Sci* 1997; **110** (Pt 7): 861.
110. Xu J and Clark RA: Extracellular matrix alters PDGF regulation of fibroblast integrins. *J Cell Biol* 1996; **132**: 239.
111. Mogford JE, Tawil N, Chen A, Gies D, Xia Y, and Mustoe TA: Effect of age and hypoxia on TGFbeta1 receptor expression and signal transduction in human dermal fibroblasts: impact on cell migration. *J Cell Physiol* 2002; **190**: 259.
112. Siddiqui A, Galiano RD, Connors D, Gruskin E, Wu L, and Mustoe TA: Differential effects of oxygen on human dermal fibroblasts: acute versus chronic hypoxia. *Wound Repair Regen* 1996; **4**: 211.
113. Falanga V, Zhou L, and Yufit T: Low oxygen tension stimulates collagen synthesis and COL1A1 transcription through the action of TGF-beta1. *J Cell Physiol* 2002; **191**: 42.
114. Steinbrech DS, Longaker MT, Mehrara BJ, Saadeh PB, Chin GS, Gerrets RP, Chau DC, Rowe NM, and Gittes GK: Fibroblast response to hypoxia: the relationship between angiogenesis and matrix regulation. *J Surg Res* 1999; **84**: 127.

115. Yamanaka M and Ishikawa O: Hypoxic conditions decrease the mRNA expression of pro α 1(I) and (III) collagens and increase matrix metalloproteinases-1 of dermal fibroblasts in three-dimensional cultures. *J Dermatol Sci* 2000; **24**: 99.
116. De Jong L and Kemp A: Stoichiometry and kinetics of the prolyl 4-hydroxylase partial reaction. *Biochim Biophys Acta* 1984; **787**: 105.
117. Siegel RC, Pinnell SR, and Martin GR: Cross-linking of collagen and elastin. Properties of lysyl oxidase. *Biochemistry* 1970; **9**: 4486.
118. Uzawa K, Grzesik WJ, Nishiura T, Kuznetsov SA, Robey PG, Brenner DA, and Yamauchi M: Differential expression of human lysyl hydroxylase genes, lysine hydroxylation, and cross-linking of type I collagen during osteoblastic differentiation *in vitro*. *J Bone Miner Res* 1999; **14**: 1272.
119. Kao WW, Berg RA, and Prockop DJ: Ascorbate increases the synthesis of procollagen hydroxyproline by cultured fibroblasts from chick embryo tendons without activation of prolyl hydroxylase. *Biochim Biophys Acta* 1975; **411**: 202.
120. Baldock C, Oberhauser AF, Ma L, Lammie D, Siegler V, Mithieux SM, Tu Y, Chow JY, Suleman F, Malfois M, Rogers S, Guo L, Irving TC, Wess TJ, and Weiss AS: Shape of tropoelastin, the highly extensible protein that controls human tissue elasticity. *Proc Natl Acad Sci U S A* 2011; **108**: 4322.
121. Berk JL, Hatch CA, Morris SM, Stone PJ, and Goldstein RH: Hypoxia suppresses elastin repair by rat lung fibroblasts. *Am J Physiol Lung Cell Mol Physiol* 2005; **289**: L931.
122. Dalton SJ, Mitchell DC, Whiting CV, and Tarlton JF: Abnormal extracellular matrix metabolism in chronically ischemic skin: a mechanism for dermal failure in leg ulcers. *J Invest Dermatol* 2005; **125**: 373.
123. Dalton SJ, Whiting CV, Bailey JR, Mitchell DC, and Tarlton JF: Mechanisms of chronic skin ulceration linking lactate, transforming growth factor- β , vascular endothelial growth factor, collagen remodeling, collagen stability, and defective angiogenesis. *J Invest Dermatol* 2007; **127**: 958.
124. Kim BC, Kim HT, Park SH, Cha JS, Yufit T, Kim SJ, and Falanga V: Fibroblasts from chronic wounds show altered TGF- β -signaling and decreased TGF- β Type II receptor expression. *J Cell Physiol* 2003; **195**: 331.
125. Kim HJ, Kim PK, Bae SM, Son HN, Thoudam DS, Kim JE, Lee BH, Park RW, and Kim IS: Transforming growth factor- β -induced protein (TGF β 1 β /beta ig-h3) activates platelets and promotes thrombogenesis. *Blood* 2009; **114**: 5206.
126. O'Meara S, Tierney J, Cullum N, Bland JM, Franks PJ, Mole T, and Scriven M: Four layer bandage compared with short stretch bandage for venous leg ulcers: systematic review and meta-analysis of randomised controlled trials with data from individual patients. *BMJ* 2009; **338**: b1344.
127. O'Brien JF, Grace PA, Perry IJ, Hannigan A, Clarke Moloney M, and Burke PE: Randomized clinical trial and economic analysis of four-layer compression bandaging for venous ulcers. *Br J Surg* 2003; **90**: 794.
128. Partsch H: Understanding the pathophysiological effects of compression. In: *European Wound Management Association, Position Document: Understanding Compression Therapy*, edited by Calne S. London: Medical Education Partnership Ltd., 2003, 2. http://ewma.org/fileadmin/user_upload/EWMA/pdf/Position_Documents/2003/Spring_2003__English_.pdf
129. Moffatt CJ: Understanding compression therapy. In: *European Wound Management Association, Position Document: Understanding Compression Therapy*, edited by Calne S. London: Medical Education Partnership Ltd., 2003, 1. http://ewma.org/fileadmin/user_upload/EWMA/pdf/Position_Documents/2003/Spring_2003__English_.pdf
130. Williams C: Leg ulcer after care: the role of compression hosiery. *Br J Nurs (Mark Allen Publishing)* 2000; **9**: 822.
131. Coull A, Tolson D, and McIntosh J: Class-3c compression bandaging for venous ulcers: comparison of spiral and figure-of-eight techniques. *J Adv Nurs* 2006; **54**: 274.
132. Clark M: Compression bandages: principles and definitions. In: *European Wound Management Association, Position Document: Understanding Compression Therapy*, edited by Calne S. London: Medical Education Partnership Ltd., 2003, 5. http://ewma.org/fileadmin/user_upload/EWMA/pdf/Position_Documents/2003/Spring_2003__English_.pdf
133. Comerota AJ, Chouhan V, Harada RN, Sun L, Hosking J, Veermansunemi R, Comerota AJ, Jr., Schlappy D, and Rao AK: The fibrinolytic effects of intermittent pneumatic compression: mechanism of enhanced fibrinolysis. *Ann Surg* 1997; **226**: 306.
134. Taradaj J, Franek A, Blaszcak E, Polak A, Chmielewska D, Krol P, and Dolibog P: Physical therapy in the treatment of venous leg ulcers: biophysical mechanisms. *Wounds-a Compend Clin Res Prac* 2012; **24**: 138.
135. Australian and New Zealand Clinical Practice Guideline for Prevention and Management of Venous Leg Ulcers: Cambridge Publishing. 2011, www.awma.com.au/publications/2011_awma_vlug.pdf
136. Back TL, Padberg FT, Jr., Araki CT, Thompson PN, and Hobson RW, 2nd.: Limited range of motion is a significant factor in venous ulceration. *J Vasc Surg* 1995; **22**: 519.
137. Dix FP, Brooke R, and McCollum CN: Venous disease is associated with an impaired range of ankle movement. *Eur J Vasc Endovasc Surg* 2003; **25**: 556.
138. Yang D, Vandongen YK, and Stacey MC: Changes in calf muscle function in chronic venous disease. *Cardiovasc Surg* 1999; **7**: 451.
139. Jull A, Parag V, Walker N, Maddison R, Kerse N, and Johns T: The prepare pilot RCT of home-based progressive resistance exercises for venous leg ulcers. *J Wound Care* 2009; **18**: 497.
140. Kan YM and Delis KT: Hemodynamic effects of supervised calf muscle exercise in patients with venous leg ulceration: a prospective controlled study. *Arch Surg* 2001; **136**: 1364.
141. Yang D, Vandongen YK, and Stacey MC: Effect of exercise on calf muscle pump function in patients with chronic venous disease. *Br J Surg* 1999; **86**: 338.
142. Barnes MD, Mani R, Barrett DF, and White JE: Changes in skin microcirculation at peritrophic sites in patients with chronic venous ulcers during leg elevation. *Phlebology* 1992; **7**: 36.
143. Karaca M and Nilsson IM: Fibrinolytic activity in hemiplegic patients. *Acta Med Scand* 1971; **189**: 325.
144. Wipke-Tevis DD and Stotts NA: Nutritional risk, status, and intake of individuals with venous ulcers: a pilot study. *J Vasc Nurs* 1996; **14**: 27.
145. Rojas AI and Phillips TJ: Patients with chronic leg ulcers show diminished levels of vitamins A and E, carotenes, and zinc. *Dermatol Surg* 1999; **25**: 601.
146. Hunt AH: The role of vitamin C in wound healing. *Br J Surg* 1941; **28**: 436.
147. Lazareth I, Hubert S, Michon-Pasturel U, and Priollet P: Vitamin C deficiency and leg ulcers. A case control study. *J Mal Vasc* 2007; **32**: 96.
148. Heinen MM, van Achterberg T, Reimer WSo, van den Kerkhof PCM, and de Laat E: Venous leg ulcer patients: a review of the literature on lifestyle and pain-related interventions. *J Clin Nurs* 2004; **13**: 355.
149. Robson MC, Cooper DM, Aslam R, Gould LJ, Harding KG, Margolis DJ, Ochs DE, Serena TE, Snyder RJ, Steed DL, Thomas DR, and Wiersma-Bryant L: Guidelines for the treatment of venous ulcers. *Wound Repair Regen* 2006; **14**: 649.