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REVIEW ARTICLE

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Recent developments in targets for ischemic foot disease

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Abstract

Diabetes is a key risk factor for ischaemic foot disease, which causes pain, tissue loss, hospital admission, and major amputation. Currently, treatment focuses on revascularisation, but many patients are unsuitable for surgery and revascularisation is frequently unsuccessful. The authors describe recent research in animal models and clinical trials investigating novel medical targets for ischaemia, including theories about impaired wound healing, animal models for limb ischaemia and recent randomised controlled trials testing novel medical therapies. Novel targets identified in animal models included stimulating mobilisation of CD34+ progenitor cells through upregulating oncostatin *M* or microRNA-181, downregulating tumour necrosis factor superfamily member 14, or activating the Wingless pathway. Within the ischaemic limb vasculature, upregulation of apolipoprotein *L* domain containing 1, microRNA-130b or long noncoding RNA that enhances endothelial nitric oxide synthase expression promoted limb blood supply recovery, angiogenesis, and arteriogenesis. Similarly, administration of soluble guanylate cyclase stimulators riociguat or praliciguat or 3-ketoacyl-CoA thiolase inhibitor trimetazidine promoted blood flow recovery. Translating pre-clinical findings to patients has been challenging, mainly due to limitations in clinically translatable animal models of human disease. Promising results have been reported for administering plasmids encoding hepatocyte growth factor or intra-arterial injection of bone marrow derived cells in small clinical trials. It remains to be seen whether these high resource therapies can be developed to be widely applicable. In conclusion, an ever-expanding list of potential targets for medical revascularisation is being identified. It is hoped that through ongoing research and further larger clinical trials, these will translate into new broadly applicable therapies to improve outcomes.

KEYWORDS

clinical trials, diabetes-related foot disease, ischaemia, mouse models, peripheral artery disease, ulcer

1 | INTRODUCTION

Ischaemia is the key risk factor for the failure of diabetes-related foot ulceration healing and increases the risk of hospital admission and major amputation.[1] Together with ischaemic gangrene and ischaemic rest pain, ischaemic ulceration constitutes one of the presentations of chronic limb threatening ischaemia (CLTI). The treatment of CLTI focuses on revascularisation by open or endovascular surgery along with treatment of associated infection and wound debridement as recommended by global guidelines.[1,2] Revascularisation is the main treatment of CLTI but is not always possible or successful with minor and major amputation still being a common outcome, thus highlighting the need for identifying additional treatments.[1,3] This narrative review discusses the pathological factors driving impaired wound healing and the findings from recent animal model studies and clinical trials to develop new therapies for CLTI and ischaemic wounds.

2 | ANIMAL MODELS OF FOOT ISCHEMIA

Animal studies of peripheral artery disease (PAD) employ surgically induced hind limb ischaemia (HLI) (Supplement Figure 1).[4,5] Most studies have used surgical ligation or excision.[5,6] Less commonly, artery occlusion was achieved by cautery or injecting particles into the artery (Supplement Figure 1).[7,8] Photochemical induction of thrombotic artery occlusion has also been used.[9] Mice are the most commonly used animal models due to their low cost and the availability of a range of genetic tools to study the effects of individual pathways. Other species used include rats, rabbits and pigs.[5] Mouse strains vary in terms of their susceptibility to ischaemia induction, with C57BL/6 being relatively resistant whilst BALB/c being very sensitive.[10] In BALB/c mice, proximal as opposed to distal artery occlusion causes more severe ischaemia.[11] In C57BL/6 mice, which is a common background for most genetically modified models, excision of the femoral artery causes a rapid reduction in limb blood supply, which recovers to normal within 4 weeks (Supplement Figure 2).[6] Placement of ameroid constrictors around the femoral artery causes gradual femoral artery narrowing over 3–5 days, and it is believed to cause less rapid increase in shear stress, more limited induction of growth factors and less severe mobilisation of inflammatory cells that limits blood flow recovery.[12] This model was recently extended by a second stage procedure performed 2 weeks after ameroid placement when the femoral artery and any collateral arteries were excised (Supplement Figure 2).[6] This two-stage procedure leads to prolonged severe ischaemia and impaired ambulation not evident after femoral excision alone, better simulating the human presentation of PAD (Supplement Figure 2).[6] It should be noted, that all these animal models have multiple limitations in simulating human PAD. For example, ischaemia induced in these animal models does not result from atherosclerosis, as in humans. Furthermore, there are multiple pathophysiological differences between humans and rodents. As a result, translating findings from animal models to patients remains challenging.

3 | TARGETING INFLAMMATION IN ISCHEMIC AND DIABETES-RELATED WOUNDS

3.1 | Rewiring macrophage polarisation

Ischaemia drives an inflammatory response that contributes to tissue perfusion recovery. Macrophages, which produce growth factors for vascular remodelling, are essential for this process. Uncontrolled macrophage activation is implicated in delayed wound healing and thus manipulation of macrophages has been proposed to promote wound healing. Iron overload has been implicated in ischaemia associated with renal failure and induces a pro-inflammatory M1 activation state in macrophages, triggering senescence in resident fibroblasts and impairing wound healing.[13,14] A study using a HLI mouse model found that the lipid mediator resolvin D1, acting through its receptor ALX/FPR2 on macrophages, promotes perfusion recovery.[15] Tissue macrophages can be instructed to resemble tumour-associated macrophages, thereby helping in suppressing inflammation, stimulating angiogenesis, and activating fibroblasts, ultimately leading to an improvement in diabetic wound healing.[16] In a study conducted on 60 individuals without diabetes and 53 patients with type 2 diabetes, we examined monocyte subsets based on CD (cluster of differentiation) 14 and CD16 markers and characterised them as M1 (CD68(+)CCR2(+)) and M2 (CX3CR1(+) CD206(+)/CD163(+)) monocytes based on in vitro phenotypes.[17] Patients with diabetes showed an imbalanced M1/M2 ratio, primarily due to a reduction in M2 monocytes. The M1/M2 ratio was correlated with waist circumference and HbA1c levels. Among diabetic patients, the decrease in M2 monocytes and increase in the M1/M2 ratio were specifically associated with microangiopathy. Additionally, there was a decrease in M2 monocytes in the bone marrow of diabetic patients, with a relative excess of M2 cells in the bloodstream. Stimulation of the bone marrow with granulocyte-colony stimulating factor mobilised M2 macrophages in diabetic individuals but not in healthy individuals. Thus, the study demonstrates that type 2 diabetes leads to a significant reduction in anti-inflammatory M2 monocytes, primarily due to dysregulation in bone marrow function. This defect may have a negative impact on microangiopathy, suggesting that the imbalance in monocyte polarisation may contribute to the development of complications in distant organs, including the wound sites.[17] To understand the implications of these findings in the diabetic ischaemic wound healing process, it should be emphasised that M2 macrophages, which are defective in diabetes, are directly implicated in angiogenesis and responses to ischaemia. M2 macrophages secrete various factors and cytokines that promote angiogenesis. In addition to growth factors, M2 macrophages produce enzymes, such as matrix metalloproteinases that facilitate the remodelling of the extracellular matrix, allowing endothelial cells to migrate and form new vessels. They also secrete cytokines such as interleukin-8 (IL-8) and tumour necrosis factor-alpha that promote endothelial cell proliferation and vessel sprouting. Furthermore, M2 macrophages can directly interact with endothelial cells through cell-cell contact and the

release of adhesive molecules. These interactions help in the stabilisation and maturation of newly formed blood vessels.[18]

Comorbidities often observed in diabetes, such as dyslipidemia, can also contribute to an imbalance in monocyte phenotype and can sustain the ischaemic component of delayed wound healing. In individuals with familial and non-familial (NFH) hypercholesterolaemia, compared to those with normocholesterolemia (CTRL), higher levels of circulating pro-inflammatory M1 monocytes were detected, marked by the CD68(+)CCR2(+) phenotype. Additionally, NFH individuals showed reduced anti-inflammatory M2 monocytes, characterised by the CX3CR1(+)CD163(+)/CD206(+) phenotype. The M1/M2 ratio was increased in hypercholesterolemic individuals and correlated with pre-treatment low density lipoprotein-cholesterol levels, and was strongly associated with the severity of atherosclerosis. This study suggests that human hypercholesterolaemia is linked to an imbalance of circulating monocytes, favouring a pro-inflammatory state. This imbalance can potentially contribute to the development of atherosclerosis, thereby sustaining the ischaemic component of delayed wound healing.[19]

Ding and colleagues analysed publicly available transcriptomics data and found downregulation of period circadian regulator 1 gene expression in monocytes from patients with CLTI compared to controls.[20] In C57BL/6 mice, an adenovirus delivering mRNA expressing period circadian regulator 1 significantly improved recovery of blood flow following femoral artery excision. This effect was associated with a switch in macrophage phenotype from M1 to M2 and increased capillary density. It should be noted that the role of monocyte-macrophage phenotype may be different in men and women. Oestrogen treatment can protect against the inflammatory response induced by M1 stimuli in macrophages, whereas the loss of oestrogen during menopause is associated with impaired M2 activation.[21]

Notably, targeting macrophage polarisation has recently reached clinical application. A novel compound (ON101) that inhibits pro-inflammatory M1 macrophages and stimulates adipose precursor cells to secrete granulate colony stimulating factor and CXCL3 to increase anti-inflammatory M2 macrophages was tested in a multicentre, phase 3 randomized clinical trial.[22] Among 236 patients with diabetes and Wagner grade 1 or 2 foot ulcers, those randomized to ON101 cream had an increased rate of healing (odds ratio 2.84) than those in the comparator group. There is a great expectation that new therapeutics directed to rewiring macrophage polarisation will also aid healing of ischaemic wounds, but to date there is no definite clinical demonstration.

3.2 | Disarming neutrophils

Neutrophils can interact with macrophages to impair wound resolution. Neutrophils release web-like structures called neutrophil extracellular traps (NETs) to trap and kill pathogens (NETosis). Neutrophil extracellular traps act as a physical barrier and contain antimicrobial components that neutralise and immobilise microbes, thereby aiding in host defence against infections. The role of NETs in the healing of DFUs has been examined in a growing number of studies in the last 10 years, making it a current hot topic in this research field.[23] A study demonstrated that neutrophils from diabetic individuals and mice are primed to produce NETs.[24] Higher levels of NETs and delayed wound healing were observed in diabetic mice, but these effects were mitigated in mice lacking peptidylarginine deiminase 4 (PAD4). Additionally, disrupting NETs with DNase 1 accelerated wound healing in both diabetic and non-diabetic mice. These findings suggest that inhibiting NETosis could be a potential strategy to improve wound healing in diabetes.[24] Some of the mechanisms that sustain NET release have been clarified, including oxidative stress, epigenetic changes driven by microRNAs and activation of the inflammasome.[25] We found that, in patients with diabetes, neutrophils are prone to release NETs. Specifically, NET components were enriched in samples collected from non-healing DFUs, and high levels of neutrophil elastase in the wounds were associated with infection and worsened ulcer outcomes.[26] Furthermore, inhibiting the activity of PAD4, an enzyme involved in NETosis, improved wound healing in diabetic mice.[26] The observation that NET components are over-represented in non-healing diabetic wounds has been confirmed by others, supporting this as a therapeutic target.[26,27]

Recently, blocking NETosis was reported to accelerate wound healing in mice by reducing endothelial-to-mesenchymal transition in the microcirculation and promoting angiogenesis.[28] This opens the possibility that NETosis is also involved in delaying wound healing in the presence of ischaemia and that, consequently, targeting NETosis is a strategy to accelerate vascular recovery. This area of research is still preliminary, as the role of NETosis in angiogenesis may vary according to the disease setting. In pulmonary arterial hypertension, NETs appear to be involved in the stimulation of angiogenesis and blocking NET release was proposed as a therapy.[29] NETing cells can also inhibit angiogenesis by secreting neutrophil elastase and α-defensins, which generate molecules that inhibit blood vessel formation and inactivate proangiogenic factors. It should be noted that a circulating proangiogenic neutrophil subpopulation has been recently discovered in both mice and humans, which is rapidly recruited to oxygen-deprived tissues by vascular endothelial growth factor A.[30,31] How NETosis interacts with the pro- or anti-angiogenic function of neutrophils remains to be elucidated.

4 | TARGETS FOR TREATING LIMB ISCHEMIA FROM FINDINGS OF RECENT RODENT STUDIES

Figure 1 and Table 1 summarise recent findings of targets that significantly impacted HLI in mice models.

4.1 | Promoting mobilisation, homing and differentiation of bone marrow derived cells

A long-standing concept in recovery from limb ischaemia has been that bone marrow-derived cells are mobilised in response to HLI and

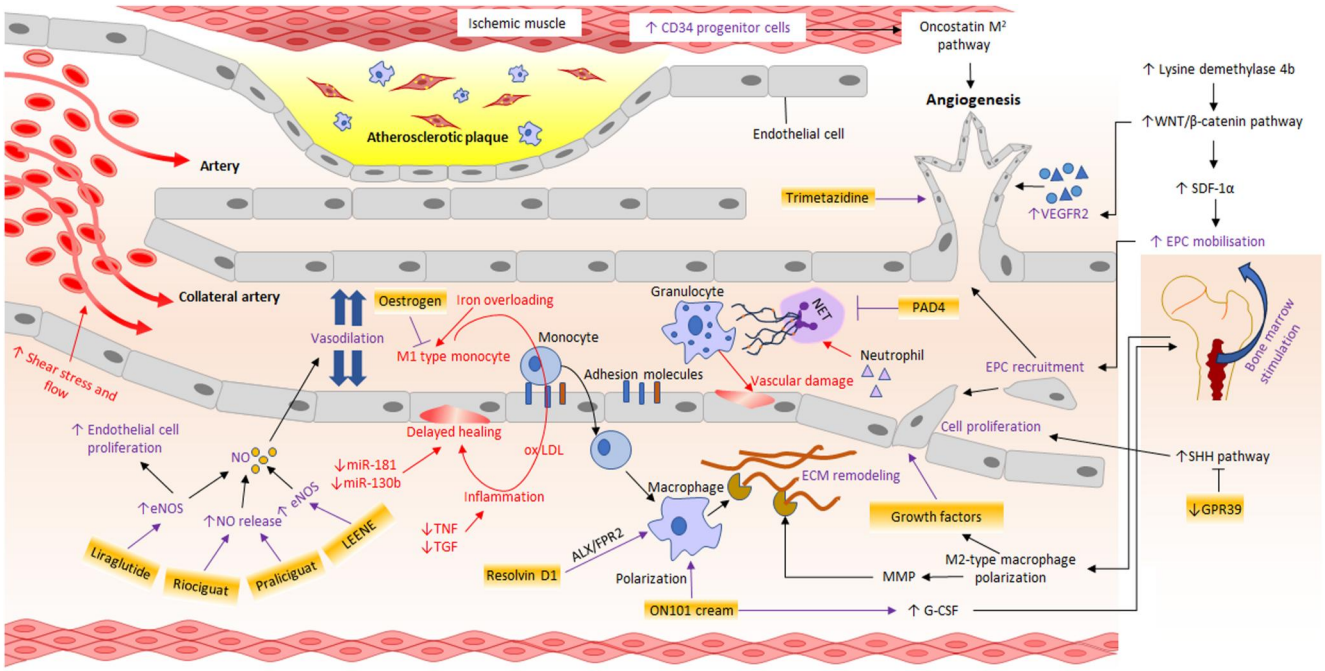

FIGURE 1 Recent findings of experimental studies on targets for novel treatments for ischaemic foot disease. Proposed mechanisms for angiogenesis and arteriogenesis and potential drug targets for medical revascularisation based on the findings of recent animal and clinical research. Beneficial and detrimental effects on revascularisation were denoted in purple and red colours respectively. Potential treatment strategies were presented in a golden yellow colour box. CD, Cluster of differentiation; eNOS, Endothelial nitric oxide synthase; G-CSF, Granulocyte colony stimulating factor; GPR39, G protein couple receptor 39; miR, MicroRNA; MMP, Matrix metalloproteinase; NET, Neutrophil extracellular traps; NO, Nitric oxide; oxLDL, Oxidised low density lipoprotein; PAD4, Peptidyl arginine deiminase 4; SDF1α, stromal cell-derived growth factor-1α; SHH, Sonic hedgehog; TGF, Transforming growth factor; TNF, Tumour necrosis factor; VEGFR, Vascular endothelial growth factor receptor; WNT, Wingless.

recruited to ischaemic tissue in response to local growth factors, where they promote new capillaries formation (angiogenesis) and remodelling and expansion of existing collateral arteries (arteriogenesis) (Figure 1).[3] A range of growth factors such as stromal cell-derived growth factor-1α (SDF1α), have been shown in rodent models to promote mobilisation of progenitor cells from the bone marrow and promote increased density of limb capillaries.[32] A study in C57BL/6 mice with HLI using single-cell RNA sequencing suggested that CD34+ progenitor cells were recruited to ischaemic muscles where they differentiate into fibroblasts and promote angiogenesis via the oncostatin M-angiopoietin-like protein pathway.[33] Oncostatin *M* blocking antibody or CD34+ cell deficiency significantly reduced blood flow recovery in C57BL/6 mice with HLI.[33] The role of this pathway is controversial because other studies demonstrated that blocking oncostatin *M* reduced the excess inflammatory myelopoiesis seen in diabetes, rescued bone marrow stem cell mobilisation, improved stem-cell transfer to ischaemic sites and facilitated recovery of limb blood supply.[34]

Hsu and colleagues identified that tumour necrosis factor superfamily 14 (TNFSF14) inhibited endothelial progenitor cell (EPC) mobilisation by downregulating SDF-1ά, significantly worsening limb blood flow in C57BL/6 mice after HLI.[32] In vitro, TNFSF14 reduced EPC proliferation and endothelial tube formation.[32] In C57BL/6 mice with streptozotocin induced diabetes and HLI, upregulating lysine demethylase 4B activated the canonical wingless (Wnt/β-catenin) pathway, which was associated with upregulation in SDF-1ά and vascular endothelial growth factor ά (VEGF). These effects significantly improved blood flow recovery compared to control mice.[35] A number of studies have also implicated microRNAs (miRs) in controlling bone marrow cell contribution to angiogenesis and arteriogenesis.[36,37] Cheng and colleagues studied miRs in patients with PAD and mice models of acute HLI secondary to Femoral artery ligation (FAL) and more gradual HLI secondary to placement of ameroid constrictors around the femoral artery (Supplement Figure 2).[36,37] They identified 22 miRs including three of the four members of the miR-181 family (*miR-181a-5p*, *miR*-181b-*5p* and *miR-181c-5p*) that were downregulated in the plasma of both patients with diabetes and PAD, and mice with HLI.[37] These miR-181 members were also downregulated within the ischaemic limbs of mice with impaired glucose tolerance compared to those with normal glucose handling.[37] C57BL/6 mice with global deficiency in alleles *miR-181a2b2 had significantly worse recovery of blood supply following HLI induction compared to wild type controls.*[37] Bone marrow transplant and cell specific transgenic studies suggested that miR-181 deficiency impaired Ly6Chi monocyte mobilisation from the bone marrow without local effects in endothelial cells. The impairment in blood flow recovery and the density of new capillaries in these mice could be rescued by infusion of Ly6Chi monocytes, illustrating the importance of these cells in angiogenesis.

TABLE 1 Drug targets from recent rodent models of hind limb ischaemia (HLI).

Citation	Model	Methods of ischaemia induction	Intervention or target	N[a]	Finding	Proposed mechanism
Mobilisation, homing and differentiation of bone marrow derived cells						
32	C57BL/6 mice	FAE	Tumour necrosis factor superfamily 14 (TNFSF14) 75, 150 or 300 μg/kg	20	Significantly worse laser Doppler assessed limb blood flow	TNFSF14 reduced SDF-1 and circulating EPCs in response to HLI and induced EPC senescence and reduced tube formation in vitro through inhibition of Akt-eNOS and lower leg capillary density
33	C57BL/6 mice	FAVL	Anti-oncostatin *M* virus or antibody	14	Significantly reduced laser Doppler perfusion at d14	CD34+ cells recruited from bone marrow differentiate to fibroblasts and promote angiogenesis via oncostatin *M* and angiopoietin like protein
33	C57BL/6 mice	FAVL	Conditional knockout of CD34+ cells	10	Significantly reduced laser Doppler perfusion at d14	
35	Male C57BL/6 mice and streptozotocin injection	FAL	Adenovirus induced lysine demethylase 4B overexpression	NR	Significantly improved laser Doppler assessed limb blood flow	Lysine demethylase 4B activated Wnt and upregulated SDF-1a and VEGFA
37	Male and female C57BL/6 mice	FAL or ameroid placement	miR-181a2b2 deficiency	NR	Significantly impaired recovery in hind limb blood supply	miR-181a2b2 deficiency impaired Ly6Chi monocyte mobilisation from the bone marrow
Local effects within the vasculature and ischaemic limb						
38	C57BL/6 mice	FAE	Apolipoprotein *L* domain containing 1 deficiency	12	Significantly worse recovery in limb blood supply	Apolipoprotein *L* domain containing 1 deficiency reduced endothelial cell proliferation and angiogenesis
36	Male and female db/db mice	FAL or ameroid placement	miR-130b mimic	18	Significantly improved laser Doppler perfusion and necrosis scores	miR-130b repressed the TGF-b superfamily member inhibin-b-A (INHBA)
39	C57BL/6 mice	FAL	Extracellular vesicles from bone marrow derived mesenchymal cells exposed to hypoxia	12	Significantly improved laser Doppler perfusion over 14 days	The vesicles upregulated miR-34c, polarised macrophages to M2 and increased capillary density
42	C57BL/6 mice administered streptozotocin plus high fat diet	FAE	Liraglutide- GLP-1 agonist	22	Significantly improved laser Doppler perfusion over 14 days	Liraglutide increased angiogenesis by promoting endothelial proliferation through upregulating eNOS
43	C57BL/6 female mice	FAL	Riociguat soluble guanylate cyclase stimulator	15	Significantly improved laser Doppler perfusion over 14 days	Riociguat increased nitric oxide release and capillary density
44	Leptin receptor-deficient mice	FAE	Praliciguat soluble guanylate cyclase stimulator	20	Significantly improved laser Doppler perfusion over 30 days	Praliciguat increased arteriolar diameter, decreased ICAM-1 and Cxcl12 expression
45	C57BL/6 mice	FAL	Deficiency in long noncoding RNA that enhances eNOS expression (LEENE)	8	Significantly worse recovery in limb blood supply	LEENE promoted transcription of genes encoding VEGFR2 and eNOS

(Continues)

TABLE 1 (Continued)

Citation	Model	Methods of ischaemia induction	Intervention or target	N[a]	Finding	Proposed mechanism
46	Male db/db mice with blood glucose >17 mMol/L	FAL	Trimetazidine 10 mg/kg/d for 2 weeks	20	Significantly improved ischaemic and functional scores	Trimetazidine upregulated VEGF-A and myogenin and downregulated ICAM-1
20	Male C57BL/6 mice	FAE	Adenovirus expressing period circadian regulator 1 mRNA	12	Significantly improved laser Doppler perfusion over 21 days	Period circadian regulator polarised macrophages to M2 associated with increased capillary density
47	Female C57BL/6 mice administered streptozotocin plus high fat diet	FAL	Deficiency in G protein–coupled receptor 39 (GPR39)	12	Significantly improved laser Doppler perfusion over 28 days	GPR39 decreased endothelial cell proliferation, migration and tube formation via blocking the sonic hedgehog pathway

Abbreviations: eNOS, endothelial nitric oxide synthase; EPC, Endothelial cell progenitor; FAE, Femoral artery excision; FAL, Femoral artery ligation; FAVL, Femoral artery and vein ligation; GLP, Glucagon-like peptide; ICAM, Intercellular adhesion molecule; N, Total sample size of intervention and control groups; R, Randomisation yes (+) or no (−); Outcome assessor blinding yes (+) or no (−); R2, Receptor 2; SDF, Stromal cell derived factor; VEGF, Vascular endothelial growth factor.

[a]Includes control group.

4.2 | Local effects within the vasculature and ischaemic limb

The growth of new capillaries and remodelling of existing collaterals is also dependent on the function of endothelial cells and vascular smooth muscle cells within the lower limb vessels.[3,5] Numerous novel local factors controlling angiogenesis and arteriogenesis have been identified in recent studies in mouse HLI models (Table 1). One of these factors is an apolipoprotein *L* domain containing 1 (Apold1), which is predominantly expressed in endothelial cells and has been shown to be upregulated in response to ischaemia. A recent study found that C57BL/6 mice deficient in Apold1 had significantly worse recovery of blood supply following HLI as a result of reduced endothelial cell proliferation and contribution to new blood vessel formation.[38] miR-130b has been found to improve limb blood supply. miR-130b was downregulated in the plasma of C57BL/6 mice and patients with limb ischaemia but upregulated in the ischaemic limb muscle compared to healthy tissue.[36] In db/db (diabetic) mice, no upregulation of miR-130b in ischaemic leg muscle was identified. In vitro, D-glucose was downregulated, whereas VEGF and hypoxia upregulated miR-130b in human umbilical endothelial cells. Endothelial cells overexpressing miR-130b had enhanced proliferation and migration in vitro. Injection of miR-130b mimics into the gastrocnemius muscle of db/db mice significantly improved blood flow recovery and reduced necrosis score following FAL but not femoral artery ameroid constriction. The improved blood supply was accompanied by increased capillary density and reduced expression of TGF-β superfamily member inhibin-b-A (INHBA). Furthermore, silencer RNA targeting INHBA injected into the gastrocnemius muscle of db/db mice after FAL significantly improved blood flow recovery and necrosis score. This suggested that miR-130b overexpression and downregulation of INHBA are targets for promoting angiogenesis. MiRs have also been suggested to be responsible for the improved limb blood supply stimulated by bone marrow-derived mesenchymal stem cells.[39] Extracellular vesicles derived from stem cells exposed to hypoxia significantly improved blood flow recovery in C57BL/6 mice. This effect was associated with upregulation in miR-34c, M2 polarisation of macrophages and increased capillary density. The effects were partially blocked by deficiency in miR-34c, suggesting an important role in promoting angiogenesis. Other approaches of promoting M2 polarisation of macrophages have also been reported to promote blood flow recovery in C57BL/6 mice as discussed earlier.[20]

Nitric oxide (NO) release promotes angiogenesis and arteriogenesis.[40,41] The glucagon-like peptide-1 (GLP-1) receptor agonist liraglutide was reported to upregulate endothelial nitric oxide synthase (eNOS) in C57BL/6 mice with hyperglycemia and was shown to improve limb blood flow.[42] Riociguat is a member of a novel class of soluble guanylate cyclase stimulators that increase NO release. Riociguat was reported to improve blood supply in C57BL/6 mice following HLI.[43] Another soluble guanylate cyclase stimulator, praliciguat, has also been reported to improve limb blood supply in a mouse model of diabetes.[44] Tang and colleagues found that levels of

long noncoding RNA that enhances eNOS expression (LEENE) were decreased in the intima of mesenteric arteries from patients with diabetes compared to controls.[45] In vitro LEENE increased expression of VEGF receptor 2 and eNOS in endothelial cells. C57BL/6 with LEENE deficiency had significantly impaired recovery of blood supply from FAL.[45] These findings suggest that LEENE expressing RNA could be a novel treatment for limb ischaemia.

Trimetazidine, a drug used to treat angina pectoris, inhibits 3-ketoacyl-CoA thiolase favouring glucose rather than fatty acid oxidation, and is proposed to be cytoprotective during ischaemia. Yang and colleagues found that trimetazidine improved semi-quantitative scores for limb ischaemia and function in hyperglycaemic db/db mice with HLI, which was associated with increased capillary density.[46] Analysis of endothelial cells from people with diabetes showed upregulation of the *G* protein-coupled receptor 39 (GPR39).[47] In vitro GPR39 acted to impair endothelial cell proliferation, migration and tube formation through blocking the sonic hedgehog pathway by binding to a repressor called suppressor of fused homologue. In C57BL/6 mice with a diabetes phenotype, an adenovirus delivering mRNA overexpressing GPR39 improved hind limb blood supply recovery.[47]

5 | TARGETS FOR TREATING LIMB ISCHEMIA FROM FINDINGS OF RECENT CLINICAL TRIALS

Twelve randomised controlled trials were identified published since 2017 that tested a range of medical and rehabilitation therapies in patients with CLTI (Table 2).[48–59] Women were under-represented, being between 0% and 38% of the enroled patients (Table 2), thus highlighting the unmet need of gender equality in this field. The interventions tested mainly aimed to promote angiogenesis or arteriogenesis using growth factors or stem cells, including plasmids designed to upregulate Hepatocyte growth factor (HGF) or SDF-1, umbilical cord blood platelet gel, bone marrow aspirates, mononuclear cells, mesenchymal stem cells and endothelial cell progenitors.[48–57] The VOYAGER sub study reported the efficacy of the anti-coagulant drug rivaroxaban at low dose in patients with PAD undergoing open surgical revascularisation, finding that the composite outcome of acute limb ischaemia, major amputation, myocardial infarction, ischaemic stroke and cardiovascular death were significantly reduced by allocation to rivaroxaban.[58] Findings were similar in the COMPASS trial.[60] Another trial tested a programme designed to improve patient self-management after major amputation for CLTI and found no effect of the intervention on musculoskeletal function but a significant improvement in the psycho-social and quality of life outcome measure.[59] The other trials had small sample sizes ranging from 20 to 155 and were thus underpowered to test the outcome assessed (Table 2).[48–57] Accepting this limitation, some promising results were reported for HGF and a number of cell therapies (Table 2). Gu and colleagues tested a HGF plasmid within two cohorts with ischaemic rest pain (n = 119) or ischaemic ulceration (n = 121) at doses ranging between 4 and 8 mg.[48] The proportion of patients with diabetes was not reported. In the cohort with rest pain, HGF plasmid significantly improved the proportion of participants with complete resolution of pain (Table 2). In the cohort with ulceration, no significant effect on the primary outcome was noted. No safety concerns were reported. An earlier trial by the same investigators reported that HGF plasmid significantly reduced pain severity.[49] In the highest dose HGF group (24 mg), there was also a significantly greater proportion of participants with complete ulcer healing in comparison to controls.[49] Of note, the administration of the growth factor required up to 32 injections into the leg repeated on three separate days that may not be well-tolerated by patients. Another open label randomised trial in 20 Italian participants with a diabetes-related foot ulcer and CLTI reported that a gel prepared from umbilical cord blood significantly improved percentage ulcer area reduction by 30 days.[51]

Sharma and colleagues reported on a placebo-controlled trial testing the effect of intra-arterial injections of bone marrow-derived stem cells.[50] Approximately 69% of the 81 participants had CLTI and approximately one third had diabetes. The intervention doubled the proportion of participants with improvement in measures of leg blood supply, including an increase in ankle brachial index of ≥0.1 and in transcutaneous oxygen pressure of ≥15% (Table 1).[50] There was a significant reduction in the rate of major amputation by comparison to controls. Liotta and colleagues reported on an open label randomised trial comparing administration of EPCs with non-enriched mononuclear cells in 40 participants with CLTI, of whom 17 had diabetes.[55] The investigators reported that both groups had significant improvement from baseline in contrast ultrasound assessed microvascular circulatory flow, rest pain, ankle brachial index and transcutaneous oxygen pressure.[55] The outcomes in the other cell therapy trials reported over the last 5 years have been less encouraging despite similar or larger sample sizes (Table 2). The reasons for this are unclear but could include heterogeneity in routes of administration, cell extraction methods, populations and outcome measures.

Three ongoing trials in people with CLTI were also identified (Supplement Table 1).[61–63] The HOPE CLTI is further testing the plasmid encoding HGF in two cohorts with rest pain (n = 150) and ischaemic ulceration (n = 240).[61] The SAIL trial is testing bone marrow derived-mesenchymal stem cells in a small trial of patients with CLTI (n = 66).[63] Finally, the GENEPAD trial is testing the value of *CYP2C19* gene sequencing prior to the choice of anti-thrombotic therapy in patients with PAD (n = 2276). This open label trial is based on the prior finding that approximately 30% of patients are unable to adequately metabolise clopidogrel to the active anti-platelet form.[62] It is hoped that these trials will shed additional light on the value of these therapies and approaches to managing people with CLTI. In addition, it is important that future trials focus on people with diabetes who have a higher risk of amputation and in whom specific pathways involved in improving blood supply to ischaemic tissues seem to be impaired, as discussed above.

TABLE 2 Recent randomised controlled trials testing medical or rehabilitation therapies for peripheral artery disease (PAD) which included patients with chronic limb threatening ischaemia (CLTI).

Cite	Intervention	N[f]	Design	Country	Female	IC	CLTI	Primary outcome	Intervention	Control	*p* value	Other outcomes
48	Plasmid encoding HGF 4 mg	61	Placebo-controlled	China	13%	0	100%	Complete pain relief at day 180	63.6%	31.0%	0.015	Pain scores significantly reduced compared to placebo in all intervention groups. No significant differences in ABI, TcO_2 or toe pressure between groups.
48	Plasmid encoding HGF 6 mg	64	Placebo-controlled	China	14%	0	100%	Complete pain relief at day 180	72.0%	31.0%	0.015	
48	Plasmid encoding HGF 8 mg	62	Placebo-controlled	China	16%	0	100%	Complete pain relief at day 180	62.5%	31.0%	0.015	
48	Plasmid encoding HGF 4 mg	61	Placebo-controlled	China	13%	0	100%	Ulcer healing at day 180	61.1%	44.4%	NS	No significant differences in ABI, TcO_2 or toe pressure between groups.
48	Plasmid encoding HGF 6 mg	60	Placebo-controlled	China	8%	0	100%	Ulcer healing at day 180	75.0%	44.4%	NS	
48	Plasmid encoding HGF 8 mg	60	Placebo-controlled	China	7%	0	100%	Ulcer healing at day 180	55.6%	44.4%	NS	
49	Plasmid encoding HGF 12 mg	100[g]	Placebo-controlled	China	19%	0	100%	Complete pain relief at day 180 ulcer healing at 6m	48.9% 42.1%	6.4% 27.3%	<0.05 NS	No significant effect on ABI, TcO_2 or toe pressure
49	Plasmid encoding HGF 18 mg	100[g]	Placebo-controlled	China	15%	0	100%	Complete pain relief at day 180 Ulcer healing at 6m	56.3% 52.1%	6.4% 27.3%	<0.05 NS	No significant effect on ABI, TcO_2 or toe pressure
49	Plasmid encoding HGF 24 mg	100[g]	Placebo-controlled	China	15%	0	100%	Complete pain relief at day 180 Ulcer healing at 6m	54.2% 66.7%	6.4% 27.3%	<0.05 <0.05	No significant effect on ABI, TcO_2 or toe pressure
53	SDF-1 plasmid 8 mg	57[h]	Placebo-controlled	USA	38%	0	100%	Wound healing score at 6m	+10	0	NS	MA rate not different
53	SDF-1 plasmid 16 mg	53[h]	Placebo-controlled	USA	38%	0	100%	Wound healing score at 6m	+10	0	NS	MA rate not different
54	Purified CD34+ cells[d]	50	Open label	China	0	0	100%	Amputations Total (36m) Minor (36m) Major (36m)	 32% 24% 8%	 16% 12% 4%	 0.185 0.462 0.552	Significantly lower pain scores in CD34+ group
55	Enriched circulating EPCs[e]	40[h]	Open label	Italy	35%	0	100%	Microcirculation TTPC TTPF	 28 20	 30 22	 NS NS	Microcirculation measures, rest pain, ABI and TcO_2 significantly improved from baseline in both groups
50	Intra-arterial autologous bone marrow-derived stem cells	81	Placebo-controlled	India	5%	31%	69%	ABI increase ≥0.1 at 6m TcO_2 increase ≥15% at 6m Mid-foot Lower-foot	85.4% 85.4% 90.2%	32.5% 42.5% 47.5%	<0.001 <0.001 <0.001	MA rate significantly reduced (0 vs. 4)
56	Concentrated bone marrow aspirates	155	Placebo-controlled	USA	NR	0	100%	Amputation free survival	80%	69%	0.22	
57	Bone marrow mononuclear cells	38	Placebo-controlled	France	18%	0	100%	Major amputation or death within 6m	18%	26%	0.48	Pain, ABI and TcO_2 not significantly different

TABLE 2 (Continued)

Cite	Intervention	N[f]	Design	Country	Female	IC	CLTI	Primary outcome	Intervention	Control	*p* value	Other outcomes
51	Umbilical cord blood platelet gel	20	Open label	Italy	35%	0	100%	Percentage wound reduction by 30 days	79%	46%	0.01	
52	High frequency spinal cord stimulation	50	Open label	Russia	16%	0	100%	Pain intensity at 3m	2.8 (2.4, 3.2)[a]	3.3 (3.0, 3.6)[a]	0.031	Improved QOL based on SF-36
58	Rivaroxaban 2.5 mg bd	1419	Placebo controlled[b]	Multiple countries	20%	63%	37%	ALI, MA, MI, IS, CVD	19.5%	23.9%	0.03	Anti-thrombotic
59	Programme to promote self-management after amputation[c]	147	Cluster randomised trial	USA	2%	0	100%	Short musculoskeletal Function assessment Patient health questionnaire	32.3 (17.9) 5.1 (4.8)	30.2 (19.4) 5.9 (6.2)	0.41 0.01	Self-management

Note: MWD: Mean change in maximum walking distance in metres (standard deviation).

Abbreviations: ABI, ankle brachial index; ALI, acute limb ischaemia; CVD, cardiovascular death; EPC, endothelial progenitor cells; HGF, hepatocyte growth factor; IS, ischaemic stroke; m, months; MA, major amputation; MI, myocardial infarction; NR, not reported; NS, not significant; QOL, quality of life; SDF, stromal cell-derived factor; SF, short-form; m: months; TcO_2, trans-cutaneous oxygen pressure.

[a]Mean visual analogue scale pain score and 95% confidence intervals.

[b]Sub-study of the main trial focusing on patients having bypass surgery.

[c]Include above knee, below knee and trans-metatarsal amputation for CLTI.

[d]Compared with peripheral blood mononuclear cells in the control group.

[e]Compared with bone marrow-derived mononuclear cells.

[f]Including the control group which in the trials of HGF consisted of only one placebo group for the three different doses. The outcomes and numbers for the placebo group have been repeated as the comparator.

[g]All part of the same trial and compared to the same one control group.

[h]More patients originally randomised but only limited numbers reported.

TABLE 3 Summary of targets identified in recent mouse models and clinical studies to upregulate or downregulate in order to improve limb blood supply.

Targets to upregulate or administer	Targets to downregulate or inhibit
Oncostatin M[33]	Tumour necrosis factor superfamily 14[32]
CD34+ cells mobilisation[33]	G protein–coupled receptor 39[47]
Lysine demethylase 4B[35]	
miR-181a2b2[37]	
Apolipoprotein *L* domain containing 1[38]	
miR-130b[36]	
Extracellular vesicles from bone marrow derived mesenchymal cells exposed to hypoxia[39]	
Liraglutide[42]	
Riociguat[43] and praliciguat[44]	
Trimetazidine[46]	
Period circadian regulator 1[20]	
Hepatocyte growth factor[48,49,61]	
Bone marrow derived stem cells[50]	
Umbilical cord blood platelet gel[51]	
High frequency spinal cord stimulation[52]	

6 | CONCLUSIONS

Diabetes is a key risk factor for ischaemic foot disease. Based on the findings of the animal studies and clinical trials summarised in this review, there are a number of novel therapy targets for improving limb blood supply and aiding the healing of ischaemic wounds as outlined in Table 3 and Figure 1. It is hoped that a range of drugs targeting these and other novel targets will be developed for medical revascularisation as adjuncts and alternatives to surgical revascularisation of ischaemic foot disease.

AUTHOR CONTRIBUTIONS

Jonathan Golledge led the writing and editing of the manuscript. Gian Paolo Fadini drafted the section on targeting inflammation. Shivshankar Thanigaimani and Kristen S. Barratt prepared the figures. All authors edited and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

ETHICS STATEMENT

None.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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