U.PORTO

FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

MESTRADO INTEGRADO EM MEDICINA

2021/2022

Fatigracy Maria dos Santos de Canha

The use of targeted angiogenic therapies for ischemic diabetic foot ulcer repair

MARÇO, 2022

Fatigracy Maria dos Santos de Canha

The use of targeted angiogenic therapies for ischemic diabetic foot ulcer repair

Mestrado Integrado em Medicina

Área: Medicina básica

Tipologia: Projeto

Trabalho efetuado sob a Orientação de:

Professora Raquel Ângela Silva Soares Lino

Trabalho organizado de acordo com as normas da revista:
Porto Biomedical Journal

MARÇO, 2022

U.PORTO
FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO

UC Dissertação/Projeto (6º Ano) - DECLARAÇÃO DE INTEGRIDADE

Eu, Fatigracy Moreira dos Santos de Cunha, abaixo assinado, nº mecanográfico 201504821, estudante do 6º ano do Ciclo de Estudos Integrado em Medicina, na Faculdade de Medicina da Universidade do Porto, declaro ter atuado com absoluta integridade na elaboração deste projeto de opção.

Neste sentido, confirmo que **NÃO** incorri em plágio (ato pelo qual um indivíduo, mesmo por omissão, assume a autoria de um determinado trabalho intelectual, ou partes dele). Mais declaro que todas as frases que retirei de trabalhos anteriores pertencentes a outros autores, foram referenciadas, ou redigidas com novas palavras, tendo colocado, neste caso, a citação da fonte bibliográfica.

Faculdade de Medicina da Universidade do Porto, 21/03/2022

Assinatura conforme cartão de identificação:

Fatig Cunha

U.PORTO
FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO

UC Dissertação/Projeto (6º Ano) – DECLARAÇÃO DE REPRODUÇÃO

NOME

Fatigreacy Maria dos Santos de Carvalho

NÚMERO DE ESTUDANTE	E-MAIL
201504821	up201504821@up.pt

DESIGNAÇÃO DA ÁREA DO PROJECTO

Medicina básica

TÍTULO ~~DISSERTAÇÃO~~/MONOGRAFIA (riscar o que não interessa)

The use of targeted angiogenic therapies for ischemic diabetic foot ulcere repair

ORIENTADOR

Prof. Doutora Raquel Ângela Silva Soares Lino

COORIENTADOR (se aplicável)

ASSINALE APENAS UMA DAS OPÇÕES:

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTE TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.	☒
É AUTORIZADA A REPRODUÇÃO PARCIAL DESTE TRABALHO (INDICAR, CASO TAL SEJA NECESSÁRIO, Nº MÁXIMO DE PÁGINAS, ILUSTRAÇÕES, GRÁFICOS, ETC.) APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.	☐
DE ACORDO COM A LEGISLAÇÃO EM VIGOR, (INDICAR, CASO TAL SEJA NECESSÁRIO, Nº MÁXIMO DE PÁGINAS, ILUSTRAÇÕES, GRÁFICOS, ETC.) NÃO É PERMITIDA A REPRODUÇÃO DE QUALQUER PARTE DESTE TRABALHO.	☐

Faculdade de Medicina da Universidade do Porto, 21/03/2022

Assinatura conforme cartão de identificação: Fatigranha

Dedicatória

Em primeiro lugar, devo agradecer à minha orientadora, a Professora Raquel Soares, pela atenção e pronta disponibilidade que teve ao longo da elaboração deste projeto e por proporcionar-me todas as ferramentas necessárias.

Um agradecimento especial aos meus pais, Luís Alberto e Élia Graça dos Santos, pelo apoio e motivação que me fizeram chegar durante este período, pelo amor incondicional e pelos sacrifícios feitos para eu estar a terminar esta etapa.

Aos meus irmãos, Alexander e Mariana Canha, pela disponibilidade que demonstraram em ouvir os meus desabafos e pela ajuda na gestão do stress.

À minha avó, Conceição Duarte, pelo amor e carinho proporcionado durante este percurso.

E finalmente, mas não menos importante, um agradecimento especial ao meu namorado, João Sousa, pelo apoio incondicional durante esta fase e pelo esforço feito para me auxiliar nos momentos mais difíceis em que me deparei no desenvolvimento deste trabalho.

Dedico, então, este trabalho, às pessoas mais importantes na minha vida, a minha família e namorado.

Resumo

Diabetes Mellitus é uma doença metabólica, que apresenta elevada prevalência a nível mundial, e é caraterizada pela hiperglicemia crónica, levando ao desenvolvimento de complicações. São, particularmente, estas complicações que causam a mortalidade em pacientes diabéticos. Esta revisão foca-se nas úlceras do pé diabético (DFUs), que são uma das complicações mais comuns da diabetes mellitus do tipo 2 (T2DM) e que acarretam significativa morbilidade, mortalidade e significativos custos de saúde associados. A cicatrização das DFUs é prejudicada pela desregulação de quase todas a fases deste processo, devido ao meio hiperglicémico presente. Embora existam atualmente terapias para gerir um doente com DFU, estas acabam por ser insuficientes. No presente trabalho, a angiogénese é destacada como parte da fase proliferativa, que, quando diminuída, desempenha um papel importante na cicatrização prejudicada de DFU e de outras feridas crónicas. Portanto, a busca por novas estratégias terapêuticas visando a angiogénese é de grande interesse. Aqui, fornecemos uma visão geral de alvos moleculares com potencial terapêutico e terapias que atuam na angiogénese. E para isto, foi realizada uma busca nas bases de dados PubMed e Scopus de artigos publicados entre 2018 a 2021, com o objetivo de verificar a angiogénese como alvo terapêutico para DFU. Fatores de crescimento, microRNAs e vias de sinalização foram investigados como alvos moleculares, e pressão negativa, oxigenoterapia hiperbárica e o uso de nanomedicina foram explorados como terapias.

The use of targeted angiogenic therapies for ischemic diabetic foot ulcer repair.

Fatigracy Canha1, Raquel Soares1,2

1Department of Biomedicine, Faculty of Medicine, University of Porto; 2i3S, Instituto de Investigação e Inovação em Saúde, Universidade do Porto

Running title: Angiogenic therapy in DFU

Corresponding author:

Raquel Soares, PhD

Department of Biomedicine

Faculty of Medicine of the University of Porto

Al Prof Hernâni Monteiro

4200-319 Porto

Portugal

Email: raqsoa@med.up.pt

Abstract

Diabetes Mellitus is a metabolic disease that has a high prevalence worldwide and is characterized by chronic hyperglycemia leading to the development of complications. In particular, it is these complications that result in huge mortality rates in diabetic patients. This work focuses on diabetic foot ulcers (DFUs), which are one of the most common complications of type 2 diabetes mellitus (T2DM) and cause significant morbidity, mortality, and healthcare costs. The healing of DFUs is hindered by deregulation of nearly all phases of this process due to the hyperglycemic environment. Although therapies currently exist to treat a patient with DFU, they are proving inadequate. In the present work, angiogenesis is highlighted as part of the proliferative phase, which, when diminished, plays an important role in the impaired healing of DFU and other chronic wounds. Therefore, the search for new therapeutic strategies targeting angiogenesis is of great interest. Here, we provide an overview of molecular targets with therapeutic potential and therapies that act on angiogenesis. To this end, a search of papers PubMed and Scopus databases from 2018 to 2021, was performed to review angiogenesis as a therapeutic target for DFU. Growth factors, miRNAs, and signaling pathways were investigated as molecular targets, and negative pressure, hyperbaric oxygen therapy, and the use of nanomedicine were explored as therapies.

Keywords: diabetes mellitus; diabetic foot ulcer; wound healing; angiogenesis; ischemic ulcer

Introduction

Diabetes mellitus is a form of metabolic disease characterized by chronic hyperglycemia resulting from resistance to insulin, inadequate insulin secretion, or both. The main types of diabetes mellitus are type 1, T1DM, (insulin-dependent diabetes mellitus), caused by autoimmune destruction of the insulin-producing beta cells of the pancreas; diabetes mellitus type 2, T2DM, (non-insulin-dependent diabetes mellitus), which is characterized by resistance of peripheral cells to the action of insulin and by inadequate secretion of insulin, and as gestational diabetes mellitus (GDM), which is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. (1, 2) According to the International Diabetes Federation, the number of people aged 20 to 79 with diabetes worldwide was 536.6 million in 2021 and is expected to increase to 783.2 million in 2045. (3) Among the countries in the European region, the Portuguese population has the third highest age-adjusted prevalence (9.8%), followed by Turkey and Germany.(4) According to WHO, diabetes is among the 10 leading causes of death, following a significant percentage increase of 70% since 2000. (5) And this chronic and progressive disease is estimated to be responsible for 12.2% of all deaths worldwide (20-79 years). (3) This mortality is mainly due to the complications that diabetes causes, rather than diabetes per se. (6)

These complications can be classified as vascular or nonvascular. Vascular disorders are the main cause of morbidity and mortality in diabetic patients. (7) Depending on the size of the affected vessels, these can be divided into microvascular, such as retinopathy or nephropathy, and macrovascular, which are atherosclerotic/thrombotic obstructions seen in coronary, cerebral, and peripheral artery disease. Non-vascular complications include forms of chronic neuropathy. (8)

In this paper, we focus on diabetic foot ulcers (DFUs), which are a common complication of diabetes mellitus, particularly T2DM, and are responsible for significant morbidity, mortality

rates, and associated healthcare costs. (9) Poor glycemic control, loss of sensation caused by peripheral neuropathy, and ischemia from peripheral arterial insufficiency often predispose patients to foot ulceration. (10) If not treated promptly, these wounds can lead to amputation. (11) It is estimated that more than two-thirds of nontraumatic lower limb amputations are preceded by an ulcer (84%). (12)

The hyperglycemic environment in diabetes promotes dysregulation of almost all healing phases in DFU. (13) After disruption of the skin barrier due to injury, the healing process begins, which is composed of four phases: Hemostasis, Inflammation, Proliferation, and Maturation/Remodeling. (14)

The initial tissue injury results in vascular damage with local hemorrhage. Initially, vasoconstriction occurs, which aims to reduce blood flow to the injured area to allow formation of a plug by platelet aggregation (primary hemostasis). Subsequently, the coagulation cascade is activated to reinforce the previously formed plug with fibrin threads (secondary hemostasis). The formed fibrin clot integrates a provisional matrix and secretes various cytokines and growth factors together with platelets to stimulate local influx of inflammatory cells. Early in the process, neutrophils are recruited to phagocyte and digest potentially infectious pathogens. Later, macrophages are recruited, which are important for the transition to the proliferative phase. (15, 16)

Macrophages are responsible for releasing growth factors that stimulate re-epithelialization, i.e., the proliferation and migration of epithelial cells on the wound surface to protect the moist environment necessary for effective healing. Due to the hypoxic environment at the wound site, these inflammatory cells also stimulate angiogenesis, a process that consists of the formation of new vessels from adjacent pre-existing ones. (15) This occurs through the activation and proliferation of endothelial cells that penetrate the underlying vascular basement membrane and enter the extracellular matrix, forming capillary buds that expand and branch to form capillary networks. (17) Angiogenesis is crucial for the supply of oxygen and nutrients and

for the formation of granulation tissue. Finally, fibroplasia occurs, which is accompanied by proliferation and migration of fibroblasts (stimulated by growth factors secreted by macrophages), synthesis of III type collagen, and other proteins required for the provisional extracellular matrix. The goal of the proliferative phase is then the formation of granulation tissue. In the final phase of normal wound healing, maturation/remodeling, type III collagen is replaced by type I collagen, which is more stable and better organized. This substitution requires a balance between degradation (matrix metalloproteinases) and synthesis (tissue inhibitors of metalloproteinases). Wound contraction also occurs, mediated by myofibroblasts arising from fibroblast differentiation, bringing the wound edges together. (15, 16)

Chronic wounds are those in which abnormal healing occurs. Normally, these are blocked in the inflammatory phase and do not progress to the proliferation and maturation/remodeling phases. (18) The hyperglycemic milieu in diabetic patients may be a reason for delayed healing. This milieu may cause increased activity of matrix metalloproteinases (MMPs), decreased conversion of the M1 (pro-inflammatory) to M2 (anti-inflammatory) phenotype of macrophages (this conversion is essential for wound closure), oxidative stress, and impaired production of growth factors. In this work, however, we will focus on angiogenesis, which, when impaired, may be a cause of delayed healing. We will see that molecular targets may present therapeutic potential and which therapies have been investigated to affect angiogenesis. (14, 16, 19)

Currently, there are several therapies for the treatment of patients with DFU, such as debridement, biological dressings, hyperbaric oxygen therapy (HBOT), platelet derivatives, wound growth factors, negative pressure wound therapy, topical antimicrobials, and others. (16). However, despite these therapies, DFU remains one of the major complications of diabetes, leading to a deterioration in patients' quality of life and an enormous burden on national health systems. (20)

Since decreased angiogenesis plays an important role in the impaired healing of DFU

and other chronic wounds (21), the search for new therapeutic strategies targeting angiogenesis is of paramount importance. Therefore, this work aims to verify angiogenesis as a targeted therapy for DFU.

Materials and Methods

Search strategy and selection criteria

A search for studies published between 2018 and 2021 was conducted in two academic databases, PubMed and Scopus. The PubMed search was performed on October 16, 2021, and to capture additional articles relevant to the present work, the Scopus search was performed on January 7, 2022. All types of studies (observational, longitudinal, review, and experimental) on the use of targeted angiogenic therapies for the treatment of ischemic diabetic foot ulcers that were conducted in humans and written in English were considered.

The query ((((("Diabetic Foot"[Mesh]) AND "Angiogenesis Inducing Agents"[Mesh]) OR "Angiogenesis Modulating Agents"[Mesh]) OR "Angiogenic Proteins"[Mesh]) AND " Wound Healing"[Mesh] was used for the search.

Results and Discussion

The PRISMA 2020 flowchart was used to illustrate the screening process. A total of 274 records were identified (230 from PubMed and 44 from Scopus), 9 of which were removed before screening because they were duplicates. Of the 265 resulting records, 101 were excluded after title and abstract analysis and 15 because full-text access was not available. Finally, 149 full texts were screened, of which 115 were excluded because they were not conducted in humans and 12 because they did not correspond to the research topic. A total of 22 studies were then included, which were subjected to a detailed reading and stripped of information relevant to the

present work (Figure 1).

Molecular targets for angiogenesis

Chronic wounds are those in which healing is impaired. Usually, they get stuck in the inflammatory phase, with impaired angiogenesis, and do not progress to the proliferation and maturation/remodelling phase. (18)

Angiogenesis is a complex multistep process, involving a huge battery of molecules such as growth factors and growth factor receptors, cytokines, hormones, transcription factors, miRNAs, matrix degradation proteases, etc. This work focuses on the angiogenic molecular targets that present therapeutic potential and on which therapies have been investigated to act on these targets. (14, 16)

Growth Factors and Cytokines

When injury occurs, the tissue receives less oxygen due to compromised vessels. In this way, angiogenesis is stimulated to receive more oxygen and nutrients needed for new tissue formation. (20) Hypoxia leads to the accumulation of hypoxia-inducible factor 1α (HIF-1α) in the cytosol of endothelial cells by inhibiting the enzymes responsible for its degradation. (22) This factor migrates to the nucleus, where it stimulates transcription of angiogenic genes such as vascular endothelial growth factor A (VEGF-A), fibroblast growth factor (FGF2), transforming growth factor beta (TGF-β1), platelet-derived growth factor (PDGF), forkhead box O1 (FOXO1), erythropoietin (EPO), and stromal cell derived factor 1 alpha (SDF-1α) induced. (22-24)

HIF-1α-induced VEGF-A is mainly secreted by macrophages, binds to specific receptors on endothelial cells membrane (VEGFR2). (25) Simultaneously, it stimulates proliferation and migration of endothelial cells to form new vessels. (22) Like VEGF, placental growth factor (PlGF), another member of VEGF family, has a pro-angiogenic effect, and its plasma concentration increases during healing. However, compared with VEGF, PlGF induces the formation of more mature and stable vessels. (20) FGF2, also known as basic fibroblast growth factor (bFGF), also stimulates proliferation of endothelial cells, and the possibility that FGF2 stimulates

angiogenesis indirectly by inducing VEGF has been noted. (22, 25) On the other hand, FOXO1 is indirectly stimulated by HIF-1 by becoming nonphosphorylated and inhibited by the P13K/Akt pathway. (22) This factor is responsible for transcription of VEGF-A and TGF-β genes. (26) TGF-β is secreted by inflammatory cells and positively regulates proliferation and migration of keratinocytes, fibroblasts, and endothelial cells. (16, 26)

PDGF family members are released from platelets and secreted by macrophages recognized in chronic wounds for a long time. This renders them the first recombinant growth factors approved for topical use to accelerate wound closure. (14) Angiopoietins (ANGPT) are potent angiogenic growth factors too. Their levels affect proliferation and survival of endothelial cells in ischemic tissues. ANGPT 1 is an agonist of the Tie2 receptor present on endothelial cells. Upon binding, dimerization of Tie2 with Tie1 occurs, leading to activation of signalling pathways through phosphorylation of specific tyrosine. ANGPT2, on the other hand, is an antagonist of the Tie2 receptor, competing with ANGPT1 in its binding. (25) SDF-1α is a chemokine involved in angiogenesis and recruitment of endothelial progenitor cells (EPCs), being also impaired in chronic wounds. (23)

Erythropoietin (EPO) is a hormone that binds to receptors on endothelial cells to promote angiogenesis and cell survival in ischemic tissues. It can also stimulate the secretion of VEGF. (25, 26)

In patients with DFU, the expression of cytokines and growth factors, such as platelet-derived growth factor (PDGF), TGF-β, and VEGF, is reduced. (14) In addition, the hyperglycemic environment leads to suppression of HIF-1. Therefore, transcription of its aforementioned target genes with angiogenic activity no longer occurs. This dysfunction eventually impairs the recovery of DFU. (14) To develop therapies that can act on angiogenesis, it is necessary to understand how dysregulation of the expression of these growth factors affects the healing of DFU.

Chen et al. performed a study in which plasma concentrations of VEGF-A and PlGF were measured and compared between diabetic patients with DFU, without DFU, and healthy

subjects (controls). In this study, it was found that plasma levels of both VEGF and PIGF were higher in patients with diabetes (with or without DFU) than in healthy subjects. Interestingly, among patients with diabetes, it was observed that the group with DFU had lower circulating VEGF and PIGF levels than the group of diabetics without DFU. (20) These findings further reveal that although angiogenic stimuli are enhanced in the diabetic patients, these do not reach DFU, implying that local impairment prevails, eventually by increased fibrosis.

One review study addressed the observed relationship between FGF2 and protein kinase 1 and 2 (ERK1/2) levels, suggesting that FGF-2 may play its role in wound healing via ERK1/2 signalling. Therefore, the use of FGF-2 alone may not be as effective in the treatment of DFU. (26) Therefore, FGF-2 has great potential to be used as a therapeutic target because it acts directly and indirectly in signalling pathways and has a potent pro-angiogenic effect. TGF-β1 is a positive predictor of the healing process. This conclusion stems from a clinical study that investigated the relationship between TGF-β1 and ulcer healing rate in diabetic patients. Lower levels of this factor were observed in patients who did not achieve complete healing in comparison to those who did. (27) This results in an increase in VEGF-A expression. [24] The effect of recombinant EPO (rhEPO) has been studied in other types of lesions, but efficacy in DFU has not yet been demonstrated. (26) Nevertheless, considering the significant pro-angiogenic action of EPO, this hormone is a promising target for angiogenesis enhancement in DFU. SDF-1α levels are reduced in patients with DFU, which impairs SDF-1α induced neovascularization by decreasing EPC recruitment. Because SDF-1α is an important factor in vasculogenesis, a process that accompanies angiogenesis, its administration has the potential to promote blood flow and tissue perfusion, which may suggest SDF-1α as a potential therapeutic target for DFU. (14)

microRNAs

MicroRNAs, or simply miRNAs, are small noncoding RNA sequences that can be used to control signalling pathways by modulating the expression of specific genes. This modulation can

occur through transcriptional suppression, when a miRNA sequence is complementary to the mRNA target sequence, and/or through mRNA degradation, by cleavage. (15) MiRNAs can be released from cells through extracellular vesicles and are easily quantified in plasma and urine. This allows monitoring of patients who benefit from targeted therapies for miRNAs. (24) There are a number of miRNAs that regulate angiogenesis, termed "angiomiRs." (23) Since angiogenesis is an essential process in DFU healing and there are miRNAs associated with this process, there may be an opportunity here to investigate and develop therapies that target these miRNAs. (15, 24)

Endothelial cells were hypothesized to be stimulated during inflammatory stress, as in diabetes, to secrete miR-191 into the plasma, where they act on zona occludens-1 (ZO -1), a protein expressed on endothelial cells of the injured area. By acting on ZO -1, they suppress angiogenesis, leading to a delay in the healing of chronic wounds in diabetic patients. To confirm the association between the effect of miR-191 and miR-200b on DFU healing, the levels of these miRNAs were compared between diabetic patients with associated peripheral arterial disease (PAD) and with chronic wounds and diabetic patients without complications. This study showed that the levels of miR-191 and miR-200b were higher in diabetics with PAD and chronic wounds compared with diabetics without complications. This suggests that these miRNAs indeed play a role in healing chronic wounds in diabetic patients. (15)

Amin et al. analysed miR-23 family expression in diabetic patients with infected DFU and diabetic patients with noninfected DFU and examined their relationship with angiogenic factors such as SDF-1α. Decreased levels of miR-23a, miR-23b, and angiogenic factor SDF-1α, were observed in DFU-infected diabetics and increased levels of miR-23c were observed in the same patients. The decrease in SDF-1α expression might be due to the influence of miRNAs such as miR-23c, which appear to be inversely related, i.e., miR-23c may negatively regulate angiogenesis through its performance in SDF-1α. (23)

Pichu et al., on the other hand, investigated the relationship between miR-210

expression and the hypoxic signalling pathway in patients with DFU, starting from the potential regulatory effect of HIF-1 on miR-210 expression. Decreased expression of the HIF-1α gene and increased levels of miR-210 in the circulation and ulcer tissue were observed in patients with type 2 diabetes mellitus compared with healthy controls. These fundings suggest an inversely proportional relationship between expression of the HIF-1α gene, which plays a role in the transcription of angiogenic factors, and miR-210. (24)

There are miRNAs that exert their functions without acting directly on endothelial cells, such as miR-205-5p, miR-15b, and miR-126. miR-126 inhibits negative mediators of VEGF. These regulate angiogenesis via mesenchymal stromal cells (MSCs). (15, 21) An injured tissue releases signalling molecules to recruit cells, including MSCs. These have the ability to secrete cytokines, growth factors, and extracellular vesicles that contain proteins, miRNAs, and/or other substances and act on target cells through paracrine signalling. (21) An et al. investigated the potential of MSCs as a cell therapy to support complete healing of DFU by inducing angiogenesis. (21)

In summary, from these miRNA studies, miR-191, miR-200, miR-466, miR-23c, miR-205-5p, and miR-15b are anti-angiogenic, whereas miR-23a, miR-23b, and miR -126 are pro-angiogenic. Therefore, inhibiting the former or enhancing the latter ones are promising angiogenic therapeutic approaches against DFU.

Angiogenic target therapies

Hyperbaric oxygen therapy (HBOT)

Hyperbaric oxygen therapy is a treatment method used to stimulate the healing process by inhalation of 100% O2 under high absolute atmospheric pressure, between 2 and 3 ATA per hour. This increases the partial pressure of O2 in the blood, leading to the development of a diffusion gradient in order to oxygenate the hypoxic areas. And this conductive environment

allows the formation of new blood vessels to supply O2, molecules and cells that support the healing process. (28, 29)

The use of this therapy is recommended due to the fact that plasma O2 solubility plays a major role in vascular complications associated with DM. (29) It is commonly used as adjuvant treatment in patients with chronic DFU. (28)

Dhamodharan et al. performed a study with the aim of investigating the molecular mechanism of HBOT in the wound healing process, focusing on the role of nuclear factor erythroid 2-related factor 2 (Nrf2) and its angiogenic markers. Nrf2 is a factor that can detach from its Keap1 suppressor and can be degraded or migrate into the nucleus to stimulate expression of its angiogenic target genes. (19, 28) For this purpose, a sample of 32 patients randomly divided into two different treatment groups that received either standard DFU treatment only or HBOT along with standard treatment for 20 days. Tissue expression of Nrf2 was assessed on days 0 and 20 of treatment in both study groups. On day 20 of treatment, significantly higher Nrf2 levels were observed in subjects receiving adjuvant HBOT [2.5 ± 0.15; p = 0.003], while no significant change was observed in those receiving standard treatment alone [1.2 ± 0.14; p = 0.06]. [13] On day 20 of treatment, an increase in the expression levels of angiogenic markers was also observed, such as FGF-2 [632.3 ± 55.7; p = 0.02] in subjects receiving HBO therapy, and VEGF [325.2 ± 53.5; p = 0.01] and PDGF [6.0 ± 0.4; p = 0.03] in both study groups. Finally, measurements of wound size were performed and demonstrated a significant reduction in wound area on day 20 of treatment (51.8%) in subjects undergoing HBO therapy, while no significant changes were seen in the standard treatment group alone. (28)

Nitric oxide (NO) is a mediator of angiogenesis and is induced by VEGF. Its synthesis is altered in patients with diabetes due to the chronic hyperglycemic environment. (29) The possible regulation of the synthesis of NO (NOS) by HBO therapy has been suggested and confirmed in the same study, the increase in the expression of endothelial NOS (eNOS) in patients who underwent HBO ($p < 0.0001$), while the other group showed no changes ($p <$

0.006). (28)

Therefore, this study suggests that HBO therapy promotes angiogenesis by increasing the expression of Nrf2 and pro-angiogenic factors, resulting in a significant reduction in wound size.

Negative pressure wound therapy (NPWT)

NPWT is another form of therapy used as an adjunct in the treatment of patients with DFU which consists of the application of subatmospheric pressure. A porous material is used on the surface of the wound to facilitate pressure transfer, and a dressing is placed over this material to create an airtight environment. A tube is connected to the dressing to drain fluid from the wound by connecting it to a vacuum system. This system allows for an increase in local blood flow and the formation of new tissue. (30)

The review carried out by Borys et al. demonstrates the therapeutic effect of this negative pressure system, which consists of tissue deformation at the macroscopic (wound contraction) and microscopic (at the cellular) levels. The micro-deformation results from the mechanical stress caused by the negative pressure, which is transmitted to the cells and leads to the induction of healing processes such as angiogenesis. The applied negative pressure creates a hypoxic and stressful environment that affects the gene expression of proangiogenic factors such as VEGF, TGF, FGF, and PDGF. (30) Several reviewed studies showed high efficacy in patients treated with negative pressure and fewer adverse effects. Among these, a randomized control trial with 350 DFU patients reported a higher healing rate in the ones treated with NPWT (43.2%) compared with those receiving standard treatment (28.9%). Similar results were described in a study of 1135 patients with DFU, in which the healing rate was 46.3% in patients who underwent NPWT compared with 32.8% for standard treatment. (30) These results reveal that standard treatment together with NPWT achieves better results in the healing of DFU than alone, angiogenesis being one of the processes induced by NPWT that support healing.

Nanomedicine applied to DFU treatment

Stimulating angiogenesis is one of the most effective strategies to promote regeneration of injured tissue. (25) Nanomedicine plays a role in healing these tissues by developing systems that transport and release angiogenic factors such as VEGF and FGF to the site of injury. (16) Therefore, there is growing interest in this area and in the development of angiogenic nanomaterials. These can be incorporated into scaffolds, biodegradable structures made of natural or synthetic materials, which guide the formation of new tissue through the slow and controlled release of angiogenic nanomaterials. (25)

Angiogenic nanomaterials capable of regenerating damaged tissue include metal-based nanoparticles such as zinc oxide nanoparticles (ZnO NPs), whose incorporation into scaffolds stimulates the expression of proangiogenic factors (FGF2 and VEGF-A). (25) Cerium oxide nanoparticles (CeO2 NPs), which stabilize the levels of HIF-1α and the expression of its target genes in endothelial cells, leading to the induction of angiogenesis, and gold NPs (Au NPs), which regulate the expression of ANGPT genes -1, ANGPT-2, and VEGF 12 and are available in different sizes, with smaller sizes (20 nm) having an antiangiogenic effect by suppressing VEGF expression. (16)

Other interesting approaches have further been developed. Graphene-based nanomaterials have proangiogenic properties when used at low concentrations, preventing the development of cellular toxicity and suppression of angiogenesis at high concentrations. Polyamine coated carbon nanotubes (CNTs) can be used to deliver miRNAs that regulate angiogenic gene expression. Finally, bioglass nanoparticles (BG -NPs), which have high biocompatibility and rapid dissolution of ions, such as Mg2+ and Cu2+, with angiogenic properties. (25)

Conclusions

Despite current treatment, DFU remains one of the most serious complications of diabetes, with high morbidity and mortality worldwide. This highlights the importance of understanding the role of angiogenic pathways in wound healing, how they are altered in DFU, and the importance of developing new therapies that are effective in healing DFU.

The state-of-the-art regarding the presence of a large variety of angiogenic growth factors and miRNAs in DFU are indicative of the relevance of angiogenesis in the pathophysiology of DFU. Although many anti-angiogenic therapeutic strategies have been established, namely HBOT and NPWT, nanotechnology currently in development to deliver specific growth factors and nucleotides targets is a promising approach for ischemic DFU repair.

Herein, we gathered the anti-angiogenic strategies currently being used in the clinic and identified novel targets and nanotechnology to further address angiogenesis, paving the way to develop more direct approaches to use in the clinical practice against this dreadful condition that causes such an immense morbidity to diabetic patients.

Acknowledgments

Assistance with the study: none.

Financial support and sponsorship: none.

Conflicts of interest: none.

Presentation: none.

References

1. Mumtaz M. Gestational diabetes mellitus. Malays J Med Sci. 2000;7(1):4-9.

2. Saeedi Borujeni MJ, Esfandiary E, Taheripak G, Codoner-Franch P, Alonso-Iglesias E, Mirzaei H. Molecular aspects of diabetes mellitus: Resistin, microRNA, and exosome. J Cell Biochem. 2018;119(2):1257-72.

3. Dianna J Magliano EJB, Beverley, Balkau NB, Elizabeth Barr, Abdul Basit, Dominika, Bhata CB, Gillian Booth, Bertrand Cariou, Juliana, Chan HC, Lei Chen, Tawanda Chivese, Dana Dabalea,, Hema Divakar DD, Bruce B Duncan, Michael Fang, Ghazal, Fazli CF, Kathryn Foti, Laercio Franco, Edward Gregg,, et al. IDF DIABETES ATLAS. 10 ed2021.

4. Raposo JF. Diabetes: Factos e Números 2016, 2017 e 2018*. Revista Portuguesa de Diabetes. 2020;15:19-27.

5. The top 10 causes of death: World Health Organization; 2020 [Available from: https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death.

6. Saeedi P, Salpea P, Karuranga S, Petersohn I, Malanda B, Gregg EW, et al. Mortality attributable to diabetes in 20-79 years old adults, 2019 estimates: Results from the International Diabetes Federation Diabetes Atlas, 9(th) edition. Diabetes Res Clin Pract. 2020;162:108086.

7. Barrett EJ, Liu Z, Khamaisi M, King GL, Klein R, Klein BEK, et al. Diabetic Microvascular Disease: An Endocrine Society Scientific Statement. J Clin Endocrinol Metab. 2017;102(12):4343-410.

8. Kibel A, Selthofer-Relatic K, Drenjancevic I, Bacun T, Bosnjak I, Kibel D, et al. Coronary microvascular dysfunction in diabetes mellitus. J Int Med Res. 2017;45(6):1901-29.

9. Everett E, Mathioudakis N. Update on management of diabetic foot ulcers. Ann Ny Acad Sci. 2018;1411(1):153-65.

10. Mishra SC, Chhatbar KC, Kashikar A, Mehndiratta A. Diabetic foot. BMJ. 2017;359:j5064.

11. Bandyk DF. The diabetic foot: Pathophysiology, evaluation, and treatment. Semin Vasc Surg. 2018;31(2-4):43-8.

12. Lim JZ, Ng NS, Thomas C. Prevention and treatment of diabetic foot ulcers. J R Soc Med. 2017;110(3):104-9.

13. Gorecka J, Kostiuk V, Fereydooni A, Gonzalez L, Luo J, Dash B, et al. The potential and limitations of induced pluripotent stem cells to achieve wound healing. Stem Cell Res Ther. 2019;10(1):87.

14. Zubair M, Ahmad J. Role of growth factors and cytokines in diabetic foot ulcer healing: A detailed review. Rev Endocr Metab Disord. 2019;20(2):207-17.

15. Ozdemir D, Feinberg MW. MicroRNAs in diabetic wound healing: Pathophysiology and therapeutic opportunities. Trends Cardiovasc Med. 2019;29(3):131-7.

16. Sharifi S, Hajipour MJ, Gould L, Mahmoudi M. Nanomedicine in Healing Chronic Wounds: Opportunities and Challenges. Mol Pharm. 2021;18(2):550-75.

17. Tonnesen MG, Feng X, Clark RA. Angiogenesis in wound healing. J Investig Dermatol Symp Proc. 2000;5(1):40-6.

18. Figueiredo A, Leal EC, Carvalho E. Protein tyrosine phosphatase 1B inhibition as a potential therapeutic target for chronic wounds in diabetes. Pharmacol Res. 2020;159.

19. Jayasuriya R, Dhamodharan U, Karan AN, Anandharaj A, Rajesh K, Ramkumar KM. Role of Nrf2 in MALAT1/ HIF-1alpha loop on the regulation of angiogenesis in diabetic foot ulcer. Free Radic Biol Med. 2020;156:168-75.

20. Chen ZC, Fu SS, Wu ZR, Chen JY, Huang YL, Wang Y, et al. Relationship between plasma angiogenic growth factors and diabetic foot ulcers. Clin Chim Acta. 2018;482:95-100.

21. An T, Chen Y, Tu YC, Lin P. Mesenchymal Stromal Cell-Derived Extracellular Vesicles in the Treatment of Diabetic Foot Ulcers: Application and Challenges. Stem Cell Rev Rep. 2021;17(2):369-78.

22. Primer KR, Psaltis PJ, Tan JTM, Bursill CA. The Role of High-Density Lipoproteins

in Endothelial Cell Metabolism and Diabetes-Impaired Angiogenesis. Int J Mol Sci. 2020;21(10).

23. Amin KN, Umapathy D, Anandharaj A, Ravichandran J, Sasikumar CS, Chandra SKR, et al. miR-23c regulates wound healing by targeting stromal cell-derived factor-1α (SDF-1α/CXCL12) among patients with diabetic foot ulcer. Microvasc Res. 2020;127:103924.

24. Pichu S, Vimalraj S, Viswanathan V. Impact of microRNA-210 on wound healing among the patients with diabetic foot ulcer. Plos One. 2021;16(7).

25. Nosrati H, Aramideh Khouy R, Nosrati A, Khodaei M, Banitalebi-Dehkordi M, Ashrafi-Dehkordi K, et al. Nanocomposite scaffolds for accelerating chronic wound healing by enhancing angiogenesis. J Nanobiotechnology. 2021;19(1):1.

26. Qi M, Zhou Q, Zeng W, Wu L, Zhao S, Chen W, et al. Growth factors in the pathogenesis of diabetic foot ulcers. Front Biosci (Landmark Ed). 2018;23(2):310-7.

27. Liu Y, Min D, Bolton T, Nube V, Twigg SM, Yue DK, et al. Increased matrix metalloproteinase-9 predicts poor wound healing in diabetic foot ulcers. Diabetes Care. 2009;32(1):117-9.

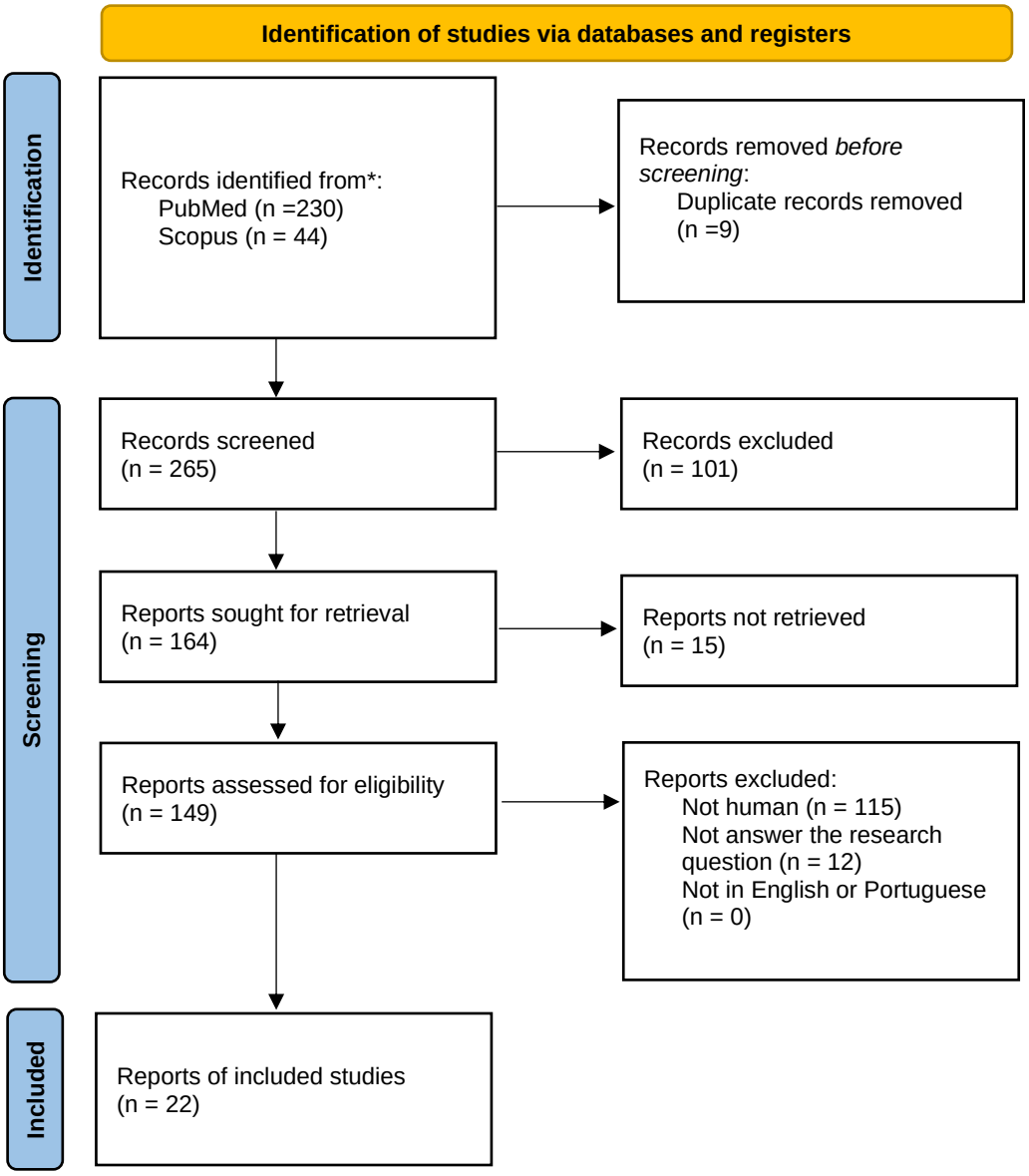
28. Dhamodharan U, Karan A, Sireesh D, Vaishnavi A, Somasundar A, Rajesh K, et al. Tissue-specific role of Nrf2 in the treatment of diabetic foot ulcers during hyperbaric oxygen therapy. Free Radic Biol Med. 2019;138:53-62.

29. Resanovic I, Zaric B, Radovanovic J, Sudar-Milovanovic E, Gluvic Z, Jevremovic D, et al. Hyperbaric Oxygen Therapy and Vascular Complications in Diabetes Mellitus. Angiology. 2020;71(10):876-85.

30. Borys S, Hohendorff J, Frankfurter C, Kiec-Wilk B, Malecki MT. Negative pressure wound therapy use in diabetic foot syndrome-from mechanisms of action to clinical practice. Eur J Clin Invest. 2019;49(4).

31. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ. 2021;372:n71.

Figure 1: PRISMA 2020 flow diagram for new systematic reviews which included searches of databases and registers only (31)



Appendices

Scale for the assessment of narrative review articles – SANRA

1) Justification of the article's importance for the readership

The importance is explicitly justified - Page 9 "In this paper, we focus on diabetic foot ulcers (DFUs), which are a common complication of diabetes mellitus, particularly T2DM, and are responsible for significant morbidity, mortality rates, and associated healthcare costs. (9)"

2) Statement of concrete aims of formulation of questions

One or more concrete aims or questions are formulated. – Page 11 "We will see that molecular targets may present therapeutic potential and which therapies have been investigated to affect angiogenesis." Page 12 "Therefore, this work aims to verify angiogenesis as a targeted therapy for DFU."

3) Description of the literature search

The literature search is described in detail, including search terms and inclusion criteria, – Page 12 "A search for studies published between 2018 and 2021 was conducted in two academic databases, PubMed and Scopus.", "All types of studies (observational, longitudinal, review, and experimental) on the use of targeted angiogenic therapies for the treatment of ischemic diabetic foot ulcers that were conducted in humans and written in English were considered." , "The query ((((("Diabetic Foot"[Mesh]) AND "Angiogenesis Inducing Agents"[Mesh]) OR "Angiogenesis Modulating Agents"[Mesh]) OR "Angiogenic Proteins"[Mesh]) AND " Wound Healing"[Mesh] was used for the search."

4) Referencing

Key statements are supported by references. – Page 11 "However, despite these therapies, DFU remains one of the major complications of diabetes, leading to a deterioration in patients' quality of life and an enormous burden on national health systems."

5) Scientific reasoning

Appropriate evidence is generally present. – Page 19 "The review carried out by Borys

et al. demonstrates the therapeutic effect of this negative pressure system, which consists of tissue deformation at the macroscopic (wound contraction) and microscopic (at the cellular) levels.", "Among these, a randomized control trial with 350 DFU patients reported a higher healing rate in the ones treated with NPWT (43.2%) compared with those receiving standard treatment (28.9%)."

6) Appropriate presentation of data

Relevant outcome data are generally presented appropriately, - Page 18 "On day 20 of treatment, significantly higher Nrf2 levels were observed in subjects receiving adjuvant HBOT [2.5 ± 0.15; p = 0.003], while no significant change was observed in those receiving standard treatment alone [1.2 ± 0.14; p = 0.06]. [13] On day 20 of treatment, an increase in the expression levels of angiogenic markers was also observed, such as FGF-2 [632.3 ± 55.7; p = 0.02] in subjects receiving HBO therapy, and VEGF [325.2 ± 53.5; p = 0.01] and PDGF [6.0 ± 0.4; p = 0.03] in both study groups."

Manuscript Preparation And Formatting Instructions

Manuscripts must be written in clear, grammatical English (see English Language Assistance above). Manuscripts not conforming to Journal format will be returned to authors for modification. Please double space the entire main body document and number each page. Do not add line numbers as the system will generate those when the PDF is built.

Title page, footnotes, abbreviations, and abstract pages must be included in the main body file. Please do not upload separate copies of these documents.

Acceptable document file types for text and tables include .DOC and .DOCX; do not submit a PDF.

Page 1:

Title Page. The following elements are required for every submission:

Title. Include a descriptive title of the work; the title should not be a sentence. No proprietary

or brand names for drugs or agents may be used in article titles. Please, include the study design in the title; for instance, “randomised controlled trial”, or “systematic review”. Titles should be as informative and complete as possible.

Authors. The full first name, middle initials, and family name of each author, as well as the name(s) of the department(s) and institution(s) to which the work should be attributed.

Address for Correspondence. A current email and full mailing address for the corresponding author must be provided.

Page 2:

Abstract. Original articles should include a structured abstract of no more than 300 words using the following headings: Background; Methods; Results; and Conclusions. They should briefly describe, respectively, the problem being addressed in the study, how the study was performed, the salient results, and what the authors conclude from the results. Conventional non-systematic, reviews should include an unstructured abstract of no more than 250 words.

Main Body: Introduction. The introduction contains a statement of the purpose of the work, the problem that stimulated it, and a brief summary of relevant published investigations.

Methods. Avoid detailed description of previously published methods and cite the appropriate reference. Include appropriate ethical and statistical information.

Results. The results should be concise, avoiding redundant tables and figures illustrating the same data.

Discussion. This section should follow the results and is used to interpret results, with minimal recapitulation of findings.

Acknowledgments: The acknowledgements section should be headed 'Acknowledgements relating to this article' and contain the following distinct statements in separate paragraphs:

· Assistance with the study. Acknowledgements should be made only to those who have made a substantial contribution to the study. Authors are responsible for

obtaining written permission from people acknowledged by name in case readers infer their

endorsement of data and conclusions. If there was no assistance state: 'Assistance with the study: none.'

· Financial support and sponsorship. You must make reference to all relevant sources of funding concerning this article. If there were no sources of funding please state: 'Financial support and sponsorship: none.'

· Conflicts of interest. You must make reference to all relevant conflicts of interest concerning this article including financial, consultant, institutional and other relationships that might lead to bias or a conflict of interest. If there are no conflicts of interest please state: 'Conflicts of interest: none.'

· Presentation (for original articles only). Presentations of preliminary data at, for example, international meetings should be acknowledged separately. If preliminary data was not previously presented please state: Presentation: none.

References: Use the Vancouver reference system as adopted by the U.S. National Library of Medicine ensuring that all journal titles conform to Index Medicus approved abbreviations. Number references consecutively in the order in which they are first mentioned in the text. Identify references in the text, tables and legends using superscripted Arabic numerals that are placed after the punctuation. References cited only in tables or in legends to figures should be numbered in accordance with the sequence established by the first identification in the text of the particular table or illustration.