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Clinical and molecular effects of Hyperbaric Oxygen Therapy in diabetic foot ulcers healing

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FMUP FACULDADE DE MEDICINA
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Esta investigação foi realizada na Consulta Multidisciplinar de Pé Diabético dos Serviços de Endocrinologia, Diabetes e Metabolismo e de Medicina Interna do Centro Hospitalar de Vila Nova de Gaia/Espinho, EPE; na Unidade de Medicina Hiperbárica do Hospital Pedro Hispano; no Centro de Medicina Subaquática e Hiperbárica do antigo Hospital da Marinha; no *Cancer Biology Group* do Instituto de Patologia e Imunologia Molecular da Universidade do Porto e no Departamento de Bioquímica da Faculdade de Medicina da Universidade do Porto.

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A candidata declara que teve uma contribuição determinante na realização do trabalho, interpretação dos resultados e discussão dos mesmos. Além disso, contribuiu activamente para a redacção dos trabalhos apresentados.

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Programa Doutoral em *Medicina e Oncologia Molecular*

Nos termos do disposto do n.º 2 do art.º 17.º do Regulamento dos Terceiros Ciclos de Estudos da Universidade do Porto, a seguir se publicita o júri de doutoramento em Medicina e Oncologia Molecular, da licenciada Daniela Maria Martins Mendes, nomeado por despacho vice-reitoral de 30 de julho de 2014, com a tese "Clinical and molecular effects of hyperbaric oxygen therapy in diabetic foot ulcers healing":

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Abstract

THE INDEPENDENT CONTRIBUTION OF DIABETIC FOOT ULCER ON LOWER EXTREMITY AMPUTATION AND MORTALITY RISK

Aims: To estimate 3-year risk for diabetic foot ulcer (DFU), lower extremity amputation (LEA) and death; determine predictive variables and assess derived models accuracy.

Material and Methods: Retrospective cohort study including all subjects with diabetes enrolled in our diabetic foot outpatient clinic from beginning 2002 until middle 2010. Data was collected from clinical records.

Results: 644 subjects with mean age of 65.1 (±11.2) and diabetes duration of 16.1 (±10.8) years. Cumulative incidence was 26.6% for DFU, 5.8% for LEA and 14.0% for death. In multivariate analysis, physical impairment, peripheral arterial disease complication history, complication count and previous DFU were associated with DFU; complication count, foot pulses and previous DFU with LEA and age, complication count and previous DFU with death. Predictive models' areas under the ROC curves ranged from 0.80 to 0.83. A simplified model including previous DFU and complication count presented high accuracy. Previous DFU was associated with all outcomes, even when adjusted for complication count, in addition to more complex models.

Conclusions: DFU seems more than a marker of complication status, having independent impact on LEA and mortality risk. Proposed models may be applicable in healthcare settings to identify patients at higher risk of DFU, LEA and death.

Keywords: Diabetes · Diabetic Foot · Foot ulcer

EFFECT OF HYPERBARIC OXYGEN THERAPY IN DIABETIC FOOT ULCER HEALING: A SYSTEMATIC REVIEW AND META-ANALYSIS

Aims: Conduct a systematic review, with meta-analysis (MA), considering hyperbaric oxygen therapy (HBOT) efficacy for diabetic foot ulcer (DFU) treatment; identify variables affecting outcome; grade overall methodological quality.

Material and Methods: We searched the National Library of Medicine, Rubicon Foundation Research Repository and hand-searched relevant scientific meeting abstract books. Two investigators independently extracted data concerning study characteristics and clinical outcomes, one assessed methodological quality.

Results: Forty studies were included: 11 randomized controlled trials (RCT), 8 non-randomized comparative trials (NRT) and 22 observational studies. The mean proportion of items satisfied, using CONSORT and STROBE, when pertinent, methodological quality reporting checklists, was 47% and 29% respectively. Wound area reduction of 92-100% in the HBOT group at 6 months was reported, but MA was not possible. Pooled difference in time to healing was 0.8 months less in HBOT group (not statistically significant). More ulcers healed with HBOT than without and the chance of healing a DFU was significantly greater with HBOT. There were also significantly fewer major amputations in those undergoing HBOT. Pooled estimates did not achieve statistical significance when RCTs alone were included in analysis. Ten studies assessed independent variables' impact on healing; only age and $TcPO_2$ were evaluated in 2 or more studies. Two models proposed to identify subjects that would benefit most of HBOT were never externally validated.

Conclusions: Although the overall methodological quality of these studies is poor, the existing evidence does suggest that HBOT is effective in DFU healing and decreasing the rate of major amputations.

Keywords: Diabetes · Diabetic Foot · Foot ulcer · Hyperbaric Oxygen Therapy · Systematic review · Therapy

MOLECULAR ENVIRONMENT CHARACTERIZATION, HYPERBARIC OXYGEN THERAPY MODULATOR EFFECT AND CLINICAL IMPACT ON DIABETIC FOOT ULCERS' HEALING

Aims: To compare, in subjects with diabetes mellitus (DM), molecular serum environment (angiogenic, vasculogenic and inflammatory markers) between those with and without active diabetic foot ulcer (DFU); assess hyperbaric oxygen therapy (HBOT) efficacy in DFU reduction and closure, serum markers modulation and microvasculature improvement.

Material and Methods: We conducted a non-randomized trial enrolling a group of patients with DM without active DFU (n=5) and one with active DFU with no significant wound evolution after 8 weeks of standard therapy. We compared those treated with HBOT (n=14) with untreated ones (n=6), due to treatment refusal or contra-indication. Endpoints (determined at 3, 6 and 12 months) included: laboratory markers, clinical outcome, DFU size, percentage of epithelialization.

Results: The sample mean age was 62 and DM duration 18 years. The majority were men, with type 2 DM, insulin-treated, with several complications and acceptable glycaemic and lipid control. After 3 months of HBOT, patients had a significant reduction in leukocyte and C-reactive protein serum levels, and of all DFU measurements. At every time points, the HBOT group achieved better outcomes (less amputation and death) and DFU reduction, with consequent increasing epithelialization percentage.

Comparison was not possible with the non-HBOT group as only one patient remained alive with active DFU and without major amputation at 3 months. Data points that after 1 month of HBOT, vessel number in the DFU tends to increase.
Conclusions: Our data reinforces the potential molecular and clinical efficacy and benefit of HBOT when added to current standard treatment of DFU.

Keywords: Diabetes · Diabetic Foot · Foot ulcer · Hyperbaric Oxygen Therapy · Therapy

IMPROVING HYPERBARIC OXYGEN THERAPY REFERRAL FOR DIABETIC FOOT ULCER TREATMENT: A NATIONWIDE MODELS' VALIDATION AND REFINEMENT STUDY

Aims: This study characterizes the population with diabetic foot ulcer (DFU) undergoing hyperbaric oxygen therapy (HBOT) in Portugal over the last 5 years.
Material and Methods: Validation and optimization of the existing models for the prediction of healing of HBOT treated DFU; through a multicentre retrospective cohort study of all patients undergoing such therapy due to DFU from 2008 to mid-2013 in Portuguese continental hyperbaric medicine centres (n=2).
Results: We included 128 individuals. 66.4% underwent HBOT in Lisbon's former Navy's Hospital. Subjects from this hospital presented less frequently retinopathy (19.6% versus 74.5%, p<0.001) and previous DFU (29.2% versus 53.5%, p=0.02) and had ulcers of shorter duration (median 1.8 versus 4.0 months, p<0.001) than Pedro Hispano's Hospital. Overall, 53.1% healed, which was more likely in non-smoking females without arterial disease, previous DFU or history of lower extremity amputation. Both completion of the planned series of treatments and increasing number of treatments had a positive impact on outcome. Available models had low predictive accuracy. We propose an optimized version of the Hawkins' model that has higher accuracy.
Conclusions: There were some differences in the patients referred to each of these facilities, but healing rates were similar. Further studies are still needed to improve referral criteria for HBOT.

Keywords: Diabetes · Diabetic Foot · Foot ulcer · Hyperbaric Oxygen Therapy · Therapy

Sumário

ÚLCERA DE PÉ DIABÉTICO EM INDIVÍDUOS COM DIABETES COMO FACTOR DE RISCO INDEPENDENTE DE AMPUTAÇÃO E MORTALIDADE

Objectivos: Estimar o risco de úlcera de pé diabético (UP), amputação do membro inferior (AMI) e morte a 3 anos; determinar as variáveis preditivas e avaliar a validade do modelos derivados.
Material e Métodos: Estudo de coorte retrospectivo incluindo todos os sujeitos com diabetes seguidos na nossa consulta de Pé Diabético entre o início de 2002 e meados de 2010. Os dados foram colhidos dos registos clínicos.
Resultados: Incluímos 644 sujeitos com idade média de 65.1 (±11.2) e duração de diabetes de 16.1 (±10.8) anos. A incidência cumulativa foi de 26.6% para UP, 5.8% para AMI e 14.0% para mortalidade. Na análise multivariada, limitação física, história de complicação de doença arterial periférica, número de complicações e UP prévia demonstraram associação com UP; número de complicações, pulsos podológicos e UP prévia com AMI e idade, número de complicações e UP prévia com morte. As áreas sob a curva ROC dos modelos derivados variaram entre 0.80 e 0.83. Um modelo simplificado incluindo apenas UP prévia e número de complicações apresentou validade elevada. UP prévia associou-se com todos os *outcomes*, mesmo quando ajustada para número de complicações ou para modelos mais complexos.
Conclusão: UP parece ser mais do que um marcador de complicações instaladas, tendo um impacto independente no risco de AMI e mortalidade. Os modelos propostos podem ser utilizados em diversos contextos clínicos para identificar os sujeitos em maior risco de UP, AMI e morte.

Palavras-Chave: Diabetes · Pé Diabético · Úlcera

EFEITO DA OXIGENOTERAPIA HIPERBÁRICA NO TRATAMENTO DE ÚLCERAS DE PÉ DIABÉTICO: UMA REVISÃO SISTEMÁTICA E META-ANÁLISE

Objectivos: Realizar uma revisão sistemática e meta-análise (MA), avaliando a eficácia da oxigenoterapia hiperbárica (OHB) no tratamento de úlceras de pé diabético (UP) em indivíduos com *Diabetes Mellitus*; identificar as variáveis preditivas; avaliar a qualidade metodológica da evidência disponível.
Material e Métodos: Foi realizada uma pesquisa electrónica nos *National Library of Medicine* e *Rubicon Foundation Research Repository* e manual dos resumos de reuniões científicas relevantes. Dois

investigadores seleccionaram os estudos e extraíram os dados e desfechos clínicos de forma independente, um avaliou a qualidade metodológica.
Resultados: Foram incluídos 40 estudos: 11 ensaios clínicos randomizados (ECR), 8 não randomizados (ECNR) e 22 estudos observacionais. A média de proporção de items reportados foi 47% e 29% para a CONSORT e a STROBE, respectivamente. A redução da área da ferida foi 92-100% no grupo OHB aos 6 meses (não foi possível MA). O grupo OHB demorou menos 0.8 meses até cicatrização (sem atingir significância estatística). As feridas com OHB cicatrizaram mais frequentemente. Ocorreram significativamente menos amputações major nos indivíduos sob OHB. As estimativas agregadas, incluindo somente os ECNR, não atingiram significância estatística. Apenas 10 estudos analisaram a associação de variáveis independentes com a cicatrização; só a idade e $TcPO_2$ foram avaliadas por 2 ou mais estudos. Os 2 modelos existentes para identificar os sujeitos que mais beneficiarão da OHB nunca foram validados externamente.
Conclusões: Embora a qualidade metodológica dos estudos seja baixa, a evidência existente sugere que a OHB é eficaz na cicatrização das UP em indivíduos com diabetes, diminuindo o risco de amputações major.

Palavras-chave: Diabetes · Pé Diabético · Úlcera · Oxigenoterapia Hiperbárica · Revisão Sistemática · Terapêutica

A OXIGENOTERAPIA HIPERBÁRICA NA EVOLUÇÃO CLÍNICA DE PATIENTES COM ÚLCERA DE PÉ DIABÉTICO ACTIVA: MELHORIA LOCAL E SISTÉMICA

Objectivos: Comparar, em indivíduos com *diabetes mellitus* (DM), com e sem úlcera de pé diabético activa (UP), o ambiente molecular sérico (marcadores angiogénicos, vasculogénicos, inflamatórios); avaliar a eficácia da oxigenoterapia hiperbárica (OHB) na redução e cicatrização de UP, modelação de marcadores séricos e melhoria da microvascularização.
Material e Métodos: Efectuámos um ensaio clínico não randomizado incluindo um grupo de participantes sem (n=5) e outro com UP sem melhoria após 8 semanas de tratamento convencional. Comparámos os sujeitos tratados com OHB (n=14) com os não tratados (n=6), por recusa ou contra-indicação. Os objectivos (aos 3, 6 e 12 meses) foram: marcadores laboratoriais, desfecho clínico, dimensões da UP e percentagem de epitelização.
Resultados: Na amostra, a idade média foi 62 e duração de DM 18 anos. A maioria eram homens, com DM tipo 2, várias complicações e controlo glicémico e lipídico aceitável. Após 3 meses de OHB, ocorreu uma redução significativa do nível sérico de leucócitos e proteína C-reactiva e de todas as dimensões da UP. Em todos os períodos, o grupo OHB apresentou melhores desfechos (menos amputações e

morte), redução da UP e consequente aumento da percentagem de epitelização. Não foi possível comparar com o grupo não-OHB visto apenas um indivíduo se manter vivo e sem amputação major aos 3 meses. Os resultados indicam que 1 mês de OHB tende a aumentar o número de vasos no leito da UP.

Conclusões: Os resultados reforçam o potencial efeito e benefício da OHB a nível molecular e clínico, quando adicionada ao tratamento convencional da UP.

Keywords: Diabetes · Pé Diabético · Úlcera · Oxigenoterapia Hiperbárica · Terapêutica

OPTIMIZAÇÃO DA REFERENCIAÇÃO DE INDIVÍDUOS COM DIABETES COM ÚLCERAS DE PÉ DIABÉTICO PARA TRATAMENTO COM OXIGENOTERAPIA HIPERBÁRICA: VALIDAÇÃO E REFINAMENTO DE MODELO COM BASE NACIONAL

Objectivos: Este estudo caracteriza a população com diabetes e úlcera de pé diabético (UP) activa que efectuaram Oxigenoterapia Hiperbárica (OHB) em Portugal nos últimos 5 anos.

Material e Métodos: Validação e optimização dos modelos existentes para a predição de cicatrização de UP tratadas com OHB, através de um estudo de coorte retrospectivo multicêntrico de todos os pacientes tratados com OHB, de 2008 a meados de 2013, nos centros de Medicina Hiperbárica em Portugal continental (n=2).

Resultados: Foram incluídos 128 indivíduos; 66.4% realizam OHB no Antigo Hospital da Marinha de Lisboa. Utentes provenientes deste hospital apresentavam menos retinopatia (19.6% *versus* 74.5%, p<0.001) e história de UP prévia (29.2% *versus* 53.5%, p=0.02) e as UP tinham menor duração (mediana 1.8 *versus* 4.0 meses, p<0.001) do que os do Hospital Pedro Hispano. No total, 53.1% das UP cicatrizaram, mais frequentemente em mulheres, não fumadoras, sem doença arterial periférica, história prévia de UP ou amputação. A realização da totalidade do tratamento prescrito e um maior número de sessões influenciaram positivamente o desfecho. Os modelos preditivos disponíveis apresentaram baixa validade, pelo que propusemos uma versão optimizada do modelo de Hawkins com melhores resultados.

Conclusões: Verificámos diferenças nas características dos indivíduos de cada instituição, mas a proporção de cicatrização foi semelhante. São necessários mais estudos para que se possa melhorar os critérios de referenciação para OHB.

Palavras-chave: Diabetes · Pé Diabético · Úlcera · Oxigenoterapia Hiperbárica · Terapêutica

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ABBREVIATIONS AND ACRONYMS

ABI	Ankle brachial index
ADA	American Diabetes Association
AGEs	Advanced Glycation End Products
ARR	Absolute Risk Reduction
ATA	Atmospheres Absolute
AUC	Area Under the Operator Receiver Operating Curve
CI	Confidence Interval
cm^3	Cubic Centimetres
CONSORT	Consolidated Standards of Reporting Trials
CRP	C-Reactive Protein
DFU	Diabetic Foot Ulcer
Dl	Decilitre
DM	Diabetes Mellitus
DPN	Diabetic Peripheral Neuropathy
ECHM	European Committee for Hyperbaric Medicine
EPCs	Endothelial Progenitor Cells
ESR	Erythrocyte Sedimentation Rate
ESRD	End Stage Renal Disease
ESWT	Extracorporeal Shockwave Therapy
Fi O_2	Fraction of Inspired Oxygen
FNH	Former Navy's Hospital
HbA1c	Glycated Haemoglobin
HbCO	Carboxyhemoglobin
HBI	Hallux brachial Index
HBOT	Hyperbaric Oxygen Therapy
HIF1	Hypoxia Inducible Factor
Hz	Hertz
kPa	Kilopascal
ICD-9	The International Classification of Diseases, 9th Revision
LEA	Lower Extremity Amputation
LR	Likelihood Ratio
MA	Meta-analysis

MEDLINE	National Library of Medicine
Ml	Millilitre
mm Hg	Millimetres of Mercury
MMPs	Metalloproteinases
MOOSE	Meta-analysis Of Observational Studies in Epidemiology
NA	Not Applicable
NNT	Number Needed to Treat
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NR	Not Reported
NRT	Non Randomized Trial
NS	No Statistical Significant Association
O_2	Oxygen
OR	Odds Ratio
PAD	Peripheral Arterial Disease
PBS	Phosphate Buffered Saline
Pg	Pico gram
PHH	Pedro Hispano's Hospital
PlGF	Placental Growth Factor
PpO_2	Partial Pressure of Oxygen
PPV	Positive Predictive Value
PRISMA	Preferred Reporting Items for systematic reviews and Meta-Analyses
RAGEs	Advanced Glycation End Products Receptors
RCT	Randomized Controlled Trial
ROC	Receiver operating characteristic
ROS	Reactive Oxygen Species
RR	Relative Risk
RRR	Relative Risk Reduction
SD	Standard Deviation
SDF 1-α	Stromal Cell Derived Factor 1- Alpha
SIGN	Scottish Intercollegiate Guideline Network
STARD	Standards for Reporting of Diagnostic Accuracy
STROBE	Strengthening of the Reporting of Observational Studies in Epidemiology
SVEFGR-1	Soluble Vascular Endothelial Growth Factor Receptor - 1

SWM	Semmes-Weinstein Monofilament
$TcPO_2$	Transcutaneous Partial Pressure of Oxygen
TNF-α	Tumor Necrosis Factor-alfa
TUC	Texas University Classification
UHMS	Undersea and Hyperbarical Medical Society
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
vs	Versus
VST	Vibration Sensation Test

List of figures

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I. List of publications

Included in the thesis

Section IV, A, 3

Martins-Mendes D, Monteiro-Soares M, Lima J, Soares R.

Diabetic foot patients' 3 and 5 year follow up: ulcer occurrence, amputation and mortality.

Rev Clin Esp, 2012, 212: 271-319

Abstract of poster oral presentation in 11th Congress of the European Federation of Internal Medicine, 24-27 October 2012, Madrid, Spain

Martins-Mendes D, Monteiro-Soares M, Boyko EJ, Ribeiro M, Barata P, Lima J, Soares R.

The independent contribution of diabetic foot ulcer on lower extremity amputation and mortality risk.

J Diabetes Complications, 2014, [Epub ahead of print]

Impact factor: 2.06

Section V

Mendes D, Fernandes T, Camacho O, Lima J, Soares R.

Clinical and molecular effects of hyperbaric oxygen in diabetic foot ulcers – Preliminary data.

Poster presentation in 37th EUBS Annual Scientific Meeting, 24-27 August 2011, Gdansk, Poland

Mendes D, Fernandes T, Camacho O, Lima J, Soares R.

Clinical and molecular effects of hyperbaric oxygen in diabetic foot ulcers – Preliminary data.

Poster presentation in 1º Encontro de Doutorandos da FMUP, 14 December 2011, Oporto, Portugal

Mendes D, Monteiro-Soares M, Lemos E, Távora A, Sobral J, Duarte I, Brandão D, Campos-Lemos J, Madureira M, Ribeiro M, Fernandes T, Camacho O, Lima J, Soares R.

Clinical and microscopic effects of hyperbaric oxygen in diabetic foot ulcers.

Poster presentation in 38th EUBS Annual Scientific Meeting, 10-13 September 2012, Belgrade, Serbia

Mendes D, Monteiro-Soares M, Fernandes T, Camacho O, Lima J, Soares R.
Clinical and microscopic effects of Hyperbaric Oxygen in diabetic foot ulcers' healing.
Oral presentation in 2º Encontro de Doutorandos da FMUP, 14 December 2012, Oporto, Portugal

Martins-Mendes D, Costa R, Moura J, Rodrigues I, Cortez A, Ribeiro M, Lima J, Soares R.
Hyperbaric oxygen therapy in clinical outcome of patients with diabetic foot ulcers: local and systemic improvement.
Submitted for publication

Section VI

Martins-Mendes D, Monteiro-Soares M, Fernandes T, Camacho O, Ribeiro M, Oliveira MJ, Lima J, Soares R.
Validação de modelo preditivo para optimizar a identificação dos utentes com diabetes e úlcera de pé diabético ativa que beneficiarão de oxigenoterapia hiperbárica.
Revista Portuguesa de Diabetes, 2014, 9 (S1)
Abstract of oral presentation in 11º Congresso Português de Diabetes, 6-9 March 2014, Vilamoura, Portugal

Martins-Mendes D, Monteiro-Soares M, Bennett M, Oliveira-Anão A, Quaresma-Guerreiro F, Fernandes T, Camacho O, Lima J, Soares R.
Improving hyperbaric oxygen therapy referral for diabetic foot ulcer treatment: a nationwide models' validation and refinement study.
Submitted for publication

In the Diabetic Foot topic but not included in this thesis

Monteiro-Soares M, Mendes D, Guimarães R et al.
Using diabetic foot ulcer development risk stratification systems for the wound healing prediction.
10th Scientific Meeting of the Diabetic Foot Study Group, 28-30 September 2012, Potsdam-Berlin, Germany

Monteiro-Soares M, Martins-Mendes D, Guimarães R et al.

Referenciação para uma consulta multidisciplinar de Pé Diabético: análise da qualidade da informação.

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Monteiro-Soares M, Martins-Mendes D, Guimarães R et al.

O impacto do controlo glicémico, avaliado através da Hba1c, na probabilidade de amputação podológico em utentes com diabetes e úlcera activa.

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Monteiro-Soares M, Martins-Mendes D, Dinis-Ribeiro M et al.

The impact of impaired renal function on the prediction of diabetic foot complications: should it be included on foot risk classifications.

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Monteiro-Soares M, Martins-Mendes D, Vaz-Carneiro A, Sampaio S, Dinis-Ribeiro M.

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Diabetes Metab Res Rev, 2014, [Epub ahead of print].

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Oxigenoterapia hiperbárica na recuperação de lesões desportivas.

Rev Medicina Desportiva, 2013, 4(5): 20–21

II. Aims and outline

With this project we aimed to study the effects of hyperbaric oxygen therapy (HBOT) in the treatment of diabetic foot ulcers (DFU), namely the molecular and clinical effects, and consequently to improve the available evidence supporting this therapy.
In order to do so we have developed 4 main studies presented throughout this thesis.

In this *Chapter II* the structure of the thesis is described, while in *Chapter III* a short comment concerning the importance of this thesis conduction is performed.

Chapter IV, *Section A,* diabetic foot epidemiology and healing process impairment are overviewed and the results of a retrospective cohort study, characterizing the population followed in our Outpatient Diabetic Foot Clinic, where the molecular studies were conducted, in terms of demographic and clinical variables; estimating the 3- and 5-year risk of DFU development, lower extremity amputation (LEA) and death; and determining causes and predictive variables for these outcomes occurrence, are presented.

Chapter IV, Section B consists in detailing HBOT mechanisms, physiologic and therapeutic effects, side effects and complications, as well as, its clinical application.

Chapter IV, Section C is composed of a systematic review and meta-analysis (MA) assessing the available evidence concerning systemic HBOT effectiveness for the DFU treatment. With this study, we aimed to a) characterize available evidence assessing HBOT effectiveness for the DFU treatment, b) assess independent variables' associated with healing, c) calculate pooled measures for minor and major LEA risk and d) identify methodological limitations of the published data.

In *Chapter V*, HBOT effectiveness in DFU is assessed. A non-randomized human trial is described, assessing molecular environment characterization, along with HBOT modulator effects in order to a)

identify and compare baseline angiogenic, vasculogenic and inflammatory markers in 3 groups of patients: 1) subjects with diabetes and without DFU, 2) with DFU and 3) with DFU treated with HBOT. In the last 2 groups, markers reassessment is performed at 3 months, to detect molecular differences due to HBOT. To understand if those effects persisted long term, in the HBOT group, a re-evaluation was carried out at 6 months. HBOT effectiveness in DFU healing was made by comparing outcome (healing, improvement, LEA and death), DFU dimensions, percentage of wound epithelialization and microvessel density analysis at different time points (3, 6 and 12 months) in the DFU groups.

Chapter VI consists in a nationwide multicentre retrospective cohort study, assessing the variables associated with DFU's outcome when treated with HBOT, validating and refining a predictive model for DFU outcome in subjects undergoing this therapy, and reporting prognostic accuracy measures.

In *Chapter VII,* main conclusions and limitations are described in a standardized manner, addressing the studies strengths and limitations, and future research to overcome them is presented.

III. Rationale

Worldwide, diabetes mellitus (DM) is one of the most frequent metabolic disorders [Wild S 2004, IDF 2013] and its global burden is attributed to its several complications, namely DFU with impaired healing, that frequently requiring LEA [Frykberg R et al, 2006; Margolis D et al, 2008; Sun J et al, 2012].

The occurrence of a DFU bodes poorly for the clinical course of patients with diabetes, with higher rates of re-ulceration, LEA, contralateral LEA and death, compared to persons with diabetes who have not experienced a DFU [Frykberg R et al, 2006].

Given the limited health care resources, it is important to optimize their allocation. To do so, an adequate stratification of subjects with diabetes by their risk of morbidity, namely DFU and LEA, as well as mortality, is crucial. Thus, identification of variables associated with these outcomes is the first step in the pathway for the creation or optimization of preventive/therapeutic programmes.

Even though the cascade of diabetic foot complications-DFU-LEA has been linked to higher mortality risk [Fortington LV, 2013], increasing number of DM complications is also associated with higher mortality [Brownrigg JR, 2012]. DFU is usually considered a marker of diabetes complication status, i. e., a marker for neuropathy and associated disease in the foot. Still, some authors hypothesized that DFU occurrence could be, *per se*, an independent predictive variable of LEA as well as mortality [Boyko EJ et al, 1996].

Nevertheless, adjustment for baseline complications was rarely conducted when assessing the impact of DFU on LEA, and of both on the mortality risk [Boyko EJ et al, 1996]. In addition, simple models for their prediction (specially using the same core variables) were seldom proposed.

Given the current state of knowledge, we considered essential to estimate the risk for DFU, LEA and death in a cohort of patients with diabetes followed in our Diabetic Foot Outpatient Clinic and determine factors that independently predict LEA and mortality. This was the first step of this thesis, in order to assess whether it was pertinent and valuable to address a treatment modality for DFU in our population.

In our sample of 644 subjects, in a high risk setting, during 2002-2010, the 3-year cumulative incidence for DFU was 26.6%, for minor LEA 2.7% and for major 3.1%, and 14.0% for death. It was possible to derive simple models for prediction of the three outcomes, and we concluded that DFU was independently associated with LEA and death.

Several mechanisms are described in the literature explaining such associations. For example, peripheral arterial disease (PAD) impairs wound healing, due to inadequate circulation, and has been independently associated with both LEA [Adler A et al, 1999] and DFU [Boyko E et al, 1999].

We observed a higher rate of DFU development (>8% annually), but a similar of LEA and death, compared to several studies [Monteiro-Soares M et al, 2011; Schaper N, 2012]. However, measures to improve these figures are still required, and so methods for DFU adequate treatment and prevention are essential.

This thesis emphasizes DFU therapy, for it is very challenging. Reported life time LEA rates range from 6.4 to 43% [Margolis D et al, 2008; Sun J et al, 2012]. Non-traumatic LEAs are up to 40 times [Fard et al, 2007] more frequent in subjects with diabetes [Boulton A et al, 2005; Frykberg R et al, 2006]. Despite this great clinical need, several authors consider the available evidence addressing DFU treatment to be very poor [Mason et al, 1999].

One of such treatments is HBOT that aims to induce several physiologic and therapeutic effects. Nevertheless, it is an expensive therapy, with limited availability, thus, identification of patients with best possible response is fundamental for proper allocation of limited healthcare resources.

Therefore, a systematic review and MA was conducted, evaluating all available evidence assessing the efficacy of systemic HBOT for DFU, identifying the independent predictors of outcome and the methodological limitations in the existing evidence. With such study, we observed that HBOT induces higher chance of DFU healing and lower risk of LEA. However, several limitations in the existing evidence were noticed, namely, small sample sizes, differences in treatment protocols, poor methodological quality and, for all these, a high heterogeneity in the calculated measures.

In addition, animal studies point that HBOT, by raising tissue oxygenation in chronically hypoxic tissues, stimulates a number of elements required for wound healing, including angiogenesis, collagen synthesis and reactivation of the oxygen-dependent phagocytic capacity of leukocytes. Combined with the demonstrated ability of even a single HBOT session to mobilise endothelial progenitor cells (EPCs) from the bone marrow in diabetic individuals, all these actions promote active healing in a chronic, non-healing DFU [Gallagher et al, 2006; Thom SR et al 2006; Thom SR et al, 2011; Yang B et al, 2007].

However, studies in humans with diabetes and active DFU are still lacking.

Hence, we considered essential to compare, in subjects with diabetes, the molecular serum environment between those with and without active DFU and assess HBOT modulation and clinical efficacy at 3, 6 and 12 months. Our results showed that HBOT was effective in improving healing. Nevertheless, we were unable to fully understand the molecular mechanisms behind our good clinical results.

With our systematic review, we detected a lack of studies addressing the factors associated with improved healing and only 2 predictive models were retrieved, without ever being externally validated, for the identification of the subjects that would benefit most of HBOT.

In Continental Portugal, there are only two Hyperbaric Medicine Centres treating patients with active DFU, one in Oporto, located in Pedro Hispano's Hospital (PHH) in Matosinhos (a public civil hospital, referral area from the north to the centre of the country) and one in Lisbon, in the former Navy's Hospital (FNH) (military hospital, referral area from the centre to the south). Recently, hyperbaric centres in the archipelagos have also started treating DFU patients. In our clinical experience, we have noticed large variations in the clinical criteria when referring patients for HBOT.

Given these disparities, we decided to characterize the Portuguese population undergoing HBOT in the last 5 years, to understand Portugal's pattern of DFU referral and subsequent outcome, and to propose an optimal simple referral model. Such goal was achieved and, despite several differences were observed between the studied institutions, healing rates were similar.

In sum, with this project we hope to have improved the evidence around DFU treatment with HBOT, in a translational manner. Firstly, assessing potential impact of HBOT in our context by evaluating diabetic foot complications development and death rates. Secondly, evaluating the available evidence about this topic to detect the possible need of further studies. As we concluded that there was a lack of biochemical and clinical studies with adequate methodological report, we performed one addressing molecular markers and clinical evolution and another improving referral protocol.

IV. Background

A. Diabetic foot

1. *Healing cascade*

Wound healing is a complex biological process involving several coordinated pathways. It is characteristically divided in four different phases: haemostasis, inflammation, proliferation and tissue remodelling [Young A, 2011], that overlap in time.

The process of healing encompasses numerous steps including haemostasis, removal of necrotic material and bacteria, inflammation terminus, extracellular matrix repair, neovascularization, epithelialization and remodeling [Li J et al, 2007; Young A, 2011]. We must emphasize that the majority of these stages are dependent on oxygen content [Eming SA et al, 2007 A; Sen C, 2008; Tandara A & Mustoe T, 2004; Young A, 2011].

Haemostasis involves arteriolar constriction and posterior dilatation, clot formation through the pathways of the clotting cascade and platelet activation. Platelets also synthesize many factors that regulate various cell types and contribute to the inflammatory phase [Li J et al, 2007; Young A, 2011].

Immediately after a lesion occurs, *inflammation* begins with different types of leukocytes being attracted to the wound and releasing several mediators, including growth factors, which begin, maintain or finish angiogenic response and also recruiting other cell types. Neutrophils, the first to arrive, ingest and destroy bacteria through phagocytosis, degranulate and release several substances to destroy microorganisms and tissue debris, and produce oxygen reactive species (ROS) that have bactericidal effect [Eming SA et al, 2007 A; Li J et al, 2007; Young A, 2011].

In the *proliferative phase*, fibroblasts invade the wound bed, proliferate, synthesize extra-cellular matrix components, namely collagen, and later differentiate to a contractile phenotype, the myofibroblast, that connects to surrounding tissues, and is involved in wound contraction [Eming SA et al, 2007 A; Li J et al, 2007; Young A, 2011].

Neovascularization can occur through angiogenesis, by activation and migration of mature resident endothelial cells, and/or vasculogenesis, from undifferentiated angioblasts or EPCs both in

embryogenesis or bone marrow-derived in adults [Stavrou D, 2008; Velasquez OC, 2007]. This is an essential process for the different phases' occurrence.

During epithelialization, the epithelial cells migrate from the edges of the wound to cover its surface [Eming SA et al, 2007 A; Li J et al, 2007; Young A, 2011].

Finally, during *tissue remodeling*, with synthesis and degradation of several components the scar is altered to achieve a structure similar to unwounded tissue [Eming SA et al, 2007 A; Young A, 2011].

Several biochemical factors are essential for an adequate healing process.

Vascular Endothelial Growth Factor (VEGF) family is composed of 7 elements: VEGF-A to F and Placental Growth Factor (PlGF). VEGF family and their receptors (VEGFR) are mediators of the embryo vascular development and of the adult angiogenesis processes (both physiological and pathological), including wound healing. VEGF-A is the most studied; its transcription is regulated by several factors such as growth factors, pro-inflammatory cytokines, hormones and cellular stress [including hypoxia – namely through action of Hypoxia Inducible Factor 1 (HIF1)] [Eming SA et al, 2007 A; Beldon P 2010].

It was studied, in transgenic mice, the role of PlGF in healing, which promotes faster wound repair, with increase of the granulation tissue vasculatization. Soluble vascular endothelial growth factor receptor-1 (sVEGFR-1), a splicing variant of transmembranar VEGFR-1, is considered a natural inhibitor of VEGF-A. Studies revealed that sVEGFR-1 concentration in chronic wounds fluid is higher than its concentration in wounds that heal [Eming SA et al, 2007 A].

In diabetic patients and animal models, the number and function of circulating EPCs is highly decreased, and these alterations are related to the cardiovascular and healing complications in diabetes [Costa C et al, 2007; Fadini GP et al, 2005; Fadini GP, 2014; Laing T et al, 2007].

Stromal Cell Derived Factor 1- Alpha (SDF1-α) is a cytokine that induces mobilization of bone-marrow derived progenitor cells, mainly through the C-X-C Chemokine receptor type 4 [Velasquez OC, 2007].

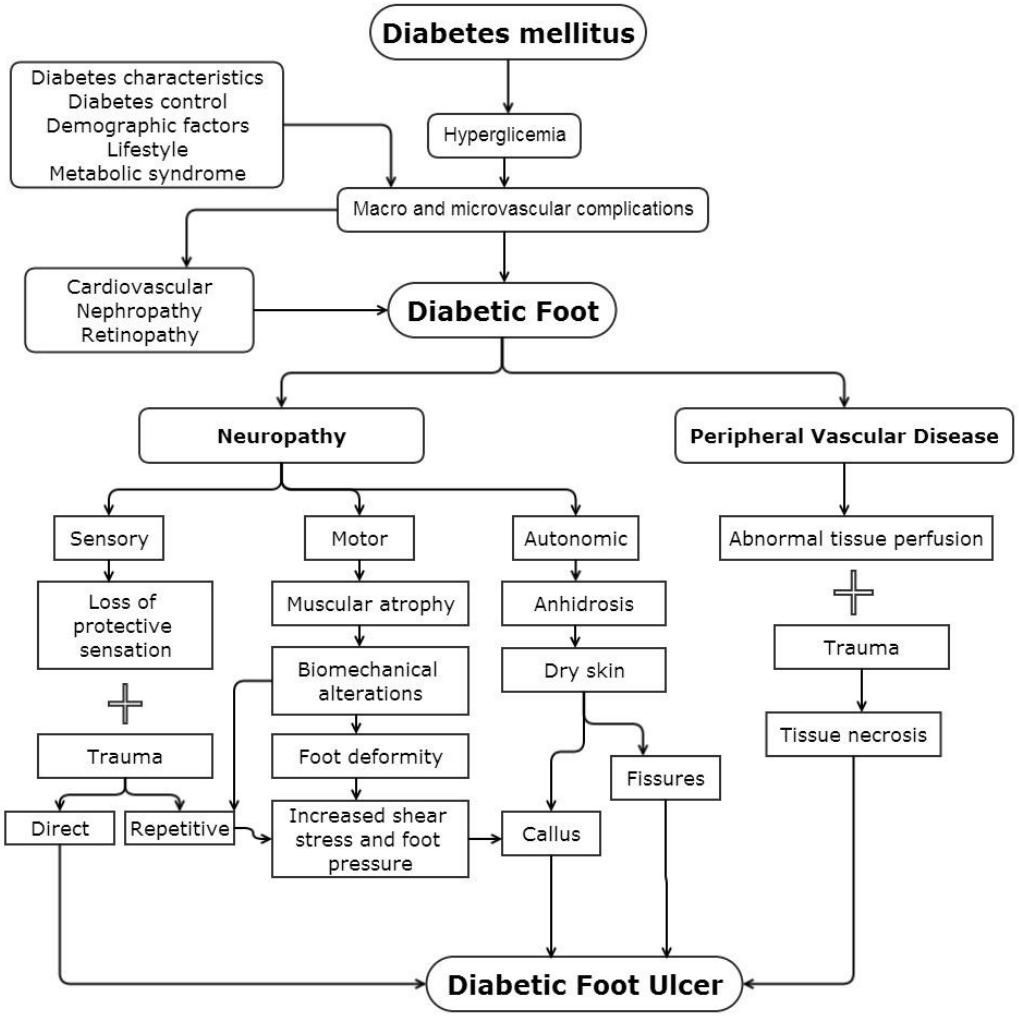
Tumor necrosis factor-alfa (TNF-α), a factor with multiple functions, plays a central role in inflammation, therefore, being an intervenient in the inflammatory phase of healing [Schreml S et al, 2010].

DM frequently leads to foot ulcers, through an already exhaustively described pathophysiologic process (Figure 1), and also to a defective wound repair due to alterations in the micro- and macro-vasculature, cellular and molecular environment, as well as to the frequent presence of infection and/or neuropathy [Brem H & Tomic-Canic M, 2007; Costa C et al, 2007; Laing et al, 2007; van Weel et al, 2008].

Diabetic patients and animal models have altered vasculature. In some territories, like retina, angiogenesis is increased, while in others, for instance distal microvasculature of the inferior limbs, it is decreased [Costa C et al, 2007; van Weel et al, 2008]. Studies have shown that diabetic mice, in whom the healing process is altered, have lower levels of VEGF-A mRNA and of the protein's intracellular processing during wound repair and decreased angiogenesis [Costa C et al, 2007; Brem H & Tomic-Canic M, 2007].

Additionally, the diabetic environment induces several molecular changes by advanced glycation end products (AGEs) that link to their receptors (RAGEs), including pathways activation, altered dermal fibroblasts and keratinocytes proliferation [Blakytny R & Jude E, 2009; Schreml S et al, 2010]

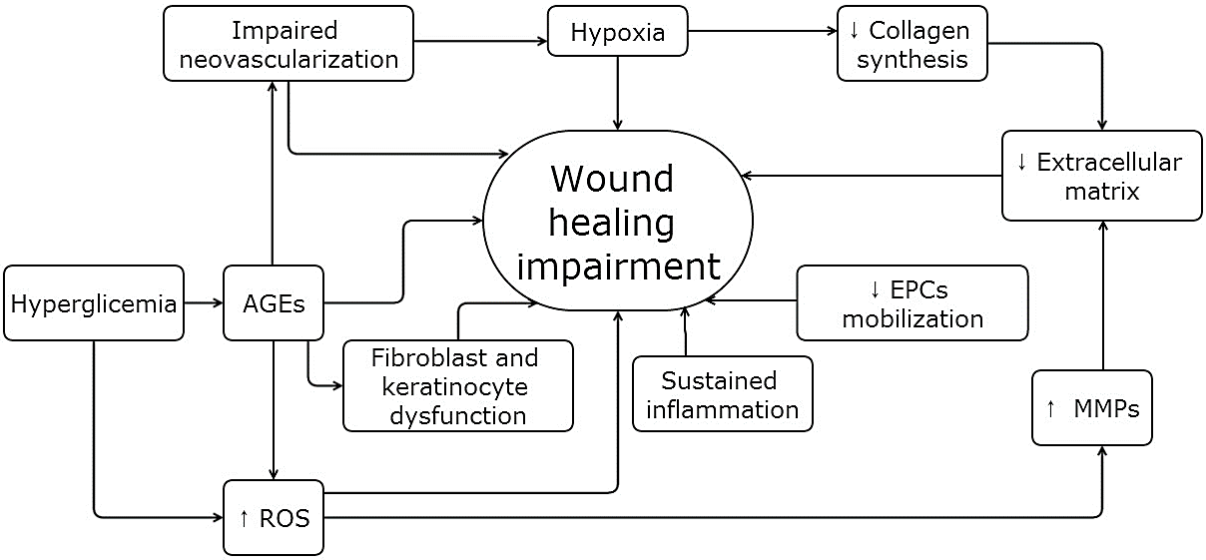
Figure 1. Diabetic foot ulcer pathophysiology



(Adapted, with permission, from Monteiro-Soares, 2010)

Local hypoxia is common. The optimum oxygen tissue pressure (pO2) is 50-100 mmHg but in many wounds it scarcely reaches 10 to 30 mmHg [Eming SA et al, 2007; van Weel et al, 2008]. On the other hand, one of the main reasons for delayed wound healing is a sustained inflammatory reaction [Eming SA et al, 2007 A; Eming SA et al, 2007 B; Schreml S et al, 2010]. For all this (Figure 2), DFUs frequently evolve to chronic wounds, which are lesions that do not heal in the usual time (4-6 weeks) with conventional therapy.

Figure 2. Healing impairment mechanism in the diabetic foot



↑: Increase; ↓: Decrease; AGEs: Advanced Glycation End product; EPCs: Endothelial Progenitor Cells; MMPs: Metalloproteinases; ROS: Reactive Oxygen Species

2. *Epidemiology of Diabetes: national and international*

Approximately 8.3% of the world adult population, corresponding to 382 million people, has DM which turns this condition in one of the most frequent metabolic disorders [IDF 2013].

Incidence and prevalence are continuously rising, consequently carrying high rates of morbid-mortality, with premature deaths, and economic burden [IDF 2013]. In fact, it was reported that 2.5% to 15% of global annual health care budgets are spent on DM, with an annual direct medical cost worldwide of around 241 billion dollars [WHO, 2013; Alavi A et al, 2014].

In Europe, it is calculated that 56 million people have DM, around 28% of deaths in people under 60 years old are due to this disease [IDF 2013] and that the majority of these could be prevented [WHO, 2013; Alavi A et al, 2014].

In Portugal, during 2012, DM caused 4880 potential years of life lost in those less than 70 years and a total of 4867 deaths were attributed to this condition, corresponding to 4.5% of global mortality [OND, 2013].

In Oporto region, DM has a prevalence of 13.9% [OND, 2009], whereas the national one is 12.9% [OND, 2013]. We must highlight that the referral area of the Centro Hospitalar de Vila Nova de Gaia/Espinho, Entidade Pública Empresarial (EPE), Diabetic Foot Outpatient Clinic, where the clinical studies of this thesis were conducted, belongs to this higher prevalence region.

Diabetic foot is one of the major DM complications and causes a considerable costs in health care and patient well-being. It is estimated that around 10 to 25% of subjects with diabetes will develop a DFU [Frykberg R et al, 2006], during their lifetime, from which 50 to 70% will recur in a 5 year period [Alavi A et al, 2014].

Chronic DFU have a great impact in the subjects' quality of life. It was stated that such individuals present from 10 to 40% lower quality of life scores when compared to the general population, being comparable to those with chronic lung disease, myocardial infarction, and breast cancer [Armstrong DG et al, 2008].

On the other hand, treating DFU represents substantial costs, with the highest impact being associated with the consequent LEA [Akhtar S et al, 2011] and rehabilitation.

In Portugal alone, 1493 LEA were performed in 2012 due to diabetic foot complications (730 major and 763 minor LEA) [OND, 2013], with major differences between the regions being noticed. In the last National Observatory report it is stated that fewer major LEA are conducted in the North region, where Oporto is included. Along this thesis some possible explanations will be discussed.

Nevertheless, several studies confirm that treating successfully a DFU is consistently more cost-effective when compared to the expenses of performing a major LEA [Alavi A et al, 2014; Morbach S, 2003].

For all this, the development and/or improvement of therapeutic options for the DFU healing are crucial.

3. *The independent contribution of diabetic foot ulcer on lower extremity amputation and mortality risk*

Material and Methods

Subjects

A retrospective cohort study was conducted including all subjects with diabetes followed in Centro Hospitalar de Vila Nova de Gaia/Espinho, Entidade Pública Empresarial (EPE), Diabetic Foot Outpatient Clinic from the 1st of January 2002 until the 31st of May 2010.

Subjects were excluded if they met any of the following criteria: active DFU at the moment of inclusion, inability to ambulate, communication or cognitive impairment (due to aphasia and/or dementia), missing data on any covariate (except for vibration sensation assessed using a tuning fork and HbA1c), follow-up period of less than 3 years, or living outside our referral area.

The Diabetic Foot Clinic is a tertiary care unit, with a multidisciplinary team and specialized diabetic foot care, treating patients from primary care institutions (usually with high risk feet and/or unavailable appropriate care in their residence area) or from other departments and hospitals.

The study was approved by the Ethics Committee of our institution and no adverse event occurred in any subject due to participation in this research.

Data collection

Clinical records were reviewed and data collected from 1st until the 30th of June 2013.

All variables were collected in the first podiatric appointment in the clinic, through a structured interview and detailed foot exam, apart from HbA1c, by one of the two department podiatrists who were experienced in the care of diabetic foot complications.

Demographic characteristics (age at the time of inclusion, gender, education level), DM type (classified according to the World Health Organization definition [WHO, 2006]), duration and treatment (diet only, oral medication or insulin), metabolic control [through glycated haemoglobin (HbA1c)], physical (inability to reach his/hers own feet [Monteiro-Soares M et al, 2012]) and/or visual impairment and smoking habits (absent, current, former) were recorded.

DM complications [retinopathy, nephropathy, neuropathy, cerebrovascular, cardiovascular, PAD and metabolic (ketoacidosis, hyperosmolar coma or other coma)] were classified in accordance to the Diabetes Complications Severity Index created by Young et al [Young BA et al, 2008], according to their protocol, using the International classification of diseases, 9th revision (ICD-9) Codes, through clinical record review.

As Young et al concluded that the accuracy of the number of complications was similar to the Complication Severity Index and as the number of complications is easier to calculate we opted to use it. Nephropathy was also staged by the American Diabetes Association (ADA) classification [ADA, 2013], using the serum creatinine value closest to the date of the first appointment.

Participants' feet were characterized using the variables more frequently described in DFU development risk stratification systems [5] and with proved association with its occurrence [Monteiro-Soares M et al, 2012], namely, the presence of deformities, onychomycosis, diabetic peripheral neuropathy (DPN) [using the Texas Verbal Questionnaire [Armstrong DG et al, 1998 A], Semmes-Weinstein monofilament (SWM) insensitivity and tuning fork vibration sensation], PAD (characterized by total foot pulses and intermittent claudication), oedema and history of previous DFU or LEA. There is a lack of studies assessing the reliability of these measurements [Monteiro-Soares M et al, 2012]. Previous DFU was collected through foot assessment, patient self-report and all were additionally confirmed by medical record review. All the above described variables in addition to visual and/or physical impairment, presence of onychomycosis and DFU occurrence were collected and defined according to the protocol previously described by Monteiro-Soares et al [Monteiro-Soares et al, 2010 B].

Vibration sensation test (VST) was assessed with a 128 Hz tuning fork applied, perpendicularly with a constant pressure, on a bony part on the dorsal side of the distal phalanx of the first toe.

This procedure was repeated twice and two incorrect answers were classified as altered sensation [Bakker K et al, 2012]. This procedure was instituted in 2008 and therefore patients that entered into the study prior to this time do not have this assessment.

Subjects were followed from the time of inclusion to death or completion of the 3-year follow-up.

Minor LEA was defined as the surgical removal of toe(s), ray(s) or forefoot. Major LEA was considered amputation of the entire foot by any level of the leg (including the ankle).

HbA1c value was not always available (n= 164) as several patients were followed for their metabolic control mainly by primary care physicians.

DFU and/or LEA occurrence and death dates were registered. Subjects were advised to contact the clinic if any lesion developed and during appointments they were asked if any DFU occurred. Furthermore, complete medical records from the hospital as well as primary care institutions were reviewed in order to detect missed events. LEA and death (date and cause) are automatically registered in the individuals' computerized clinical file. Death causes were collected using the ICD-9 codes.

Statistical analysis

Association between variables and outcomes (DFU, LEA or death) was conducted using univariate logistic regression. Values of $p \leq 0.05$ were considered as statistically significant and ≤ 0.1 as pertinent for initial inclusion into the predictive models. Multivariate analysis to estimate odds ratios (OR) for amputation and mortality in relation to DFU adjusted for covariates was performed using logistic regression analysis employing a backwards stepwise algorithm approach. In addition, all multivariable models included age, gender and diabetes duration.

After the model creation for each outcome, a multivariable score was computed for each subject using the β coefficient values and the actual values for the covariates for those subjects. The ability of the score to discriminate between patients who did and did not develop the outcomes of interest was assessed using the area under the receiver operating characteristic curve (AUC) with the 95% confidence interval (CI).

All statistical analyses were conducted using the programme IBM SPSS, version 20.0 (Chicago, IL, USA). Missing and indeterminate results were excluded from analysis.

Results

Participant characteristics

In this study, 644 subjects were included and followed for a median of 36 months (range 1-36).

At baseline, patients had a mean age of 65.1 (±11.2) years; mean diabetes duration of 16.1 (±10.8) years; and mean HbA1c of 7.8% (±3.7). The majority had type 2 DM and less than half were on insulin. More than half were female; over 80% were undereducated (primary school level or less) and over a quarter had some form of impairment (visual and/or physical). The most frequent complications were PAD related (63.0%) and the least frequent was metabolic complication history (3.6%). Forty-one percent of our population had a history of previous DFU (Table 1).

Cumulative incidence at 3 years for DFU and the outcomes of interest was as follows: DFU 26.6% (95% CI 23.2-30.0), recurrent DFU 34.5% (95% CI 27.4-48.4), minor LEA 2.7% (95% CI 1.4-4.0), major LEA 3.1% (95% CI 1.8-4.4), total LEA 5.8% (95% CI 3.9-7.5) and death 14.0% (95% CI 11.3-16.7).

Table 1: Participants baseline characteristics

Variables	Values (n= 644)
SUBJECT CHARACTERIZATION	
Age [mean (SD)]	65.1 (11.2)
Female gender [n (%)]	339 (52.6)
Analphabetic or primary school [n (%)]	529 (82.2)
Visual impairment [n (%)]	248 (38.5)
Physical impairment [n (%)]	237 (36.8)
Past or present smoker [n (%)]	134 (20.8)
DM AND ITS COMPLICATIONS	
Type 2 [n (%)]	629 (97.7)
Duration (in years) [mean (SD)]	16.1 (10.8)
Insulin use [n (%)]	260 (40.4)
HbA1c (in %) [mean (SD)][a]	7.8 (3.7)
Cardiovascular complications history [n (%)][b]	219 (34.0)
Retinopathy [n (%)][b]	297 (46.1)
Laser photocoagulation [n (%)]	211 (32.8)
Nephropathy [n (%)][b]	98 (15.2)
4-5 stage in ADA classification [n (%)]	37 (5.8)
PDV complications history [n (%)][b]	406 (63.0)
Neuropathy complications history [n (%)][b]	340 (52.8)
Metabolic complications history [n (%)][b]	23 (3.6)
Complications count [mean (SD)][b]	1.7 (1.1)
FOOT CHARACTERIZATION	
Foot deformity [n (%)]	503 (78.1)
Oedema [n (%)]	165 (25.6)
Onychomycosis [n (%)]	379 (58.9)
Total foot pulses ≤ 2 [n (%)]	241 (37.4)
Intermittent claudication [n (%)][c]	180 (28.2)
DPN symptoms [n (%)]	395 (61.3)
Altered SWM sensation [n (%)][d]	309 (49.6)
Altered VST [n (%)][e]	134 (33.9)
Previous DFU [n (%)]	264 (41.0)
Previous LEA [n (%)]	74 (11.5)

[a]: 164 missing values, [b]: Using the Young *et al* (2008) proposed complications' classification,[c]: 7 indeterminate values, [d]: 21 indeterminate values,[e]:249 indeterminate/missing values, HbA1c: Glycated Hemoglobin, ADA: American Diabetes Association, DFU: Diabetic Foot Ulcer, DM: Diabetes Mellitus, DPN: Diabetic Peripheral Neuropathy, LEA: Lower Extremity Amputation, SD: Standard Deviation, SWM: Semmes-Weinstein Monofilament, VST: Vibration Sensation Test

DFU development risk variables

In univariate analysis, variables associated with DFU occurrence were age, gender, visual impairment, physical impairment, DM duration, retinopathy, nephropathy, PAD complications history, neuropathy complications history, complication count, and all foot characteristic variables except oedema.

In multivariate analysis only physical impairment, PAD complications history, complications count and previous DFU remained statistically significant (Table 2). Using these variables we were able to create a model that discriminated between those patients who did and did not develop a DFU with AUC value of 0.80 (Figure 3). Considering a simplified model that included complications count and previous DFU only, the AUC value was 0.79 (CI 95% 0.76-0.83) (Figure 4).

Previous DFU history remained associated with greater risk of incident DFU ($p<0.001$) even when adjusted for age, gender, visual and physical impairment, diabetes type and duration, PAD complications history, complication count and previous LEA.

Table 2: Variables association with diabetic foot ulcer, lower extremity amputation and death occurrence

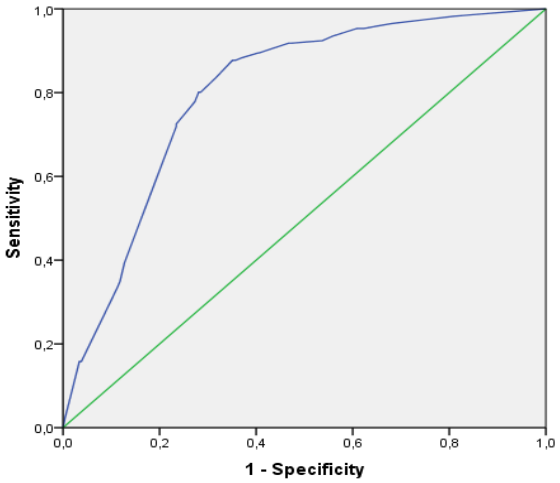
Variables	DFU (n= 171)		LEA (n= 37)		Death (n= 90)	
	Univariate OR (95% CI)	**Multivariate OR (95% CI)**	**Univariate OR (95% CI)**	**Multivariate OR (95% CI)**	**Univariate OR (95% CI)**	**Multivariate OR (95% CI)**
SUBJECT CHARACTERIZATION						
Age	*1.02 (1.01-1.04)*	NS	1.03 (0.99-1.07)	-	*1.13 (1.09-1.16)*	1.12 (1.09-1.15)
Female gender	*0.49 (0.35-0.70)*	NS	*0.47 (0.23-0.93)*	NS	0.65 (0.41-1.02)	-
Analphabetic or primary school	1.55 (0.95-2.55)	-	1.85 (0.64-5.32)	-	1.10 (0.61-2.00)	-
Visual impairment	*1.60 (1.12-2.28)*	NS	*2.20 (1.12-4.30)*	NS	*1.73 (1.11-2.71)*	NS
Physical impairment	*2.38 (1.67-3.41)*	1.73 (1.15-2.59)	*2.11 (1.09-4.12)*	NS	*1.99 (1.27-3.11)*	NS
Past or present smoker	1.06 (0.78-1.44)	-	1.05 (0.47-2.36)	-	0.92 (0.61-1.39)	-
DM AND ITS COMPLICATIONS						
Type 2	2.39 (0.53-10.69)	NS	NA [a]	-	NA[a]	-
Duration (in years)	*1.03 (1.01-1.05)*	NS	1.02 (0.99-1.05)	-	*1.03 (1.01-1.05)*	NS
Insulin use	0.99 (0.70-1.43)	-	1.64 (0.80-3.39)	-	0.94 (0.65-1.60)	-
HbA1c (in %) [b]	1.05 (0.97-1.14)	-	0.99 (0.84-1.16)	-	1.02 (0.97-1.08)	-
Cardiovascular complications history [c]	1.32 (0.91-1.89)	-	*1.91 (0.98-3.72)*	NS	*2.43 (1.55-3.81)*	NS
Retinopathy [c]	*1.62 (1.14-2.31)*	NS	1.57 (0.81-3.08)	-	1.08 (0.69-1.69)	-
Laser photocoagulation	*1.63 (1.14-2.35)*	NS	1.61 (0.82-3.15)	-	0.71 (0.43-1.18)	-
Nephropathy [c]	*1.71 (1.08-2.69)*	NS	*2.56 (1.22-5.38)*	NS	1.63 (0.93-2.86)	-
4-5 stage in ADA classification	*2.81 (1.44-5.50)*	NS	*2.81 (1.03-7.69)*	-	*2.44 (1.14-5.23)*	NS

PAD complications history [c]	*11.03 (6.09-19.96)*	2.52 (1.17-5.45)	*23.06 (3.14-169.31)*	NS	*3.69 (2.03-6.68)*	NS
Neuropathy complications history [c]	*3.14 (2.14-4.60)*	NS	*3.45 (1.55-7.67)*	NS	1.48 (0.94-2.34)	-
Metabolic complications history [c]	0.57 (0.19-1.71)	-	NA[d]	-	2.26 (0.87-5.89)	-
Complication count [c]	*2.03 (1.69-2.43)*	1.31 (1.03-1.67)	*2.17 (1.57-3.01)*	1.74 (1.15-2.62)	*1.76 (1.42-2.18)*	1.50 (1.17-1.94)
FOOT CHARACTERIZATION						
Foot deformity	*2.03 (1.25-3.25)*	NS	0.74 (0.35-1.57)	-	0.85 (0.50-1.43)	-
Oedema	1.10 (0.74-1.63)	-	0.93 (0.43-2.01)	-	1.55 (0.96-2.51)	-
Onychomycosis	*1.75 (1.21-2.53)*	NS	0.58 (0.30-1.12)	-	*1.99 (1.22-3.25)*	NS
Total foot pulses ≤ 2	*3.43 (2.39-4.94)*	NS	*8.04 (3.47-18.62)*	4.17 (1.76-9.88)	*2.51 (1.59-3.94)*	NS
Intermittent claudication [e]	*1.70 (1.12-2.47)*	NS	*2.03 (1.03-3.98)*	NS	1.10 (0.67-1.80)	-
DPN symptoms	*1.52 (1.05-2.20)*	NS	1.53 (0.74-3.14)	-	0.94 (0.59-1.48)	-
Altered SWM sensation [f]	*3.16 (2.16-4.64)*	NS	*3.25 (1.50-7.02)*	NS	1.30 (0.82-2.04)	NS
Altered vibration sensation test[g]	*3.54 (2.21-5.68)*	NS	*5.03 (1.74-14.61)*	NS	*2.38 (1.31-4.33)*	NS
Previous DFU	*8.74 (5.80-13.17)*	4.54 (2.79-7.38)	*10.35 (3.97-26.93)*	5.54 (2.09-14.72)	*2.46 (1.56-3.87)*	1.73 (1.04-2.88)
Previous LEA	*5.12 (3.09-8.47)*	NS	*4.22 (2.02-8.82)*	NS	0.72 (0.33-1.56)	-

-: Not included in the multivariate analysis, [a]: Model extrapolated values due to reduced number of subjects with diabetes type 1 and no event occurrence in such group, [b]: 164 missing values, [c]: Using the Young *et al* (2008) proposed complications' classification, [d]: Model extrapolated values due to reduced number of subjects with history of metabolic complications and no event occurrence in such group, [e]: 7 indeterminate values, [f]: 21 indeterminate values, [g]: 249 missing values, HbA1c: Glycated Hemoglobin, ADA: American Diabetes Association, CI: Confidence Interval, DFU: Diabetic Foot Ulcer, DM: Diabetes Mellitus, DPN: Diabetic Peripheral Neuropathy, FU: Follow Up, LEA: Lower Extremity Amputation, NA: Not Applicable, NS: No Statistical significant association was observed, OR: Odds Ratio, SD: Standard Deviation, SWM: Semmes-Weinstein Monofilament

Figure 3: Receiver operating characteristic curve of predictive models for diabetic foot ulcer (a), lower extremity amputation (b) and death (c) occurrence

(a)



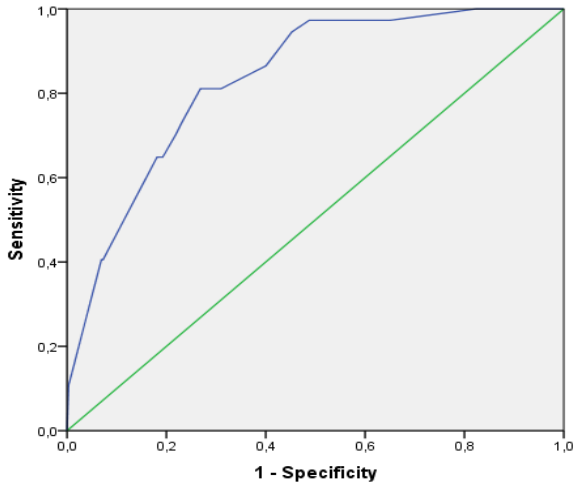
Model score calculation

-3.29 + 0.55 x Physical impairment + 0.93 x Peripheral arterial disease complication history presence + 0.27 x Number of complications count + 1.51 x Previous diabetic foot ulcer presence

Area under the curve

0.80 (Confidence Interval 95% 0.76-0.84)

(b)



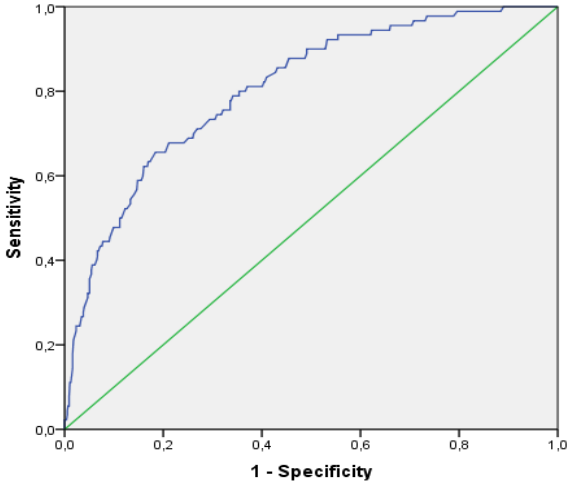
Model score calculation

-6.01 + 0.55 x Number of complications count + 1.43 x ≤2 foot pulses (out of 4) presence + 1.71 x Previous diabetic foot ulcer

Area under the curve

0.83 (Confidence Interval 95% 0.78-0.89)

(c)



Model score calculation

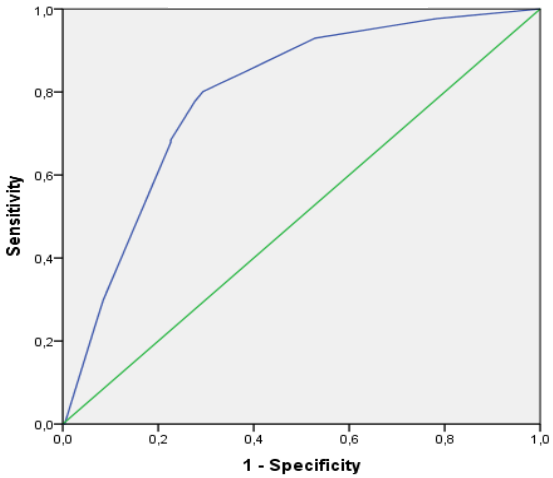
-10.70 + 0.11 x Age (in years) + 0.41 x Number of complications count + 0.55 x Previous diabetic foot ulcer

Area under the curve

0.81 (Confidence Interval95% 0.76-0.85)

Figure 4: Receiver operating characteristic curve of predictive models for diabetic foot ulcer (a), lower extremity amputation (b) and death (c) occurrence using only complication count and previous diabetic foot ulcer

(a)



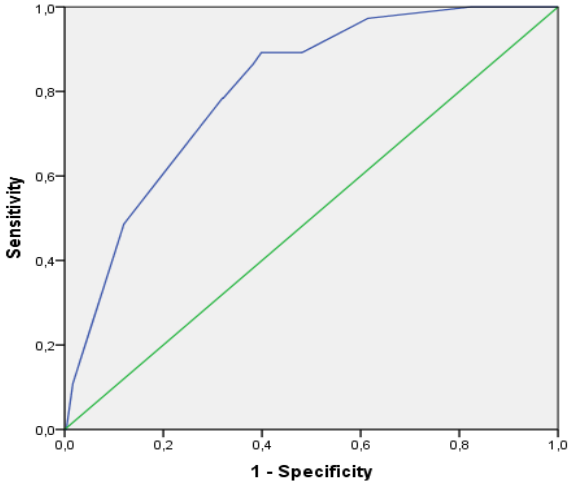
Model score calculation

-2.86 + 0.46 x Number of complications count + 1.84 x Previous diabetic foot ulcer presence

Area under the curve

0.79 (Confidence Interval 95% 0.76-0.83)

(b)



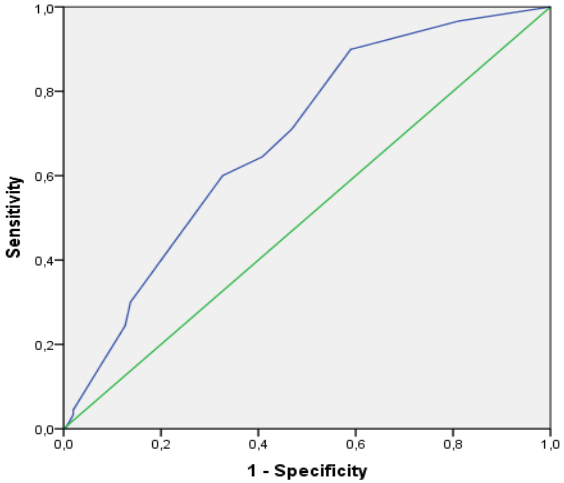
Model score calculation

-5.35 + 0.61 x Number of complications count + 1.91 x Previous diabetic foot ulcer

Area under the curve

0.81 (Confidence Interval 95% 0.74-0.87)

(c)



Model score calculation

-3.02 + 0.49 x Number of complications count + 0.51 x Previous diabetic foot ulcer

Area under the curve

0.69 (Confidence Interval 95% 0.63-0.74)

LEA occurrence risk variables

In univariate analysis, variables associated with LEA were gender, visual and physical impairment, cardiovascular complications history, nephropathy, PAD complications history, neuropathy complications history, complication count, two or fewer of four foot pulses, intermittent claudication, altered SWM sensation and VST, and previous foot complications (DFU and/or LEA).

In multivariate analysis only complication count, two or fewer of four foot pulses and previous DFU maintained statistical significance (Table 2), producing a score with an AUC value of 0.83 for the discrimination between those who did or did not experience an incident LEA (Figure 3). When using a simplified model, including only complications count and previous DFU, the AUC value was 0.81 (CI 95% 0.74-0.87) (Figure 4).

Once more, when adjusting for age, gender, physical impairment, diabetes duration, complication count, total foot pulses ≤2 and previous LEA, previous DFU maintained a statistically significant association with LEA risk (p=0.001).

Death occurrence risk variables

In univariate analysis, variables associated with death were age, visual and/or physical impairment, DM duration, cardiovascular complications history, end-stage renal disease, PAD complications history, complication count, onychomycosis, foot pulses, altered VST and previous DFU.

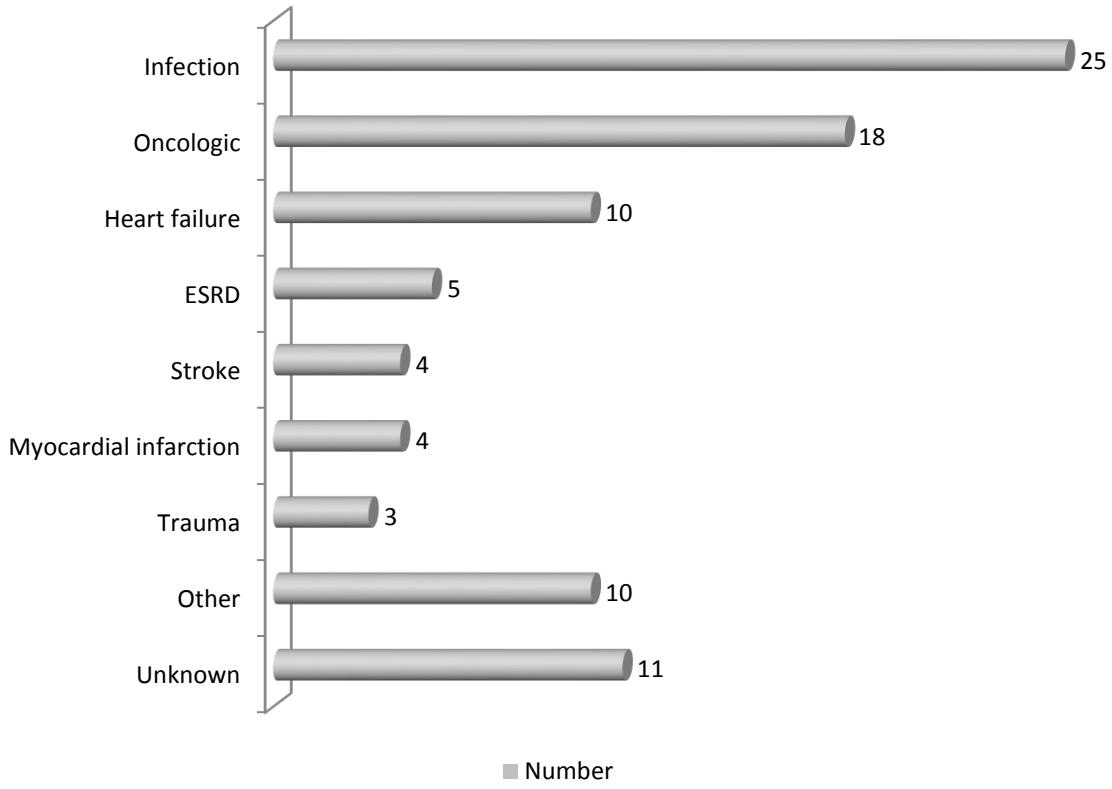
Age, complication count and previous DFU were the only variables that remained statistically significant in multivariate analysis (Table 2). The resultant predictive model yielded an AUC value of 0.81 in the discrimination between patients who did and did not die during follow-up (Figure 3). However, using the simplified model including complications count and previous DFU the AUC value dropped to 0.69 (CI 95% 0.63-0.74) (Figure 4).

Once again, DFU history was associated with a higher mortality rate independent of age, gender, visual and physical impairment, diabetes duration, complication count and previous LEA (p<0.05) (*data not shown*).

We must highlight that patients developing a DFU during follow-up also had a significantly higher death rate (OR 1.75, CI 95% 1.09-2.79), although the same was not observed when adjusting for previous DFU (OR 1.18, CI 95% 0.70-1.99) or among those who had an LEA during follow-up (OR 2.09, CI 95% 0.95-4.58).

The most frequent causes of death were infections (27.8%), oncologic disease (20%), and heart failure (9%) (Figure 5).

Figure 5: Causes of death



ESRD: End-Stage Renal Disease

ICD-9 codes verified

Infection 52, 421, 464, 466, 480-488, 490-508, 519, 590, 595, 681, 682, 785

Oncologic 151, 153, 154, 157, 161, 162, 171-174, 185, 188, 189, 191, 203

Heart failure 428

ESRD 250.4, 585

Stroke 434, 436

Myocardial infarction 410

Trauma 800-804, 820-829

Discussion

Several investigations have assessed all-cause mortality in type 2 DM with the derivation and validation of multivariate models [Hayes AJ et al, 2013; Yang EX et al, 2008]. However, and despite the proved impact of DFU on mortality risk [Monteiro-Soares M et al, 2011; Robbins JM et al, 2008], it was not included in such models.

On the other hand, DFU's link with death occurrence has rarely been adjusted for other pertinent variables (such as age and baseline complications presence) [Boyko EJ et al, 1996].

Therefore, we have conducted this study assessing DFU impact on LEA and death risk, in a large cohort of consecutively enrolled subjects (n= 644), using the Strengthening of the Reporting of Observational Studies in Epidemiology (STROBE) [Vandenbroucke J et al, 2007] and STARD [Bossuyt PM et al, 2003] checklists as the

basis for its development and reporting, and conducting adequate statistical adjustment. Moreover, due to the cohort design, observers were blind to outcome occurrence when collecting baseline data. We observed that the different outcomes on which we focused shared several common predictive variables in univariate analysis, such as physical impairment; cardiovascular and PAD complications history, complications count; total foot pulses number, altered VST and previous DFU. However, few remained statistically associated in multivariate analysis, and different predictors of the outcomes of interest were seen across the three models for the outcomes DFU, LEA, and death, with the exception of complications count and previous DFU.

On the other hand, the 3 derived models (using 3 to 5 variables) for each outcome were able to produce high AUC values (from 0.81 to 0.83). A simplified model that included complications count and previous DFU only retained high AUC values for DFU and LEA occurrence (0.79 and 0.81, respectively) but dropped to 0.69 in the case of death. This may be explained greatly by the fact that advancing age is highly and directly linked to death.

This 2 variable model is very simple, uses easily collected data from a clinical appointment, can be employed in every clinical setting, from primary to tertiary care, to identify subjects at higher risk of developing DFU and/or LEA. This may, consequently, lead to increased surveillance of such individuals in order to prevent these complications from occurring. The simplified model to predict death under performs compared to the full model that includes age, so the full model should be used for the prediction of this outcome because it is more accurate.

In the multivariate analysis, previous DFU maintained statistical significance for all the outcomes (even when using a broad group of variables for statistical adjustment).

Surprisingly, LEA only achieved statistical significance in the multivariate analysis for DFU occurrence prediction. It was not associated with higher risk of death (not even in the univariate analysis). During follow-up, 10.8% of subjects with a past history of LEA died, in comparison to 24.3% of those requiring any type of LEA during follow-up and 35% in the case of a major LEA.

In 1996, Boyko et al [Boyko EJ et al, 1996] assessed the relationship of DFU and mortality also adjusting the risk of death for some variables. However, 98% of the population were men and they tested a smaller range of variables. Cusick et al [Cusick M et al, 2005], in 2005, also conducted a multivariate analysis evaluating the association between mortality and several diabetes complications in patients with type 1 and type 2 diabetes, although all patients had retinopathy. Moreover, in both articles evaluation of complications was made by assessing the presence or absence of each one at baseline while we in contrast used the validated complication count proposed by Young et al [Young BA et al, 2008] (its accuracy was considered similar to the Complication Severity Index).

There is substantial literature on mechanisms to explain many of the associations we describe between the outcomes of interest and predictors. PAD (or diminished foot pulses as its correlate) has been independently associated with both LEA [Adler A et al, 1999] and DFU [Boyko E et al, 1999], probably due to impairment in wound healing due to inadequate circulation. Diabetes complication count and physical impairment signal greater disease severity, which has also been shown to predict a higher risk of DFU, LEA, and death [Boyko E et al, 1999; Cusick M et al, 2005; Young BA et al, 2008]. Previous DFU is an instance of a diabetes complication signalling high disease burden of specific importance in the development of foot complications, such as future DFU and amputation [Adler A et al, 1999; Boyko E et al, 1999]. In addition, and not surprisingly, the higher disease burden also predicts greater mortality [Boyko EJ et al, 1999; Cusick M et al, 2005; Ramsey S et al, 1999].

Limitations of our study include its retrospective nature, the exclusion of patients outside our direct referral area, the presence and exclusion from analysis of missing data of VST and HbA1c values, as well as indeterminate results for intermittent claudication and SWM sensation.

We must emphasize that, due to the selected design, all the assessed patient-related events (i.e. inclusion, follow-up and determination of outcomes) occurred prior to the research being undertaken. The VST exam only started in the middle of 2008. Regarding HbA1c values, our hospital is a tertiary care centre for diabetic foot care, but nevertheless a recent HbA1c value (within less than 3 months) was not always available.

Even though there are works addressing the impact of depression in the mortality of patients with DFU [Ismail K et al, 2007; Winklei K et al, 2012], this variable is not collected in our daily practice and therefore was not available in the subjects' clinical file for incorporation into prediction models.

We have observed several indeterminate results when assessing intermittent claudication due to the presence of patients that have extremely reduced ambulation and/or symptoms similar to DPN. SWM sensation test result in some patients was difficult to assess due to the presence of several callus/dry skin and patients' automatic and constantly positive response (even when false positive test points were being conducted). In 23 patients, where hallux or transmetatarsal LEA was present in both feet VST was not possible to conduct.

We have decided to use the complication count proposed by Young et al [Young BA et al, 2008], instead of the Complication Severity Index. This choice was due to the fact that both report equal accuracy and the first was easier to apply and interpret in our population.

Our data reveals a high rate of DFU development (> 8% annually) [Singh N et al, 2005], consistent with our high risk referral practice from which we selected study participants, of whom 41.0% had previous DFU.

Our mortality rate is in accordance with the ones described in the literature, namely in the Eurodiale study [Shaper N 2012]. In addition, our population has a high rate of comorbidities (13% cardiovascular disease and 63% PVD). Conversely, our LEA rate is inferior to Eurodiale results [Shaper N 2012], as it would be expected, since we started with a population without active DFU while they included only patients with active DFU.

The referral nature of the study setting, high prevalence of type 2 diabetes (97.7%), and low education level (82.2% primary school level or less) may limit the generalizability of these results to dissimilar populations.

As stated in the methods section, foot related variables were registered at the first podiatric appointment by one of 2 podiatrists with high experience in diabetic foot using a standardized form. We must highlight that both the professionals and form remained relatively unchanged during the study period. Variables that were collected by clinical interview may present information bias.

To overcome this limitation we have searched the clinical file and the national data platform in order to get access to the subjects' most complete and accurate information. For all this and the long study period we believe that misclassification bias may have occurred. However, due to the selected type of study (a cohort) we believe that it was not differential.

Given the retrospective nature of the study we present several variables with missing data, as presented in the tables. However, we must emphasize that there was no missing data for the variables included in the models. Therefore, AUC values and respective 95% CI were calculated using the entire sample. On the other hand, we must highlight that we believe to have identified the great majority (if not all) the outcome events. We have conducted a broad search in the Hospitals' and Health Data Platform (a program with access to data regarding all public healthcare institutions), in which is registered automatically all occurrences of LEA and death.

We encouraged subjects to contact our service if any DFU occurred, thus enhancing our ability to capture this outcome.

We only used the ICD-9 codes when considering the cause of death and grouped them, acknowledging the potential limitations of the existing codes.

We conclude that DFU occurrence has a major and independent impact on LEA and death, even when adjusted for baseline complications. Thus the history of a DFU is a marker for poorer outcomes in patients with diabetes in this population. These findings also suggest that DFU prevention may be a potential path for better survival and diminished morbidity in persons with diabetes. New studies are needed in order to better understand this link. In our opinion, DFU presence implies a decrease of the subjects' mobility and general well-being and, consequently, of the quality of life, higher infection risk

and inflammatory, immune and physiologic changes. All of these most certainly lead to a higher mortality risk.

These models were obtained in a high risk context. So they should be tested in primary care to assess if they are clinically relevant and valid enough *per se*, or if they should be added to pre-existing models/classifications.

B. Hyperbaric Oxygen therapy

HBOT consists in the administration of oxygen in a hyperbaric chamber, at pressures higher than the atmospheric pressure, usually between 1.5 and 3.0 ATA (atmospheres absolute), but can reach 6 ATA if needed [UHMS, 2014].

Historically, the first reference to hyperbaric therapy is from 1662, when the British clergyman Henshaw intended to treat several diseases using a compartment, the "*domicilium*", which was pressurized or depressurized. However, it was not until the 19th century that European and North American scientific communities regained interest in this treatment. Still, its widespread use for several conditions with little or none scientific base and/or results led to disbelief [Albuquerque-Sousa 2007].

In the mid-20th century HBOT was introduced in clinical practice, with scientific support after the experimental studies by Boerema, where fatally anaemic (bled) pigs were maintained alive for 45 minutes with HBOT. These experiments have drawn attention to the potential of this therapy and basic and clinical research have increased ever since [Albuquerque-Sousa 2007]. In fact, in the last decades there has been a high number of publications addressing it. For example, when searching the currently larger electronic repository [National Library of Medicine (MEDLINE)] for "hyperbaric oxygen therapy" we retrieved 11193 results [http://www.ncbi.nlm.nih.gov/pubmed/?term=hyperbaric+oxygen+treatment in 15/04/2014].

Nowadays, this treatment modality has been diffused worldwide. In Europe, several countries have multiple hyperbaric centres. Portugal has five located in: the FNH in Lisbon, PHH, Funchal's Hospital, Ponta Delgada's Hospital and Horta's Hospital.

On the other hand, the quality of the available evidence for several clinical indications is still scarce and with several limitations (as explained in the section 3 of this Chapter: Indications and contra-indications).

1. *Hyperbaric chamber*

A **hyperbaric chamber** is a therapeutic unit constituted by a hermetic structure with a compartment that can be pressurized up to 6 ATA [Latham E et al, 2013]. There are two types of chambers for HBOT:

- Type A (Multiplace) – has the capacity to treat several patients together; is pressurized with air; medicinal gases [oxygen (O_2), helium, nitrogen] are administered in a closed circuit (through masks or hoods); many have facilities to treat critical care patients, including ventilated ones (Figure 6, Figure 7, Figure 8 and Figure 9).

- Type B (Monoplace) – smaller; pressurized with 100% O_2 or gas mixtures; in some models, the patient may breath the medicinal gases through a mask; can be used for treatment or for transport of patients in hyperbaric conditions to other therapeutic units (for example, in diving accidents).

Figure 6: Pedro Hispano's Hospital multiplace chamber with capacity for 16 seating patients

Figure 7: Former Navy's Hospital chambers with total capacity of 24 seating patients

Figure 8: Patients undergoing treatment for different pathologies

Figure 9: Monitoring panel

2. *Effects*

HBOT effects that can be divided in physiologic and therapeutic, will be described in more detail bellow [Albuquerque-Sousa JG, 2002; Albuquerque-Sousa J, 2007; Bakker DJ, 2000; Bonomo SR et al, 1998; Boykin R, 2000; Boykin R & Baylis C, 2007; Cianci P et al, 2008; Desola J et al, 1998; Gallagher KA et al, 2006; Goldstein LJ et al, 2006; Gurdol F et al, 2010; Hills BA, 1999; Hopf H et al, 2005; Knighton et al, 1986; Latham E et al, 2013; Liu ZJ & Velazquez OC, 2008; Mathieu D et al, 2006; Milovanova T et al, 2009; Nylander G et al, 1984; Roeckl-Wiedmann I et al, 2005; Sheilkh AY et al, 2000; Soares R, 2009; Thom SR, 2011; Thom SR et al, 2011; Velazquez O, 2007].

Physiologic effects – depend on the increase of the environmental pressure *per se* and, on the other hand, on the elevation of oxygen partial pressure. They include:

- Volumetric effects – according to the Boyle-Mariotte law, the increase of environmental pressures decreases the volume of all hollow organic cavities that are not in contact with the respiratory tract (bladder, gastro-intestinal tract) and of air/emboligenous bubbles, in an inversely proportional relation. This effect is reversible with the re-establishment of the atmospheric pressure.
- Dissolution effects – under the Henry's law, when breathing pure oxygen (O_2) in hyperbaric conditions there is a progressive increase of the arterial O_2 pressure that reach values higher than 2000 mm Hg at a 3 ATA pressure. So, the O_2 volume dissolved and carried in the plasma, that is minimal at atmospheric pressure, increases more than 22 times. Consequently, venous O_2 pressure can reach 600 mm Hg and the tissue pressure 400 mm Hg. This high driving O_2 pressure improves tissue oxygenation, even in relatively poorly perfused areas and restores hypoxic tissue to more normal levels. The body protects itself from this excessive amount of O_2 through the production of free radicals, on which effects HBOT acts as modulator, and by dose-dependent peripheral vasoconstriction. Despite the decrease in blood flow caused by this vasoconstriction, plasma hyperoxia maintains O_2 supply, thus it is a non-hypoxic vasoconstriction. This hyperoxic vasoconstriction favours oedema reabsorption and consequent reduction of interstitial pressure, improving O_2 delivery to the tissues. The anti-oedema effect of hyperoxia may be further enhanced by a direct osmotic effect of intravascular O_2 at these high tensions. Hills has estimated an effect of as much as an 8.4% increase in oncotic pressure, which is established because O_2 diffusing into the tissues is instantly metabolised, maintaining the steep concentration gradient across the vascular wall.

Therapeutic effects consist in**:**

- Direct effects – the arterial, venous and tissue hyperoxia and, specially, the great increase and availability of plasmatic O_2 provide a possible therapeutic effect in diseases where tissue

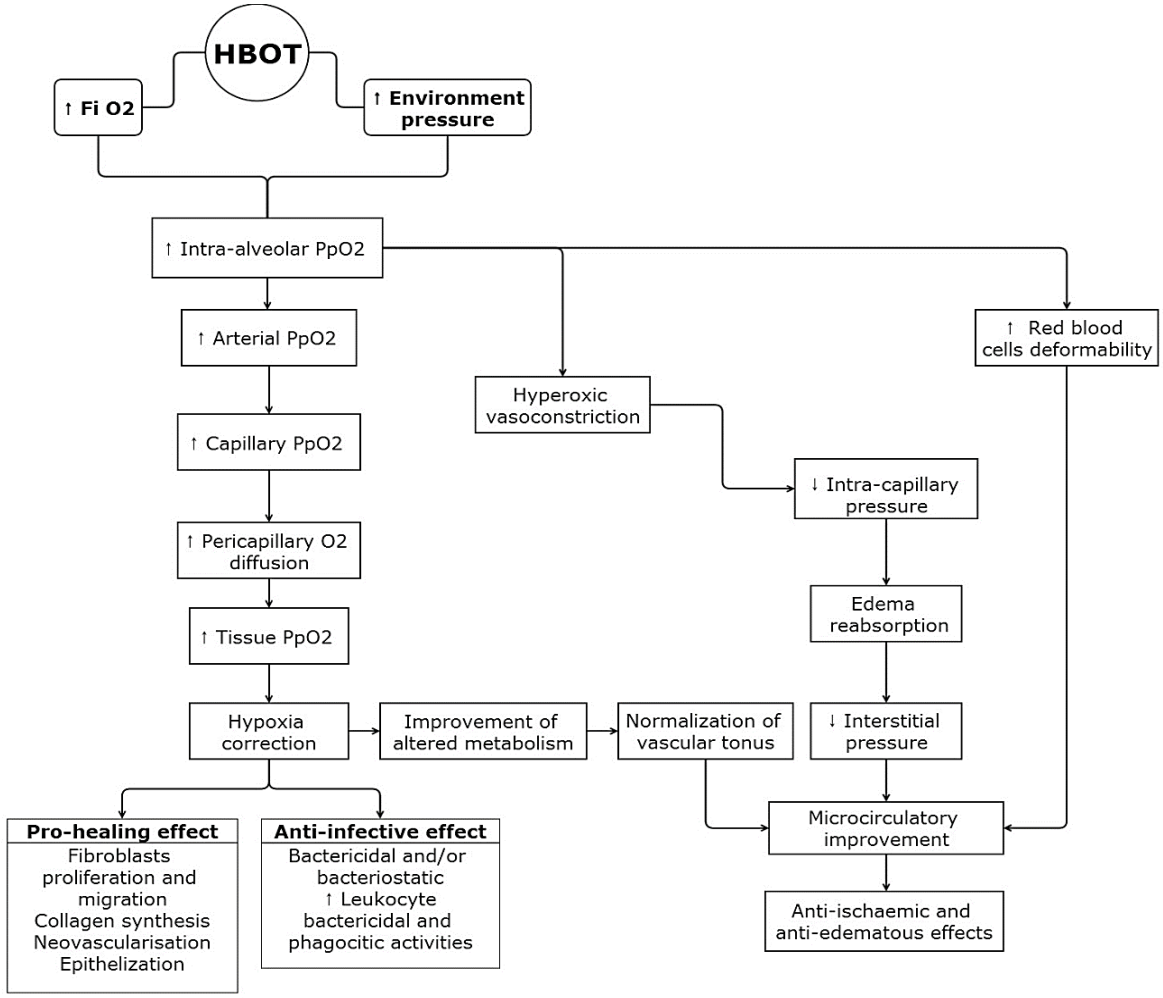
hypoxia, whether systemic or local, is a predominant etiopathogenic factor or when there is an oxygen-dependent physiopathologic mechanism. The dissolved O_2 is not affected by hematologic limitations or metabolic conditions that, in several situations, limit the transfer or use of erythrocyte O_2; it is delivered to tissues, like terminal ischemic tissues, by capillarity, being transferred through a simple diffusion gradient.

- Indirect effects – depending on the condition's physiopathology there are specific therapeutic actions:
    - Decrease of bubbles volume in gaseous embolism and decompression sickness: the increase of O_2 partial pressure and the reduction of the nitrogen partial pressure to zero accelerates the absorption of gaseous emboli through a concentration gradient till their elimination.
    - "Robin-Hood" effect: the hyperoxic vasoconstriction is a physiological mechanism of defense against hyperoxia, whereby blood flow is preferentially maintained to hypoxic zones and reduced in normoxic areas.
    - Micro-neovascularisation and neo-collagen synthesis stimulation: HBOT favors proline hydroxylation and granulation tissue production in situations where, due to hypoxia, they were decreased (namely, diabetic microangiopathy, irradiated tissues or advanced arteriopathies), and increases the synthesis of several growth factors that stimulate angiogenesis. On the other hand, there is evidence that local hyperoxia/hypoxia interchange is an angiogenic stimulus.
    - Reactivation of the leukocyte O_2 dependent phagocytic ability: the higher tissue O_2 content reinforces leukodiapedesis and phagocytic capacity of the activated neutrophils.
    - Increase in leukocyte bactericidal power: through rise in intra-leukocyte synthesis of superoxide radicals due to the activation of the oxidative pathway linked to nicotinamide adenine dinucleotide phosphate.
    - Synergism with some antibiotics: hypoxia and anaerobiosis contribute to reduced action of several antimicrobials, like aminoglycosides, vancomycin, cotrimoxazole, fluoroquinolones, nitrofurantoin and rifamycins. The elevation of tissue O_2 tension, through HBOT, restores the normal activity of many of these antibiotics, increasing, in some cases, its antimicrobial power. This, however, is not a universal effect, varying with antibiotic and/or microorganism involved. HBOT prolongs the post-antibiotic effect, enhancing efficacy and duration of action of some antibiotics. As HBOT also

improves wound neovascularisation, it likewise increases antimicrobial's local bioavailability.

- Bacteriostatic action on several non-sporulated anaerobic and some aerobic microorganisms: HBOT should be considered an adjuvant treatment in situations when conventional therapy (antibiotics) has failed.
- Bactericidal action on some non-sporulated strict anaerobic microorganisms: in particular of the *Clostridium* gender, due to their lack of anti-oxidant systems.
- *Clostridium* toxin production block: toxin production is dependent on the existence of low redox potential. The rise of the later by HBOT stops toxin production.
- Faster elimination of carboxyhemoglobin (HbCO): HbCO half-life under environmental conditions is 520 minutes, when breathing 100% oxygen at the atmospheric pressure lowers to 80 minutes and with hyperbaric oxygen at 3 ATA it is reduced to 23 minutes.
- Attenuation of the reperfusion injury: inappropriate leukocyte activation is responsible for much of the damage linked to reperfusion (an indirect injury mechanism). HBOT reduces it by preventing this activation.
- Recruitment of bone marrow-derived EPCs: the hyperoxia caused by HBOT increases nitric oxide synthase (NOS) activity and evidence points that it stimulates the mobilization of EPCs from the bone marrow by a mechanism mediated by nitric oxide (NO).

Figure 10. Hyperbaric oxygen therapy mechanisms of action in wound healing



(Adapted, with permission, from Albuquerque e Sousa, 2006)

↑: Increase; ↓: Decrease; Fi: Fraction of Inspired Oxygen; O_2: Oxygen; PpO_2: Partial Pressure of Oxygen

From the exposed, due to the increase in the dissolved O_2 in the plasma, hyperoxic vasoconstriction with plasma volume derived from the healthy areas to the regions with local hypoxia, stimulation of micro-neovascularisation and production of collagen, improvement in leukocyte phagocytic and bactericidal capacities, direct bactericidal or bacteriostatic effect of the higher O_2 content on some bacterial agents, and synergism with some antimicrobial drugs, summarized in Figure 10, HBOT is used to treat chronic non-healing DFU that are typically ischemic and/or infected.

Side effects – they are uncommon but include [Mathieu D et al, 2006; Neuman T & Thom S, 2008]:

- Barotraumatic lesions: can occur in the ear, paranasal sinuses, lung and, less often, teeth, eyes or gastrointestinal hollow viscera, especially if preventive measures are not adopted.

- Central nervous system oxygen toxicity: ranges from simple symptoms like nausea, dizziness, headache, light-headedness to, when an excessively high O_2 pressure is reached, generalized tonic-clonic seizures that stop when the O_2 mask is removed with consequent decrease in the inspired oxygen partial pressure and may have a transitory post-ictal aura.
- Toxic O_2 effects on the respiratory tract: in case of continuous or prolonged exposures to hyperbaric O_2 (more than 10 hours continuously or 200 cumulative). The first sign is a reduction in vital capacity.
- Effects on the eye:
    - Transient myopia: it is not a true refraction change but the effect on the visual function is alike. However it is temporary and reverts a few days after finishing the treatment.
    - In patients with cataracts their evolution may be accelerated. Data indicates that there is no new cataract formation in unaffected eyes using the usual treatment schedules.
- Claustrophobia

Using treatment plans with duration inferior to 3 hours and a maximum pressure of 3 ATA, HBOT is considered a safe treatment, being the occurrence of side effects very rare.

3. *Indications and contra-indications*

The 7th European Consensus Conference on Hyperbaric Medicine of the European Committee for Hyperbaric Medicine (ECHM) held in 2004 in Lille, France, aimed, among other objectives, to review the agreement on HBOT indications based in the available evidence [ECHM, 2004].

The type of indication was classified using a 3 grade scale according to the strength of the recommendation, and the level of evidence that supported each recommendation was classified from A to F (A – at least 2 concordant, large, double-blind, controlled randomized studies with no or little methodological bias; B – double-blind controlled, randomized studies but with methodological flaws, studies with only small samples, or only a single study; C – consensus opinion of experts; D – only uncontrolled studies with no consensus opinion of expert; E – no evidence of beneficial action, or methodological or interpretation bias preclude any conclusion; F – existing evidence favors not to use HBOT).

- **Type I recommendations** – pathologies in which HBOT is strongly recommended and contributes to change the prognosis

  Acute:

  - Carbon monoxide intoxication (Level B)
  - Crush syndrome (Level B)
  - Decompression accident (Level C)
  - Gas embolism (Level C)
  - Anaerobic or mixed bacterial anaerobic infections (Level C)

  Chronic:

  - Prevention of osteoradionecrosis after dental extraction (Level B)
  - Osteoradionecrosis (mandible) (Level B)
  - Soft tissue radionecrosis (cystitis) (Level B)

- **Type II recommendations** – HBOT is recommended and may improve outcome

  Acute:

  - Compromised skin graft and musculocutaneous flap (Level C)
  - Sudden deafness (Level C)

  Chronic:

  - Diabetic foot lesions (Level B)
  - Osteoradionecrosis (other bones) (Level C)
  - Radio-induced proctitis/enteritis (Level C)
  - Radio-induced lesions of soft tissues (Level C)
  - Surgery and implant in irradiated tissue (preventive action) (Level C)
  - Ischemic ulcer (Level C)
  - Refractory chronic osteomyelitis (Level C)
  - Neuroblastoma stage IV (Level C)

- **Type III recommendations** – HBOT is optional

  Acute:

  - Post anoxic encephalopathy (Level C)
  - Post-vascular procedure reperfusion syndrome (Level C)
  - Limb replantation (Level C)

- Burns > 20% of body surface area and 2nd degree (Level C)
- Acute ischemic ophthalmological disorders (Level C)

Chronic:

- Larynx radionecrosis (Level C)
- Radio-induce CNS lesion (Level C)
- Selected non-healing wounds secondary to inflammatory processes (Level C)
- Pneumatosis cystoides intestinalis (Level C)

- **Other indications** – were considered not recommended as they had a level of evidence classified as D to F

The Undersea and Hyperbarical Medical Society (UHMS) and Food and Drugs Administration also considered as indication for HBOT severe anemia when there is inability to transfuse red blood cells and intracranial abscess [UHMS, 2014].

Being a medical treatment, HBOT also has some contraindications [Latham E et al, 2013; Neuman T et al, 2002; Wang J et al 2002], that can be classified in two groups:

- **Absolute:**
  - Presence of an untreated pneumothorax
  - Current treatment with bleomycin, cisplatin, disulfiram and mafenide acetate
- **Relative:**
  - History spontaneous pneumothorax
  - Recent thoracic, ocular and/or otorhinolaryngologic surgery
  - Severe acute or chronic sinus infections
  - Upper respiratory infection
  - Asthma
  - Chronic obstructive pulmonary disease, especially emphysema
  - Congenital spherocytosis
  - High fever
  - Epilepsy
  - Pregnancy

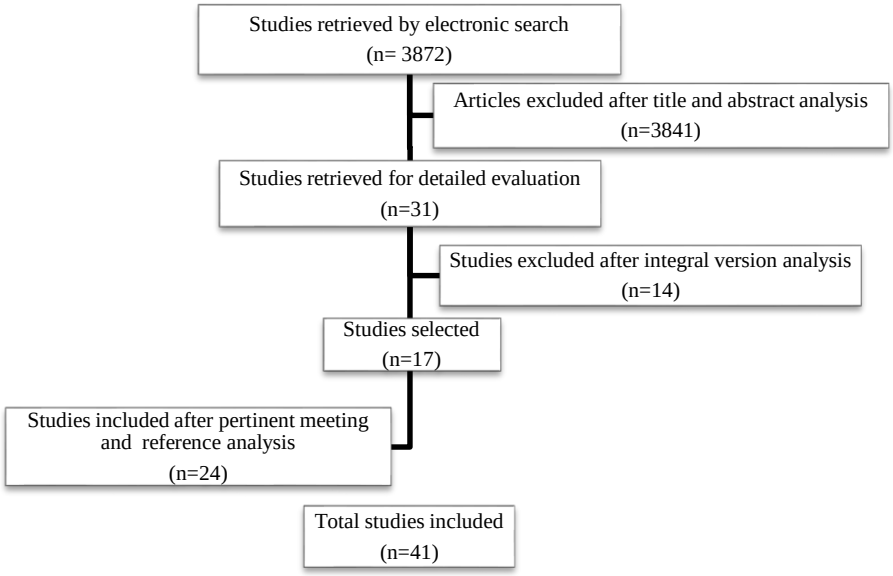
C. *Effect of Hyperbaric Oxygen therapy in diabetic foot ulcer healing: a systematic review and meta-analysis*

1. *Material and methods*

Search strategy and article selection process

A sensitive search in the MEDLINE (PubMed) database was conducted in December 2012 for studies addressing HBOT effectiveness for chronic DFU, using the query outlined in Figure 11.

Figure 11: Systematic review flow diagram of article selection process



Article selection process, using the following query in MEDLINE: *("Hyperbaric Oxygenation/economics"[Mesh] OR "Hyperbaric Oxygenation/ standards"[Mesh] OR "Hyperbaric Oxygenation/statistics and numerical data"[Mesh] OR "Hyperbaric Oxygenation/therapy"[Mesh] OR "Hyperbaric Oxygenation/trends"[Mesh] OR "Hyperbaric Oxygenation/utilization"[Mesh]) AND "Diabetic Foot/blood"[Mesh] OR "Diabetic Foot/classification"[Mesh] OR "Diabetic Foot/economics"[Mesh] OR "Diabetic Foot/enzymology"[Mesh] OR "Diabetic Foot/immunology"[Mesh] OR "Diabetic Foot/therapy"[Mesh])*and the following terms in other search engines*: "Diabetic Foot" AND/OR "Hyperbaric Oxygen Therapy"*

This search retrieved 3,872 studies. These results were examined by two independent investigators (Daniela Martins-Mendes and Matilde Monteiro-Soares) using the following inclusion criteria: (1) *type*

of study: randomized controlled trials (RCT), non-randomized trials (NRT), cohorts, case–control studies and case series; (2) *population:* subjects with diabetes and active DFU; (3) *results:* reported healing and/or LEA proportions and/or median healing time in subjects treated with systemic HBOT, eventual association between systemic HBOT and DFU healing and/or between independent variables' and improved outcome in those receiving HBOT; (4) *outcomes*: DFU total healing, LEA rates, area reduction or time to heal and; (5) published in the following *languages:* English, French, Italian, Portuguese or Spanish.

Disagreements were resolved by a third investigator (Pedro Barata). In the first phase, inclusion was based on the title and abstract (when available). All included titles at this stage (n= 31) were then retrieved as full articles and a final assessment made. At the conclusion of this process 17 articles were included. We then extended the search by examining the citation lists for each of the 17 included articles and of previous reviews on this topic [Kranke P et al, 2006; Kranke P et al, 2012; Löndahl M, 2012; Medical Advisory Secreteriat, 2005; Tiaka E et al, 2012], hand-searching the abstracts and proceedings of major meetings in the fields of both Diabetology and Hyperbaric Medicine (European Association for the Study of Diabetes, International Diabetes Foundation, European Underwater and Baromedical Society, South Pacific Underwater Medicine Society and Undersea and Hyperbaric Medical Society) and searched an on-line database of scholarly works in Hyperbaric Medicine (The Rubicon Foundation Research Repository). Using the same procedure described above, when a new study abstract was identified, a search was made and/or an email sent in order to ascertain if a full paper had been produced and published. If there was a full publication [Baroni G et al, 1987; Bishop A & Mudge E, 2012; Çerkes N et al, 1994; Doctor N et al, 1992; Faglia E et al, 1998; Hawkins G et al, 2006; Oriani G et al, 1992; Otto G et al, 2000; Wang CJ et al, 2009; Wattel F et al , 1991; Wattel F et al , 1995], we included it in our review, if not the study was included in abstract or poster form only [Buyukcakir C et al, 1998; Fife C et al, 1997; Hawkins G et al, 2005; Jovanovic T et al, 2011; Kawashima M et al, 2006; Mathieu D et al, 1997; Mendes D et al, 2012; Perdrizet G et al, 2007; Ramon Y et al, 1999; Subbotina N et al, 2002; Zivkovic M et al, 1999]. These searches yielded a further 24 articles and we have included 41 studies in this review (Figure 11).

Data collection and analysis

The following data were collected: (1) *article identification:* title, author(s), publication date, journal; (2) *methods:* study design, setting, sources and methods of participant selection, inclusion and exclusion criteria, sample size, HBOT regimen and number of sessions; (3) *results:* study participants' characteristics, outcome prevalence (healing, minor, major or total LEA), methods of statistical analysis, HBOT efficacy regarding reduction of LEA risk, DFU area and time until complete healing, and/or association between independent variables and outcome; and (4) *quality assessment*: assessed (by Matilde Monteiro-Soares) through the number of items fulfilled in two study quality checklists

[STROBE for observational studies [Vandenbroucke J et al, 2007] and CONSORT (Consolidated Standards of Reporting Trials) for RCTs [Schulz K et al, 2010]]. Due to a lack of an implemented checklist for NRT we have decided to evaluate such studies by also using the STROBE checklist.

MA was conducted for healing and LEA proportion using Microsoft Excel [Neyeloff J et al, 2012], while Review Manager 5.0 software was used for risk measures. Concerning HBOT effectiveness, estimates were made using pooled mean differences, when DFU area reduction and time until complete healing was reported and healing proportion in each group. Relative risk (RR) and absolute risk reduction (ARR) were used when DFU total healing or LEA proportions were reported. Relative risk reduction (RRR) and number needed to treat (NNT) were calculated for each study, using the formulas 1-RR and 1/RR, respectively [Bender R et al, 2005]. A pooled estimate was also calculated when heterogeneity was low, applying the referred formula and using the pooled RR and ARR, respectively [Bender R et al, 2005]. For all estimates, 95% CI were determined.

Heterogeneity tests were conducted (Chi-square test based Q-statistic and I^2 statistic) and, when evident (the first >10 or the second >20%), a random effects model (DerSimonian and Laird method) was used. Otherwise, the fixed effect model (Mantel-Haenszel's method) was selected. Funnel plots were constructed to assess possible publication bias.

A $p<0.05$ was considered statistically significant, and all p values were two sided.

2. *Results*

Included studies characterization

A total of 41 studies assessing HBOT impact on DFU healing were included: 11 RCT, 8 NRT, 10 cohort studies and 12 case series (Table 3).

The only studies reporting double blinding were Lee [Lee CT et al, 2004] and Löndahl [Löndahl M et al, 2010; Löndahl M et al, 2011].

In Kalani [Kalani M et al, 2002], only the first 14 participants were randomized while the remaining were allocated according to HBOT availability.

In two studies from the same group of authors, HBOT was compared to extracorporeal shockwave therapy (ESWT) [Kranke P et al, 2006; Wang CJ et al, 2011] instead of standard DFU treatment. In the latter of these two studies (2011), a second course of therapy when the ulcer had improved but not healed completely was permitted, according to decision of the attending physician.

In several studies, the selection process (n= 15), participants' age (n= 9), gender (n= 16), HbA1c (n= 27), diabetes duration (n= 21), ankle-brachial index (ABI) (n= 34), transcutaneous partial pressure of

Oxygen ($TcPO_2$) (n= 31), wound severity (n= 25), setting (n= 17) and mean follow-up (n= 19) were not reported (Table 5).

Where reported at all, most studies selected patients with DFU by simple consecutive inclusion. The most frequent inclusion criterion was chronic DFU, with a minimum duration of 4 [Albuquerque-Sousa J, 2005; Duzgun A et al, 2008] to 12 weeks [Hawkins G et al, 2005; Hankins G et al, 2006; Kessler L et al, 2003; Löndahl M et al, 2010; Löndahl M et al, 2011; Ramon Y et al, 1999; Wang CJ et al, 2011], while the most frequent selection criteria for control subjects were those refusing, abandoning or unable to receive treatment.

Sample size ranged from 10 [Zamboni W et al, 1997] to 1006 [Fife C et al, 1997] participants, with a total number of 4,347 (mean 106; median 36).

The mean age of participants in each study was not always reported, but ranged from something more than 51 years [Fife C et al, 2007] to 75 years [Perdrizet G et al, 2007]. Most were men with diabetes for more than five years. Generally, patients included had DFU Wagner classification grades III-V.

ABI and $TcPO_2$ values were rarely reported, and when they were, the data was highly variable (ABI 0.64 [Faglia E et al, 1996; Faglia E et al, 1998] to 1.26 [Wang CJ et al, 2009]; $TcPO_2$ 12 [Mendes D et al, 2012] to 60mm Hg [Zamboni W et al, 1997]).

Most studies were undertaken in hospital-based hyperbaric facilities; 14 studies used multiplace chambers and 6 monoplace chambers (21 did not specify). When described, all used pressures of 2 to 3 ATA and provided oxygen at 100%.There were only 3 multicentre studies, all from the same group [Otto G et al, 2000; Fife C et al, 1997; Fife C et al, 2007].

The duration of each HBOT session varied from 45 [Doctor N et al, 1992] to 120 minutes [Zamboni W et al, 1997; Chen CE et al, 2010; Zgonis T et al, 2005; Kaya A et al, 2009], most commonly 90 minutes, once or twice daily, from 4 [Doctor N et al, 1992] to 7 days per week [Çerkes N et al, 1994].

Table 3: Characteristics of included studies ordered by study type, quality assessment and sample size

Study	Selection process	Inclusion criteria	Sample size (n)	Controls' selection process	Participants' characterization							Setting	HBOT characterization		Mean follow up (months)
					Mean (SD) Age [years]	Male (%)	Mean (SD) HbA1c [%]	Mean (SD) diabetes duration [years]	Mean (SD) ABI [mmHg]	Mean (SD) $TcP0_2$ [mmHg]	Wagner Grade		Regimen	Number of sessions (mean)	
RANDOMIZED CONTROLLED TRIALS															
Löndahl M et al, 2010&2011	NR	Full-thickness DFU > 12 weeks, followed in a diabetes foot clinic for > 8 weeks	I: 38 C: 37	NA	I: 69 C: 68	I: 78 C: 84	I: 7.8 C: 8.1	I: 20 C: 23	NR	NR	I: 24% grade II, 75% grade III-V C: 27% grade II, 73% grade III-V	NR	85-min daily x 5 days/8-10 weeks sessions, max 40, multi chamber, 2.5 ATA	80% > 35 sessions in total	12
Duzgun A et al, 2008	Consecutive inclusion	Subjects >18 years with active DFU > 4 weeks	I: 50 C: 50	NA	I: 58 (11) C: 63 (9)	*I: 74 C:54*	I: 8.0 (1.9) C: 8.7 (2.9)	I: 17 (6) C: 16 (6)	NR	NR	I: 12% grade II, 88% grade III-V C: 24% grade II, 46% grade III-V	University Hospital	1-2 x 90-min daily sessions, mono chamber, 2-3 ATA	NR	23 (3)
Wang C et al, 2011	NR	Chronic DFU > 12 weeks	I: 38 C: 39	NA	I: 62 (14) C: 61 (14)	NR	I: 8.1 (1.8) C: 8.8 (2.2)	I: 6[a] C: 6[a]	I: 0.91 (0.27) C: 1.07 (0.10)	NR	NR	NR	90-min daily x 5 days/week sessions, max 20 x 2, multi chamber, 2.5 ATA	Total 20 or 40	I: 11 (5) C: 14 (4)
Abidia A et al, 2003	Consecutive inclusion	DFU > 1cm and < 10 cm diameter, > 6 weeks, ABI < 0.8 or HBI <0.7	I: 9 C: 9	NA	I: 72 (13*)* C: 70 (7)	I: 67 C:33	I: 12.7 (1.2) C: 12.5 (1.7)	I: 13 (10) C: 10 (6)	NR	NR	I: 78% grade II, 11% grade III-V C: 89% grade II, 11% grade III-V	Hull Royal Infirmary	90-min daily x 5 days/week sessions, multi chamber, 2.4 ATA	Total 30	max 12
Wang C et al, 2009	NR	Chronic DFU	I: 36 C: 34	NA	I: 59 (13) C: 63 (10)	NR	I: 9.1 (1.2) C: 8.8 (2.1)	I: 19 (20) C: 23 (21)	I: 1.26 (0.27) C: 1.22 (0.19)	NR	NR	Hospital	90-min daily x 5 days/week sessions, max 20, multi chamber, 2.5 ATA	Total 20	I: 12 (2) C: 12 (2)
Faglia E et al, 1996	Consecutive inclusion	Subjects hospitalized for DFU	I: 33 C: 35	NA	I: 62 (10) C: 66 (9)	I:77 C:64	I: 9.3 (2.5) C:8.5 (2.3)	I: 16 (10) C: 19 (9)	I: 0.65 (0.28) C: 0.64 (0.25)	I: 23. (11) C: 21 (11)	I: 12% grade II, 88% grade III - IV C: 15% grade II, 85% grade III-V	Hospital (Diabetes Unit)	90-min daily sessions, multi chamber, 2.5 ATA	39	NR
Kessler L et al, 2003	Consecutive inclusion	DFU no favourable evolution ≥ 3 months	I: 14 C: 13	NA	I: 60 (10) C:68 (11)	I: 71 C:69	I: 9.4 (2.4) C: 8.1 (1.4)	I: 18 (13) C: 22 (13)	NR	I: 46 (18) C:45 (24)	I-III	University Hospital	2 x 90-mindaily x 5 days/week sessions, multi chamber, 2.5 ATA	20	1

Kalani M et al, 2002	NR	DFU > 2 months with $TcPO_2$< 40 mmHg, not eligible for vascular intervention	I: 17 C: 21	NA	*I:54 (14)* *C:65 (11)*	I: 71 C:86	I: 7.1 (1.5) C: 7.3 (1.4)	I: 28 (12) C: 26 (17)	NR	I:22 (12) C:25 (10)	NR	NR	90-min x 5 days/week sessions, mono chamber, 250 kPa	Total ranged 40-60	36
Lee C 2004 (abstract)	Consecutive inclusion	DFU after debridement or minor LEA	I: 20 C: 12	NA	NR	NR	NR	NR	NR	NR	NR	Hospital (HBOT medicine dept)	NR	NR	All 24
Doctor N et al, 1992	Consecutive inclusion	Chronic DFU	30	NA	I: 56 (NR) C: 60 (NR)	I: 75 C: 67	NR	I: 10 C: 11	NR	NR	NR	Hospital	45-min x 4/2 week sessions, mono chamber, 3.0 ATA	4	NR
NON-RANDOMIZED TRIAL															
Zamboni W et al, 1997	Consecutive inclusion	Insulin dependent diabetics with chronic DFU	I: 5 C: 5	Refused treatment	I: 64 (4) C: 54 (3)	I: 80 C: 80	NR	NR	NR	I: 53 (4) C: 60 (2)	NR	NR	120-min x 5 days/week sessions, mono chamber, 2.0 ATA	Total 30	4-6
Faglia E et al, 1998	Consecutive inclusion	Patients hospitalized for DFU	I: 51 C: 64	Various extemporaneous reasons	*I: 61 (10)* *C: 65 (10)*	73	NR	17 (9)	0.64 (0.25)	28 (13)	11% grade II, 89% grade III-V	Hospital	90-min 5 days/week to daily sessions, chamber type NR, 2.2-2.5 ATA	32 (11)	NR
Chen C et al, 2010[b]	NR	DFU	I: 21 C: 21	Abandoned treatment	I: 66 (21) C: 68 (11)	I: 52 C: 52	I: 9.8 (1.9) C: 9.2 (2.9)	I:13 (8) C:12 (10)	I: 0.76 (0.30) C: 0.71 (0.31)	NR	I: 100% grade III-V C: 100% grade III-V	NR	120-min daily x 5 days/week sessions + intermittent schedule at weekend, multi chamber, 2.5 ATA	I: 15 C: 13	I: 23 (10) C: 3 (2)
Baroni G et al 1987	Consecutive inclusion	Necrotic DFU	I: 18 C: 10	Refused treatment	I: 58 (7) C: 59 (8)	I: 60 C: 61	I: 8.9 (1.6) C: 8.8 (1.2)	I: 16 (7) C: 14 (6)	NR	NR	NR	Metabolic unit	90-min daily sessions, multi chamber, 2.5 or 2.8 ATA	34 (22)	14 (10)
Oriani G et al, 1990	Consecutive inclusion	Major ulcero-necrotic lesions	I: 62 C: 18	Refused treatment	I: 53 (12) C: 58 (8)	I: 58 C: 67	*I: 9.5* *C: 8.2*	I: 14 (10) C: 16 (6)	NR	NR	All grade IV or V	NR	5-6 days/week sessions multi chamber, 2.5 or 2.8 ATA	72 (29)	NR
Perdrizet G et al, 2007 (abstract)	Consecutive inclusion	NR	I: 25 C: 25	Matched for age and gender	I: 64 (14) C: 75 (12)	I: 56 C: 68	NR	NR	I: 1.1 (0.5) C: 0.8 (0.6)	I: 31 (18) C: 42 (22)	NR	NR	NR	All 30	C: 10 I: 12

Mendes D et al, 2012 (poster)	Consecutive inclusion	Chronic DFU (> 8 weeks)	I: 9 C: 4	Refused treatment or had contra-indication	60 (11)	I: 89 C: 100	8.5 (2.0)	NR	NR	12 (9)	100% grade III or IV	NR	90-min daily sessions, chamber type NR, 2.1 ATA	Total ranged 56-111	Up to 12
Albuquerque J, 2005 (retrosp)	Consecutive inclusion	Chronic DFU (>4weeks)	I: 55 C: 41	Refused treatment	I: 61 (13) C: 64 (14)	I: 74 C: 66	NR	I: 22 (10) C: 20 (8)	NR	NR	I: 14% grade II, 86% grade III-V C: 10% grade II, 90% grade III-V	Hospital	90-min daily x 5 days/week sessions, multi chamber, 2.5 ATA	54 (31)	50 (ranged 17-120)
PROSPECTIVE COHORT STUDIES															
Hawkins G et al, 2006	Consecutive inclusion	Chronic wounds (> 3 months)	I: 40	NA	I: 66	NR	NR	NR	NR	NR	NR	HBOT unit	NR	23 (10)	Up to 12
Mathieu D et al, 1997 (abstract)	Consecutive inclusion	DFU	I: 29	NA	I: 61 (16)	NR	NR	NR	NR	I: > 11	NR	HBOT unit	2 x 90-min daily x 5 days/week sessions, chamber type NR 2.5 ATA	NR	Max 6 weeks
Wattel F et al, 1991	Consecutive inclusion	DFU	I: 59	NA	I: 60 (13)	I: 58	NR	NR	NR	NR	NR	HBOT unit	2 x 90-min daily x 5 days/week sessions, chamber type NR, 2.5 ATA	29 (19)	NR
Hawkins G et al, 2005 (poster)	Consecutive inclusion	Chronic DFU (>12 weeks)	I: 43	NA	NR	NR	NR	NR	NR	NR	NR	Hospital	NR	NR	NR
Ong M et al, 2008	Consecutive inclusion	DFU	I: 45	NA	I: 59	NR	NR	NR	NR	NR	NR	HBOT medicine centre	90-min daily sessions, chamber type NR, 2.5 ATA	20[a]	NR
Ramon Y et al, 1999 (abstract)	Consecutive inclusion	Chronic DFU (> 3 months)	I: 26	NA	NR	NR	NR	NR	NR	NR	NR	NR	90-min daily x 6 days/week, chamber type NR, 2.5 ATA	>61	NR
RETROSPECTIVE COHORT STUDIES															
Zgonis T et al, 2006	NR	Subjects undergoing partial foot amputation	I: 35	NA	NR	NR	NR	NR	NR	NR	NR	NR	120-min daily sessions, mono chamber, 2 ATA	Total ranged 16-20	Total ranged 1.5-7
Otto G et al, 2000	NR	Diabetic patients from HBOT facilities	I: 180	NA	I: > 62	NR	NR	I: > 17	NR	NR	NR	Multicentre HBOT facilities	NR	NR	NR
Fife C et al, 2007	NR	Diabetic patients from HBOT facilities	I: 971	NA	I: > 51	I: 58	NR	I: > 21	NR	NR	NR	Multicentre HBOT facilities	≥ 5 days/week sessions, chamber type NR, 2.0-2.4 ATA	Mean ranged 24-34	NR

Fife C et al, 1997 (abstract)	NR	Diabetic patients from hyperbaric facilities	I: 1006	NA	NR	NR	NR	NR	NR	NR	NR	Multicentre HBOT facilities	NR	NR	NR
CASE SERIES															
Bishop A et al, 2012	Consecutive inclusion	Diabetic patients with DFU treated with HBO	I: 30	NA	I: 63	I: 20	NR	I: 17	NR	NR	NR	HBOT medicine centre	NR	40	All 3
Mathieu D et al, 1991	Consecutive inclusion	DFU	I: 59	NA	I: 60 (13)	I: 58	NR	NR	NR	I: >13	NR	HBOT unit	90-min x 2 daily x 5 days/week sessions, chamber type NR, 2.5 ATA	29 (19)	3 (2) weeks
Kaya A et al, 2009	Consecutive inclusion	Diabetic patients with DFU treated with HBO	I: 184	NA	I: 60 (11)	I: 72	I: 8.5 (2.1)	I: 15 (9)	NR	NR	I: 31% grade II, 69% grade III-V	NR	120-min x 1-2 daily x 6 days/week sessions, multi chamber, 2.4 ATA	39	All 12
Cianci P et al, 1997	Blindly selected from a larger group of consecutive inclusion	DFU	I: 41	NA	I: 64	NR	NR	NR	NR	NR	Average Wagner score: 4	Hospital	NR	NR	NR
Çerkeş N et al, 1994	NR	Diabetic patients with DFU treated with HBO	I: 30	NA	I: 62 (12)	I: 73	NR	NR	NR	NR	NR	NR	90-min x 1 – 2 daily x 7 days/week sessions multi chamber, 2.4 – 3 ATA	36	1.5
Subbotina N etal, 2002 (abstract)	NR	Chronic DFU	I: 191	NA	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Jovanovic T et al, 2011 (abstract)	Consecutive inclusion	DFU	I: 69	NA	I: 58	I: 81	NR	NR	NR	NR	NR	HBOT medicine centre	90-min daily x 5 days/week sessions, chamber type NR, 2.5 ATA	Total ranged 25-40	NR
Oriani G et al, 1992	NR	Diabetic patients with gangrenous DFU treated with HBOT	I: 151	NA	I: >68	I: 63	NR	NR	NR	NR	I: 100% grade IV-V	Hospital	90-min daily sessions, multi chamber, 2.5-2.8 ATA	40	NR

Wattel F et al, 1995	Consecutive inclusion	Chronic DFU (>6 weeks without revascularization surgery indication)	I: 40	NA	NR	NR	NR	NR	NR	NR	NR	NR	90-min x 2 daily x NR days/week sessions, chamber type NR, 2.5 ATA	NR	NR
Zivkovic M et al, 1999 (abstract)	NR	DFU	I: 41	NA	I: 61	I: 66	NR	I: 15	NR	NR	I: 63% grade I-III, 37% grade IV-V	NR	60-min x 1-2 daily x NR days/week sessions, mono chamber, 2 – 2.4 ATA	22	NR
Kawashima M et al, 2006 (abstract)	NR	Diabetic patients treated with HBOT	I: 87	NA	NR	NR	NR	NR	NR	NR	I: 72% grade I-III, 28% grade IV-V	NR	60-min sessions, chamber type NR, 2.0 ATA	NR	NR
Buyukcakir C et al, 1998 (abstract)	NR	Diabetic patients treated with HBOT	I: 21	NA	NR	NR	NR	NR	NR	NR	NR	NR	90-min daily sessions, multi chamber, 2.5 ATA	NR	NR

In italic: statistically significant difference between groups, [a] Median, [b] intervention group: > 10 HBOT sessions and control group: < 10 HBOT sessions, ABI: Ankle Brachial-Index, ATA: Atmosphere Absolute Pressure, C: Control Group, cm: Centimetre, dept: Department, DFU: Diabetic Foot Ulcer, HbA1c: Glycated Haemoglobin, HBI: Hallux Brachial Index, HBOT: Hyperbaric Oxygen Therapy, I: Intervention Group, kPa: Kilopascal, max: Maximum, min: Minutes, mmHg: Millimetres of Mercury, n: Sample Size, NR: Not Reported, NA: Not Applicable, retrosp: Retrospective, SD: Standard Deviation, $TcPO_2$: Transcutaneous Partial Pressure of Oxygen

Each RCT was assessed for methodological quality by compliance with the CONSORT checklist [Schulz K et al, 2010]. There was an adequate summary, background, objectives or hypothesis, eligibility criteria, participant flow, numbers of participants and results description and interpretation were described in more than 90% of the studies. On the other hand, adequate descriptions of trial design, methods or outcomes changes, interim analyses, method used and person responsible to generate random allocation sequence, description of the similarity of interventions, reason for the trial's end, ancillary analyses and where to access the full trial protocol were reported in less than 10% of the studies. From a total of 37 items, completeness ranged from 8 (22%) [Doctor N et al, 1992; Lee Ct el al, 2004] to 21 (57%) [Löndahl M et al, 2010], with a mean of 18 (47%) (Table 4).

In a similar analysis using the STROBE checklist [Vandenbroucke J et al, 2007], more than 90% of studies reported key elements of study design and results. In contrast, less than 10% described the matching criteria, any potential sources of bias, the reason for non-participation of the control subjects, details of missing data and a number of other items. From a total of 34 items, completeness varied from 4 (12%) [Buyukcakir C et al, 1998; Kawashima M et al, 2006] to 16 (47%) [Bishop A & Mudge E, 2012], with a mean of 10 (29%) (Table 5, Table 6, Table 7).

Table 4: Randomized controlled trials methodological quality assessment using the CONSORT checklist

Topic / Item \ Study reference		Löndahl M, 2010	Duzgun A, 2008	Wang C, 2011	Abidia A, 2003	Wang C, 2009	Faglia E, 1996	Kessler L, 2003	Löndahl M, 2011	Kalani M, 2002	Lee C, 2004	Doctor N, 1992	Total n (%)
Title and abstract	1a												4 (36)
	1b												10 (91)
Introduction	2a												10 (91)
Background and objectives	2b												10 (91)
Methods	3a												0 (0)
Trial design	3b												0 (0)
Participants	4a												10 (91)
	4b												4 (36)
Interventions	5												9 (82)
Outcomes	6a												9 (82)
	6b												0 (0)
Sample size	7a												3 (27)
	7b												0 (0)
Randomisation	8a												6 (55)
Sequence generation	8b												1 (9)
Allocation concealment	9												3 (27)
Implementation	10												0 (0)
Blinding	11a												2 (18)
	11b												1 (9)
Statistical methods	12a												7 (64)
	12b												0 (0)
Results	13a												10 (91)
Participant flow	13b												5 (45)
Recruitment	14a												8 (73)
	14b												0 (0)
Baseline data	15												9 (82)
Numbers analysed	16												10 (91)
Outcomes and estimation	17a												10 (91)
	17b												7 (64)
Ancillary analyses	18												1 (9)
Harms	19												4 (36)
Discussion													
Limitations	20												6 (15)
Generalizability	21												2 (18)
Interpretation	22												10 (91)
Other information													
Registration	23												3 (27)
Protocol	24												0 (0)
Funding	25												6 (55)
Total present items (possible 37)		21	20	20	20	19	16	15	14	14	8	8	

We have ordered studies by their CONSORT checklist number of fulfilled items and by sample size (the same criteria as Table 3). Dark grey represent an absent item, light grey represent a present item. n: Number

Table 5: Non-randomized trials methodological quality assessment using the STROBE checklist

Topic / Item \ Study ref		Zamboni W, 1997	Faglia E,1998	Chen C,2010	BaroniG, 1987	Albuquerque J, 2005	Oriani G,1990	Perdrizet G,2007	Mendes D, 2012	Total n (%)
Title and abstract	1a									1(12)
	1b									6 (75)
Introduction										
Background	2									6(75)
Objectives	3									4 (50)
Methods										
Study design	4									8 (100)
Setting	5									3 (38)
Participants	6a									7(88)
	6b									1 (12)
Variables	7									2 (25)
Data sources	8									1(12)
Bias	9									0 (0)
Study size	10									0 (0)
Quantitative Variables	11									3(38)
Statistical methods	12a									3(38)
	12b									0 (0)
	12c									0 (0)
	12d									0 (0)
	12e									0 (0)
Results	13a									2(25)
Participants	13b									0 (0)
	13c									0 (0)
Descriptive data	14a									7 (88)
	14b									0 (0)
	14c									2(25)
Outcome data	15									8 (100)
Main results	16a									5 (62)
	16b									0 (0)
	16c									0 (0)
Other analyses	17									0 (0)
Discussion										
Key results	18									8 (100)
Limitations	19									4 (50)
Interpretation	20									1(12)
Generalizability	21									0 (0)
Other information										
Funding	22									1(12)
Total present items (possible 34)		13	12	12	10	10	8	8	8	

We have ordered studies by their STROBE checklist number of fulfilled items and by sample size (the same criteria as Table 3). Dark grey represent an absent item, light grey represent a present item. n: Number

Table 6: Cohort studies methodological quality assessment using the STROBE checklist

Topic / Item \ Study ref		Zgonis T, 2005	Hawkins G, 2006	Otto G, 2000	Mathieu D, 1997	Fife C, 2007	Wattel F, 1991	Hawkins G, 2005	Ong M, 2008	Fife C, 1997	Ramon Y, 1999	Total n (%)
Title and abstract	1a											2 (20)
	1b											6 (60)
Introduction												
Background	2											8 (80)
Objectives	3											8 (80)
Methods												
Study design	4											10 (100)
Setting	5											3 (30)
Participants	6a											7 (70)
	6b											0 (0)
Variables	7											4 (40)
Data sources	8											2 (20)
Bias	9											0 (0)
Study size	10											1 (10)
Quantitative Variables	11											1 (10)
Statistical methods	12a											6 (60)
	12b											0 (0)
	12c											0 (0)
	12d											0 (0)
	12e											0 (0)
Results	13a											5 (50)
Participants	13b											0 (0)
	13c											0 (0)
Descriptive data	14a											4(40)
	14b											0 (0)
	14c											2(20)
Outcome data	15											10(100)
Main results	16a											7 (70)
	16b											0 (0)
	16c											0 (0)
Other analyses	17											0 (0)
Discussion												
Key results	18											10(100)
Limitations	19											4 (40)
Interpretation	20											5 (50)
Generalizability	21											1 (10)
Other information												
Funding	22											1 (10)
Total present items (possible 34)		15	13	13	12	12	11	9	7	7	6	

We have ordered studies by their STROBE checklist number of fulfilled items and by sample size (the same criteria as Table 3). Dark grey represent an absent item, light grey represent a present item. n: Number

Table 7: Case series methodological quality assessment using the STROBE checklist

Topic / Item \ Study ref		Bishop A, 2012	Mathieu D, 1991	Kaya A, 2009	Cianci P, 1997	Çerkeş N, 1994	Subbotina N, 2002	Jovanovic T, 2011	Oriani G, 1992	Wattel F, 1995	Zivkovic M, 1999	Kawashima M, 2006	Buyukcakir C, 1998	Total n (%)
Title and abstract	1a													1 (8)
	1b													6 (50)
Introduction														
Background	2													10 (83)
Objectives	3													7 (58)
Methods														
Study design	4													12 (100)
Setting	5													2 (16)
Participants	6a													9 (75)
	6b													0 (0)
Variables	7													0 (0)
Data sources	8													1 (8)
Bias	9													1 (8)
Study size	10													4 (33)
Quantitative Variables	11													1 (8)
Statistical methods	12a													6 (50)
	12b													0 (0)
	12c													0 (0)
	12d													0 (0)
	12e													0 (0)
Results	13a													10 (83)
Participants	13b													2 (16)
	13c													0 (0)
Descriptive data	14a													7 (58)
	14b													0 (0)
	14c													4 (33)
Outcome data	15													7 (58)
Main results	16a													3 (25)
	16b													0 (0)
	16c													0 (0)
Other analyses	17													1 (8)
Discussion														
Key results	18													12 (100)
Limitations	19													2 (16)
Interpretation	20													3 (25)
Generalizability	21													1 (8)
Other information														
Funding	22													2 (16)
Total present items (possible 34)		16	13	12	11	11	9	8	7	7	5	4	4	

We have ordered studies by their STROBE checklist number of fulfilled items and by sample size (the same criteria as Table 3). Dark grey represent an absent item, light grey represent a present item. n: Number

HBOT efficacy

Mean wound area reduction

Only 3 studies reported crude data [Abidia A et al, 2003; Kessler L et al, 2003; Mendes D et al, 2012] for this outcome. Abidia [Abidia A et al, 2003] achieved 100% healing in the HBOT group and 95% in the control group, although no standard deviation (SD) was available. Kessler [Kessler L et al, 2003] observed no difference in the reduction of wound area between groups (61.9±23.3 in the HBOT group *vs* 55.1 ±21.5% in the controls). Zamboni et al [Zamboni W et al, 1997] reported that a higher area reduction occurred in the HBOT group, but no data was available. Mendes [Mendes D et al, 2012] reported a 92% area reduction (± 12%) in the HBOT group, but all control subjects had an LEA so there was no comparator.

Time to complete healing

Five studies concluded that subjects in the HBOT group took the same [Kalani M et al, 2002] or less time to achieve healing in DFU subjects [Abidia A et al, 2003; Albuquerque-Sousa J, 2005; Baroni G et al, 1987; Lee CT et al, 2004]. Abidia [Abidia A et al, 2003] and Albuquerque [Albuquerque-Sousa J, 2005] reported a shorter healing time in the HBOT groups (6 *vs* 9 and 45 *vs* 55 months, respectively), but SD was not reported. Lee et al [Lee CT et al, 2004] reported a shorter healing time in the HBOT group, but no crude data was available. The combination of the remaining data from Kalani's RCT [Kalani M et al, 2002] and Baroni's NRT [Baroni G et al, 1987], suggests a pooled mean difference of 0.8 months shorter healing time in the HBOT group, but this was not statistically significant (95%CI -2.5 to +0.9, I^2=0%) (Table 8).

Table 8: Diabetic foot ulcer time until complete healing comparison between subjects treated with or without Hyperbaric Oxygen therapy: meta-analysis

Study	Time to outcome (months)	Time until complete healing (months)		
		HBOT [mean (SD)]	Control [mean (SD)]	Mean difference (95% CI)
RANDOMIZED CONTROLLED TRIALS				
Abidia A et al, 2003	6	6[a] (2-18)	9[a] (3-60)	NE
Kalani M et al, 2002	36	15 (7)	15 (4)	0.0 (-3.7, 3.7)
NON RANDOMIZED TRIALS				
Baroni G et al, 1987 [b]	14	2 (1)	3 (3)	-1.0 (-2.9,0.9)
Albuquerque J, 2005	50[c]	45 (NR)	55 (NR)	NE
			Pooled estimate	-0.8 (-2.5,0.9)
			Q statistic	0.22
			I^2 statistic	0 %

[a] median, [b] 80% or more granulating tissue or partial/ complete epithelialization, [c] mean, CI: Confidence Interval, HBOT: Hyperbaric Oxygen Therapy, NE: Not Estimable, NR: Not Reported, SD: Standard Deviation

We have ordered studies by their type, CONSORT or STROBE checklist number of fulfilled items and by sample size (the same criteria as Table 3)

Proportion of ulcers healed

A total of 15 RCT or NRT [Abidia A et al, 2003; Albuquerque-Sousa J, 2005; Baroni G et al, 1987; Chen CE et al, 2010; Duzgun A et al, 2008; Kalani M et al, 2002; Kessler L et al, 2003; Lee CT et al, 2004; Löndahl M et al, 2010; Löndahl M et al, 2011; Mendes D et al, 2012; Perdrizet G et al, 2007; Wang CJ et al, 2009; Wang CJ et al, 2011; Zamboni W et al, 1997] compared the proportion of healed DFU with or without HBOT. In all, except Wang [Wang CJ et al, 2009; Wang CJ et al, 2011], the chance of healing tended to be higher in the intervention group.

Because of clear clinical heterogeneity (both Wang studies [Wang CJ et al, 2009; Wang CJ et al, 2011] did not compare HBOT with standard care, but rather with extra-corporeal shock wave therapy), we excluded these studies and recalculated our pooled measures. The resultant pooled analysis suggests that DFUs are about twice as likely to heal in the HBOT group (RR 2.19, 95% CI1.05 to 4.57).

Including all available studies, one can observe that the proportion healed was significantly higher in those undergoing HBOT (pooled estimate of 58.3% *vs* 18.5%) (Table 9).

Table 9: Diabetic foot ulcer healing rate comparison between subjects treated with or without Hperbaric Oxygen therapy: meta-analysis

Study	Time to outcome (months)	Healing proportion				Association measures	
		HBOT		Control		RR	ARR
		n	% (CI 95%)	n	% (CI 95%)	(CI 95%)	(CI 95%)
RANDOMIZED CONTROLLED TRIALS							
Löndahl M et al, 2010& 2011	12	25	65.8 (40.0, 91.6)	12	32.4 (14.1, 50.8)	2.0 (1.2, 3.4)	0.3 (0.1, 0.6)
Duzgun A et al, 2008	23	33	66.0 (43.5, 88.5)	0	0.0 (NE)	67.0 (4.2, 1064.2)	0.7 (0.5, 0.8)
Wang C et al, 2011	≥ 11	11	28.9 (11.8, 46.0)	31	79.5 (51.5, 100.0)	0.4 (0.2, 0.6)	-0.5 (-0.7, -0.3)
Abidia A et al, 2003	6	5	55.6 (6.8, 100.0)	2	22.2 (0.0, 53.0)	2.5 (0.6, 9.7)	0.3 (0.1, 0.6)
Wang C et al, 2009	12	8	22.2 (6.8, 37.6)	11	32.4 (13.2, 51.5)	0.7 (0.3, 1.5)	-0.1 (-0.3, 0.1)
Kessler L et al, 2003	1	2	14.3 (0.0, 34.1)	0	0.0 (NE)	4.7 (0.2, 89.0)	0.1 (-0.07, 0.4)
Kalani M et al, 2002	36	13	76.5 (34.9, 100.0)	10	47.6 (18.1, 77.1)	1.6 (1.0, 2.7)	0.3 (-0.09, 0.8)
Lee C et al, 2004	24	16	80.0 (40.8, 100.0)	8	66.7 (20.5, 100.0)	1.2 (0.8, 1.9)	0.1 (-0.2, 0.4)
Pooled estimate			47.1 (29.2, 65.0)		25.9 (14.3, 37.6)	1.4 (0.7, 2.7)	0.2 (-0.1, 0.5)
Q statistic			29.2		72.5	41.6	110.4
I^2 statistic			76%		90%	83%	94%
NON-RANDOMIZED TRIALS							
Zamboni W et al, 1997	NR	5	100.0 (12.3, 100.0)	1	20.0 (0.0, 59.2)	3.7 (0.9, 3.4)	0.8 (0.4, 1.2)
Chen C et al, 2010[a]	>6	16	76.2 (38.8, 100.0)	7	33.3 (8.6, 58.0)	2.3 (1.2, 4.4)	0.4 (0.2, 0.7)
Baroni G et al, 1987[b]	14	16	88.9 (45.3, 100.0)	1	10.0 (0.0, 29.6)	8.9 (1.4, 57.5)	0.8 (0.6, 1.0)

Perdrizet G et al, 2007	2.5	14	56.0 (26.7, 85.3)	8	32.0 (9.8, 54.2)	1.8 (0.9, 3.4)	0.2 (-0.03, 0.5)
Mendes D et al, 2012	6	5	55.6 (6.8, 100.0)	0	0.0 (NE)	5.5 (0.4, 80.9)	0.6 (0.2, 1.0)
Albuquerque J, 2005	50[c]	14	25.4 (12.1, 38.8)	1	2.4 (0.0, 7.2)	10.4 (1.4, 76.2)	0.2 (0.1, 0.4)
Pooled estimate			*59.7 (33.7, 85.7)*		*13.0 (1.7, 24.3)*	*3.2 (2.1, 5.0)*	*0.5 (0.3, 0.7)*
Q statistic			16.4		12.8	6.8	23.1
I^2 statistic			69%		61%	27%	78%
PROSPECTIVE COHORT STUDIES							
Hawkins G et al, 2006	NR	31[d, e]	77.5 (50.2, 100.0)		NA		
Wattel F et al, 1991	NR	48	81.4 (58.3, 100.0)		NA		
Hawkins G et al, 2005	NR	21[d, e]	48.8 (27.9, 69.7)		NA	Not Estimable	
Ong M et al, 2008	NR	32[b, f]	71.1 (46.5, 95.7)		NA		
Ramon Y et al, 1999	NR	19	73.1 (40.2, 100.0)		NA		
RETROSPECTIVE COHORT STUDIES							
Zgonis T et al, 2006	1.5-7	28	80.0 (50.4, 100.0)		NA	Not Estimable	
CASE SERIES REPORT							
Bishop A et al, 2012	3	8	26.7 (81.9, 45.1)		NA		
Mathieu D et al, 1991	0.7	52	88.1 (64.2, 100.0)		NA		
Kaya A et al, 2009	NR	115	62.5 (51.1, 73.9)		NA		
Cianci P et al, 1997	NR	27	65.8 (41.0, 90.7)		NA		
Çerkeş N et al, 1994	1.5	19	63.3 (34.8, 91.8)		NA		
Subbotina N et al, 2002	NR	69	36.1 (27.6, 44.6)		NA		
Jovanovic T et al, 2011	NR	42	60.9 (42.5, 79.3)		NA	Not Estimable	
Wattel F et al, 1995	NR	36	90.0 (60.6, 100.0)		NA		
Kawashima M et al, 2006	NR	48	55.2 (39.6, 70.8)		NA		
Buyukcakir C et al, 1996	NR	15	71.4 (35.3, 100.0)		NA		
Pooled estimate			63.6 (53.6, 73.5)		NA		
Q statistic			58.1				
I^2 statistic			74%				
TOTAL							
Pooled estimate			*58.3 (49.9, 66.8)*		*18.5 (11.1, 25.8)*	*2.0 (1.2, 3.3)*	*0.3 (0.1, 0.5)*
Q statistic			120.4		88.8	60.7	141.6
I^2 statistic			76%		85%	79%	91%

[a] Intervention group: > 10 HBOT sessions and control group: < 10 HBOT sessions, [b] 80% or more granulating tissue or partial/ complete epithelialization, [c] Mean, [d] 1 month after HBOT, [e] Substantially or completely healed, [f] 1 week after HBOT, ARR: Absolute Risk Reduction, CI: Confidence Interval, HBOT: Hyperbaric Oxygen Therapy, NA: Not Applicable, NNT: Number Needed to Treat, NR: Not Reported, RR: Relative Risk, RRR: Relative Risk Reduction. We have ordered studies by their type, CONSORT or STROBE checklist number of fulfilled items and by sample size (the same criteria as Table 3)

Minor lower extremity amputation proportion

For Doctor [Doctor N et al, 1992] it was not possible to calculate risk measures because the number of subjects in each group was not given.

In 2 NRTs, no minor LEA occurred and in another 2 only global LEA proportion was reported. Pooled data from both RCTs and NRTs failed to show any statistical difference in the risk of minor LEA (Table 10).

Proportion of patients requiring lower limb extremity amputation

Major LEA (includes all amputations above the mid-foot level) risk was significantly lower in the HBOT group when pooling both RCTs and NRTs, but not significantly so when including RCTs only.

The risk of receiving a major LEA was significantly lower in those receiving HBOT (RR with HBOT 0.4 (CI 95% 0.1, 1.3) for RCTs, 0.4 (CI 95% 0.2, 0.5) for NRT and 0.3 (CI 95% 0.2, 0.5) combining all studies. Overall, when including all available studies for pooled estimate calculation, both major (5.3% *vs* 22.4%) and overall LEA (14.6 *vs* 37.2%) were significantly less frequent in the HBOT group (Table 10).

Table 10: Lower extremity amputation proportion comparison between subjects treated with or without Hyperbaric Oxygen therapy: meta-analysis

Study	Time to outcome (months)	LEA proportion									Association measures Minor LEA		Association measures Major LEA		Association measures Global LEA	
		HBOT					Control									
		Minor		Major		Global	Minor		Major		Global					
		n	% (CI 95%)	n	% (CI 95%)	% (CI 95%)	n	% (CI 95%)	n	% (CI 95%)	% (CI 95%)	RR (CI 95%)	ARR (CI 95%)	RR (CI 95%)	ARR (CI 95%)	RR (CI 95%)	ARR (CI 95%)
RANDOMIZED CONTROLLED TRIALS																	
Löndahl M et al, 2010& 2011	12	4	10.5 (0.2, 20.8)	3	7.9 (0.0, 16.8)	18.4 (4.8, 32.1)	4	10.8 (0.2, 21.4)	1	2.7 (0.0, 8.0)	13.5 (1.7, 25.3)	1.0 (0.3, 2.8)	0.0 (-0.1, 0.1)	2.9 (0.3, 26.8)	0.05 (-0.05, 0.2)	1.4 (0.5, 3.0)	0.05 (-0.1, 0.2)
Duzgun A et al, 2008	23	4	8.0 (0.2, 15.8)	0	0.0 (NE)	8.0 (0.2, 15.8)	24	48.0 (28.8, 67.2)	17	34.0 (17.8, 50.2)	82.0 (56.9, 100.0)	0.2 (0.06, 0.4)	-0.4 (-0.6,-0.2)	0.03 (0.0, 0.5)	-0.3 (-0.5, -0.2)	0.1 (0.04, 0.2)	-0.7 (-0.9, -0.6)
Abidia A et al, 2003	6	1	11.1 (0.0, 32.9)	1	11.1 (0.0, 32.9)	22.2 (0.0, 53.0)	0	0.0 (NE)	1	11.1 (0.0, 32.9)	11.1 (0.0, 32.9)	3.0 (0.1, 65.2)	0.1 (-0.2, 0.4)	1.0 (0.07, 13.6)	0.0 (-0.3, 0.3)	2.0 (0.2, 18.3)	0.1 (-0.2, 0.4)
Faglia E et al, 1996	NR	21	63.6 (36.4, 90.8)	3	9.1 (0.0, 19.4)	72.7 (43.6, 100.0)	12	34.3 (14.9, 53.7)	11	31.4 (12.8, 50.0)	65.7 (38.8, 92.6)	1.7 (1.0, 2.8)	0.2 (0.01, 0.5)	0.3 (0.08, 0.8)	-0.2 (-0.4, -0.06)	1.1 (0.8, 1.5)	0.07 (-0.2, 0.3)
Kalani M et al, 2002	36	0	0.0 (NE)	2	11.8 (0.0, 28.1)	11.8 (0.0, 28.1)	0	0.0 (NE)	7	33.3 (8.6, 58.0)	33.3 (8.6, 58.0)	Not Estimable	0.0 (-0.1, 0.1)	0.4 (0.07, 13.6)	-0.2 (-0.5, 0.04)	0.4 (0.08, 1.5)	-0.2 (-0.5, 0.04)
Lee C et al, 2004	24	4	20.0 (0.4, 39.6)	0	0.0 (NE)	20.0 (0.4, 39.6)	4	33.3 (0.7, 66.0)	0	0.0 (NE)	33.3 (0.7, 66.0)	0.6 (0.2, 2.0)	-0.1 (-0.4, 0.2)	Not Estimable		0.6 (0.2, 2.0)	-0.1 (-0.5, 0.2)
Doctor N et al, 1992	NR	4	Not Estimable	2	Not Estimable		2	Not Estimable	7	Not Estimable		Not Estimable					
Pooled estimate			13.8 (3.4, 24.2)		*2.0 (0.0, 4.8)*	21.7 (8.3, 35.0)		16.7 (5.2, 28.3)		*15.6 (4.9, 26.4)*	38.9 (0.1, 62.4)	*0.7 (0.3, 2.2)*	-0.04 (-0.2, 0.1)	0.4 (0.1, 1.3)	-0.2 (-0.3, 0.04)	0.6 (0.2, 1.8)	-0.2 (-0.5, 0.2)
Q statistic			27.3		8.3	19.0		39.6		28.7	33.6	19.3	29.0	8.2	34.0	37.6	81.2
I^2 statistic			82%		40%	74%		87 %		82%	85%	79%	83%	51%	85%	87%	94%
NON RANDOMIZED TRIALS																	
Zamboni W et al, 1997	NR	0	0.0 (NE)	0	0.0 (NE)	0.0 (NE)	0	0.0 (NE)	0	0.0 (NE)	2.0 (0.0, 14.4)	Not Estimable		Not Estimable		Not Estimable	
Faglia E et al, 1998	NR	0	0 (NE)	7	13.7	13.7	0	0.0 (NE)	20	31.2	31.2			0.4	-0.2	0.4	-0.2

					(3.6, 23.9)	(3.6, 23.9)				(17.6, 44.9)	(17.5, 44.9)			(0.2, 1.0)	(-0.3, -0.03)	(0.2, 1.0)	(-0.32, -0.03)
Chen C et al, 2010[a]	>6	0	0 (NE)	4	19.0 (0.4, 37.7)	19.0 (0.4, 37.7)	0	0.0 (NE)	10	47.6 (18.1, 77.1)	47.6 (18.1, 77.1)			0.4 (0.1, 1.1)	-0.3 (-0.5, -0.01)	0.4 (0.2, 1.1)	-0.3 (-0.6, -0.01)
Baroni et al, 1987[b]	14	0	0 (NE)	2	11.1 (0.0, 26.5)	11.1 (0.0, 26.5)	0	0.0 (NE)	4	40.0 (0.8, 79.2)	40.0 (0.8, 0.79)			0.3 (0.06, 1.3)	-0.3 (-0.6, 0.05)	0.3 (0.06, 1.3)	-0.3 (-0.6, 0.05)
Oriani G et al, 1990	NR		3			4.8 (0.0, 10.3)		6			33.3 (6.7, 60.0)			Not Estimable		0.2 (0.04, 0.5)	-0.3 (-0.5, -0.06)
Perdrizet G et al, 2007	2.5		4			16.0 (0.3, 31.7)		8			32.0 (9.8, 54.2)			Not Estimable		0.5 (0.2, 1.4)	-0.2 (-0.4, 0.07)
Mendes D et al, 2012	6	0	0 (NE)	0	0.0 (NE)	1.1 (0.0, 8.0)	1	25.0 (0.0, 74.0)	3	75.0 (0.0, 100.0)	100.0 (2.0, 100.0)	0.2 (0.01, 3.4)	-0.2 (-0.6, 0.2)	0.07 (0.0, 1.1)	-0.7 (-1.2, -0.3)	0.06 (0.0, 0.8)	-1.0 (-1.3, -0.7)
Albuquerque J, 2005	50[c]	7	12.7 (3.3, 22.2)	8	14.5 (4.4, 24.6)	27.3 (13.5, 41.1)	10	24.4 (9.3, 39.5)	17	41.5 (21.8, 61.2)	65.8 (41.7, 90.7)	0.4 (0.2, 1.3)	-0.2 (-0.3, 0.04)	0.4 (0.2, 0.7)	-0.3 (-0.5,-0.09)	0.4 (0.3, 0.7)	-0.4 (-0.6, -0.2)
Pooled estimate			0.8 (0.0, 2.5)		8.9 (2.7, 15.1)	*10.0 (4.1, 16.1)*		1.5 (0.0, 4.6)		32.0 (13.0, 50.9)	*36.0 (18.6, 53.4)*	0.5 (0.2, 1.1)	-0.13 (-0.3, 0.02)	*0.4 (0.2, 0.5)*	*-0.3 (-0.4, -0.1)*	*0.4 (0.3, 0.5)*	*-0.4 (-0.5, -0.2)*
Q statistic			6.8		9.0	17.1		11.0		20.6	30.2	0.5	0.3	1.7	6.5	4.8	26.9
I^2 statistic			26%		45%	59%		54%		76%	77%	0%	0%	0%	38%	0%	78%
PROSPECTIVE COHORT STUDIES																	
Wattel F et al, 1991	NR		11		19	18.6 (7.6, 29.7)		Not applicable				Not Estimable					
CASE SERIES REPORT																	
Bishop A et al, 2012	3		1			3.3 (0.0, 9.9)		Not applicable				Not Estimable					
Mathieu D et al, 1991	0.7		7			11.9 (3.1, 20.6)											
Kaya A et al, 2009	NR	29	15.8 (10.0, 21.5)	9	4.9 (1.7, 8.1)	20.6 (14.1, 27.2)											
Cianci P et al, 1997	NR	0	0 (NE)	1	2.4 (2.3, 7.2)	2.4 (0.0, 7.2)											
Çerkeş N et al, 1994	1.5	6	20.0 (4.0, 36.0)	5	16.7	36.7											

					(2.0, 31.3)	(15.0, 58.3)										
Jovanovic T et al, 2011	NR	9	13.0 (4.5, 21.6)	0	0.0 (NE)	13.0 (4.5, 21.6)										
Oriani G et al, 1992	NR		NR	21	13.9 (8.0, 19.8)	13.9 (8.0, 19.8)										
Wattel F et al, 1995	NR		14			35.0 (16.7, 53.3)										
Zivkovic M et al, 1999	NR	6	14.6 (2.9, 26.3)	4	9.8 (0.2, 19.3)	24.4 (9.3, 39.5)										
Buyukcakir C et al, 1996	NR	0	0.0 (NE)	6	28.6 (5.7, 51.4)	28.6 (5.7, 51.4)										
Pooled estimate			8.6 (2.8, 14.4)		7.2 (2.4, 11.9)	15.9 (10.0, 21.8)										
Q statistic			43.8		40.8	46.1										
I^2 statistic			88%		85%	78%										
TOTAL																
Pooled estimate			5.2 (2.7, 7.6)		*5.3 (3.1, 7.5)*	*14.6 (10.6, 18.7)*	5.7 (1.9, 9.6)	*22.4 (12.4, 32.3)*	*37.2 (24.0, 50.5)*	0.6 (0.3, 1.6)	-0.07 (-0.2, 0.1)	*0.3 (0.2, 0.5)*	*-0.2 (-0.4, -0.09)*	*0.4 (0.2, 0.7)*	*-0.3 (-0.4, -0.09)*	
Q statistic			83.7		67.9	87.8	53.8	66.3	64.1	24.1	30.3	9.5	40.0	53.7	107.6	
I^2 statistic			80%		74%	73%	80%	83%	80%	75%	80%	5%	77%	78%	89%	

[a] intervention group: > 10 HBOT sessions and control group: < 10 HBOT sessions, [b] 80% or more granulating tissue or partial/ complete epithelialization, [c] mean, ARR: Absolute Risk Reduction, CI: Confidence Interval, HBOT: Hyperbaric Oxygen Therapy, min: Minor, maj: Major, NA: Not Applicable, NNT: Number Needed To Treat, NR: Not Reported, RR: Relative Risk, RRR: Relative Risk Reduction

We have ordered studies by their type, CONSORT or STROBE checklist number of fulfilled items and by sample size (the same criteria as Table 3)

Predictors of healing with HBOT

Of the reported patient characteristics, age [Chen CE et al, 2010; Wattel F et al, 1991], gender [Hawkins G et al, 2006; Wattel F et al, 1991], diabetes duration [Chen CE et al, 2010], insulin use [Wattel F et al, 1991; Fife C et al, 2007], nephropathy [Wattel F et al, 1991] and retinopathy [Wattel F et al, 1991] failed to have a statistically significant impact on the chance of healing when considered in isolation. On the other hand, when chronological age and the duration of diabetes were combined [Otto G et al, 2000; Fife C et al, 1997; Fife C et al, 2007] there was a significant association with the chance of healing, as there was with renal failure (dialysis dependent or a history of kidney transplant) [Fife C et al, 2007] and smoking habits (> 10 packs-year) [Otto G et al, 2000; Fife C et al, 1997; Fife C et al, 2007].

Only Chen et al [Chen CE et al, 2010] analysed biochemical parameters and found no association with healing.

As to other potential predictors, ABI [Chen CE at al, 2010; Löndahl M et al, 2011], large vessel alteration [Wattel F et al, 1991], laser Doppler flowmetry [Mathieu D et al, 1997], microangiopathy [Wattel F et al, 1991], neuropathy [Wattel F et al, 1991; Zamboni W et al, 1997], presence of a palpable *dorsalis pedal* pulse [Ong M, 2008], previous LEA [Zamboni W et al, 1997], surgeries [Chen CE et al, 2010] and toe blood pressure [Löndahl M et al, 2011] were not associated with DFU healing.

Ramon et al [Ramon Y et al, 1999] did not report if statistical analysis was done, nonetheless, they concluded that subjects with severe peripheral arterial disease had less chance of healing.

Even though in some studies it did not achieve statistical significance [Hawkins G et al, 2006; Wattel F et al, 1991], higher $TcPO_2$ values, when measured close to the wound at sea level or under HBOT conditions, were associated with wound healing in both in univariate and multivariate analyses [Fife C et al, 2007; Löndahl M et al, 2011; Mathieu D et al, 1997; Otto G et al, 2000; Wattel F et al, 1991; Zgonis T et al, 2005].

Neither the DFUr area nor duration were associated with chance of healing in a single study [Hawkins G et al, 2006]. DFUs arising following trauma appeared to have a better outcome in Fife 1997 [Fife C et all, 1997], while higher Wagner grade [Fife C et al, 1997; Fife C et al, 2007; Otto G et al, 2000] and the product of the total wound(s) volume (cm^3) and Wagner grade value [Fife C et al, 1997] were associated with non-healing.

Only ten of the included studies in our systematic review sought any association between wound healing and any of these potential associated factors (Table 11).

Table 11: Variables association with complete diabetic foot ulcer healing in subjects undergoing Hyperbaric Oxygen therapy

Association / Variable	Present		Absent	
	Univariate analysis	**Multivariate analysis**	**Univariate analysis**	**Multivariate analysis**
Patient characteristics				
Age			Chen C et al, 2010 [62];Fife C et al, 2007 [67]; Wattel F et al, 1991 [31]	
Age + diabetes duration	↓ Fife C et al, 1997 [43]	↓ Otto G et al, 2000 [42];Fife C et al 2007 [67]		
Gender			Hawkins G et al, 2006 [30];Wattel F et al, 1991 [31]	
Diabetes Duration			Chen C et al, 2010 [62];Fife C et al, 2007 [67]	
Insulin use			Wattel F et al, 1991 [31];Fife C et al, 2007[67]	
Nephropathy			Wattel F et al, 1991 [31]	
Renal failure	↓ Fife C et al, 2007[67]			
Retinopathy			Wattel F et al, 1991 [31]	
Smoking habits	↓ Fife C et al,2007 [67];Fife C et al,1997 [43]	↓Otto G et al, 2000 [42];Fife C et al, 2007[67]		
Analytic parameters				
Albumin			Chen C et al, 2010 [62]	
CRP			Chen C et al, 2010 [62]	
ESR			Chen C et al, 2010 [62]	
Haemoglobin			Chen C et al, 2010 [62]	
HbA1c			Chen C et al, 2010 [62]	
Leucocyte count			Chen C et al, 2010 [62]	
Total lymphocyte			Chen C et al, 2010 [62]	
Foot characteristics				
ABI			Löndahl M et al, 2011 [53];Chen C et al, 2010 [62]	Löndahl M et al, 2011 [53]
Large vessel alteration			Wattel F et al, 1991[31]	
Laser Doppler flowmetry			Mathieu D et al, 1997 [40]	
Microangiopathy			Wattel F et al, 1991 [31]	
Neuropathy			Wattel F et al, 1991 [31];Fife C et al,2007[67]	
Palpable *dorsalis* pedal pulse			Ong G et al, 2008 [65]	
Previous amputation			Fife C et al,2007[67]	
Surgeries			Chen C et al, 2010 [62]	
$TcPO_2$				
Air			Mathieu D et al, 1997 [40]	
- At wound	↑Löndahl M et al, 2011[53]; Zgonis T et al, 2005 [66]	↑Löndahl M et al, 2011 [53]; Otto G et al, 2000 [42];Fife C et al, 2007[67]	Hawkins G et al, 2006[30];Wattel F et al, 1991 [31]	
- At reference point			Wattel F et al, 1991 [31]	
HBOT	↑Mathieu D et al, 1997[40]			
- At wound	↑ Wattel F et al, 1991 [31];Fife C et al,2007[67]			
- At reference point			Wattel F et al, 1991 [31]	
Toe Blood Pressure			Löndahl M et al, 2011 [53]	Löndahl M et al, 2011 [53]
DFU characteristics				
Area			Hawkins G et al, 2006 [30]	
Duration			Hawkins G et al, 2006 [30]	
Traumatic aetiology	↑ Fife C et al,1997 [43]			
Wagner grade	↓ Fife C et al, 2007 [67]; Fife C et al, 1997 [43]	↓Otto G et al, 2000 [42]; Fife C et al,2007 [67]		
All wound volume * Wagner grade	↓ Fife C et al,1997 [43]			

↓: impairs healing, ↑: improves healing, ABI: ankle brachial index, CRP: C-Reactive Protein, DFU: Diabetic Foot Ulcer, ESR: Erythrocyte Sedimentation Rate, HbA1c: Glycated Haemoglobin, $TcPO_2$: Transcutaneous Partial Pressure of Oxygen

We have ordered studies by their type, CONSORT or STROBE checklist number of fulfilled items and by sample size (the same criteria as Table 3)

Hawkins [Hawkins G et al, 2006], considering outcome 1 month after HBOT, suggested a model including DFU area, DFU duration, and $TcPO_2$, but these variables were not significant in their univariate analysis. Fife [Fife C et al, 2007] proposed a model, through multiple regression analysis, to predict outcome of patients undergoing HBOT and identify patients that would most benefit with this therapy. The score includes all the variables that were significantly associated with complete or partially healing in their multivariate analysis: the number of HBOT sessions, $TcPO_2$, function for pack-years of smoking, maximal Wagner grade, age + duration of diabetes and interruption of the treatment regimen. For both models no prognostic accuracy measures were reported or possible to calculate.

Publication bias assessment

As for all outcomes we could not retrieve more than 10 studies within each type, we considered that funnel plot analysis would not be helpful.

3. *Discussion*

This study was designed to further establish or refute the case for the use of HBOT as an adjunctive treatment for DFU. We have made a systematic search for all relevant clinical studies and combined them where possible for statistical analysis.

The fact that more than half of the included studies were retrieved outside the MEDLINE Indexed search indicates that the majority of the presented works were either never published in full, or only in the "Grey" literature. To the best of our knowledge, this is the first study both to meta-analyse all the available studies for HBOT efficacy on DFU healing and to evaluate the methodological quality of those studies. In addition, no systematic review of factors or models to identify patients that would most benefit from HBOT has been done.

Our review suggests that overall, the addition of HBOT to the care of the DFU results in a higher chance of healing [RR 2.2], perhaps over a shorter [0.8 months less (95%CI -2.5 to + 0.9)] and a lower risk of major amputation when compared with standard care.

Regarding the average reduction in the area of a DFU only few studies with incomplete data assessed this outcome and further research is needed if this is to be regarded as an important outcome.

In terms of LEA, in our opinion the main goal is to reduce the chance of a patient requiring a major LEA. Such amputations carry a considerable burden in terms of the individuals' quality of

life, physical limitation and increased risk of future contralateral lesions. According to our results, HBOT is effective in reducing the chance of needing a major LEA.

Our conclusions are based on all the available clinical evidence and for that reason they represent the best estimate we can make at this time of the true clinical impact of HBOT on this important complication of diabetes. However, our pooled estimates of effect are based on both randomised and non-randomised evidence, and should be treated with caution for a number of reasons discussed below. Our approach has several strengths and weaknesses.

Although much of the data are derived from non-randomised sources, selection procedures when described were by consecutive case inclusion, which potentially improves the validity of our results. Such an inclusive sampling technique means there is less chance of selection factors biasing the outcome over the broad spectrum of patients with DFU. No patient is selected based on baseline characteristics or healing potential and, when the study period is sufficiently long, seasonal or other time-related factors should be accounted for [Hulley S & Cummings S, 1988]. On the other hand, when HBOT administration depends upon a physician decision, patient refusal or the presence of a contraindication, there remains a considerable possibility of selection bias, leading to a confounded result.

Participant characteristics were poorly described in general, especially in cohort studies and case series, and nine of the studies are abstracts or posters with very limited information and limited peer review.

Inclusion criteria usually included chronic DFU. The period of time these ulcers were present prior to enrolment varied greatly across the included studies, and this may have a considerable impact on the effectiveness of HBOT. We would postulate that those ulcers present for long periods would be more resistant to all attempts to heal, including HBOT. Unfortunately, the data reported in the included studies precluded any attempt to test this hypothesis.

In more than half of the studies, total sample size was less than 50 subjects, which is probably responsible for the fact that few studies achieved statistically significant results and carried wide CI. Only 3 studies, conducted by the same group of authors in the same institutions, were multicentre. Studies with such small sample sizes and done in single institutions represent low evidence levels as they are more prone to bias and diminished generalizability. Multicentre studies usually include larger samples, from a wider range of population groups and different locations and present the possibility of comparing the result from different centers, which improves generalizability.

There was considerable variation in the course of HBOT delivered across these studies. The mean number of sessions ranged from 4 to 111, durations of each session from 45 to 120

minutes, the frequency (once or twice per day, from 4 to 7 days per week), and pressure (2 to 3 ATA). It is possible these factors are responsible for some of the heterogeneity between trial results. It is crucial to define the optimal HBOT protocol if standardize clinical care is to be widely implemented. In our opinion, the data available did not allow us to propose such a protocol; further research designed especially for this purpose is needed so that a consensus could be reached.

The methodological quality of reporting was generally very poor in the included studies, with important methodological information absent from most studies. It is perhaps surprising that even the RCTs are so poorly reported, given the time and efforts required to plan and complete them. Authors should consider available checklists to improve reporting quality and, consequently, results' evaluation and protocol replication.

Even though RCTs (corresponding only to 27% of the available evidence on this topic) represent the highest level of evidence, they present the various limitations described above. The question of whether to include only RCTs or also observational studies in MA is still under debate and is addressed in the Shrier study [Shrier I et al, 2007]. We have decided to include all types of studies in order to globally evaluate available evidence and to try and understand where the results differ according to methodology. We hope our pooled estimates can also be useful when estimating sample sizes for future cost-benefit studies.

In general, it is expected that NRTs will give larger estimates for the effect of any intervention. For all this, we decided not only to calculate all measures aggregating all available evidence, but also by study type. When considering RCTs in isolation, no statistical significance was achieved for the chance of healing, minor or total LEA proportions, in contrast to positive findings when NRTs were included in the pooled analyses.

The largest RCT included in this review enrolled 50 subjects in each arm [Duzgun A et al, 2008]. However, using our pooled estimates of effect, we calculate that the sample size required to detect statistically significant differences (for an 80% power at the 0.05 significance level) it would be necessary to include 65 subjects in each arm for major LEAs and 114 for total LEAs. All the reported RCTs seem to be underpowered in this respect.

All these factors could explain why RCTs did not achieve statistical significance, though pooled measures consistently tended to favour HBOT.

Despite the fact that association measures for minor and major LEAs did not reveal a substantial difference between study types, we noted that the NRT generally reported lower rates of minor LEA with HBOT, while the RCTs presented lower rates of major LEA with HBOT. We do not know

the reason for this. It may be a product of different inclusion criteria or comparator therapy or simply a chance finding.

While it is possible to draw a range of conclusions from the data presented here, overall there does seem to exist some support for the continued use of HBOT in the care of selected patients with DFUs. The question as to which patients are most likely to benefit remains of great importance. The analysis of those factors most strongly associated with either better or worse outcome has been rarely undertaken, and then most often with fewer than 100 subjects included and different variables assessed in each study. Only three factors have been included in more than one study: age, age+ duration of diabetes and $TcPO_2$. Interestingly, while no statistical association between age and healing is apparent, there is such an association with the function of age + diabetes duration, and this deserves further investigation.

$TcPO_2$is another commonly discussed predictor of healing. Across these studies the O_2 tensions have been measured at different sites and environmental pressure resulting in conflicting results. Smart reviewed this area and concluded that peri-wound $TcPO_2$ measured while breathing air at environmental pressure confirms hypoxia, but does not predict DFU outcome with HBOT, whereas when measured under hyperbaric conditions and breathing 100% oxygen a $TcPO_2$ superior to 200 mmHg is a useful healing predictor [Smart D et al, 2006]. We have only retrieved 3 studies in the latter circumstances [Doctor N et al, 1992; Oriani G et al, 1990; Wattel F et al, 1995] and, in all, higher values were associated with improved healing, in their univariate analysis.

Additionally, multivariate analysis was conducted only in 3 studies. Furthermore, only two articles attempted to create predictive models for this purpose. Hawkins et al [Hawkins G et al, 2006] used variables with no significant association with healing in univariate analysis and Fife et al [Fife C et al, 2007] used post-HBOT variables (number of sessions and treatment interruption) that cannot be used to predict the outcome in patients presenting for treatment. Neither predictive model has been externally validated.

This study was conducted respecting Meta-analysis Of Observational Studies in Epidemiology (MOOSE) [Stroup D et al, 2000] and Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [Liberati A et al, 2009] checklists which were created to help develop and assess systematic reviews and MA including observational and RCT studies, respectively.

There are a few limitations in our study, namely the exclusion of one Chinese article [Lee SS et al, 1997] for linguistic limitations, methodological quality assessment by only one of the researchers and a high level of heterogeneity between the included studies.

Publication bias was not observed across the limited sample size for the majority of the assessed outcomes.

In summary, using the SIGN (Scottish Intercollegiate Guideline Network) system for grading recommendations [Harbour R et al, 2001], we conclude that HBOT prevents major LEA with an A grade. However, for the remaining outcomes (time until complete healing, healing proportion and minor and total LEA) the grade lowers to a B.

Despite a high number of studies assessing HBOT effect on DFU healing, this topic is still far from allowing unequivocal conclusions to be drawn. Thus, future research is crucial and should include therapy protocol definition improvement, use institutions' available data for retrospective cohort studies creating and/or validating models for a more rational HBOT allocation, conduction of RCTs with larger sample sizes and improved methodological reporting and cost-effectiveness assessment. Scientific and medical societies in this area should consider creation of a specific checklist to enhance quality reporting and stimulate multicentre studies to allow a new and higher recommendation level.

V. Molecular environment characterization, Hyperbaric Oxygen therapy modulator effect and clinical impact on diabetic foot ulcers healing

A. Material and Methods

1. *Study design and participants selection*

A non-randomized clinical trial was conducted at Centro Hospitalar de Vila Nova de Gaia/Espinho, EPE (Entidade Pública Empresarial) Diabetic Foot Outpatient Clinic, which is a tertiary care unit with a multidisciplinary team.

The clinical trial was registered in the Brazilian Clinical Trial Registry (Registro Brasileiro de Ensaios Clínicos) number UTN U1111-1146-8232 and approved by the Ethics Committee of our institution.

Subjects with active DFU, a full-thickness skin defect distal to the malleoli requiring more than 15 days to heal [Boyko EJ et al, 1996], that, after 8 weeks of standard treatment (including angioplasty and/or revascularization surgery if needed), had no significant wound improvement (no healing or ulcer area reduction < 30%) were consecutively proposed to HBOT.

Participants were included from the 1st October 2010 until the 31st December 2012.

HBOT proposal was performed in accordance to the multidisciplinary team and financial department approval. Team decision relied on selecting patients that would most benefit of healing, excluding namely patients bedridden and dependent on third person for daily life activities. Therefore, HBOT was used, depending on hospital economic resources and availability of the HBOT facility, as last resource in people that despite maximized macrovascular blood flow had no healing.

These participants were divided in two groups: HBOT – treated with HBOT and NHBOT – patients that refused the treatment or had a contra-indication.

Both groups with DFU were compared with a group of non-DFU diabetic subjects that were participating in an educative program on diabetes in our department.

HBOT was performed in the referral area HBOT center (Unidade de Medicina Hiperbárica of PHH) according to the used treatment protocol - 80 minutes at 2.4 ATA, once a day, five days a week, up to a maximum of 100 sessions.

Standard care was conveyed in the DFU groups by a team independent of the investigators. In case of multiple ulcers, evolution of the larger DFU was evaluated through the decrease in the wound measures.

All subjects were followed for twelve months to evaluate healing in the DFU groups and the potential occurrence of lesions in the non-DFU subjects.

2. *Participants and diabetic foot ulcer characteristics*

At enrollment, the following demographic characteristics were collected: age at the time of inclusion; gender; DM type (classified according to the World Health Organization definition [WHO,2006]), duration (in years) and treatment (oral anti-diabetic agents or insulin); metabolic control [through HbA1C]; smoking habits; presence of any DM related complications (namely, retinopathy, nephropathy, neuropathy, cerebrovascular, cardiovascular and/or PAD and metabolic), were registered in accordance to the definition used in the Diabetes Complications Severity Index created by Young et al [Young BA et al, 2008].

For the foot characterization we used the presence of PAD, when only one or fewer pedal pulses were palpable on the DFU foot [Monteiro-Soares M et al, 2010 B] and/or the ankle-brachial index (ABI) was inferior to 0.8 [Armstrong DG et al, 1998 B]; $TcPO_2$, determined by measuring once at 2 points peri-DFU and reporting the highest value; DPN, defined as inability to feel the SWM at one or more of 4 sites in the foot [Smieja M et al, 1999]; and previous DFU or LEA. We also recorded the ulcer area (in cm2), reported duration (in months), location, Texas University classification (TUC) [Armstrong DG et al, 1998 B], number of DFUs and the presence or absence of infection.

DFU photographic and dimensional records (area, maximum and mean depths and volume) were performed using a digital wound measurement device (Aranz Medical Silhouette Mobile TM), at baseline, and if still with active DFU, at months 3, 6 and at the end of follow-up (12 months). Based on the area measurements, percentage of epithelialization was calculated on the same endpoints.

Considering the HBOT description, the total number of sessions, whether or not the planned number of sessions was completed and side-effects were registered.

We have considered complete healing when the DFU presented full epithelialization without the need of further dressing [Younes N & Albsoul A, 2004]. Minor LEA was defined as amputation distal to or including the forefoot and major LEA was considered amputation above or by the ankle [Beckert S et al, 2006].

Clinical outcome occurrence was also assessed at 3, 6 and 12 months.

3. *Laboratory and molecular analysis*

In all three groups (non-DFU, HBOT, NHBOT) blood samples were collected at baseline and 3 months after, in the HBOT group a sample was drawn also at 6 months. Additionally, in the DFU groups ulcer debridement material was collected at 0 and 1 months. Blood samples were collected to EDTA (ethylenediamine tetraacetic acid) or gel and clot activator tubes.

Full blood count was performed using an automated hematology analyzer XE 2100 or 5000 from the Sysmex Corporation.

Serum was analysed for glucose, urea, creatinine, total proteins, albumin, lipid profile, uric acid and C-reactive protein (CRP) with the Cobas 8000c701 (Roche).

HbA1c was determined through high pressure chromatography in the Horiba Medical G7 device.

Microalbuminuria level was evaluated by immunoturbidimetry with Cobas 6000 c501 analyzer (Roche).

Angiogenic (VEGF, PlGF), vasculogenic (SDF1-α) and inflammatory (TNF-α) markers were determined by enzyme-linked immunoabsorbent assay (ELISA) multiplex using Quantikine ELISA Immunoassay kits, according to manufacturer instructions.

Ulcer bed tissue was collected, when DFU debridement was performed, for histological and immunohistochemistry studies. The tissue specimens were fixed in 10% neutral-buffered formalin solution and paraffin-embedded. Three-micrometer sections were stained with hematoxylin and eosin (H&E) or used for capillary endothelial cells immunostaining. Endogenous peroxidase activity was blocked with 4% hydrogen peroxide in phosphate buffered saline (PBS) for 30 minutes at room temperature. To retrieve antigen, sections were placed in 10 mM citrate buffer (pH = 6) and heated at 98°C. After blocking with 10% bovine serum albumin (BSA) in PBS for 1 hour, sections were incubated with primary antibody against CD31 (1:100) (Abcam, Cambridge, UK) overnight at 4°C. Then, anti-rabbit secondary antibody (1:200) (Santa Cruz Biotechnology, USA) was applied for 30 minutes. Avidin Biotin Complex (ABC) complex method

(Vectastain ABC kit, Vector, Burlingame, CA, USA) was used according to the manufacturer's instructions. The antigen-antibody reaction was developed using diaminobenzidine (DAB) (DAB substract kit, Abcam, Cambridge, UK) as peroxidase substrate, rendering CD31 positive cells with a brown staining. Sections were counterstained with hematoxylin (Sigma-Aldrich, Portugal), dehydrated and coverslipped. Cluster of differentiation 31 (CD31)-expressing microvessels were counted in the three most vascularized areas with magnification of 200 x, and the data were averaged and normalized to the total area of the tissue section. Any positive-staining endothelial cell or endothelial cell cluster that was separated from adjacent microvessels was considered an individual vessel [Soares R et al, 2004].

All laboratory/molecular studies were performed by investigators blinded to the subjects' group allocation.

4. *Statistical analysis*

Continuous variables will be described by mean and SD, in the case of having normal distribution, or median and range otherwise. Normality of the distribution will be assessed through the histogram analysis.

Comparison between 2 groups will be conducted with t-test for independent samples or Mann-Whitney U test, according to the variable distribution. For the comparison between 2 moments in the same group we will use the t-test for paired samples or Wilcoxon signed rank test, when pertinent. For the comparison between 3 moments, the Wilcoxon signed rank or the Friedman test were used, according to the variable distribution.

For differences' evaluation between variables in the 3 groups, the One-Way ANOVA test (using the Bonferroni correction) or the Kruskall-Wallis test will be applied.

For categorical variables description, frequency and percentage will be used and, for association analysis, the X^2 or Fisher's exact test (when applicable).

All tests were two sided, p values less than 0.05 were considered as statistically significant.

Statistical analysis was performed using the IBM SPSS version 22.0 (Chicago, IL, USA).

B. Results

1. *Participants characteristics*

We included a total of 25 patients: 5 in the non-DFU, 6 in the NHBOT DFU and 14 in the HBOT DFU groups described in Table 12.

The DFU participants were allocated not to undergo HBOT due to refusal of treatment in 5 patients and 1 for presenting a contra-indication.

The mean age in our sample was 62 years and DM duration 18 years, and the majority were men, with type 2 DM treated with insulin, with visual and physical impairment. Concerning DM complications, most had retinopathy, PAD complications and neuropathy. The mean complications count was 4 [Young BA et al, 2008] and the non-DFU group had significantly less PAD complications and tended to present less frequently the remaining complications.

The non-DFU group was composed mainly by female subjects by way of chance, such difference presented statistical significance.

Concerning the total foot pulses number, non-DFU persons had a significantly higher number comparing to both DFU groups.

Intermittent claudication was significantly more common in the non-HBOT DFU participants, as they tended to present more PAD complications, ischemia diagnosed by pulses palpation and statistically significant less frequently DPN diagnosed by SWM.

Almost all DFUs reached the bone and were infected and ischemic. Several were post-minor LEA and 25% were located in the toes.

Table 12. Participants baseline characteristics

Variables	Global (n=25)	Non-DFU (n=5)	NHBOT DFU (n=6)	HBOT DFU (n=14)	p value
SUBJECT CHARACTERIZATION					
Age [mean (SD)]	62 (12)	68 (10)	63 (11)	61 (13)	0.6*,a
Male gender [n (%)]	18 (72)	1 (20)	6 (100)	11 (79)	**0.009†/0.006¥**
Visual impairment [n (%)]	19 (76)	3 (60)	5 (83)	11 (79)	0.6†/0.5¥
Physical impairment [n (%)]	16 (64)	3 (60)	4 (67)	9 (64)	0.7†/0.9¥
Past or present smoker [n (%)]	13 (52)	1 (20)	4 (67)	8 (57)	0.3†/0.3¥
DM AND ITS COMPLICATIONS					
Type 2 [n (%)]	23 (92)	4 (80)	6 (100)	13 (93)	0.5†/0.5¥
Duration (in years) [mean (SD)]	18 (9)	26 (19)	15 (7)	20 (10)	0.3*, a
Insulin use [n (%)]	17 (68)	3 (60)	5 (83)	9 (64)	0.6†/0.9¥
Cardiovascular complications history [n (%)]	7 (28)	1 (20)	1 (17)	5 (36)	0.6†/0.4¥
Retinopathy complications history [n (%)]	21 (84)	3 (60)	6 (100)	12 (86)	0.2†/0.3¥
Nephropathy complications history [n (%)]	12 (48)	1 (20)	5 (83)	6 (43)	*0.1†*/0.7¥
Cerebrovascular complications history [n (%)]	3 (12)	0 (0)	0 (0)	3 (21)	0.3†/0.1¥
PAD complications history [n (%)]	18 (72)	1 (20)	6 (100)	13 (93)	**0.001†/0.003¥**
Neuropathy complications history [n (%)]	22 (88)	5 (100)	4 (67)	13 (93)	0.2†/1.0¥
Metabolic complications history [n (%)]	11 (44)	2 (40)	2 (33)	7 (50)	0.8/†/0.6¥
Complications count [mean (SD)]	4 (1)	3 (1)	4 (2)	4 (2)	0.1*, a
DFU FOOT CHARACTERIZATION					
Foot deformity [n (%)] b	14 (61)	3 (60)	2 (14)	9 (64)	0.7†/0.8¥
Total foot pulses ≤ 1 [n (%)]	20 (80)	1 (20)	6 (100)	13 (93)	**0.001†/0.003¥**
ABI < 0.8 [n (%)]	5 (75)	NA	2 (33)	3 (21)	0.6γ
$TcPO_2$ [median (range)]	18 (67)	NA	24 (34)	16 (67)	0.6 α
Intermittent claudication [n (%)]	12 (48)	0 (0)	5 (83)	7 (50)	**0.02†**/0.2¥
DPN symptoms [n (%)]	23 (92)	5 (100)	5 (83)	13 (93)	0.6†/0.8¥
Altered SWM sensation [n (%)] c	17 (81)	3 (60)	3 (60)	11 (100)	*0.07†/**0.04¥***
Previous DFU [n (%)]	15 (60)	1 (20)	6 (100)	8 (57)	**0.03†**/0.4¥
Previous LEA [n (%)]	7 (28)	0 (0)	3 (50)	4 (29)	0.2†/0.4¥
DFU CHARACTERIZATION					
Texas grade					
III (Bone or joint) [n (%)]	25 (100)	NA	6 (100)	14 (100)	1.0γ
Texas stage					
B (Infection) [n (%)]	1 (5)	NA	0 (0)	1 (7)	1.0γ
D (Infection plus ischemia) [n (%)]	19 (95)	NA	6 (100)	13 (93)	
Located at toes [n (%)]	5 (25)	NA	3 (50)	2 (14)	0.1γ

*: One-Way ANOVA test with Bonferroni correction, †: Chi-square test for association, ¥: Chi-square test for tendency, α: Mann-Whitney U test, γ: Fisher's exact test, a: No statistical difference between groups, b: in 2 subjects it was not applicable, c: in 4 subjects it was not possible to conduct, ABI: Ankle-brachial index, DFU: Diabetic foot ulcer, DPN: Diabetic peripheral neuropathy, HbA1c: Glycated haemoglobin, HBOT: Hyperbaric oxygen therapy, LEA: Lower extremity amputation, NA: Not applicable, NHOTB: No hyperbaric oxygen therapy, PAD: Peripheral arterial disease, SD: Standard deviation, SWM: Semmes-Weistein monofilament, $TcPO_2$: Transcutaneous Partial Pressure of Oxygen

2. *Laboratory markers*

Regarding glycaemic control and lipid profile, at baseline, patients were acceptably controlled, with a mean HbA1c of 7.9-8.5%, total cholesterol around 170 mg/dl and triglycerides ranging from 112 to 146 mg/dl (Table 13).

The HBOT DFU group had significantly less microalbuminuria at baseline comparing to non-HBOT DFU subjects.

DFU subjects presented normal total proteins, albumin and uric acid (results not shown).

After 3 months of therapy, HBOT DFU individuals presented significantly lower leukocyte and CRP level, and tended to have lower VEGF levels.

For the remaining laboratory markers, we did not observe statistically significant differences.

TNF-α results were not presented as the most of the serum values were undetectable (< 0.00 pg/ml).

3. *Clinical outcome*

In the non-HBOT DFU group, at the 3rd month, there were four LEA (three major and one midfoot), from whom one patient died in the early post-operative period and another from lung cancer. In addition, one subject died after refusing major LEA (Table 14).

In comparison, all HBOT DFU participants improved or presented complete healing at months 3 and 6, and none required LEA. However three were dead at the 12th month for causes non DFU related.

Therefore, we achieved statistically significant differences between groups at all endpoints. Besides having a poorer outcome, the deaths in the NHBOT DFU group occurred earlier (month 3 *vs* month 12).

None in the non-DFU group developed DFU.

Table 13. Laboratory markers

Variables	Global			Non DFU (n=5)	NHBOT DFU patients (n=6)		Paired samples tests p value	HBOT DFU patients (n=14)			Paired samples tests p value	Independent samples tests p value	
	M0 (n=25)	M3 (n=20)	M6 (n=14)	M0	M0	M3		M0	M3	M6		M0	M3
Glucose (mg/dl) [mean (SD)]	172 (75)	213 (82)[a]	211 (75)	165 (60)	194 (110)	236[a] (109)	0.3[ε]	165 (64)	200 (68)	211 (75)	0.2*,[b]	0.5[ж]	0.4[ж]
HbA1C (in %) [mean (SD)]	8.1 (1.7)	8.3 (1.8)[a]	8.2 (1.6)	8.5 (1.5)	8.3 (2.1)	9.5 (2.9)[a]	0.2[ε]	7.9 (1.7)	7.8 (1.1)	8.2 (1.6)	0.8*,[b]	0.6[ж]	0.3[ж]
Haemoglobin (g/dl) [mean (SD)]	12 (1)	12 (2)[a]	12 (2)	13 (2)	12 (1)	11 (2)[a]	0.3[ε]	12 (1)	12 (2)	12 (2)	0.8*,[b]	0.8[ж]	0.6[ж]
Leukocytes (x10^3/dl) [mean (SD)]	9.5 (3.4)	10.0 (3.7)[a]	7.9 (1.8)	6.6 (0.9)	11.5 (4.1)	12.5 (4.4)	0.4[ε]	9.4 (3.0)	8.7 (2.7)	7.9 (1.8)	0.3*,[b]	0.2[ж]	**0.03**[ж]
Platelets (x10^3/dl) [mean (SD)]	267 (71)	265 (79)[a]	229 (76)	236 (34)	256 (57)	305 (90)[a]	0.2[ε]	277 (81)	244 (67)	229 (76)	0.2*,[b]	0.6[ж]	0.1[ж]
C-reactive protein (mg/dl) [median (range)]	0.8 (15.7)	0.3 (22.5)[a]	0.3 (11.2)	0.7 (0.6)	2.1 (11)	11.8 (22.4)[a]	0.1[*]	0.7 (15.7)	0.3 (5.4)	0.3 (11.2)	0.3[ϫ]	0.2[α]	**0.03**[α]
Total cholesterol (mg/dl) [mean (SD)]	170 (54)	153 (37)[a]	151 (24)	180 (68)	163 (68)	168 (57)[a]	0.5[ε]	170 (46)	147 (26)	151 (24)	0.2*,[b]	0.8[ж]	0.3[ж]
LDL cholesterol (mg/dl) [mean (SD)]	101 (47)	84 (32)[a]	83 (24)	98 (56)	97 (57)	97 (53)[a]	0.5[ε]	104 (43)	79 (20)	83 (24)	0.1*,[b]	0.8[ж]	0.3[ж]
HDL cholesterol (mg/dl) [mean (SD)]	44 (10)	41 (12)[a]	42 (17)	55 (10)	43 (10)	36 (13)[a]	0.1[ε]	41 (9)	43 (11)	42 (17)	0.9*,[b]	0.7[ж]	0.3[ж]
Triglycerides (mg/dl) [mean (SD)]	130 (70)	143 (85)[a]	131 (70)	146 (119)	112 (44)	177 (82)[a]	0.1[ε]	133 (60)	130 (86)	131 (70)	0.8*,[b]	0.5[ж]	0.3[ж]
Creatinine (mg/dl) [median (range)]	0.8 (7.3)	0.9 (8.2)[a]	0.9 (2.2)	0.6 (0.8)	1.3 (7.3)	1.6 (8.2)[a]	0.2[*]	1.0 (1.8)	0.9 (2.9)	0.9 (2.3)	0.8[ϫ]	0.7[α]	1.0[α]
Urea (mg/dl) [mean (SD)]	55 (23)	69 (47)[a]	63 (34)	42 (7)	66 (33)	92 (65)[a]	0.1[ε]	54 (21)	57 (32)	63 (34)		0.5[ж]	0.3[ж]
Microalbuminuria (mg/dl) [n (%)]	12 (48)	12 (63)[a]	7 (64)	0 (0)	5 (83)	4 (80)[a]	NA	7 (50)	8 (57)	7 (64)	NA	**0.02**[†]/0.2[¥]	0.6[ν]
VEGF (pg/ml) [median (range)]	70 (268)	66 (355)[a]	40 (131)	37 (101)	189 (236)	99 (344)[a]	0.9[*]	72 (268)	56 (129)	40 (131)	0.6[ϫ]	0.6[α]	*0.07*[α]
PlGF (pg/ml) [median (range)]	12.6 (66.5)	8.5 (65.8)[a]	10.5 (51.2)	6.8 (8.3)	3.1 (14.7))	7.4 (12.1)[a]	0.7[*]	14.8 (65.7)	9.6 (64.0))	10.5 (51.2)	0.4[ϫ]	0.2[α]	0.4[α]
SDF1- α (pg/ml) [mean (SD)]	1911 (642)	2030 (713)[a]	1739 (2136)	1992 (470)	2297 (514)	2062 (1054)[a]	0.5[ε]	1716 (691)	2017 (562)	1739 (2136)	0.8*,[b]	*0.08*[жc]	0.9[ж]

[ε]: Student's t test for paired samples, [ж]: Student's t test for independent samples, [α]: Mann-Whitney U test, [*]: Wilkoxon signed ranks test, *: One-Way ANOVA test with Bonferroni correction, [ϫ]: Friedman test, [†]: Chi-square test for association, [¥]: Chi-square test for tendency, [ν]: Fisher's exact test, [a]: 1 missing value, [b]: No statistical difference between groups, %: Percentage, DFU: Diabetic Foot Ulcer, dl: Deciliter, g: Gram, HbA1c: Glycated Hemoglobin, HBOT: Hyperbaric Oxygen Therapy, HDL: High Density Lipoprotein, LDL: Low Density Lipoprotein, M0: Month 0, M3: Month 3, M6: Month 6, mg: Milligram, ml: Millilitre, NA: Not Applicable, NHOTB: No Hyperbaric Oxygen Therapy, pg: Pico gram; PlGF: Placental Growth Factor, SD: Standard Deviation, SDF1- α: Stromal Cell-derived Factor 1 Alpha, VEGF: Vascular Endothelial Growth Factor

Table 14. Clinical outcome

Variables	Global (n=20)			NHBOT DFU patients (n=6)			HBOT DFU patients (n=14)			Comparison between groups Fisher's exact test p value		
	M3	M6	M12	M3	M6	M12	M3	M6	M12	M3	M6	M12
Complete healing / improvement [n (%)]	15 (75)	15 (75)	12 (60)	1 (17)	1 (17)	1 (17)	14 (100)	14 (100)	11 (79)	**<0.001**	**<0.001**	**0.02**
LEA or death [n (%)]	5 (25)	5 (25)	8 (40)	5 (83)	5 (83)	5 (83)	0 (0)	0 (0)	3 (21)			

HBOT: Hyperbaric oxygen therapy, LEA: Lower extremity amputation, M0: Month 0, M6: Month 6, M12: Month 12, NHBOT: No hyperbaric oxygen therapy

4. *Diabetic foot ulcer characterization*

At baseline, DFUs had equivalent dimensions in the two groups (Table 15).

As only one subject remained alive and without major LEA at the 3rd month in the non-HBOT DFU, no comparison was possible between baseline and this moment. Nevertheless, this patient seems to present a larger and deeper ulcer when comparing to the median values in the HBOT DFU group.

All measurements presented or tended to present significantly lower values at the 3rd month of HBOT when comparing to baseline.

Table 15. Diabetic foot ulcer characteristics at baseline and third month of follow-up

Variables	Global (n=20)		NHBOT DFU patients (n=6)		Paired samples tests p value	HBOT DFU patients (n=14)		Paired samples tests p value	Independent samples tests p value	
	M0	M3	M0	M3		M0	M3		M0	M3
Baseline ulcer area (in cm^2) [median (range)]	11.0 (31.1)[a]	2.7 (24.8)[b]	12.1 (16.1)	13.1 (23.5)[b]	NP	7.3 (31.1)[a]	2.7 (16.3)	**0.001**[*]	0.4[α]	NP
Mean depth (in mm) [median (range)]	2.3 (11.6)[c]	1.7 (6.5)[d]	1.8 (4.7)	3.5 (3.6)[b]	NP	2.3 (11.6)[c]	1.4 (6.5)[a]	*0.1*[*]	0.6[α]	NP
Maximum depth (in mm) [median (range)]	4.6 (18.3)[c]	3.3 (10.7)[d]	3.6 (8.2)	7.9 (5.2)[b]	NP	4.6 (18.1)[c]	3.2 (10.7)[a]	**0.03**[*]	0.3[α]	NP
Volume (in cm^3) [median (range)]	1.2 (24.8)[c]	0.4 (13.8)[d]	1.5 (10.4)	7.0 (13.6)[b]	NP	1.2 (24.5)[c]	0.4 (10.7)[a]	**0.006**[*]	0.8[α]	NP

[*]: Wilkoxon signed ranks test, [α]: Mann-Whitney U test, [a]: 1 missing values, [b]: 4 missing values, [c]: 2 missing values, [d]: 5 missing values, cm^2: Squared Centimetre, cm^3: Cubic Centimetre, DFU: Diabetic Foot Ulcer, DFU: Diabetic Foot Ulcer, HBOT: Hyperbaric Oxygen Therapy, M0: Month 0, M3: Month 3, mm: Millimetre, NHBOT: No Hyperbaric Oxygen Therapy, NP: Not Possible

Considering long-term follow-up, the remaining NHBOT DFU subject healed at the 6^{th} month, so, once again, it was not possible to perform any comparison (Table 16).

In the HBOT DFU group, all measurements improved at months 6 and 12 ($p \leq 0.006$).

Table 16. Diabetic foot ulcer characteristics at baseline, 6th and 12th month of follow-up

Variables	Global (n=20)			NHBOT DFU patients (n=6)			Paired samples tests p value	HBOT DFU patients (n=14)			Paired samples tests p value
	M0	M6	M12	M0	M6	M12		M0	M6	M12	
Baseline ulcer area (in cm^2) [median (range)]	11.0 (31.1)[a]	0.4 (6.3)[b]	0.0 (1.9)[c]	12.1 (16.1)	0.0 (0.0)[b]	0.0 (0.0)[b]	NP	7.3 (31.1)[a]	0.4 (6.3)	0.0 (1.9)[d]	**<0.001** [¤]
Mean depth (in mm) [median (range)]	2.3 (11.6)[e]	0.7 (2.2)[f]	0.0 (2.7)[g]	1.8 (4.7)	0.0 (0.0)[b]	0.0 (0.0)[b]	NP	2.3 (11.6)[e]	0.9 (2.2)[e]	0.4 (2.7)[f]	**0.001** [¤]
Maximum depth (in mm) [median (range)]	4.6 (18.3)[c]	1.3 (4.3)[g]	0.0 (4.7)[h]	3.6 (8.2)	0.0 (0.0)[b]	0.0 (0.0)[b]	NP	4.6 (18.1)[e]	1.7 (4.3)[e]	1.3 (4.7)[f]	**0.006** [¤]
Volume (in cm^3) [median (range)]	1.2 (24.8)[e]	0.0 (6.2)[g]	0.0 (0.3)[h]	1.5 (10.4)	0.0 (0.0)[b]	0.0 (0.0)[b]	NP	1.2 (24.5)[e]	0.0 (6.2)[e]	0.0 (0.3)[f]	**0.001** [¤]

[¤]: Friedman test, [α]: Mann-Whitney U test, [a]: 1 missing values, [b]: 5 missing values, [c]: 8 missing values, [d]: 3 missing values, [e]: 2 missing values, [f]: 4 missing values, [g]: 7 missing values, [h]: 9 missing values, cm^2: Squared Centimetre, cm^3: Cubic Centimetre, DFU: Diabetic Foot ulcer, HBOT: Hyperbaric Oxygen Therapy, M0: Month 0, M3: Month 3, mm: Millimetre, NP: Not Possible, NHOTB: No Hyperbaric Oxygen Therapy

5. *Percentage of epithelialization*

As described above, in the non-HBOT DFU group there was only one subject remaining with active DFU at month 3, presenting a median percentage of epithelialization of 79.4%. The DFU was completely healed by month 6th and remained 100% epithelialized at month 12.

In opposition, in the HBOT DFU group, significant and gradual improvement was observed in all three time points (median percentage of epithelialization of 64.4%, 85.7% and 100.0% at months 3, 6 and 12 respectively, p=0.001).

6. *Immunohistochemistry*

As DFU bed tissue was gathered only when debridement was performed, not all collected samples presented viable tissue. So, immunohistochemistry for CD31-expressing microvessels samples paired by subject, were only possible in 3 and 6 patients in the NHBOT DFU and HBOT DFU groups, respectively.

In the HBOT group the median number of vessels tended to increase after 1 month (617 *vs* 709), whereas the opposite occurred in the NHBOT DFU patients (746 *vs* 680), not achieving, however, statistical significance (Figure 12). Two examples are given on the table below (Table 17).

Figure 12. Boxplot of number of vessels at baseline and 1 month in patients undergoing or not Hyperbaric Oxygen therapy

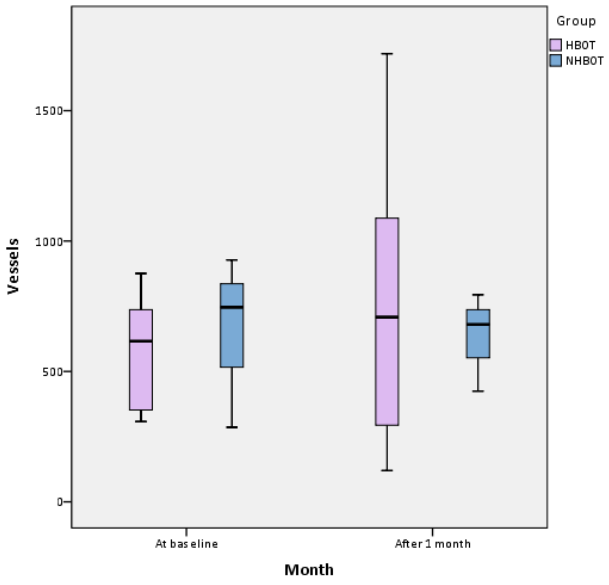
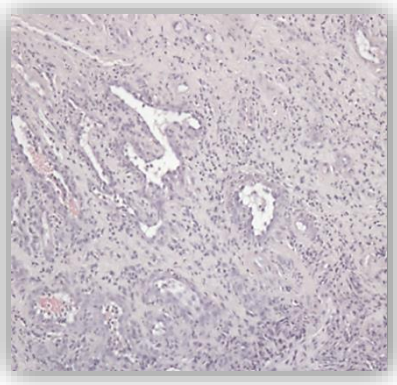
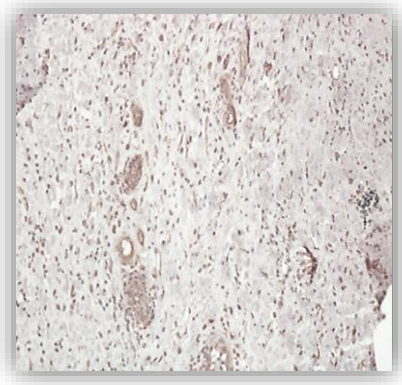
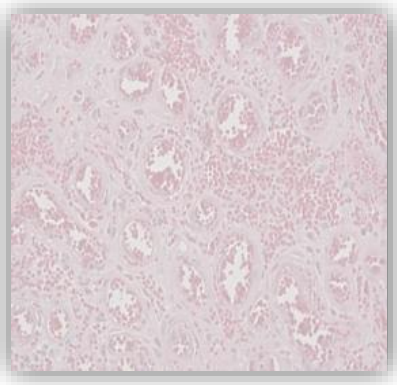
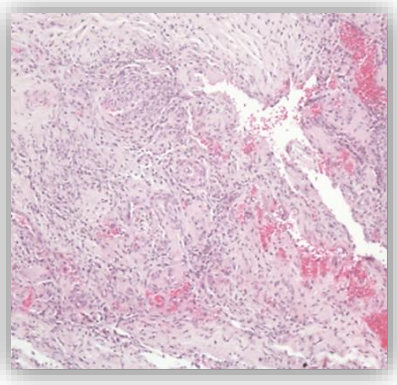
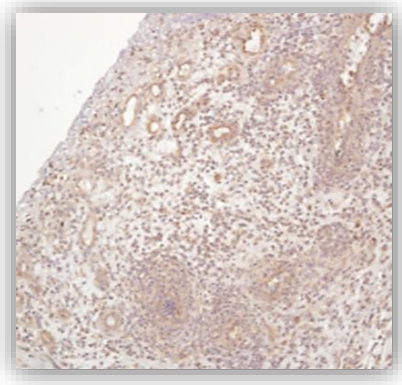
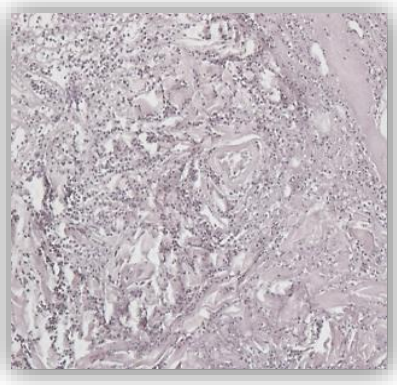
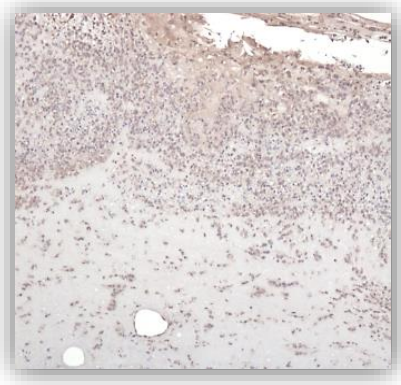


Table 17. Example images of wound bed histology and immunohistochemistry at baseline and month 1 in one patient of each study group with active diabetic foot ulcer

	HBOT DFU		**NHBOT DFU**	
	HE, 20x10	*CD 31, 20x10*	*HE, 20x10*	*CD 31, 20x10*
M0				
M1				

CD 31: Cluster of differentiation 31, DFU: Diabetic Foot ulcer, HBOT: Hyperbaric Oxygen Therapy, HE: Haematoxylin-eosin, M0: Month 0, M1: Month 1, NHOTB: No Hyperbaric Oxygen Therapy

C. Discussion

Several studies have addressed HBOT impact on DFU healing (see Section IV C). However, this is the first study to simultaneously evaluate and compare biochemical markers and clinical outcome in subjects with no DFU and with DFU undergoing or not HBOT.

We have concluded that, in those undergoing HBOT, merely 3 months of therapy, induced a significantly lower leukocyte and CRP level. These findings support the evidence of an anti-microbial and anti-inflammatory effect of HBOT [Albuquerque-Sousa JG, 2007; Thom SR, 2011].

In addition, VEGF levels diminished, to values similar to those in the non-DFU group, but statistical significance was not achieved. From the results, we can see that DFU patients have higher levels of VEGF when compared to non-DFU. The decrease of VEGF with HBOT may be related to a correction of the hypoxia and thus tendency to return to a "normal" diabetic non-DFU molecular serum environment.

In the HBOT group, SDF1-α increased at month 3, returning to baseline levels at month 6. This fact may have occurred as in the first endpoint all subjects and, at the latter, none were receiving HBOT. So, we hypothesize that HBOT augments SDF1-α values, with possible rise in the number of circulating EPCs, but such effect seems to stop with the suspension of treatment.

Even though, TNF-α is a main mediator of inflammation we were unable to quantify it in the serum. Further studies, determining TNF-α expression in the wound bed are needed.

Our sample presented a mean age, gender, DM duration and HbA1c, similar to the populations reported by several studies included in our systematic review (Table 3). Median $TcPO_2$ was inferior than in most studies. Regarding depth, we included deeper DFUs than RCT but similar to NRT.

At every time points, the HBOT group achieved better outcomes when compared to those with DFU without undergoing such adjunctive therapy. We observed less major LEA and death rates. Assessing all DFU measures, a high DFU reduction and an increasing epithelialization percentage were observed along all the study period in the HBOT group. Comparison was not possible with the non-HBOT group, as only one subject remained alive and without major LEA. Analyzing our systematic review results (Table 3), we noticed that our clinical outcomes rates were equivalent (within the 95% CI) to those reported by the long term, at least 12 months, to RCT and NRT for both groups.

During a mean number of HBOT sessions of 86, it was observed a gradual DFU improvement that continued after the prescribed therapy program completion. Comparing to the available

literature, we observed that our patients received more sessions, this may have affected positively the results.

After one month, we noticed that the median number of vessels in the HBOT group tended to increase, while the opposite occurred in the non-HBOT DFU patients (without statistical significance).

Infection and PAD are considered by several classifications as the most important factors, along with depth, for DFU prognostic assessment [Monteiro-Soares M et al, 2014]. HBOT improves circulation by stimulating micro-vascularization and optimizes oxygen delivery ameliorating leucocytes function and anti-bacterial effect. We believe that such mechanisms are responsible for such good clinical outcomes and are translated in our results.

We must highlight that only one subject suffered a minor side effect due to HBOT (ear barotrauma), that responded to treatment.

The authors decided to conduct a non-randomized trial design. Although it represents a more feasible methodology in clinical practice, it presents several limitations.

DFU control group, that is, non-HBOT subjects, was selected by refusal or contra-indication, instead of using randomization techniques. This may induce group differences. Comparing baseline characteristics, we observed that the main difference was that there was significantly more history of previous DFU in the non-HBOT DFU individuals than in the HBOT DFU, this might be one of the possible reasons for them to refuse HBOT.

A small sample size was achieved, due to limited selection of adequate patients and financial constraints. Nevertheless, significant results were obtained for several measures.

Due to ethical reasons, were not able to perform DFU biopsy. So, we only collected small fragments resulting from wound debridement. Therefore, not all collected samples were representative of the DFU bed tissue, limiting the evaluation of microvessel density. Despite this, it appears that HBOT DFU subjects tend to have an increase in vessel number after 1 month of therapy, with the opposite occurring in the non-HBOT group.

In order to attempt to overcome the described study weaknesses, selection of DFU individuals for HBOT was performed by a team, independent of the study, including only patients with optimized standard care and still no DFU improvement; all laboratory/molecular and microvessel density evaluation analysis were performed by investigators blinded to the group allocation; clinical end-points were objective (percentage of ulcer healing, amputation and death) and all DFU measurements were performed using a digital laser measuring device.

Due to the defined criteria, our diabetic foot clinic team referred for HBOT mainly DFUs that reached the bone, were infected, ischemic (grade III and stage D in the UTC) and after minor LEA

has occurred. This fact may limit the results' generalizability and it may diminish the measure magnitude of effect. So, if such clinical results were observed under HBOT, it is expected even better outcome in a less severely affected population.

In sum, our data reinforces the potential molecular and clinical efficacy and benefit of HBOT when added to current standard treatment of DFU. But further studies are required, particularly, evaluation of the HBOT modulator effect on DFU bed tissue using an animal model; increase the total number of patients in whom angiogenic, vasculogenic and inflammatory markers are studied not only in serum but also in the DFU bed. It is also crucial to define the optimal HBOT timing after revascularization for DFU treatment, since revascularization improves macrovascular disease and HBOT seems to ameliorate microvasculature, and referral.

VI. Improving hyperbaric oxygen therapy referral for diabetic foot ulcer treatment: a nationwide models' validation and refinement study

A. Material and methods

1. *Participants selection*

A multicentre retrospective cohort study was conducted in all Portuguese hyperbaric medicine centres treating diabetic foot patients (n=2) including all subjects that underwent HBOT for DFU treatment from January 1, 2008 to June 30, 2013.

Participants with only one appointment, associated auto-immune diseases, pressure and malleoli ulcers, under treatment at the time of data collection and/or missing data concerning outcome (healing, non-healing, LEA) were excluded.

The study was approved by the Ethics Committee of both institutions.

2. *Data collection*

Clinical records were reviewed and data collected from July 1 to the August 31, 2013.

Demographic characteristics (age at the time of inclusion, gender), DM type (classified according to the World Health Organization definition [WHO, 2006]), duration (in years) and treatment (oral medication or insulin), metabolic control (through HbA1C), smoking habits (in packs-year) and the character and severity of any DM complications (retinopathy, laser photocoagulation; nephropathy, dialysis; neuropathy; cerebrovascular and/or cardiovascular disease) were recorded.

Factors of interest specific to the DFU were: the presence of PAD; $TcPO_2$; ABI; DPN and previous DFU or LEA. We also collected the ulcer area (in cm^2), reported duration (in months), location, Wagner grade [Wagner F, 1981], number of DFUs and the presence or absence of infection. In the case of multiple DFUs, only the largest was considered.

PAD was considered as present when the foot with DFU presented one or fewer pedal pulses [Monteiro-Soares M et al, 2010]. $TcPO_2$ was determined by measuring once at 2 points peri-DFU and reporting the highest value.

DFU area was calculated multiplying the 2 larger axes. Time zero for DFU duration estimation was considered the last major surgical debridement or LEA. In the absence of either, we calculated the actual duration of DFU.

Considering HBOT, we recorded the total number of sessions, whether or not the planned number of sessions was completed and outcome (complete healing *vs* not healed or LEA).

Complete healing was considered as DFUs full epithelialization without the need of further treatment [Younes N & Albsoul A, 2004]. Minor LEA was defined as the surgical removal of toe(s), ray(s) or forefoot. Major LEA was considered amputation of any part at or above the entire foot.

3. *Existing models of healing prediction*

We conducted a systematic review (Section IV, C) in order to retrieve all studies proposing predictive models for DFU healing with HBOT. We identified only two: Fife 2007 [Fife C et al, 2007] and Hawkins 2006 [Hawkins G et al, 2006].

Using the Fife model we can calculate the odds of healing with HBOT compared to the odds of healing without HBOT by using the following formula: Log (OR) = 0.99 + 0.21 x [Ln (HBOT number of sessions + 1)] + 0.004 x ($TcPO_2$ in mmHg) – 0.04 x (RAMP) – 0.15 x (modified Wagner) – 0.04 x (age in years x diabetes duration in years) – 0.19 x (1 if interrupted treatment or 0 if no interruption occurred) where RAMP is a function for pack-years of smoking (those with ≤10 pack-years = 0; those with >10 pack years = number of pack years – 10) [9]. In Fife's article [Fife C et al, 2007], the authors made use of the Wagner grading scale modified by Kominsky, which varies from II (superficial) to VI (gangrene of the entire foot).

Hawkins' model for the odds of healing with HBOT is: Log (OR) =2.30 – 0.09 x (DFU area in cm2) – 0.11 x (DFU duration in months) + 0.06 ($TcPO_2$ in mmHg).

Both models were applied to all subjects.

4. *Statistical analysis*

For continuous variables, we used Student's t-test to compare groups when the data appeared acceptably normally distributed (using both histogram and Kolmogorov-Smirnov testing) and the Mann-Whitney U-test when the assumption of normality was not appropriate. Categorical variables were compared using the X^2 test or Fisher's exact test, when applicable.

Multivariate analysis, and consequent variables' adjustment and predictive models' proposal, was performed using logistic regression.

All statistical analyses were conducted using the program IBM SPSS, version 20.0 (Chicago, IL, USA). All tests were two sided, p values less than 0.05 were considered statistically significant and less than 0.1 as pertinent for inclusion in the predictive models.

As Fife's model uses post-treatment variables (such as HBOT number of sessions and treatment interruption) and our goal was to create a model using only pre-treatment variables), we decided just to validate this and refine the one proposed by Hawkins, if pertinent.

Therefore, we started by using all the variables included in the Hawkins model and those considered pertinent for inclusion in our univariate analysis. Missing and indeterminate results were excluded from analysis.

For the resultant optimized predictive model, a prognostic accuracy assessment was made including sensitivity, specificity, predictive values, likelihood ratios (LR), AUC and respective 95% CI calculation, comparing the CI and evaluating if there was an overlap to identify statistically significant differences.

B. Results

1. *Hyperbaric Oxygen therapy facilities and program characterization*

The center of the FNH (Centro de Medicina Subaquática e Hiperbárica) has been treating DFU patients since January 1990, it has two connected multiplace chambers with capacity to treat 24 individuals simultaneously. The treatment program performed for DFU is 75 minutes at 2.5 ATA, once a day, five days a week.

The center in the north of Portugal (Unidade de Medicina Hiperbárica of PHH) is equipped with a 16 place chamber, and started treating subjects with DFU in January 2008. The therapy protocol used is 80 minutes at 2.4 ATA, once a day, five days a week.

2. *Participants characteristics*

This study included 128 patients (85 from the FNH). The majority were males (73.4%), with a mean age of 62.9 (SD 11.8) years and 62.7% were active or past smokers. Most had type DM, with a mean duration of 18.2 (SD 9.9) years, and more than half were on insulin, with a mean HbA1c of 8.6% (SD 1.8). The most frequent DM complications were retinopathy [44.7%, of whom more than half (27.7% of the total) had received laser photocoagulation] and nephropathy (34.6%; 12.2% on dialysis).

Most of the subjects had PAD (84.8%) and DPN (89.5%). We observed that in PHH, DPN was diagnosed using the SWM, while in the FNH the procedure was never described.

The mean $TcPO_2$ was 33.5 (range 1-76) and ABI 0.78 (SD 0.28) (data only available from PHH).

The median baseline DFU area was 8.2 (range 0.3-60.0) cm^2 with a median duration of 2.5 (0-36) months. The majority were post-LEA (54.7%) and were infected (89.8%). DFUs were classified as Wagner grade III-V in 71.7% of cases. Multiple DFUs were present in 22.1% of patients.

After a mean of 55 HBOT sessions (SD 34.1), 16 (12.5%) subjects required LEA and in 44 (34.4%) the DFU remained unhealed. The majority of patients (66.4%) completed the prescribed treatment. The most common reasons for failure to complete were the withdrawal of health administration approval and worsening of the clinical condition.

Subjects from PHH presented more often with retinopathy ($p < 0.001$), previous DFU ($p = 0.02$) and longer DFU duration ($p < 0.001$) than those from FNH. However, PAD was more frequent in those from FNH ($p < 0.001$). No differences were observed between facilities in the remaining variables (Table 18).

Table 18: Patient characteristics according to Hyperbaric Oxygen therapy unit

Variables	Total (n=128)	Former Navy's Hospital (n= 85)	Matosinhos Hospital (n= 43)	p value
DEMOGRAPHIC AND DIABETES CHARACTERISTICS				
Male [n (%)]	94 (73.4)	65 (76.5)	29 (67.4)	0.30 *
Age (in years) [mean (SD)]	62.9 (11.8)	64.1 (12.1)	60.7 (11.1)	0.13†
Diabetes duration (in years) [mean (SD)]	18.2 (9.9) [a]	17.7 (10.9) [a]	18.7 (8.8)	0.64†
Age+ Diabetes duration [mean (SD)]	1123.3 (655.7) [a]	1109.5 (721.3) [a]	1139.2 (578.3)	0.83†
HbA1C (in %) [mean (SD)]	8.6 (1.8) [b]	8.9 (2.1)[c]	8.6 (1.7)[d]	0.64†
Type 2 diabetes [n (%)]	113 (89.0)[e]	74 (88.1) [e]	39 (90.7)	0.77 *
Insulin use [n (%)]	75 (65.8)[f]	45 (63.4) [f]	30 (69.8)	0.55 *
Smoking habits (in pack-year)[median (range)]	30.0 (0.0-100.0) [g]	38.0 (0.0-100.0)[g]	20.0 (0.0-96.0)	0.12†
MACRO AND MICROVASCULAR COMPLICATIONS				
Retinopathy [n (%)]	42 (44.7)[h]	10 (19.6) [h]	32 (74.5)	**<0.001** #
Laser photocoagulation [n (%)]	26 (27.7)	4 (7.8)	22 (51.2)	
Nephropathy [n (%)]	34 (34.6)[i]	15 (27.3) [i]	19 (44.2)	0.19 #
Dialysis [n (%)]	12 (12.2)	6 (10.9)	6 (14.0)	
Previous stroke history [n (%)]	14 (14.6)[j]	5 (9.4) [j]	9 (20.9)	0.15 *
Previous myocardial infarction history [n (%)]	28 (28.9)[k]	19 (35.2) [k]	9 (20.9)	0.18 *
FOOT CHARACTERISTICS				
0 or 1 foot pulses	106 (84.8)[l]	74 (89.1) [m]	32 (76.2) [e]	**<0.001** #
$TcPO_2$ [median (range)]	33.5 (1.0-76.0)[n]	NP	33.5 (1.0-76.0)[o]	NP
ABI [mean (SD)]	0.78 (0.28)[p]	NP	0.78 (0.28)[d]	NP
DPN [n (%)]	17 (89.5)[q]	8 (80.0) [r]	9 (100.0) [s]	0.47 *
Previous DFU [n (%)]	42 (38.9)[t]	19 (29.2) [t]	23 (53.5)	**0.02 ***
Previous LEA [n (%)]	27 (25.0)[t]	14 (21.5) [t]	13 (30.2)	0.37 *
DFU CHARACTERISTICS				
Area (in cm^2) [median (range)]	8.2 (0.3-60.0)[u]	9.8 (0.4-60.0)[e]	6.4 (0.3-54.0)[v]	0.35§
Duration (in months) [median (range)]	2.5 (0-36)[w]	1.8 (0.0-24.0)[w]	4.0 (1.0-36.0)	**<0.001**§
Multiple DFU [n (%)]	29 (22.7)	20 (23.5)	9 (20.9)	0.83 *
Located at toes *[n (%)]*	19 (14.8)	14 (16.5)	5 (11.6)	0.60*
Post-LEA [n (%)]	70 (54.7)	51 (60.0)	19 (44.2)	*0.09**
Wagner grade III-V [n (%)]	91 (71.7)[e]	62 (73.8) [e]	29 (67.4)	0.54 *
Infection [n (%)]	115 (89.8)	75 (88.2)	43 (93.0)	0.54*
HBOT CHARACTERISTICS AND OUTCOME				
Complete healing [n (%)]	68 (53.1)	45 (52.9)	23 (53.5)	1.00 *
Number of sessions [mean (SD)]	54.9 (34.1)	52.1 (32.0)	60.4 (37.6)	0.19 †
Completed treatment [n (%)]	85 (66.4)	56 (65.9)	32 (74.4)	0.42 *

*: Fisher's exact test; †: t-test for independent samples; # X^2 test for association and trend; § Mann-Whitney test; [a]: 35 missing values; [b]:83 missing values; [c]: 77 missing values; [d]: 6 missing values;[e]: 1 missing value; [f]: 14 missing values; [g]: 51 missing values; [h]: 34 missing values; [i]: 30 missing values; [j]: 32 missing values; [k]: 31 missing values; [l]:3 missing values; [m]: 2 missing values; [n]:104 missing values; [o]: 19 missing values; [p]:91 missing values; [q]:109 missing values; [r]: 75 missing values; [s]: through Semmes-Weinstein monofilament and with 34 missing values; [t]: 20 missing values; [u]: 6 missing values; [v]: 5 missing values; [w]: 27 missing values; HbA1C: Glycated Hemoglobin; ABI: Ankle-Brachial Index; DFU: Diabetic Foot Ulcer; DPN: Diabetic Peripheral Neuropathy; LEA: Lower Extremity Amputation; NP: Not Possible; SD: Standard Deviation; $TcPO_2$: Transcutaneous Oxygen Tension

3. *Predictive variables for diabetic foot ulcer healing*

Univariate analysis (Table 19)

Univariate analysis suggested that the patient characteristics associated with healing were female gender (p = 0.03), lower median duration of smoking (pack years, p = 0.01), no PAD (p = 0.002), no previous DFU (p = 0.01) and no LEA (p = 0.04).

No macro or microvascular complication had an impact on healing (p > 0.05).

Smaller (p = 0.002) and more superficial DFU (p = 0.03) healed more frequently.

Ulcers that healed received more HBOT sessions on average (p = 0.07), while those that completed their planned treatment were more likely to heal (p < 0.001).

Table 19: Patient characteristics by outcome

Variables	Complete healing (n=68)	Non-healing / LEA (n=60)	p value
DEMOGRAPHICS			
Male [n (%)]	44 (64.7)	50 (83.3)	**0.03 ***
Age (in years) [mean (SD)]	61.4 (12.1)	64.7 (11.4)	0.12 †
Diabetes duration (in years) [mean (SD)]	19.5 (10.5) [a]	16.5 (8.9) [b]	0.15 †
Age * Diabetes duration [mean (SD)]	1178.3 (697.6) [a]	1050.3 (596.6) [b]	0.35 †
HbA1c (in %) [mean (SD)]	8.8 (1.7) [c]	8.5 (1.8) [d]	0.62 †
Type 2 diabetes [n (%)]	57 (85.1) [e]	56 (93.3)	0.17 *
Insulin use [n (%)]	42 (67.7) [f]	33 (63.5) [g]	0.69 *
Smoking habits (in pack-year) [median (range)]	0.0 (0.0-90.0) [h]	40.0 (0.0-100.0) [b]	**<0.001** §
MACRO AND MICROVASCULAR COMPLICATIONS			
Retinopathy [n (%)]	19 (35.9) [a]	23 (56.1) [i]	0.13 #
Laser photocoagulation [n (%)]	11 (20.8)	14 (36.6)	
Nephropathy [n (%)]	16 (28.5) [j]	18 (42.9) [k]	0.30 #
Dialysis [n (%)]	5 (8.9)	7 (16.7)	
Previous stroke history [n (%)]	9 (16.7) [l]	5 (11.9) [k]	0.57 *
Previous myocardial infarction history [n (%)]	14 (25.5) [m]	14 (33.3) [k]	0.50 *
FOOT CHARACTERISTICS			
0 or 1 foot pulses [n (%)]	49 (74.2) [n]	57 (96.8) [e]	**0.002 #**
$TcPO_2$ [median (range)]	21.0 (1.0-76.0) [o]	37.0 (3.0-67.0) [p]	0.52 §
ABI [mean (SD)]	0.9 (0.3) [q]	0.7 (0.3) [d]	0.15 †
DPN [n (%)]	8 (80.0) [r]	9 (100.0) [q]	0.47 *
Previous DFU [n (%)]	16 (27.1) [s]	26 (53.1) [t]	**0.01 ***
Previous LEA [n (%)]	10 (16.9) [s]	17 (34.7) [t]	**0.04 ***
DFU CHARACTERISTICS			
Area (in cm^2) [median (range)]	6.1 (0.3-41.0) [n]	12.0 (0.8-60.0) [u]	**0.002 §**
Duration (in months) [median (range)]	2.0 (0.0-30.0) [v]	3.0 (0.0-36.0) [w]	0.14 §
Multiple DFU [n (%)]	15 (22.1)	14 (23.3)	1.00 *
Located at toes [n (%)]	13 (19.1)	6 (10.0)	0.21*
After LEA [n (%)]	33 (48.5)	37 (61.7)	0.16*
Wagner grade III-V [n (%)]	43 (63.2)	48 (81.4) [e]	**0.03 ***
Infection [n (%)]	58 (85.3)	57 (95.0)	*0.08 **

HBOT CHARACTERISTICS			
Matosinhos' Hospital [n (%)]	23 (33.8)	20 (33.3)	1.00 *
Number of sessions [mean (SD)]	60.1 (32.8)	40.0 (3.0-120.0)	*0.07 †*
Completed treatment [n (%)]	65 (95.6)	23 (38.3)	**<0.001 ***

*: Fisher's exact test; †: t-test for independent samples; # X^2 test for association and trend; § Mann-Whitney test; [a]: 15 missing values; [b]: 20 missing values; [c]: 43 missing values; [d]: 40 missing values; [e]: 1 missing values; [f]: 6 missing values; [g]: 8 missing values; [h]: 31 missing values; [i]: 19 missing values; [j]: 12 missing values; [k]: 18 missing values; [l]: 14 missing values; [m]: 13 missing values; [n]: 2 missing values; [o]: 59 missing values; [p]: 45 missing values; [q]: 51 missing values; [r]: 58 missing values; [s]: 9 missing values; [t]: 11 missing values; [u]: 4 missing values; [v]: 17 missing values; [w]: 10 missing values; HbA1C: Glycated Hemoglobin; ABI: Ankle-Brachial Index; CI: Confidence Interval; DFU: Diabetic Foot Ulcer; DPN: Diabetic Peripheral Neuropathy; LEA: Lower Extremity Amputation; SD: Standard Deviation; $TcPO_2$: Transcutaneous Oxygen Tension

Multivariate analysis

Following univariate analysis, we performed a multivariate analysis including all factors identified as potentially predictive of healing (p-value on univariate analysis < 0.1) plus the elements in the Hawkins model. We substituted the $TcPO_2$ value in the Hawkins model with PAD (defined as ≤1 pedal pulse in the affected foot) because of the high number of missing values for $TcPO_2$ in our data. Using a backward stepwise elimination approach, we have derived the best model. Twenty-eight subjects were excluded from analysis due to missing data.

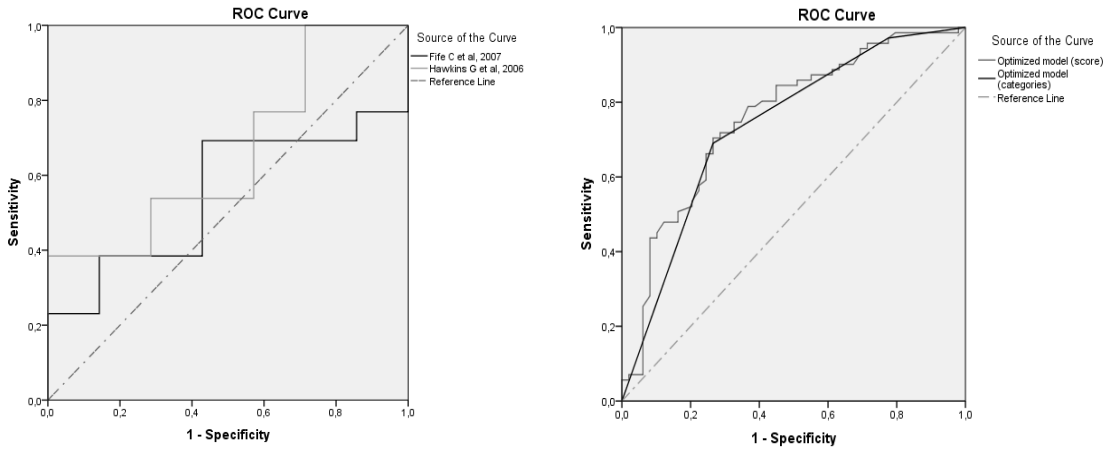
Using this methodology, we propose an optimized model, derived from the one proposed by Hawkins in 2006 (3), with a score calculation based on the following equation: Score = 2.96– 1.34 x (≤ 1 pedal pulse) – 0.04 x (DFU area in cm2) – 0.84 x (DFU graded as Wagner III-V) – 1.15 x (previous DFU – yes= 1, no = 0).

Through receiver operating characteristic (ROC) curves analysis, we have also proposed the creation of three diagnostic categories for DFU healing and determined cut-off values to maximize predictive ability: healing not probable for values < -0.91, probable for values from -0.91 to 0.36 and highly probable to heal with HBOT for values > 0.36.

4. *Predictive model accuracy*

Figure 13 shows the ROC curves for the existing models (Fife 2007 and Hawkins 2006) compared with our optimized models.

Figure 13: Predictive models receiver operating characteristic curve



The left image represents the Fife 2007 (in black) and Hawkins 2006 (in grey) score area under the receiver operating characteristic curve. The right image represents the optimized model scores (in blue) and categories (in green) area under the receiver operating characteristic curve.

The AUC values are higher for the latter [0.51% (95% CI 0.24-0.78) in Fife 2007, 0.63% (95% CI 0.37-0.90) in Hawkins 2007 *vs* 0.78% (95% CI 0.69-0.87)] in optimized model). However, these differences are not statistically significant (Table 20).

Table 20: Predictive models area under the receiver operating characteristic curve

Model	**AUC**	**95% CI**
Fife C et al, 2007 [a]	0.51	0.24-0.78
Hawkins G et al, 2006	0.63	0.37-0.90
Optimized model [c]		
Score	0.78	0.69-0.87
Categories	0.75	0.65-0.85

[a]: 104 missing values; [b]: 107 missing values; [c]: 28 missing values; AUC: Area Under the Receiver Operating Characteristic Curve; CI: Confidence Interval

Our model had good accuracy values, with LR values having a potential small to moderate effect on clinical decision, with negative LR smaller than 0.5 and positive LR superior to 1.3 [Fritz J & Wainner R, 2001]. Predictive values (negative and positive) were superior to 60% (Table 21).

Table 21: Optimized models prognostic accuracy measures

	Subjects n (%)	Healed n (%)	Accuracy measures					
			Sens % (CI 95%)	Spec % (CI 95%)	LR+ % (CI 95%)	LR- % (CI 95%)	PPV % (CI 95%)	NPV % (CI 95%)
OPTIMIZED MODEL [a]								
Highly probable	48 (48.0)	38 (79.2)	67.9 (55.6-80.1)	77.3 (64.9-90.0)	3.0 (1.7-5.3)	0.4 (0.3-0.6)	79.2 (67.7-90.7)	65.4 (52.5-78.3)
Highly probable+ Probable	87 (87.0)	54 (62.1)	96.4 (91.6-100.0)	25.0 (12.2-37.8)	1.3 (1.1-1.5)	0.1 (0.03-0.6)	62.1 (51.9-72.3)	81.6 (65.0-100.0)

[a]: 28 missing values; CI: Confidence Interval; Spec: Specificity; Sens: Sensitiviy; LR-: Negative Likelihood Ratio; LR+: Positive Likelihood Ratio; NPV: Negative Predictive Value; PPV: Positive Predictive Value

C. Discussion

Standardized referral systems are essential for optimal resource allocation and prognostic estimation, especially concerning expensive adjunctive therapeutics (such as HBOT in chronic DFU).

This is the first study evaluating all subjects treated over a period of five years with HBOT for DFUs in continental Portugal.

The sample size is limited (n= 128), and this reduces the power of our analysis to draw definitive conclusions. There are potentially two main reasons for this small sample size. First, HBOT is a poorly understood and promoted treatment modality, and second, even when considered, it is a treatment of last resort given the financial constraints within the healthcare system in Portugal.

Furthermore, we have excluded subjects with associated auto-immune diseases, pressure and malleoli wounds as these ulcers have different clinical and pathophysiological characteristics than the "standard" DFU, with potential impact on healing rates.

The review period corresponds with the start of DFU treatment at PHH HBOT unit, while the FNH unit has been operating since 1989. This may in part explain the fact that 2/3 of the included subjects were treated at the FNH.

Our population is similar to those reported in the available literature [Abidia A et al, 2003; Albuquerque-Sousa J, 2005; Baroni G et al, 1987; Chen CE et al, 2010; Doctor N et al, 1992; Duzgun A et al, 2008; Faglia E et al, 1996; Faglia E et al, 1998; Kalani M et al, 2002; Kessler L et al, 2003; Löndahl et al, 2010; Löndahl et al, 2011; Oriani G et al, 1990; Wang CJ et al, 2009;

Wang CJ et al, 2011; Zamboni W et al, 1997]; i e, mainly men, above 60 years, with type 2 DM, for around 15 years and an HbA1c greater than 8%.

In our population almost 30% presented with a Wagner wound grade of I or II, which suggests our population had less severe DFU than the majority of previously reported studies [Albuquerque-Sousa J, 2005; Duzgun A et al, 2008; Faglia E et al, 1996; Faglia E et L, 1998; Oriani G et al, 1990] but comparable to the largest randomized controlled trial published in this field [Löndahl M et al, 2010].

The majority of our subjects presented diminished palpable pulses (84.8%) and infected DFU (89.8%) with a median duration of 2.5 months.

We observed a higher percentage of PAD in the FNH participants. This might be due to the fact that in the North (referral area of PHH), there is a lower PAD prevalence [Menezes JD et al, 2009], but it is also possible that vascular surgeons take a more active approach to chronic DFU patients, including a higher use of angioplasty techniques and revascularization surgery (personal communication).

Conversely, DFU duration was longer at the PHH (median difference of 2.2 months). This is a more recent unit and so HBOT is less widespread among referral healthcare institutions, thus professionals tend to send only patients with persisting DFU despite all other treatments. Additionally, we considered the last major surgical intervention as time 0 for duration estimation, and in the FNH there was a higher rate of post-LEA wounds with immediate referral for HBOT.

We observed that subjects from PHH tended to present higher rates of DM-related complications (retinopathy, nephropathy and stroke). It is not clear if subjects from the North of the country tend to present more complications (although DM duration and HbA1c values were similar) or if the detection rate is superior [OND 2013].

However, despite these differences between centres, the healing rates, number of HBOT sessions and proportion who completed the planned treatment are similar.

Eleven studies [Chen CE et al, 2010; Fife C et al, 1997; Fife C et al, 2007; Hawkins G et al, 2006; Löndahl M et al, 2010; Löndahl M et al, 2011; Mathieu D et al 1997; Ong M, 2008; Otto G et al, 2000; Wattel F et al, 1991; Zgonis T et al, 2005] have reported patient factors that may be predictive of healing in DFU treated with HBOT. In our population, clinical factors associated with DFU healing were female gender, lower smoking pack-years, smaller and more superficial DFU as well as absence of PAD, previous DFU and/or LEA. Some of the previous studies have similarly reported both smoking habit and Wagner grade as predictive [Fife C et al, 1997; Fife C et al, 2007; Otto G et al, 2000].

In contrast to Fife 2003, we could not demonstrate an impact of (age + diabetes duration) [Fife C et al, 1997; Fife C et al, 2007; Otto G et al, 2000], renal failure [Fife C et al, 2007] or previous LEA on outcome.

Similarly, in contrast to Hawkins, we observed a statistically significant predictive ability for gender, DFU area and duration.

During the follow-up period, 12.5% of our subjects required an LEA and in 34.4% DFU persisted unhealed immediately after the last HBOT session. These data are in accordance with several studies, including the two largest randomized controlled trials [Çerkes N et al, 1994; Cianci P & Hunt T, 1997; Duzgun A et al, 2008; Kaya A et al, 2009; Löndahl M et al, 2010; Löndahl M et al, 2011].

Because the practice in Portugal is to continue wound care in the referring facility, we believe our results confirm a true benefit from HBOT, rather than reflecting any change in wound care management in a specialized unit. Our data also suggest this benefit is greater if the prescribed course of HBOT is completed. While this seems likely, based on common sense, it may also be biased through the tendency for those doing well to keep attending, while those seeing little or no progress may be less inclined to do so. It is worth noting that the mean number of sessions was 55, which is slightly higher than most studies have reported [Abidia A et al, 2003; Baroni G et al, 1987; Faglia E et al, 1996; Faglia E et al, 1998; Kessler L et al, 2003; Wang CJ et al, 2009; Wang CJ et al, 2011; Zamboni W et al, 1997].

Of the two previously reported predictive models, we observed that both had low accuracy (with the AUC value inferior to 0.63), and we considered that optimization of one or both these models was appropriate. Ultimately, we have excluded Fife's model because it also used post-treatment variables (number of sessions and episodes of treatment interruption), making it unsuitable for pre-HBOT decision making – including when it is appropriate to refer for HBOT.

We have therefore optimized the Hawkins model using logistic regression, by including two additional variables (Wagner grade and previous DFU) and removing DFU duration variable. Due to the number of missing values and in order to increase the optimized models' application in daily clinical care, we decided to replace the variable TcPO2 by the number of palpable pedal pulses.

In this way, we proposed a model easy to apply and tending to produce higher accuracy measures in comparison to the Hawkins' baseline model.

Due to the low sample size and high number of missing values in the models' included variables, CI were wide and no statistical differences were detected between the original and the optimized models' AUC values. For this reason, we were also unable to compare other accuracy measures.

Evaluating the retrospective performance of our optimized model, we observe that it is characterised by low specificity and LR but also by potentially useful sensitivity and predictive values. We therefore consider that a larger prospective study for model validation and

refinement is appropriate, and that such a study should be conducted prospectively to avoid the pitfalls of retrospective data collection.

VII. Conclusion

A. Main Conclusions

The independent contribution of DFU on LEA and mortality risk

1. Cumulative incidence at 3 years was
    a. DFU 26.6% (95% CI 23.2-30.0),
    b. recurrent DFU 34.5% (95% CI 27.4-48.4),
    c. minor LEA 2.7% (95% CI 1.4-4.0),
    d. major LEA 3.1% (95% CI 1.8-4.4),
    e. total LEA 5.8% (95% CI 3.9-7.5) and
    f. death 14.0% (95% CI 11.3-16.7).
2. Therefore, we have a
    a. high rate of DFU development,
    b. mortality rate in accordance with the Eurodiale study [Schaper N, 2012] and a
    c. LEA rate inferior to Eurodiale results [Schaper N, 2012], as they included only patients with active DFU.
3. In multivariate analysis
    a. physical impairment, PAD complication history, complication count and previous DFU were associated with DFU,
    b. complication count, foot pulses and previous DFU with LEA and age, and
    c. complication count and previous DFU with death.
4. These predictive models' AUC ranged from 0.80 to 0.83 and may be applicable in different healthcare settings to identify patients at higher risk of DFU, LEA and death.
5. Previous DFU was associated with all outcomes, even when adjusted for complication count, in addition to more complex models (including age, gender, visual and physical impairment, diabetes type and duration, PAD complications history, complication count

and previous LEA. So, DFU seems more than a marker of complication status, having independent impact on LEA and mortality risk.

6. The most frequent causes of death were infections and oncologic disease.

Effect of HBOT in DFU healing: a systematic review and meta-analysis

1. Forty studies were included
    a. 11 RCT,
    b. 8 NRT,
    c. 22 observational studies.
2. Mean proportion of items satisfied, using CONSORT and STROBE methodological quality reporting checklists, was low.
    a. Thus, important information was absent in most studies.
    b. Even RCTs were inadequately reported and conducted (specially, regarding sample size).
3. The majority of the included studies were retrieved outside the MEDLINE Indexed search, which points out that the many works were either never published in full, or only in the "Grey" literature.
4. Participant characteristics were poorly described in general and total sample size, in more than half of the studies, was less than 50 subjects.
5. There was considerable variation in HBOT protocols across these studies, namely in sessions number, duration, frequency and pressure.
6. Substantial DFU area reduction was reported in the HBOT group at 6 months, but MA was not possible.
7. Time to healing was lower in HBOT group although not reaching statistical significance.
8. DFU healed more often with HBOT.
9. Major amputations were significantly less frequent in those undergoing HBOT.
10. A small amount of studies assessed independent variables' impact on healing with only age and $TcPO_2$ being evaluated in 2 or more studies.
11. We retrieved only two models to identify subjects that would benefit most of HBOT.
    a. Hawkins et al [Hawkins G et al, 2006] included variables that were not associated with healing in univariate analysis;
    b. Fife et al [Fife C et al, 2007] used post-HBOT variables;
    c. neither has been externally validated.

12. Using the SIGN system for grading recommendations [Harbour R & Miller J, 2001], for major LEA prevention HBOT presents A grade. However, for the remaining DFU related outcomes the grade lowers to B.

Molecular environment characterization, HBOT modulator effect and clinical impact on DFU healing

1. Almost all DFUs proposed for HBOT by our diabetic foot clinic team reached the bone, were infected, ischemic and post-minor LEA.
2. After 3 months of therapy, HBOT DFU individuals presented:
    a. significantly lower leukocyte and CRP level,
    b. tended to have lower VEGF levels and
    c. remaining laboratory markers, did not reveal statistically significant differences.
3. At every time points, the HBOT group achieved better outcomes
    a. less amputation and death,
    b. higher DFU reduction,
    c. increasing epithelialization percentage.
    d. Comparison was not possible with the non-HBOT group.
4. The median number of vessels in the HBOT group tended to increase after 1 month, while the opposite occurred in the NHBOT DFU patients (without statistical significance).
5. Our data reinforced the potential molecular and clinical efficacy and benefit of HBOT when added to current standard treatment of DFU.

Improving HBOT referral for DFU treatment: a nationwide models' validation and refinement study

1. Lisbon's FNH was responsible for the treatment of 66.4% of the population and patients from this hospital presented less frequently retinopathy or previous DFU and had ulcers of shorter duration.
2. The majority of all subjects presented chronic, infected and ischemic DFUs.
3. During the study period, LEA was necessary in 12.5% of cases, DFU persisted unhealed immediately after the last HBOT session in 34.4% and 53.1% healed.
    a. Complete healing was more likely in non-smoking females without arterial disease, previous DFU or history of LEA.
    b. Completion of the planned treatment and higher number of sessions had a positive impact on outcome.

c. Healing rates, number of HBOT sessions and proportion of who completed the planned treatment are similar between institutions.

4. Available models, by Fife et al and Hawkins et al, demonstrated low predictive accuracy (with the AUC < 0.63).
5. We proposed an optimized version of the Hawkins' et al model with higher accuracy by including two additional variables (Wagner grade and previous DFU) and removing DFU duration variable.

B. Main Strengths and Limitations

The independent contribution of DFU on LEA and mortality risk

Strengths

1. We have conducted a study
    a. assessing DFU impact on LEA and death risk,
    b. in a large cohort of consecutively enrolled subjects,
    c. using the STROBE [Vandenbroucke J et al, 2007] and STARD [Bossuyt PM et al, 2003] checklists as the basis for its development and reporting,
    d. conducting adequate statistical adjustment.
2. Due to the cohort design,
    a. observers were blind to outcome occurrence when collecting baseline data and
    b. all the assessed patient-related events occurred prior to the research being undertaken.
3. For the variables included in the models we had no missing data.
4. We have confidence in having identified the great majority of outcome occurrence by applying a comprehensive use of the available data platforms.

Limitations

1. Retrospective nature.
2. Exclusion of patients outside our direct referral area.
3. Presence and exclusion from analysis of missing data regarding VST and HbA1c values.
4. Limited generalizability of the results since it was a high risk referral practice, with great prevalence of type 2 DM, and low education level.

5. Variables collected by clinical interview may present information bias, namely non differential misclassification bias.

Effect of HBOT in DFU healing: a systematic review and meta-analysis

Strengths

1. First study simultaneously meta-analyzing all the available studies for HBOT efficacy on DFU healing and evaluating the methodological quality of those studies.
2. A systematic review of factors or models to identify patients that would most benefit from HBOT had never been done.
3. MOOSE [Stroup D et al, 2000] and PRISMA [Liberati A et al, 2009] checklists were used for study planning and reporting.
4. As our conclusions were based on all the available clinical evidence, currently, represent the best possible estimate of the true clinical impact of HBOT in DFU treatment. This approach potentially improved the validity of our results, diminished the chance of selection bias.

Limitations

1. The inclusion of non-randomized studies, may lead to participant selection bias, with consequent confounded result.
2. Studies frequently presented small sample sizes, including RCTs (under powering their results) and were conducted in single institutions, representing low evidence levels and diminished generalizability.
3. As there was a great variation in the HBOT protocols, high heterogeneity was observed in several MA measures.
4. Methodological quality assessment was made by only one of the researchers.

Molecular environment characterization, HBOT modulator effect and clinical impact on DFU healing

Strengths

1. First study concurrently evaluating and comparing biochemical markers and clinical outcome in DFU patients undergoing or not HBOT,
2. with prospective design and
3. including baseline molecular and clinical characterization and comparison with non-DFU subjects.

4. All laboratory/molecular and microvessel density evaluation analysis were performed by investigators blinded to the group allocation.
5. Selection of DFU individuals for HBOT was performed by a team, independent of the study, including only patients with optimized standard care and still no DFU improvement.
6. Clinical end-points were objective: percentage of ulcer healing, amputation, death.
7. All DFU measurements were performed using a digital laser measuring device.

Limitations

1. Allocation was not randomized.
2. Allocation occultation limitations, since the principal investigator and patients were not blinded.
3. Small sample size.
4. DFU bed tissue was not representative in several collected samples.

Improving HBOT referral for DFU treatment: a nationwide models' validation and refinement study:

Strengths

1. First study
    a. evaluating all subjects treated over a period of five years with HBOT for DFUs in continental Portugal and
    b. validating and optimizing predictive model for HBOT referral.
2. Proposed a model including easily collectable and available variables.
3. Our optimized model, presented potentially useful sensitivity and predictive values.

Limitations

1. Retrospective nature.
2. The sample size was limited and the missing values in the models' included variables, resulted in wide CI and no statistical differences between the original and the optimized models' AUC values.

C. *Future research*

The independent contribution of DFU on LEA and mortality risk

1. Derived models should be tested in primary care to assess if they are clinically relevant and valid enough *per se*, or if they should be added to pre-existing models/classifications.
2. The link between DFU history and poorer outcomes in patients with diabetes needs further studies to better understand it.

Effect of HBOT in DFU healing: a systematic review and meta-analysis

1. To optimize available evidence in the future there should be:
    a. therapy protocol definition improvement,
    b. RCTs with larger sample sizes and improved methodological reporting,
    c. cost-effectiveness assessment and
    d. creation of a specific checklist to enhance quality reporting and stimulate multicentre studies by consensus of scientific and medical societies of the area.

Molecular environment characterization, HBOT modulator effect and clinical impact on DFU healing

1. Study, in an animal model, more precisely and adequately, the HBOT modulator effect on DFU bed tissue.
2. Increase the total number of patients in whom angiogenic, vasculogenic and inflammatory markers are studied.
3. Develop a study to define optimal HBOT timing after revascularization for DFU treatment, since revascularization improves macrovascular disease and HBOT seems to ameliorate microvasculature.
4. Conduct an RCT with the same outcomes in a larger multicentre sample.

Improving HBOT referral for DFU treatment: a nationwide models' validation and refinement study

1. To conduct a study to assess HBOT knowledge and attitudes of Primary and Hospital Care Physicians in Portugal.
2. Analyze the impact of educational interventions about HBOT, in the same settings, in referral improvement.

3. Perform a larger multicenter prospective study for predictive model's validation and refinement.

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IX. Annex

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The independent contribution of diabetic foot ulcer on lower extremity amputation and mortality risk

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ABSTRACT

Aims: To estimate 3-year risk for diabetic foot ulcer (DFU), lower extremity amputation (LEA) and death; determine predictive variables and assess derived models accuracy.
Material and Methods: Retrospective cohort study including all subjects with diabetes enrolled in our diabetic foot outpatient clinic from beginning 2002 until middle 2010. Data were collected from clinical records.
Results: 644 subjects with mean age of 65.1 (± 11.2) and diabetes duration of 16.1 (± 10.8) years. Cumulative incidence was 26.6% for DFU, 5.8% for LEA and 14.0% for death. In multivariate analysis, physical impairment, peripheral arterial disease complication history, complication count and previous DFU were associated with DFU; complication count, foot pulses and previous DFU with LEA and age, complication count and previous DFU with death. Predictive models' areas under the ROC curves ranged from 0.80 to 0.83. A simplified model including previous DFU and complication count presented high accuracy. Previous DFU was associated with all outcomes, even when adjusted for complication count, in addition to more complex models.
Conclusions: DFU seems more than a marker of complication status, having independent impact on LEA and mortality risk. Proposed models may be applicable in healthcare settings to identify patients at higher risk of DFU, LEA and death.

1. Introduction

Diabetes mellitus (DM) is one of the most frequent metabolic disorders, with an estimate of 371 million people living with this condition worldwide (IDF.org). Incidence and prevalence are rising, carrying high costs (more than 471 billion US dollars in 2012) and rates of morbid-mortality, with premature deaths (IDF.org; WHO.org). Around 4.8 million people died in 2012 due to diabetes, half of them were under 60 years (IDF.org; WHO.org).

The diabetic foot is one of the major complications of this disease, with an estimated 10% to 25% of diabetic patients developing a diabetic foot ulcer (DFU) in their lifetimes (Frykberg, Zgonis, Armstrong, et al., 2006), causing a considerable burden in health care and patient well-being (IDF.org; Monteiro-Soares, Boyko, Ribeiro, Ribeiro, & Dinis-Ribeiro, 2011; Singh, Armstrong, & Lipsky, 2005).

The occurrence of a DFU bodes poorly for the clinical course of patients with diabetes, with higher rates of re-ulceration, LEA, contralateral LEA and death compared to persons with diabetes who have not experienced a DFU (Frykberg et al., 2006).

Abbreviations: ABI, Ankle–brachial Index; ADA, American Diabetes Association; AUC, Area Under the Receiver Operating Characteristic Curve; ARR, Absolute Risk Reduction; CI, Confidence Interval; CRP, C-reactive Protein; DFU, Diabetic Foot Ulcer; DM, *Diabetes mellitus*; DPN, Diabetic Peripheral Neuropathy; ESR, Erythrocyte Sedimentation Rate; HbA1c, Glycated Haemoglobin; HR, Hazard Ratio; Hz, Hertz; ICD-9, The International Classification of Diseases, 9th Revision; LEA, Lower Extremity Amputation; LR, Likelihood Ratio; mmHg, Millimetres of Mercury; NNT, Number Needed to Treat; PAD, Peripheral Arterial Disease; RR, Relative Risk; RRR, Relative Risk Reduction; SD, Standard Deviation; SWM, Semmes–Weinstein Monofilament; $TcPO_2$, Transcutaneous Partial Pressure of Oxygen; VST, Vibration Sensation Test; WHO, World Health Organization.

Conflict of interest: The authors state that they do not present any conflict of interest.

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Given the limited health care resources, it is important to optimize their allocation. To do so, an adequate stratification of subjects with diabetes by their risk of morbidity, namely DFU and LEA, as well as mortality, is crucial. Thus, identification of variables associated with these outcomes is the first step in the pathway for the creation or optimization of preventive/therapeutic programmes.

Even though the cascade of diabetic foot complications–DFU–LEA has been linked to higher mortality risk (Fortington et al., 2013), an increasing number of DM complications are also associated with higher mortality (Brownrigg et al., 2012). DFU is usually considered a marker of diabetes complication status, *i.e.*, a marker for neuropathy and associated vascular disease in the foot. Still, some authors hypothesized that DFU occurrence could be *per se* an independent predictive variable of LEA as well as mortality (Boyko, Ahroni, Smith, & Davignon, 1996).

Nevertheless, adjustment for baseline complications was rarely conducted when assessing the impact of DFU on LEA, and of both on the mortality risk (Boyko et al., 1996). In addition, simple models for their prediction (specially using the same core variables) were seldom proposed.

Given the current state of knowledge, we considered it essential to 1) estimate the risk at 3 years for DFU, LEA and death in a cohort of patients with diabetes followed in our Diabetic Foot Outpatient Clinic, 2) determine factors that independently predict LEA and mortality using multivariate analysis and 3) determine the ability of the models to discriminate between those who did and did not experience the outcomes of interest.

2. Subjects

A retrospective cohort study was conducted including all subjects with diabetes followed in Centro Hospitalar de Vila Nova de Gaia/Espinho, Entidade Pública Empresarial, Diabetic Foot Outpatient Clinic from the 1st of January 2002 until the 31st of May 2010. Subjects were excluded if they met any of the following criteria: active DFU at the moment of inclusion, inability to ambulate, communication or cognitive impairment (due to aphasia and/or dementia), missing data on any covariate (except for vibration sensation assessed using a tuning fork and HbA1c), follow-up period of less than 3 years, or outside our referral area.

The Diabetic Foot Clinic is a tertiary care unit, with a multidisciplinary team and specialized diabetic foot care, treating patients from primary care institutions (usually with high risk feet and/or unavailable appropriate care in their residence area) or from other departments and hospitals.

The study was approved by the Ethics Committee of our institution and no adverse event occurred in any subject due to participation in this research.

3. Material and methods

3.1. Data collection

Clinical records were reviewed and data collected from 1st until the 30th of June 2013.

All variables were collected in the first podiatric appointment in the clinic, through a structured interview and detailed foot exam, apart from HbA1c, by one of the two department podiatrists who were experienced in the care of diabetic foot complications.

Demographic characteristics (age at the time of inclusion, gender, education level), DM type (classified according to the WHO definition (WHO.org)), duration and treatment (diet only, oral medication or insulin), metabolic control (through HbA1c), physical (inability to reach his/hers own feet (Monteiro-Soares, Boyko, Ribeiro, Ribeiro, & Dinis-Ribeiro, 2012)) and/or visual impairment and smoking habits (absent, current, former) were recorded.

DM complications [retinopathy, nephropathy, neuropathy, cerebrovascular, cardiovascular, peripheral arterial disease and metabolic (ketoacidosis, hyperosmolar coma or other coma)] were classified in accordance to the Diabetes Complications Severity Index created by Young et al. (2008), according to their protocol, using ICD-9 Codes, through clinical record review.

As Young et al. concluded that the accuracy of the number of complications was similar to the Complication Severity Index and as the number of complications is easier to calculate we opted to use it. Nephropathy was also staged by the American Diabetes Association (ADA) classification (American Diabetes Association, 2013), using the serum creatinine value closest to the date of the first appointment.

Participants' feet were characterized using the variables more frequently described in DFU development risk stratification systems (Monteiro-Soares et al., 2011) and with proved association with its occurrence (Monteiro-Soares et al., 2012), namely, the presence of deformities, onychomycosis, diabetic peripheral neuropathy (DPN) [using the Texas Verbal Questionnaire (Armstrong, Peters, Athanasiou, & Lavery, 1998), Semmes–Weinstein monofilament (SWM) insensitivity and tuning fork vibration sensation], peripheral arterial disease (PAD) (characterized by total foot pulses and intermittent claudication), oedema and history of previous DFU or LEA. There is a lack of studies assessing the reliability of these measurements (Monteiro-Soares et al., 2012). Previous DFU was collected through foot assessment, patient self-report and all were additionally confirmed by medical record review. All the above described variables in addition to visual and/or physical impairment, presence of onychomycosis and DFU occurrence were collected and defined according to the protocol previously described by Monteiro-Soares and Dinis-Ribeiro (2010).

Vibration sensation test (VST) was assessed with a 128 Hz tuning fork applied, perpendicularly with a constant pressure, on a bony part on the dorsal side of the distal phalanx of the first toe. This procedure was repeated twice and two incorrect answers were classified as altered sensation (Bakker, Apelqvist, & Schaper, 2012). This procedure was instituted in 2008 and therefore patients entered into the study prior to this time do not have this assessment.

Subjects were followed from the time of inclusion to death or completion of the 3-year follow-up.

Minor LEA was defined as the surgical removal of toe(s), ray(s) or forefoot. Major LEA was considered amputation of the entire foot by any level of the leg (including the ankle).

HbA1c value was not always available ($n = 164$) as several patients were followed for their metabolic control mainly by primary care physicians.

DFU and/or LEA occurrence and death dates were registered. Subjects were advised to contact the clinic if any lesion developed and during appointments they were asked if any DFU occurred. Furthermore, complete medical records from the hospital as well as primary care institutions were reviewed in order to detect missed events. LEA and death (date and cause) are automatically registered in the individuals' computerized clinical file. Death causes were collected using the ICD-9 codes.

3.2. Statistical analysis

Association between variables and outcomes (DFU, LEA or death) was conducted using univariate logistic regression. Values of $p \leq 0.05$ were considered as statistically significant and ≤ 0.1 as pertinent for initial inclusion into the predictive models. Multivariate analysis to estimate odds ratios for and mortality in relation to DFU adjusted for covariates was performed using logistic regression analysis employing a backwards stepwise algorithm approach. In addition, all multivariable models included age, gender and diabetes duration.

After the model creation for each outcome, a multivariable score was computed for each subject using the β coefficient values and the actual values for the covariates for those subjects. The ability of the score to discriminate between patients who did and did not develop

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Table 1
Participants' baseline characteristics.

Variables	Values (n = 644)
Subject characterization	
Age [mean (SD)]	65.1 (11.2)
Female gender [n (%)]	339 (52.6)
Analphabetic or primary school [n (%)]	529 (82.2)
Visual impairment [n (%)]	248 (38.5)
Physical impairment [n (%)]	237 (36.8)
Past or present smoker [n (%)]	134 (20.8)
DM and its complications	
Type 2 [n (%)]	629 (97.7)
Duration (in years) [mean (SD)]	16.1 (10.8)
Insulin use [n (%)]	260 (40.4)
HbA1c (in %) [mean (SD)][a]	7.8 (3.7)
Cardiovascular complications history [n (%)][b]	219 (34.0)
Retinopathy [n (%)][b]	297 (46.1)
Laser photocoagulation [n (%)]	211 (32.8)
Nephropathy [n (%)][b]	98 (15.2)
4–5 stage in ADA classification [n (%)]	37 (5.8)
PVD complications history [n (%)][b]	406 (63.0)
Neuropathy complications history [n (%)][b]	340 (52.8)
Metabolic complications history [n (%)][b]	23 (3.6)
Complications count [mean (SD)][b]	1.7 (1.1)
Foot characterization	
Foot deformity [n (%)]	503 (78.1)
Oedema [n (%)]	165 (25.6)
Onychomycosis [n (%)]	379 (58.9)
Total foot pulses ≤ 2 [n (%)]	241 (37.4)
Intermittent claudication [n (%)][c]	180 (28.2)
DPN symptoms [n (%)]	395 (61.3)
Altered SWM sensation [n (%)][d]	309 (49.6)
Altered VST [n (%)][e]	134 (33.9)
Previous DFU [n (%)]	264 (41.0)
Previous LEA [n (%)]	74 (11.5)

HbA1c: Glycated Haemoglobin, ADA: American Diabetes Association, DFU: Diabetic Foot Ulcer, DM: Diabetes Mellitus, DPN: Diabetic Peripheral Neuropathy, LEA: Lower Extremity Amputation, SD: Standard Deviation, SWM: Semmes–Weinstein Monofilament, VST: Vibration Sensation Test.

[a] 164 missing values.
[b] Using the Young et al. (2008) proposed complications' classification.
[c] 7 indeterminate values.
[d] 21 indeterminate values.
[e] 249 indeterminate/missing values.

the outcomes of interest was assessed using the area under the receiver operating characteristic curve (AUC) with the 95% confidence interval (CI).

All statistical analyses were conducted using the programme IBM SPSS, version 20.0 (Chicago, IL, USA).

Missing and indeterminate results were excluded from analysis.

4. Results

4.1. Participant characteristics

In this study, 644 subjects were included and followed for a median of 36 months (range 1–36).

At baseline, patients had a mean age of 65.1 (±11.2) years; mean diabetes duration of 16.1 (±10.8) years; and mean HbA1c of 7.8% (±3.7). The majority had type 2 DM and less than half were on insulin. More than half were female; over 80% were undereducated (primary school level or less) and over a quarter had some form of impairment (visual and/or physical). The most frequent complications were PAD related (63.0%) and the least frequent was metabolic complication history (3.6%). Forty-one percent of our population had a history of previous DFU (*See* Table 1).

Cumulative incidence at 3 years for DFU and the outcomes of interest was as follows: DFU 26.6% (95% CI 23.2–30.0), recurrent DFU 34.5% (95% CI 27.4–48.4), minor LEA 2.7% (95% CI 1.4–4.0), major LEA 3.1% (95% CI 1.8–4.4), total LEA 5.8% (95% CI 3.9–7.5) and death 14.0% (95% CI 11.3–16.7).

4.2. DFU development risk variables

In univariate analysis, variables associated with DFU occurrence were age, gender, visual impairment, physical impairment, DM duration, retinopathy, nephropathy, PAD complications history, neuropathy complications history, complication count, and all foot characteristic variables except oedema.

In multivariate analysis only physical impairment, PAD complications history, complications count and previous DFU remained statistically significant (*See* Table 2). Using these variables we were able to create a model that discriminated between those patients who did and did not develop a DFU with an Area Under the Receiver Operating Characteristic Curve (AUC) value of 0.80 (See Fig. 1). Considering a simplified model that included complications count and previous DFU only, the AUC value was 0.79 (CI 95% 0.76–0.83) (*See* Fig. 2).

Previous DFU history remained associated with greater risk of incident DFU ($p < 0.001$) even when adjusted for age, gender, visual and physical impairment, diabetes type and duration, PAD complications history, complication count and previous LEA.

4.3. LEA occurrence risk variables

In univariate analysis, variables associated with LEA were gender, visual and physical impairment, cardiovascular complications history, nephropathy, PAD complications history, neuropathy complications history, complication count, two or fewer of four foot pulses, intermittent claudication, altered SWMS and VST, and previous foot complications (DFU and/or LEA).

In multivariate analysis only complication count, two or fewer of four foot pulses and previous DFU maintained statistical significance (*See* Table 2), producing a score with an AUC value of 0.83 for the discrimination between those who did or did not experience an incident LEA (*See* Fig. 1). When using a simplified model, including only complications count and previous DFU, the AUC value was 0.81 (CI 95% 0.74–0.87) (*See* Fig. 2).

Once more, when adjusting for age, gender, physical impairment, diabetes duration, complication count, total foot pulses ≤2 and previous LEA, previous DFU maintained a statistically significant association with LEA risk ($p = 0.001$).

4.4. Death occurrence risk variables

In univariate analysis, variables associated with death were age, visual and/or physical impairment, DM duration, cardiovascular complications history, end-stage renal disease, PAD complications history, complication count, onychomycosis, foot pulses, altered VST and previous DFU.

Age, complication count and previous DFU were the only variables that remained statistically significant in multivariate analysis (*See* Table 2). The resultant predictive model yielded an AUC value of 0.81 in the discrimination between patients who did and did not die during follow-up (*See* Fig. 1). However, using the simplified model including complications count and previous DFU the AUC value dropped to 0.69 (CI 95% 0.63–0.74) (*See* Fig. 2).

Once again, DFU history was associated with a higher mortality rate independent of age, gender, visual and physical impairment, diabetes duration, complication count and previous LEA ($p < 0.05$) (data not shown).

We must highlight that patients developing a DFU during follow-up also had a significantly higher death rate (OR 1.75, CI 95% 1.09–2.79), although the same was not observed when adjusting for

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Table 2
Variables' association with diabetic foot ulcer, lower extremity amputation and death occurrence.

Variables	DFU (n = 171)		LEA (n = 37)		Death (n = 90)	
	Univariate OR (95% CI)	Multivariate OR (95% CI)	Univariate OR (95% CI)	Multivariate OR (95% CI)	Univariate OR (95% CI)	Multivariate OR (95% CI)
Subject characterization						
Age	*1.02 (1.01–1.04)*	NS	1.03 (0.99–1.07)	–	*1.13 (1.09–1.16)*	1.12 (1.09–1.15)
Female gender	*0.49 (0.35–0.70)*	NS	*0.47 (0.23–0.93)*	NS	0.65 (0.41–1.02)	–
Analphabetic or primary school	1.55 (0.95–2.55)	–	1.85 (0.64–5.32)	–	1.10 (0.61–2.00)	–
Visual impairment	*1.60 (1.12–2.28)*	NS	*2.20 (1.12–4.30)*	NS	*1.73 (1.11–2.71)*	NS
Physical impairment	*2.38 (1.67–3.41)*	1.73 (1.15–2.59)	*2.11 (1.09–4.12)*	NS	*1.99 (1.27–3.11)*	NS
Past or present smoker	1.06 (0.78–1.44)	–	1.05 (0.47–2.36)	–	0.92 (0.61–1.39)	–
DM and its complications						
Type 2	2.39 (0.53–10.69)	NS	NA [a]	–	NA[a]	–
Duration (in years)	*1.03 (1.01–1.05)*	NS	1.02 (0.99–1.05)	–	*1.03 (1.01–1.05)*	NS
Insulin use	0.99 (0.70–1.43)	–	1.64 (0.80–3.39)	–	0.94 (0.65–1.60)	–
HbA1c (in %) [b]	1.05 (0.97–1.14)	–	0.99 (0.84–1.16)	–	1.02 (0.97–1.08)	–
Cardiovascular complications history [c]	1.32 (0.91–1.89)	–	*1.91 (0.98–3.72)*	NS	*2.43 (1.55–3.81)*	NS
Retinopathy [c]	*1.62 (1.14–2.31)*	NS	1.57 (0.81–3.08)	–	1.08 (0.69–1.69)	–
Laser photocoagulation	*1.63 (1.14–2.35)*	NS	1.61 (0.82–3.15)	–	0.71 (0.43–1.18)	–
Nephropathy [c]	*1.71 (1.08–2.69)*	NS	*2.56 (1.22–5.38)*	NS	1.63 (0.93–2.86)	–
4–5 stage in ADA classification	*2.81 (1.44–5.50)*	NS	*2.81 (1.03–7.69)*	–	*2.44 (1.14–5.23)*	NS
PAD complications history [c]	*11.03 (6.09–19.96)*	2.52 (1.17–5.45)	*23.06 (3.14–169.31)*	NS	*3.69 (2.03–6.68)*	NS
Neuropathy complications history [c]	*3.14 (2.14–4.60)*	NS	*3.45 (1.55–7.67)*	NS	1.48 (0.94–2.34)	–
Metabolic complications history [c]	0.57 (0.19–1.71)	–	NA[d]	–	2.26 (0.87–5.89)	–
Complication count [c]	*2.03 (1.69–2.43)*	1.31 (1.03–1.67)	*2.17 (1.57–3.01)*	1.74 (1.15–2.62)	*1.76 (1.42–2.18)*	1.50 (1.17–1.94)
Foot characterization						
Foot deformity	*2.03 (1.25–3.25)*	NS	0.74 (0.35–1.57)	–	0.85 (0.50–1.43)	–
Edema	1.10 (0.74–1.63)	–	0.93 (0.43–2.01)	–	1.55 (0.96–2.51)	–
Onychomycosis	*1.75 (1.21–2.53)*	NS	0.58 (0.30–1.12)	–	*1.99 (1.22–3.25)*	NS
Total foot pulses ≤ 2	*3.43 (2.39–4.94)*	NS	*8.04 (3.47–18.62)*	4.17 (1.76–9.88)	*2.51 (1.59–3.94)*	NS
Intermittent claudication [e]	*1.70 (1.12–2.47)*	NS	*2.03 (1.03–3.98)*	NS	1.10 (0.67–1.80)	–
DPN symptoms	*1.52 (1.05–2.20)*	NS	1.53 (0.74–3.14)	–	0.94 (0.59–1.48)	–
Altered SWM sensation [f]	*3.16 (2.16–4.64)*	NS	*3.25 (1.50–7.02)*	NS	1.30 (0.82–2.04)	NS
Altered vibration sensation test [g]	*3.54 (2.21–5.68)*	NS	*5.03 (1.74–14.61)*	NS	*2.38 (1.31–4.33)*	NS
Previous DFU	*8.74 (5.80–13.17)*	4.54 (2.79–7.38)	*10.35 (3.97–26.93)*	5.54 (2.09–14.72)	*2.46 (1.56–3.87)*	1.73 (1.04–2.88)
Previous LEA	*5.12 (3.09–8.47)*	NS	*4.22 (2.02–8.82)*	NS	0.72 (0.33–1.56)	–

–: Not included in the multivariate analysis. HbA1c: Glycated Haemoglobin, ADA: American Diabetes Association, CI: Confidence Interval, DFU: Diabetic Foot Ulcer, DM: Diabetes Mellitus, DPN: Diabetic Peripheral Neuropathy, FU: Follow Up, LEA: Lower Extremity Amputation, NA: Not Applicable, NS: Not statistical Significant association observed, OR: Odds Ratio, SD: Standard Deviation, SWM: Semmes–Weinstein Monofilament.

[a] Model extrapolated values due to reduced number of subjects with diabetes type 1 and no event occurrence in such group.
[b] 164 missing values.
[c] Using the Young et al. (2008) proposed complications' classification.
[d] Model extrapolated values due to reduced number of subjects with history of metabolic complications and no event occurrence in such group.
[e] 7 indeterminate values.
[f] 21 indeterminate values.
[g] 249 missing values.

previous DFU (OR 1.18, CI 95% 0.70–1.99) or among those who had an LEA during follow-up (OR 2.09, CI 95% 0.95–4.58).

The most frequent causes of death were infections (27.8%), oncologic disease (20%), and heart failure (9%) (*See* Fig. 3).

5. Discussion

Several investigations have assessed all-cause mortality in type 2 DM with the derivation and validation of multivariate models (Hayes, Leal, Gray, Holman, & Clarke, 2013; Yang et al., 2008). However, and despite the proved impact of DFU on mortality risk (Monteiro-Soares et al., 2011; Robbins et al., 2008), it was not included in such models.

On the other hand, DFU's link with death occurrence has rarely been adjusted for other pertinent variables (such as age and baseline complications presence) (Boyko et al., 1996).

Therefore, we have conducted this study assessing DFU impact on LEA and death risk, in a large cohort of consecutively enrolled subjects (n = 644), using the STROBE (Vandenbroucke et al., 2007) and STARD (Bossuyt, Reitsma, Bruns, et al., 2003) checklists as the basis for its development and reporting, and conducting adequate statistical adjustment. Moreover, due to the cohort design, observers were blind to outcome occurrence when collecting baseline data.

We observed that the different outcomes on which we focused shared several common predictive variables in univariate analysis, such as physical impairment; cardiovascular and PAD complications history, complications count; total foot pulses number, altered VST and previous DFU. However, few remained statistically associated in multivariate analysis, and different predictors of the outcomes of interest were seen across the three models for the outcomes DFU, LEA, and death, with the exception of complications count and previous DFU. On the other hand, the 3 derived models (using 3 to 5 variables) for each outcome were able to produce high AUC values (from 0.81 to 0.83). A simplified model that included complications count and previous DFU only retained high AUC values for DFU and LEA occurrence (0.79 and 0.81, respectively) but dropped to 0.69 in the case of death. This may be explained greatly by the fact that advancing age is highly and directly linked to death.

This 2 variable model is very simple, uses easily collected data from a clinical appointment, can be employed in every clinical setting, from primary to tertiary care, to identify subjects at higher risk of developing DFU and/or LEA. This may, consequently, lead to increased surveillance of such individuals in order to prevent these complications from occurring. The simplified model to predict death under performs compared to the full model that includes age, so the full model should be used for the prediction of this outcome because it is more accurate.

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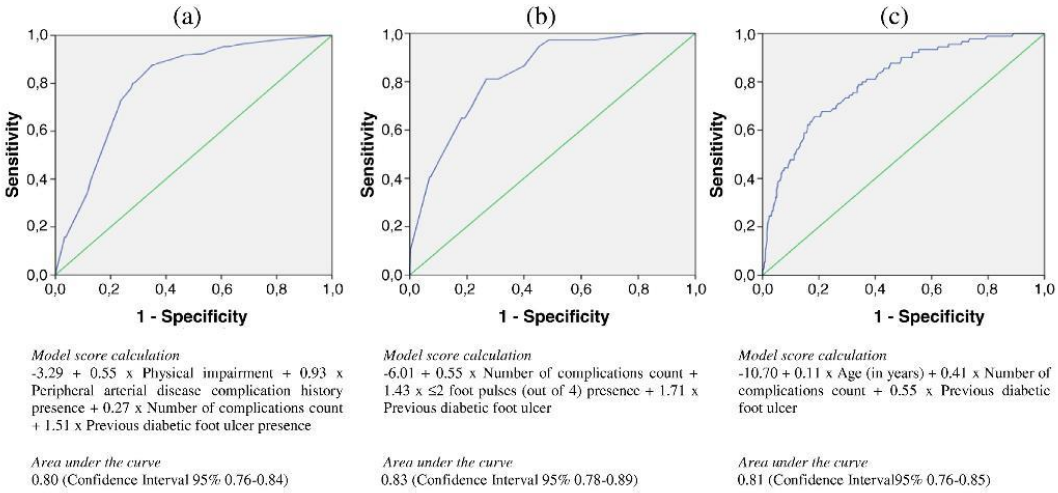

Fig. 1. Receiver operating characteristic curve of predictive models for diabetic foot ulcer (a), lower extremity amputation (b) and death (c) occurrence.

In the multivariate analysis, previous DFU maintained statistical significance for all the outcomes (even when using a broad group of variables for statistical adjustment).

Surprisingly, LEA only achieved statistical significance in the multivariate analysis for DFU occurrence prediction. It was not associated with higher risk of death (not even in the univariate analysis). During follow-up, 10.8% of subjects with a past history of LEA died, in comparison to 24.3% of those requiring any type of LEA during follow-up and 35% in the case of a major LEA.

In 1996, Boyko et al. (1996) assessed the relationship of DFU and mortality also adjusting the risk of death for some variables. However, 98% of the population were men and they tested a smaller range of variables. Cusick, Meleth, Agrón, et al. (2005), also conducted a multivariate analysis evaluating the association between mortality and several diabetes complications in patients with type 1 and type 2 diabetes, although all patients had retinopathy. Moreover, in both articles evaluation of complications was made by assessing the presence or absence of each one at baseline while we in contrast used the validated complication count proposed by Young et al. (2008) (its accuracy was considered similar to the Complication Severity Index).

There is substantial literature on mechanisms to explain many of the associations we describe between the outcomes of interest and predictors. Peripheral arterial disease (or diminished foot pulses as its correlate) has been independently associated with both LEA (Adler, Boyko, Ahroni, & Smith, 1999) and DFU (Boyko et al., 1999), probably due to impairment in wound healing due to inadequate circulation. Diabetes complication count and physical impairment signal greater disease severity, which has also been shown to predict a higher risk of DFU, LEA, and death (Boyko et al., 1999; Cusick et al., 2005; Young et al., 2008). Previous DFU is an instance of a diabetes complication signalling high disease burden of specific importance in the development of foot complications such as future DFU and amputation (Adler et al., 1999; Boyko et al., 1999). In addition, and

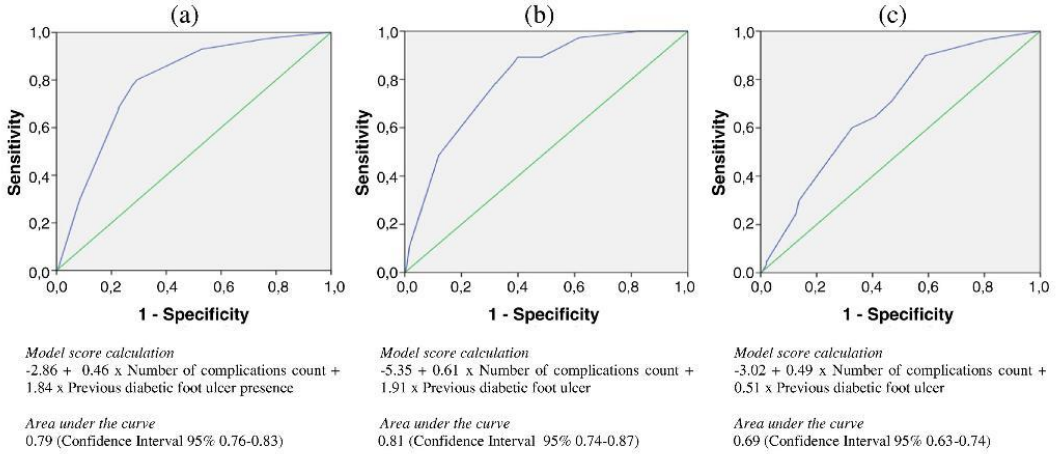

Fig. 2. Receiver operating characteristic curve of predictive models for diabetic foot ulcer (a), lower extremity amputation (b) and death (c) occurrence using only complication count and previous diabetic foot ulcer.

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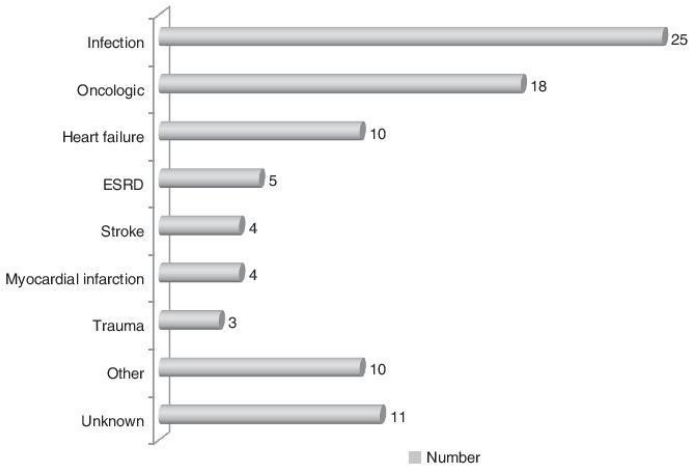

Fig. 3. Causes of death. ESRD: End-Stage Renal Disease. ICD-9 codes verified. Infection 52, 421, 464, 466, 480–488, 490–508, 519, 590, 595, 681, 682, 785. Oncologic 151, 153, 154, 157, 161, 162, 171–174, 185, 188, 189, 191, 203. Heart failure 428. ESRD 250.4, 585. Stroke 434, 436. Myocardial infarction 410. Trauma 800–804, 820–829.

not surprisingly, the higher disease burden also predicts greater mortality (Boyko et al., 1996; Cusick et al., 2005; Ramsey, Newton, Blough, et al., 1999).

Limitations of our study include its retrospective nature, the exclusion of patients outside our direct referral area, the presence and exclusion from analysis of missing data of VST and HbA1c values, as well as indeterminate results for intermittent claudication and SWM sensation.

We must emphasize that, due to the selected design, all the assessed patient-related events (i.e. inclusion, follow-up and determination of outcomes) occurred prior to the research being undertaken.

The VST exam only started in the middle of 2008. Regarding HbA1c values, our hospital is a tertiary care centre for diabetic foot care, but nevertheless a recent HbA1c value (within less than 3 months) was not always available.

Even though there are works addressing the impact of depression in the mortality of patients with DFU (Ismail, Winkley, Stahl, Chalder, & Edmonds, 2007; Winkley, Sallis, Kariyawasam, et al., 2012), this variable is not collected in our daily practice and therefore was not available in the subjects' clinical file for incorporation into prediction models.

We have observed several indeterminate results when assessing intermittent claudication due to the presence of patients that have extremely reduced ambulation and/or symptoms similar to DPN. SWM sensation test result in some patients was difficult to assess due to the presence of several callus/dry skin and patients' automatic and constantly positive response (even when false positive test points were being conducted). In 23 patients, where hallux or transmetatarsal LEA was present in both feet VST was not possible to conduct.

We have decided to use the complication count proposed by Young et al. (2008), instead of the Complication Severity Index. This choice was due to the fact that both report equal accuracy and the first was easier to apply and interpret in our population.

Our data reveal a high rate of DFU development (>8% annually) (Singh et al., 2005), consistent with our high risk referral practice from which we selected study participants, of whom 41.0% had previous DFU.

Our mortality rate is in accordance with the ones described in the literature, namely in the Eurodiale study (Schaper, 2012). In addition, our population has a high rate of comorbidities (13% cardiovascular disease and 63% PVD). Conversely, our LEA rate is inferior to Eurodiale results (Schaper, 2012), as it would be expected, since we started with a population without active DFU while they included only patients with active DFU

The referral nature of the study setting, high prevalence of type 2 diabetes (97.7%), and low education level (82.2% primary school level or less) may limit the generalizability of these results to dissimilar populations.

As stated in the methods section, foot related variables were registered at the first podiatric appointment by one of 2 podiatrists with high experience in diabetic foot using a standardized form. We must highlight that both the professionals and form remained unchanged during the study period. Variables that were collected by clinical interview may present information bias. To overcome this limitation we have searched the clinical file and the national data platform in order to get access to the subjects' most complete and accurate information. For all this and the long study period we believe that misclassification bias may have occurred. However, due to the selected type of study (a cohort) we believe that it was not differential.

Given the retrospective nature of the study we present several variables with missing data, as presented in the tables. However, we must emphasize that there was no missing data for the variables included in the models. Therefore, AUC values and respective 95% CI were calculated using the entire sample. On the other hand, we must highlight that we believe to have identified the great majority (if not all) the outcome events. We have conducted a broad search in the Hospitals' and Health Data Platform (a program with access to data regarding all public healthcare institutions), in which is registered automatically all occurrences of LEA and death. We encouraged subjects to contact our service if any DFU occurred, thus enhancing our ability to capture this outcome.

We only used the ICD-9 codes when considering the cause of death and grouped them, acknowledging the potential limitations of the existing codes.

We conclude that DFU occurrence has a major and independent impact on LEA and death, even when adjusted for baseline complications. Thus the history of a DFU is a marker for poorer outcomes in patients with diabetes in this population. These findings also suggest that DFU prevention may be a potential path for better survival and diminished morbidity in persons with diabetes. New studies are needed in order to better understand this link. In our

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opinion, DFU presence implies a decrease of the subjects' mobility and general well-being and, consequently, of the quality of life, higher infection risk and inflammatory, immune and physiologic changes. All of these most certainly lead to a higher mortality risk.

These models were obtained in a high risk context. So they should be tested in primary care to assess if they are clinically relevant and valid enough *per se*, or if they should be added to pre-existing models/classifications.

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ORIGINAL ARTICLE SUBMITTED FOR PUBLICATION

Title

Improving hyperbaric oxygen therapy referral for diabetic foot ulcer treatment: a nationwide models' validation and refinement study

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Short running title

Hyperbaric oxygen therapy in diabetic foot

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Diabetes · Diabetic Foot · Foot ulcer · Hyperbaric oxygen therapy

ABSTRACT

This study characterizes the population with diabetic foot ulcer (DFU) undergoing hyperbaric oxygen therapy (HBOT) in Portugal over the last 5 years and validates and optimizes the existing models for the prediction of healing of HBOT treated DFU; through a multicentre retrospective cohort study of all patients treated with HBOT from 2008 to mid-2013 in Portuguese continental hyperbaric medicine centres (n=2). We included 128 individuals. 66.4% underwent HBOT in Lisbon's former Navy's Hospital (FNH). Subjects from this hospital presented less frequently retinopathy (19.6% versus 74.5%, $p<0.001$) or previous DFU (29.2% versus 53.5%, $p=0.02$), and had ulcers of shorter duration (median 1.8 months versus 4.0 months, $p<0.001$) than Pedro Hispano's Hospital. Overall, 53.1% healed, which was more likely in non-smoking females without arterial disease, previous DFU or history of lower extremity amputation (LEA). Both completion of the planned series of treatments and increasing number of treatments had a positive impact on outcome. Available models had low predictive accuracy. We propose an optimized version of the Hawkins' model that has higher accuracy. There were some differences in the patients referred to each of these facilities, but healing rates were similar. Further studies are still needed to improve referral criteria for HBOT.

INTRODUCTION

With an estimated 371 million people with *Diabetes mellitus* (DM) around the world, this pathology is considered one of the most frequent metabolic disorders [1].

Hyperbaric oxygen therapy (HBOT) is indicated in chronic diabetic foot ulcers (DFU) with evidence revealing it can decrease the risk of consequent major lower extremity amputation (LEA) [2]. Nevertheless, it is an expensive treatment with limited availability, so identification of patients with best possible response is fundamental for proper allocation of limited healthcare resources.

In Continental Portugal there are only two Hyperbaric Medicine Centres treating patients with active DFU, one in Oporto, located in Pedro Hispano's Hospital (PHH) in Matosinhos (a public civil hospital, referral area from the north to the centre of the country) and one in Lisbon, in the former Navy's Hospital (FNH) (military hospital, referral area from the centre to the south).

In our experience we have noted large variations in the clinical criteria in referring patients for HBOT. To the best of our knowledge, there is no established and validated model specifically created for identifying those patients with DFU who are appropriate for referral to HBOT.

Two models have been reported with the aim of predicting DFU healing for patients undergoing HBOT (Hawkins 2006 [3] and Fife 2007 [4]). However, neither have been externally validated, nor accuracy measures reported. Furthermore, Fife's model includes post-therapeutic variables (namely HBOT session number and treatment interruption) that make it unsuitable for the purpose of clinical decision-making.

Given this uncertainty, we decided to 1) characterize the Portuguese population undergoing HBOT; 2) compare the characteristics of patients and outcomes between the two facilities; 3) identify the subjects with better healing and 4) validate and optimize the existing models. The main goal is to understand Portugal's pattern of DFU referral and subsequent outcome, and to propose an optimal simple referral model.

METHODS

Participants' selection

A multicentre retrospective cohort study was conducted in all Portuguese hyperbaric medicine centres treating diabetic foot patients (n=2) including all subjects that underwent HBOT for DFU treatment from January 1, 2008 to June 30, 2013.

Participants with only one appointment, associated auto-immune diseases, pressure and malleoli ulcers, under treatment at the time of data collection and/or missing data concerning outcome (healing, non-healing, LEA) were excluded.

The study was approved by the Ethics Committee of both institutions.

Data collection

Clinical records were reviewed and data collected from July 1 to the August 31, 2013.

Demographic characteristics (age at the time of inclusion, gender), DM type (classified according to the World Health Organization definition [5]), duration (in years) and treatment (oral medication or insulin), metabolic control [through glycated haemoglobin (HbA1C)], smoking habits (in packs-year) and the character and severity of any DM complications (retinopathy, laser photocoagulation; nephropathy, dialysis; neuropathy; cerebrovascular and/or cardiovascular disease) were recorded.

Factors of interest specific to the DFU were: the presence of peripheral vascular disease (PAD); transcutaneous partial pressure of oxygen ($TcPO_2$); ankle-brachial index (ABI); diabetic peripheral neuropathy (DPN) and previous DFU or LEA. We also collected the ulcer area (in cm^2), reported duration (in months), location, Wagner grade [7], number

of DFUs and the presence or absence of infection. In the case of multiple DFUs, only the largest was considered.

PAD was considered as present when the foot with DFU presented one or fewer pedal pulses [6]. $TcPO_2$ was determined by measuring once at 2 points peri-DFU and reporting the highest value.

DFU area was calculated multiplying the 2 larger axes. Time zero for DFU duration estimation was considered the last major surgical debridement or LEA. In the absence of either, we calculated the actual duration of DFU.

Considering HBOT, we recorded the total number of sessions, whether or not the planned number of sessions was completed and outcome (complete healing *vs.* not healed or LEA).

Complete healing was considered as DFUs full epithelisation without the need of further treatment [8]. Minor LEA was defined as the surgical removal of toe(s), ray(s) or forefoot. Major LEA was considered amputation of any part at or above the entire foot.

Existing models of healing prediction

We conducted a systematic review in order to retrieve all studies proposing predictive models for DFU healing with HBOT. We identified only two: Fife 2007 [4] and Hawkins 2006 [3].

Using the Fife model we can calculate the odds of healing with HBOT compared to the odds of healing without HBOT by using the following formula: Log (OR) = 0.99 + 0.21 x [Ln (HBOT number of sessions + 1)] + 0.004 x ($TcPO_2$ in mmHg) – 0.04 x (RAMP) – 0.15 x (modified Wagner) – 0.04 x (age in years x diabetes duration in years) – 0.19 x (1 if interrupted treatment or 0 if no interruption occurred) where RAMP is a function for pack-years of

smoking (those with ≤10 pack-years = 0; those with >10 pack years = number of pack years – 10) [9]. In Fife's article [4], the authors made use of the Wagner grading scale modified by Kominsky, which varies from II (superficial) to VI (gangrene of the entire foot).

Hawkins' model for the odds of healing with HBOT is: Log (OR) =2.30 – 0.09 x (DFU area in cm^2) – 0.11 x (DFU duration in months) + 0.06 ($TcPO_2$ in mmHg).

Both models were applied to all subjects.

Statistical analysis

For continuous variables, we used Student's t-test to compare groups when the data appeared acceptably normally distributed (using both histogram and Kolmogorov-Smirnov testing) and the Mann-Whitney U-test when the assumption of normality was not appropriate. Categorical variables were compared using the X^2 test or Fisher's exact test, when applicable.

Multivariate analysis, and consequent variables' adjustment and predictive models' proposal, was performed using logistic regression.

All statistical analyses were conducted using the programme IBM SPSS, version 20.0 (Chicago, IL, USA). All tests were two sided, p values less than 0.05 were considered statistically significant and less than 0.1 as pertinent for inclusion in the predictive models.

As Fife's model uses post-treatment variables (such as HBOT number of sessions and treatment interruption) and our goal is to create including model using only pre-treatment variables) we decided to validate and refine the one proposed by Hawkins if pertinent.

Therefore, we started by using all the variables included in the Hawkins model and those considered pertinent for inclusion in our univariate analysis. Missing and indeterminate results were excluded from analysis.

For the resultant optimized predictive model, a prognostic accuracy assessment was made including sensitivity, specificity, predictive values, likelihood ratios (LR), AUC and respective 95% confidence intervals (CI) calculation, comparing the CI and evaluating if there was overlap to see if there were statistically significant differences.

RESULTS

HBOT facilities and program characterization

The center of the FNH (Centro de Medicina Subaquática e Hiperbárica) has been treating DFU patients since January 1990, it has two connected multiplace chambers with capacity to treat 24 individuals simultaneously. The treatment program performed for DFU is 75 minutes at 2.5 ATA, once a day, five days a week.

The centre in the north of Portugal (Unidade de Medicina Hiperbárica of PHH) is equipped with a 16 place chamber, and started treating subjects with DFU in January 2008. The therapy protocol used is 80 minutes at 2.4 ATA, once a day, five days a week.

Patient characteristics (*See table 1*)

This study included 128 patients (85 from the FNH). The majority were males (73.4%), with a mean age of 62.9 (SD11.8) years and 62.7% were active or past smokers. Most had type DM, with a mean duration of 18.2 (SD 9.9) years, and more than half were on insulin, with a mean HbA1c of 8.6% (SD 1.8). The most frequent DM complications were retinopathy [44.7%, of whom more than half (27.7% of the total) had received laser photocoagulation] and nephropathy (34.6%; 12.2% on dialysis).

Most of the subjects had PAD (84.8%) and DPN (89.5%). We observed that in PHH, DPN was diagnosed using the Semmes-Weinstein monofilament, while in the FNH the procedure was never described.

The mean $TcPO_2$ was 33.5 (range 1-76) and ABI 0.78 (SD 0.28) *(data only available from PHH)*.

The median baseline DFU area was 8.2 (range 0.3-60.0) cm^2 with a median duration of 2.5 (0-36) months. The majority were post-LEA (54.7%) and were infected (89.8%). DFUs were classified as Wagner grade III-V in 71.7% of cases. Multiple DFUs were present in 22.1% of patients.

After a mean of 55 HBOT sessions (SD 34.1), 16 (12.5%) subjects required LEA and in 44(34.4%) the DFU remained unhealed. The majority of patients (66.4%) completed the prescribed treatment. The most common reasons for failure to complete were the withdrawal of health administration approval and worsening of the clinical condition.

Subjects from PHH presented more often with retinopathy ($p < 0.001$), previous DFU ($p = 0.02$) and longer DFU duration ($p < 0.001$) than those from FNH. However, PAD was more frequent in those from FNH ($p < 0.001$). No differences were observed between facilities in the remaining variables *(See table 1).*

Predictive variables for DFU healing *(See table 2)*

Univariate analysis

Univariate analysis suggested that the patient characteristics associated with healing were female gender ($p = 0.03$), lower median duration of smoking (pack years, $p = 0.01$), no PAD ($p = 0.002$), no previous DFU ($p = 0.01$) and no LEA ($p = 0.04$).

No macro or microvascular complication had an impact on healing ($p > 0.05$).

Smaller ($p = 0.002$) and more superficial DFU ($p = 0.03$) healed more frequently.

Ulcers that healed received more HBOT sessions on average ($p = 0.07$), while those that completed their planned treatment were more likely to heal ($p < 0.001$).

Multivariate analysis

Following univariate analysis, we performed a multivariate analysis including all factors identified as potentially predictive of healing (p-value on univariate analysis < 0.1) plus the elements in the Hawkins model. We substituted the $TcPO_2$ value in the Hawkins model with PAD (defined as ≤1 pedal pulse in the affected foot) because of the high number of missing values for PtcO2 in our data. Using a backward stepwise elimination approach, we have derived the best model. Twenty-eight subjects were excluded from analysis due to missing data.

Using this methodology, we propose an optimized model, derived from the one proposed by Hawkins in 2006 (3), with a score calculation based on the following equation: Score = 2.96– 1.34 x (≤ 1 pedal pulse) – 0.04 x (DFU area in cm^2) – 0.84 x (DFU graded as Wagner III-V) – 1.15 x (previous DFU – yes= 1, no = 0).

Through ROC curves analysis, we have also proposed the creation of three diagnostic categories for DFU healing and determined cut-off values to maximize predictive ability: healing not probable for values < -0.91, probable for values from -0.91 to 0.36 and highly probable to heal with HBOT for values > 0.36.

Predictive model accuracy

F*igure 1* shows the receiver operating characteristic (ROC) curves for the existing models (Fife 2007 and Hawkins 2006) compared with our optimized model. The AUC values are higher for the latter [0.51% (95% CI 0.24-0.78) in Fife 2007, 0.63% (95% CI 0.37-0.90) in Hawkins 2007 *vs*. 0.78% (95% CI 0.69-0.87)] in optimized model). However, these differences are not statistically significant (*See tables 3).*

Our model had good accuracy values, with LR values having a potential small to moderate effect on clinical decision, with negative LR smaller than 0.5 and positive LR superior to 1.3 [10]. Predictive values (negative and positive) were superior to 60% *(See table 4).*

DISCUSSION

Standardized referral systems are essential for optimal resource allocation and prognostic estimation, especially concerning expensive adjunctive therapeutics (such as HBOT in chronic DFU).

This is the first study evaluating all subjects treated over a period of five years with HBOT for DFUs in continental Portugal.

The sample size is limited (n= 128), and this reduces the power of our analysis to draw definitive conclusions. There are potentially two main reasons for this small sample size. First, HBOT is a poorly understood and promoted treatment modality, and second, even when considered, it is a treatment of last resort given the financial constraints within the healthcare system in Portugal.

Furthermore, we have excluded subjects with associated auto-immune diseases, pressure and malleoli wounds as these ulcers have different clinical and pathophysiological characteristics than the 'standard' DFU, with potential impact on healing rates.

The review period corresponds with the start of DFU treatment at PHH HBOT unit, while the FNH unit has been operating since 1989. This may in part explain the fact that 2/3 of the included subjects were treated at the FNH.

Our population is similar to those reported in the available literature [11-26]; i e, mainly men, above 60 years, with Type-II diabetes for around 15 years and an HbA1c greater than 8%.

In our population almost 30% presented with a Wagner wound grade of I or II, which suggests our population had less severe DFU than the majority of previously reported studies [13,17,22,25,26] but comparable to the largest randomized controlled trial published in this field [11].

The majority of our subjects presented diminished palpable pulses (84.8%) and infected DFU (89.8%) with a median duration of 2.5 months.

We observed a higher percentage of PAD in the FNH participants. This might be due to the fact that in the North (referral area of PHH), there is a lower PAD prevalence [27], but it is also possible that vascular surgeons take a more active approach to chronic DFU patients, including a higher use of angioplasty techniques and revascularization surgery (personal communication).

Conversely, DFU duration was longer at the PHH (median difference of 2.2 months). This is a more recent unit and so HBOT is less widespread among referral healthcare institutions, thus professionals tend to send only patients with DFU persisting despite all other treatment. Additionally, we considered the last major surgical intervention as time 0 for duration estimation, and in the FNH there was a higher rate of post-LEA wounds with immediate referral for HBOT.

We observed that subjects from PHH tended to present higher rates of DM-related complications (retinopathy, nephropathy and stroke). It is not clear if subjects from the North of the country tend to present more complications (although DM duration and HbA1c values were similar) or if the detection rate is superior [28].

However, despite these differences between centres, the healing rates, number of HBOT sessions and proportion who completed the planned treatment are similar.

Eleven studies [3,4,9,11,12,23,29-33] have reported patient factors that may be predictive of healing in DFU treated with HBOT. In our population, clinical factors associated with DFU healing were female gender, lower smoking pack-years, smaller and more superficial DFU as well as absence of PAD, previous DFU and/or LEA. Some of the previous studies have similarly reported both smoking habit and Wagner grade as predictive [4,9,29].

In contrast to Fife 2003, we could not demonstrate an impact of (age + diabetes duration) [4,9,29] , renal failure [4] or previous LEA on outcome. Similarly, in contrast to

Hawkins, we observed a statistically significant predictive ability for gender, DFU area and duration.

During the follow-up period, 12.5% of our subjects required an LEA and in 34.4% DFU persisted unhealed immediately after the last HBOT session. These data are in accordance with several studies, including the two largest randomized controlled trials [11-13, 34-36].

Because the practice in Portugal is to continue wound care in the referring facility, we believe our results confirm a true benefit from HBOT, rather than reflecting any change in wound care management in a specialized unit. Our data also suggest this benefit is greater if the prescribed course of HBOT is completed. While this seems likely, based on common sense, it may also be biased through the tendency for those doing well to keep attending, while those seeing little or no progress may be less inclined to do so. It is worth noting that the mean number of sessions was 55, which is slightly higher than most studies have reported [14-18,21,22,24].

Of the two previously reported predictive models, we observed that both had low accuracy (with the AUC < 0.63), and we considered that optimization of one or both these models was appropriate. Ultimately, we have excluded Fife's model because it also used post-treatment variables (number of sessions and episodes of treatment interruption), making it unsuitable for pre-HBOT decision making – including when it is appropriate to refer for HBOT.

We have therefore optimized the Hawkins model using logistic regression, by including two additional variables (Wagner grade and previous DFU) and removing DFU duration variable. Due to the number of missing values and in order to increase the optimized

models' application in daily clinical care, we decided to replace the variable $TcPO_2$ by the number of palpable pedal pulses.

In this way, we proposed a model easy to apply and tending to produce higher accuracy measures in comparison to the Hawkins' baseline model.

Due to the low sample size and high number of missing values in the models' included variables, confidence intervals were wide and no statistical differences were detected between the original and the optimized models' AUC values. For this reason, we were also unable to compare other accuracy measures.

Evaluating the retrospective performance of our optimized model, we observe that it is characterised by low specificity and LR but also by potentially useful sensitivity and predictive values. We therefore consider that a larger prospective study for model validation and refinement is appropriate, and that such a study should be conducted prospectively to avoid the pitfalls of retrospective data collection.

Abbreviations

ABI	Ankle–brachial Index
AUC	Area Under the Receiver Operating Characteristic Curve
CI	Confidence Interval
DFU	Diabetic Foot Ulcer
DM	*Diabetes mellitus*
DPN	Diabetic Peripheral Neuropathy
FNH	Former Navy's Hospital
HbA1c	Glycated Haemoglobin
HBOT	Hyperbaric Oxygen Therapy
LEA	Lower Extremity Amputation
LR	Likelihood Ratio

OR	Odds Ratio
PHH	Pedro Hispano’s Hospital
PAD	Peripheral Arterial Disease
ROC	Receiver operating characteristic
SD	Standard Deviation
$TcPO_2$	Transcutaneous Partial Pressure of Oxygen

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Table 1. Patient characteristics according to Hyperbaric Oxygen Therapy Unit

Variables	Total (n=128)	Former Navy's Hospital (n= 85)	Matosinhos Hospital (n= 43)	p value
DEMOGRAPHIC AND DIABETES CHARACTERISTICS				
Male [n (%)]	94 (73.4)	65 (76.5)	29 (67.4)	0.30 *
Age (in years) [mean (SD)]	62.9 (11.8)	64.1 (12.1)	60.7 (11.1)	0.13†
Diabetes duration (in years) [mean (SD)]	18.2 (9.9) [a]	17.7 (10.9) [a]	18.7 (8.8)	0.64†
Age+ Diabetes duration [mean (SD)]	1123.3 (655.7) [a]	1109.5 (721.3) [a]	1139.2 (578.3)	0.83†
HbA1C (in %) [mean (SD)]	8.6 (1.8) [b]	8.9 (2.1)[c]	8.6 (1.7)[d]	0.64†
Type 2 diabetes [n (%)]	113 (89.0)[e]	74 (88.1) [e]	39 (90.7)	0.77 *
Insulin use [n (%)]	75 (65.8)[f]	45 (63.4) [f]	30 (69.8)	0.55 *
Smoking habits (in pack-year)[median (range)]	30.0 (0.0-100.0) [g]	38.0 (0.0-100.0)[g]	20.0 (0.0-96.0)	0.12†
MACRO AND MICROVASCULAR COMPLICATIONS				
Retinopathy [n (%)]	42 (44.7)[h]	10 (19.6) [h]	32 (74.5)	**<0.001** #
Laser photocoagulation [n (%)]	26 (27.7)	4 (7.8)	22 (51.2)	
Nephropathy [n (%)]	34 (34.6)[i]	15 (27.3) [i]	19 (44.2)	0.19 #
Dialysis [n (%)]	12 (12.2)	6 (10.9)	6 (14.0)	
Previous stroke history [n (%)]	14 (14.6)[j]	5 (9.4) [j]	9 (20.9)	0.15 *
Previous myocardial infarction history [n (%)]	28 (28.9)[k]	19 (35.2) [k]	9 (20.9)	0.18 *
FOOT CHARACTERISTICS				
0 or 1 foot pulses	106 (84.8)[l]	74 (89.1) [m]	32 (76.2) [e]	**<0.001** #
$TcPO_2$ [median (range)]	33.5 (1.0-76.0)[n]	NP	33.5 (1.0-76.0)[o]	NP
ABI [mean (SD)]	0.78 (0.28)[p]	NP	0.78 (0.28)[d]	NP
DPN [n (%)]	17 (89.5)[q]	8 (80.0) [r]	9 (100.0) [s]	0.47 *
Previous DFU [n (%)]	42 (38.9)[t]	19 (29.2) [t]	23 (53.5)	**0.02 ***
Previous LEA [n (%)]	27 (25.0)[t]	14 (21.5) [t]	13 (30.2)	0.37 *

DFU CHARACTERISTICS				
Area (in cm^2) [median (range)]	8.2 (0.3-60.0)[u]	9.8 (0.4-60.0)[e]	6.4 (0.3-54.0)[v]	0.35§
Duration (in months) [median (range)]	2.5 (0-36)[w]	1.8 (0.0-24.0)[w]	4.0 (1.0-36.0)	**<0.001**§
Multiple DFU [n (%)]	29 (22.7)	20 (23.5)	9 (20.9)	0.83 *
Located at toes *[n (%)]*	19 (14.8)	14 (16.5)	5 (11.6)	0.60*
Post-LEA [n (%)]	70 (54.7)	51 (60.0)	19 (44.2)	*0.09**
Wagner grade III-V [n (%)]	91 (71.7)[e]	62 (73.8) [e]	29 (67.4)	0.54 *
Infection [n (%)]	115 (89.8)	75 (88.2)	43 (93.0)	0.54*
HBOT CHARACTERISTICS AND OUTCOME				
Complete healing [n (%)]	68 (53.1)	45 (52.9)	23 (53.5)	1.00 *
Number of sessions [mean (SD)]	54.9 (34.1)	52.1 (32.0)	60.4 (37.6)	0.19 †
Completed treatment [n (%)]	85 (66.4)	56 (65.9)	32 (74.4)	0.42 *

*: Fisher's exact test; †: t-test for independent samples; # X^2 test for association and trend; § Mann-Whitney test; [a]: 35 missing values; [b]:83 missing values; [c]: 77 missing values; [d]: 6 missing values;[e]: 1 missing value; [f]: 14 missing values; [g]: 51 missing values; [h]: 34 missing values; [i]: 30 missing values; [j]: 32 missing values; [k]: 31 missing values; [l]:3 missing values; [m]: 2 missing values; [n]:104 missing values; [o]: 19 missing values; [p]:91 missing values; [q]:109 missing values; [r]: 75 missing values; [s]: through Semmes-Weinstein monofilament and with 34 missing values; [t]: 20 missing values; [u]: 6 missing values; [v]: 5 missing values; [w]: 27 missing values; HbA1C: Glycated Hemoglobin; ABI: Ankle-Brachial Index; DFU: Diabetic Foot Ulcer; DPN: Diabetic Peripheral Neuropathy; LEA: Lower Extremity Amputation; NP: Not Possible; $TcPO_2$: Transcutaneous Oxygen Tension; SD: Standard Deviation

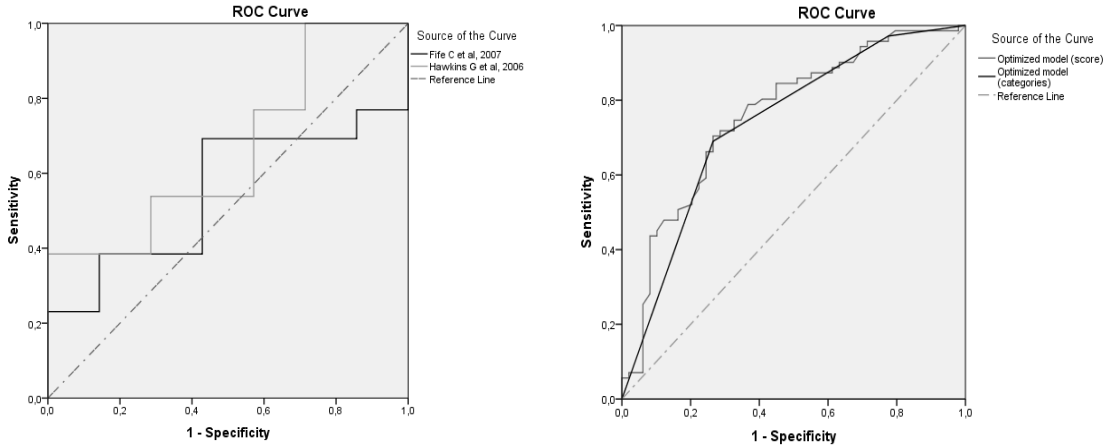
Table 2. Patient characteristics by outcome

Variables	Complete healing (n=68)	Non-healing / LEA (n=60)	p value
DEMOGRAPHICS			
Male [n (%)]	44 (64.7)	50 (83.3)	**0.03 ***
Age (in years) [mean (SD)]	61.4 (12.1)	64.7 (11.4)	0.12 †
Diabetes duration (in years) [mean (SD)]	19.5 (10.5) [a]	16.5 (8.9) [b]	0.15 †
Age * Diabetes duration [mean (SD)]	1178.3 (697.6) [a]	1050.3 (596.6) [b]	0.35 †
HbA1c (in %) [mean (SD)]	8.8 (1.7) [c]	8.5 (1.8) [d]	0.62 †
Type 2 diabetes [n (%)]	57 (85.1) [e]	56 (93.3)	0.17 *
Insulin use [n (%)]	42 (67.7) [f]	33 (63.5) [g]	0.69 *
Smoking habits (in pack-year) [median (range)]	0.0 (0.0-90.0) [h]	40.0 (0.0-100.0) [b]	**<0.001** §
MACRO AND MICROVASCULAR COMPLICATIONS			
Retinopathy [n (%)]	19 (35.9) [a]	23 (56.1) [i]	0.13 #
Laser photocoagulation [n (%)]	11 (20.8)	14 (36.6)	
Nephropathy [n (%)]	16 (28.5) [j]	18 (42.9) [k]	0.30 #
Dialysis [n (%)]	5 (8.9)	7 (16.7)	
Previous stroke history [n (%)]	9 (16.7) [l]	5 (11.9) [k]	0.57 *
Previous myocardial infarction history [n (%)]	14 (25.5) [m]	14 (33.3) [k]	0.50 *
FOOT CHARACTERISTICS			
0 or 1 foot pulses [n (%)]	49 (74.2) [n]	57 (96.8) [e]	**0.002 #**
$TcPO_2$ [median (range)]	21.0 (1.0-76.0) [o]	37.0 (3.0-67.0) [p]	0.52 §
ABI [mean (SD)]	0.9 (0.3) [q]	0.7 (0.3) [d]	0.15 †
DPN [n (%)]	8 (80.0) [r]	9 (100.0) [q]	0.47 *
Previous DFU [n (%)]	16 (27.1) [s]	26 (53.1) [t]	**0.01 ***
Previous LEA [n (%)]	10 (16.9) [s]	17 (34.7) [t]	**0.04 ***
DFU CHARACTERISTICS			
Area (in cm^2) [median (range)]	6.1 (0.3-41.0) [n]	12.0 (0.8-60.0) [u]	**0.002 §**
Duration (in months) [median (range)]	2.0 (0.0-30.0) [v]	3.0 (0.0-36.0) [w]	0.14 §
Multiple DFU [n (%)]	15 (22.1)	14 (23.3)	1.00 *

Located at toes [n (%)]	13 (19.1)	6 (10.0)	0.21*
After LEA [n (%)]	33 (48.5)	37 (61.7)	0.16*
Wagner grade III-V [n (%)]	43 (63.2)	48 (81.4) [e]	**0.03 ***
Infection [n (%)]	58 (85.3)	57 (95.0)	*0.08 **
HBOT CHARACTERISTICS			
Matosinhos' Hospital [n (%)]	23 (33.8)	20 (33.3)	1.00 *
Number of sessions [mean (SD)]	60.1 (32.8)	40.0 (3.0-120.0)	*0.07 †*
Completed treatment [n (%)]	65 (95.6)	23 (38.3)	**<0.001 ***

*: Fisher's exact test; †: t-test for independent samples; # X^2 test for association and trend; § Mann-Whitney test; [a]: 15 missing values; [b]: 20 missing values; [c]: 43 missing values; [d]: 40 missing values; [e]: 1 missing values; [f]: 6 missing values; [g]: 8 missing values; [h]: 31 missing values; [i]: 19 missing values; [j]: 12 missing values; [k]: 18 missing values; [l]: 14 missing values; [m]: 13 missing values; [n]: 2 missing values; [o]: 59 missing values; [p]: 45 missing values; [q]: 51 missing values; [r]: 58 missing values; [s]: 9 missing values; [t]: 11 missing values; [u]: 4 missing values; [v]: 17 missing values; [w]: 10 missing values; HbA1C: Glycated Hemoglobin; ABI: Ankle-Brachial Index; CI: Confidence Interval; DFU: Diabetic Foot Ulcer; DPN: Diabetic Peripheral Neuropathy; LEA: Lower Extremity Amputation; $TcPO_2$: Transcutaneous Oxygen Tension; SD: Standard Deviation;

Figure 1. Predictive models' receiver operating characteristic curve



The left image represents the Fife 2007 (in black) and Hawkins 2006 (in grey) score area under the receiver operating characteristic curve. The right image represents the optimized model scores (in blue) and categories (in green) area under the receiver operating characteristic curve.

Table 3. Predictive models' area under the receiver operating characteristic curve

Model	**AUC**	**95% CI**
Fife C et al, 2007 [a]	0.51	0.24-0.78
Hawkins G et al, 2006	0.63	0.37-0.90
Optimized model 1[c]		
Score	0.78	0.69-0.87
Categories	0.75	0.65-0.85

[a]: 104 missing values; [b]: 107 missing values; [c]: 28 missing values; AUC: Area Under the Receiver Operating Characteristic Curve; CI: Confidence Interval

Table 4. Optimized models' prognostic accuracy measures

	Subjects	Healed	Accuracy measures					
			Sens	Spec	LR+	LR-	PPV	NPV
	n (%)	n (%)	% (CI 95%)	% (CI 95%)	% (CI 95%)	% (CI 95%)	% (CI 95%)	% (CI 95%)
OPTIMIZED MODEL 1 [a]								
Highly probable	48 (48.0)	38 (79.2)	67.9 (55.6-80.1)	77.3 (64.9-90.0)	3.0 (1.7-5.3)	0.4 (0.3-0.6)	79.2 (67.7-90.7)	65.4 (52.5-78.3)
Highly probable+ Probable	87 (87.0)	54 (62.1)	96.4 (91.6-100.0)	25.0 (12.2-37.8)	1.3 (1.1-1.5)	0.1 (0.03-0.6)	62.1 (51.9-72.3)	81.6 (65.0-100.0)

[a]: 28 missing values; CI: Confidence Interval; Spec: Specificity; Sens: Sensitiviy; LR-: Negative Likelihood Ratio; LR+: Positive Likelihood Ratio; NPV: Negative Predictive Value; PPV: Positive Predictive Value

ORIGINAL ARTICLE SUMBMITTED FOR PUBLICATION

Title

Hyperbaric oxygen therapy in clinical outcome of patients with diabetic foot ulcers: local and systemic improvement

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ABSTRACT

Oxygenation of diabetic foot ulcer (DFU) by hyperbaric oxygen therapy (HBOT) promotes wound healing. However, human studies regarding HBOT local and systemic efficacy are scarce. Herein, we aim to 1) compare serum biochemical and angiogenic biomarkers in patients with and without active DFU; 2) assess HBOT efficacy in DFU reduction and closure, in serum markers modulation and in local microvasculature. We conducted a non-randomized trial enrolling a group of patients without active DFU (n=5) and one with active DFU (n=20). We compared those treated with HBOT (n=14) with untreated ones (n=6). Endpoints (at 3, 6 and 12 months) included lab markers, clinical outcome, DFU size. The groups were identical with a sample mean age of 62 yrs. and 18-year DM duration, mostly men, with type 2 DM, insulin-treated, with several complications and acceptable glycaemic and lipid control. Patients had significant serum leukocyte, C-reactive protein levels, and all DFU measurements reduction 3 months after HBOT. At every time-points, HBOT group achieved less amputation and death rates and DFU reduction with increasing epithelialization percentage. Microvessels in the DFU were increased upon 1 month HBOT. Our data reinforces the potential molecular and clinical efficacy of HBOT when added to current standard DFU treatment.

INTRODUCTION

Worldwide, diabetes mellitus (DM) is one of the most frequent metabolic disorders (1,2) and its global burden is attributed to its several complications, namely diabetic foot ulcers (DFU) with impaired healing that frequently require lower extremity amputation (3,4). DM leads to defective wound repair due to alterations in the micro- and macro-vasculature, cellular and molecular environment, where neuropathy and frequent infection also play a role (4-6).

Wound healing is divided in four different phases: haemostasis, inflammation, proliferation and tissue remodelling (7), which mostly depend on oxygen content (7-10). Therefore, tissue vascularization to support oxygen availability is of paramount importance for the whole wound healing process.

In adults, neovascularisation can occur through angiogenesis, by migration and in situ differentiation of mature resident endothelial cells, and/or from bone marrow-derived undifferentiated angioblasts or endothelial progenitor cells (EPC) (11,12).

Interestingly enough, in diabetic organs like retina, angiogenesis is exacerbated, while in others, for instance distal microvasculature of the inferior limbs, it is decreased (6,13).

DFUs frequently evolve to chronic wounds, which are lesions that do not heal in the usual time (4-6 weeks) with conventional therapy. Local hypoxia is common in this situation with tissue oxygen pressure (pO_2) scarcely reaching 10 to 30 mmHg, while optimum values range from 50 to 100 mmHg (8,13). These features led to the use of hyperbaric oxygen therapy (HBOT), which consists in the administration of oxygen in an hyperbaric chamber, at pressures usually between 1,5 and 3 ATA (atmospheres absolute) (152,0 – 304,0 kilopascal (kPa)). In agreement, a recent systematic review demonstrated that HBOT was associated with a significant reduction in the risk of major LEA in subjects with chronic DFU (14). These findings are also corroborated by animal studies that point out that HBOT stimulates healing namely by improving neovascularisation through several mechanisms, including growth factors synthesis in the wound bed and recruitment of EPC (4,6,15,16). However, studies in human subjects with diabetes and active DFU are still lacking.

Given the current state of knowledge, the aim of the current study are: 1) to quantify systemic angiogenic, vasculogenic and biochemical markers in subjects with diabetes with and without active DFU; 2) to examine the efficacy of HBOT through analysis of its impact in a) ulcer reduction

and closure, b) serum markers modulation and c) the microvasculature improvement of the ulcer bed.

Material and Methods

Study design and participants selection

A non-randomized clinical trial was conducted at Centro Hospitalar de Vila Nova de Gaia/Espinho, EPE (Entidade Pública Empresarial) Diabetic Foot Outpatient Clinic, which is a tertiary care unit with a multidisciplinary team. The clinical trial was registered in the Brazilian Clinical Trial Registry (Registro Brasileiro de Ensaios Clínicos) number UTN U1111-1146-8232 and approved by the Ethics Committee of our institution.

Subjects with active DFU, a full-thickness skin defect distal to the malleoli requiring more than 15 days to heal (17), that, after 8 weeks of standard treatment (including angioplasty and/or revascularization surgery if needed), had no significant wound improvement (no healing or ulcer area reduction < 30%) were consecutively proposed to HBOT.

Participants were included from the October 1st, 2010 until the December 31st, 2012.

HBOT proposal was performed in accordance to the multidisciplinary team and financial department approval. Team decision relied on selecting patients that would most benefit of healing, excluding namely patients bedridden and dependent on third person for daily life activities. Therefore, HBOT was used, depending on hospital economic resources and availability of the HBOT facility, as last resource in people that despite maximized macrovascular blood flow had no healing. These participants were divided in two groups: HBOT – treated with HBOT and non-HBOT – patients that refused the treatment or had a contra-indication.

Both groups with DFU were compared with a group of non-DFU diabetic subjects that were participating in an educative program on diabetes in our department.

HBOT was performed in the referral area HBOT center (Unidade de Medicina Hiperbárica of PHH) according to the used treatment protocol - 80 minutes at 2.4 ATA (243.2 kPa), once a day, five days a week, up to a maximum of 100 sessions.

Standard care was conveyed in the DFU groups by a team independent of the investigators. In case of multiple ulcers, evolution of the larger DFU was evaluated through the decrease in the wound measures.

All subjects were followed for twelve months to evaluate healing in the DFU groups and the potential occurrence of lesions in the non-DFU subjects.

Participants and diabetic foot ulcer characteristics

At enrollment, the following demographic characteristics were collected: age at the time of inclusion; gender; DM type (classified according to the World Health Organization definition (18)), duration (in years) and treatment (oral anti-diabetic agents or insulin); metabolic control (through glycated haemoglobin (HbA_{1c})); smoking habits; presence of any DM related complications (namely, retinopathy, nephropathy, neuropathy, cerebrovascular, cardiovascular and/or PAD and metabolic), were registered in accordance to the definition used in the Diabetes Complications Severity Index created by Young et al (19).

For the foot characterization we used the presence of PAD, when only one or fewer pedal pulses were palpable on the DFU foot (20) and/or the ankle-brachial index (ABI) was inferior to 0.8 (21); TcPO2, determined by measuring once at 2 points peri-DFU and reporting the highest value; DPN, defined as inability to feel the SWM at one or more of 4 specific sites on the foot (22); and previous DFU or LEA. We also recorded the ulcer area (in cm^2), reported duration (in months), location, Texas University classification (TUC) (21), number of DFUs and the presence or absence of infection.

DFU photographic and dimensional records (area, maximum and mean depths and volume) were performed using a digital wound measurement device (Aranz Medical Silhouette Mobile TM), at baseline, and if still with active DFU, at months 3, 6 and at the end of follow-up (12 months). Based on the area measurements, percentage of epithelialization was calculated at the same endpoints.

Considering the HBOT description, the total number of sessions, whether or not the planned number of sessions was completed and side-effects were registered. Complete healing was considered whenever the DFU presented full epithelialization without the need of further dressing (23). Minor LEA was defined as amputation distal to or including the forefoot and major LEA was considered amputation above or by the ankle (24).

Clinical outcome occurrence was also assessed at 3, 6 and 12 months.

Laboratory analysis of serum parameters

In all three groups (non-DFU, HBOT, non-HBOT) blood samples were collected at baseline and 3 months after, in the HBOT group a sample was drawn also at 6 months. Additionally, in the DFU groups ulcer debridement material was collected at 0 and 1 months. Blood samples were collected to EDTA (ethylenediamine tetraacetic acid) and gel and clot activator tubes.

Full blood count was performed using an automated hematology analyzer XE 2100 or 5000 (Sysmex Corporation, Norderstedt, Germany).

Serum was analysed for glucose, urea, creatinine, total proteins, albumin, lipid profile, uric acid and C-reactive protein (CRP) with the Cobas 8000c701 (Roche Diagnostics; Hitachi High-Technologies Corporation, Tokyo, Japan).

HbA_{1c} was determined through high pressure chromatography in the Horiba Medical G7 device (Horiba Medical; Horiba ABX SAS, Kyoto, Japan).

Microalbuminuria level was evaluated by immunoturbidimetry with Cobas 6000 c501 analyzer (Roche Diagnostics; Hitachi High-Technologies Corporation, Tokyo, Japan).

Angiogenic (VEGF, PlGF) and vasculogenic (SDF1-α) markers were determined by enzyme-linked immunoabsorbent assay (ELISA) multiplex using Quantikine ELISA Immunoassay kits (R&D Systems, Abingdon, United Kingdom (UK)), according to manufacturer instructions.

Immunohistochemistry assays

Ulcer bed tissue was collected, when DFU debridement was performed, for histological and immunohistochemistry studies. The tissue specimens were fixed in 10% neutral-buffered formalin solution and paraffin-embedded. Three-micrometer sections were stained with hematoxylin and eosin (H&E) or used for capillary endothelial cells immunostaining. Endogenous peroxidase activity was blocked with 4% hydrogen peroxide in phosphate buffered saline (PBS) for 30 minutes at room temperature. To retrieve antigen, sections were placed in 10 mM citrate buffer (pH = 6) and heated at 98°C. After blocking with 10% bovine serum albumin (BSA) in PBS for 1 hour, sections were incubated with primary antibody against Cluster of differentiation 31 (CD31) (1:100) (Abcam, Cambridge, UK) overnight at 4°C. Then, anti-rabbit secondary antibody (1:200) (Santa Cruz Biotechnology, USA) was applied for 30 minutes. Avidin Biotin Complex (ABC) complex method (Vectastain ABC kit, Vector, Burlingame, CA, USA) was used according to the

manufacturer's instructions. The antigen-antibody reaction was developed using diaminobenzidine (DAB) (DAB substract kit, Abcam, Cambridge, UK) as peroxidase substrate, rendering CD31 positive cells with a brown staining. Sections were counterstained with hematoxylin (Sigma-Aldrich, Portugal), dehydrated and coverslipped. CD31-expressing microvessels were counted in the three most vascularized areas with magnification of 200 x, and the data were averaged and normalized to the total area of the tissue section. Any positive-staining endothelial cell or endothelial cell cluster that was separated from adjacent microvessels was considered an individual vessel (25).

Every laboratory and molecular studies were performed by investigators blinded to the subjects' group allocation.

Statistical analysis

Continuous variables will be described by mean and standard deviation (SD), in the case of having normal distribution, or median and range otherwise. Normality of the distribution will be assessed through the histogram analysis.

Comparison between 2 groups will be conducted with t-test for independent samples or Mann-Whitney U test, according to the variable distribution. For the comparison between 2 time-points in the same group we used the t-test for paired samples or Wilcoxon signed rank test, when pertinent. For the comparison between 3 time-points, the Wilcoxon signed rank or the Friedman test were used, according to the variable distribution.

For differences' evaluation between variables in the 3 groups, the One-Way ANOVA test (using the Bonferroni correction) or the Kruskall-Wallis test were applied.

For categorical variables description, frequency and percentage were used and, for association analysis, the X^2 or Fisher's exact test (when applicable).

All tests were two sided, p values less than 0.05 were considered as statistically significant.

Statistical analysis was performed using the IBM SPSS version 22.0 (Chicago, IL, USA).

Results

Participants characteristics

A total of 25 patients were included in the study: 5 in the non-DFU, 6 in the non-HBOT DFU and 14 in the HBOT DFU groups described in Table 1. The DFU participants were allocated not to undergo HBOT due to refusal of treatment in 5 patients and 1 for presenting a contra-indication. There was no significant difference between groups regarding mean age and disease duration.

Taken the whole group of patients into account, the mean age in our sample was 62 years and DM duration 18 years, and the majority were male, with type 2 DM treated with insulin, with visual and physical impairment. Concerning DM complications, most had retinopathy, PAD complications and neuropathy. The mean complications count was 4 and the non-DFU group had significantly less PAD complications and tended to present less frequently the remaining complications.

The non-DFU group was composed mainly by female subjects by way of chance, such difference presented statistical significance.

Concerning the total foot pulses number, non-DFU persons had a significantly higher number comparing to both DFU groups. Intermittent claudication was significantly more common in the non-HBOT DFU participants, as they tended to present more PAD complications, ischemia diagnosed by pulses palpation and statistically significant less frequently DPN diagnosed by SWM.

Almost all DFUs reached the bone and were infected and ischemic. Several were post-minor LEA and 25% were located in the toes.

Laboratory markers

At the beginning the study, patients presented a mean HbA_{1c} of 7.9-8.5%, total cholesterol around 170 mg/dl and triglycerides ranging from 112 to 146 mg/dl (Table 2). These findings indicated that glycaemic control and lipid profile were acceptably controlled in the three groups of patients.

Non DFU patients did not exhibit microalbuninuria. Interestingly, the HBOT DFU group had significantly less microalbuminuria at baseline comparing to non-HBOT DFU subjects.

DFU subjects presented normal total proteins, albumin and uric acid serum levels (data not shown).

After 3 months of therapy, HBOT DFU individuals presented significantly lower leukocyte and CRP level, and tended to have lower VEGF levels, when compared to NHBOT DFU subjects.

No statistically significant differences were found for the remaining laboratory markers (values are described in Table 2).

Clinical outcome

Three months after HBOT, there were significant differences in the clinical outcome between DFU subjects who underwent HBOT as compared to non-treated patients. There were four LEA (three major and one midfoot) in the non-HBOT DFU group. One patient died in the early post-operative period and another from lung cancer. In addition, one subject died after refusing major LEA (Table 3). In contrast, all HBOT DFU participants improved or presented complete healing upon 3 and 6 months treatment, and none required LEA. However, three patients died 12 months later due to DFU non-related causes. Altogether these findings reveal that statistically significant differences between groups at every endpoint were observed.

Diabetic foot ulcer characterization

We next examined DFU features in NHBOT and HBOT patientsAt baseline, DFUs had equivalent dimensions in the two groups of patients (Table 4). As only one subject remained alive and without major LEA at the 3rd month in the non-HBOT DFU, no comparison was possible between baseline at this time-point. Nevertheless, this patient exhibited a larger and deeper ulcer when comparing to the median values in the HBOT DFU group.

All measurements (mean depth, maximum depth and volume of DFUs), presented or tended to present significantly lower values at the 3rd month of HBOT when comparing to baseline.

Considering long-term follow-up, the remaining non-HBOT DFU subject healed 6 months after the beginning of the study, again, impeding the comparison between groups (Table 5).

In the HBOT DFU group, all measurements improved at months 6 and 12 (p=0.006 for maximum depth; p=0.001 for mean depth and DFU volume).

Percentage of epithelialization

To determine whether complete healing was achieved, epithelialization was then addressed in the two DFU groups. The only subject remaining with active DFU at month 3 presented a median percentage of epithelialization of 79.4%. The DFU was completely healed at 6 months and remained 100% epithelialized at 12 months.

Conversely, in the HBOT DFU group, significant and gradual improvement was observed in all three time points (median percentage of epithelialization of 64.4%, 85.7% and 100.0% at months 3, 6 and 12 respectively, p=0.001) for all subjects.

Vessel density evaluation

To examine whether HBOT improved microvasculature within the DFU, microvessel density was assessed. As DFU bed tissue was gathered only when debridement was performed, not all collected samples presented viable tissue. Therefore, immunohistochemistry for CD31-expressing microvessels samples paired by subject, were only possible in 3 and 6 patients in the non-HBOT DFU and HBOT DFU groups, respectively.

In the HBOT group the mean number of vessels tended to increase after 1 month (617 vs 709/three 200x magnification fields). In contrast, a slight reduction was observed in the number of microvessels for the non-HBOT DFU patients (746 vs 680/three 200x magnification fields), not achieving, however, statistical significance (Figure 1). Figure 2A-2D illustrate the immunohistochemistry results.

Discussion

Several studies have addressed HBOT impact on DFU healing (14). However, this is the first study to simultaneously evaluate and compare biochemical markers and clinical outcome in subjects with no DFU and with DFU undergoing or not HBOT.

Our sample presented a mean age, gender, DM duration and HbA_{1c}, similar to the populations reported in randomized controlled trial (RCT) (26-33) and non-randomized trial (NRT) (34-37). Median TcPO2 was lower than in most studies (31,34,38,39). Regarding depth, we included deeper DFUs than RCT (21,28,31) but similar to NRT (34,36,37,40). Therefore, our subjects present comparable or worse baseline prognosis to the ones in previous studies from the literature.

Interestingly, at every time-point, the HBOT group achieved better outcomes when compared to those with DFU without undergoing such adjunctive therapy. Less major LEA rates, less death rates and longer survival rates were observed.

Assessing all DFU measures, a high DFU reduction and an increasing epithelialization percentage were observed along the whole study period in the HBOT group. Analysing the RCT and NRT in the literature, we noticed that our clinical outcome rates were equivalent (within the 95% CI) to those reported by the long term, at least 12 months, to RCT and NRT for both groups (26-28,32,35,38-40).

During a mean number of 86 HBOT sessions, a gradual DFU improvement, continuing after the prescribed therapy program completion, was observed. Comparing to the available literature (26,28,35,37-40), we realized that our patients were exposed to a higher number of sessions, which may have positively affected the results.

Our findings showed that in patients submitted to merely 3 months of HBOT exhibited a significantly lower leukocyte and CRP level. These findings support the evidence that HBOT results in a reduction in infection and inflammatory response (41,42).

Another effect widely suggested in the literature is the improvement of the reduced vascularization within DFU tissues. Consequently, we examined whether HBOT influenced systemic VEGF levels. Our results indicated that DFU patients have higher levels of VEGF when compared to non-DFU. Serum VEGF diminished to values similar to those in the non-DFU group in the HBOT group, although not reaching statistical significance. Interestingly, these VEGF levels decreased upon HBOT. Since VEGF is up-regulated by hypoxia and by inflammatory

environment, VEGF reduction may be explained by a decrease in hypoxia as well as in inflammatory factors, which altogether led to “normalized” VEGF levels.

SDF1-α is involved in the recruitment of circulating bone marrow-derived EPCs, which are known to be decreased in diabetes. We further investigated this growth factor in sera of non-DFU and DFU individuals submitted or not to HBOT. In the HBOT group, SDF1-α increased at month 3, returning to baseline levels at month 6. Since at 3 months all subjects received HBOT, and at 6 months, all had finished the treatment, we hypothesize that HBOT augments SDF1-α values, with a possible rise in the number of circulating EPCs, but this effect halts with treatment suspension (43).

DFU healing is accompanied by tissue vascularization. To further investigate the effect of HBOT in the tissue, we evaluated the number of vessels within the DFU throughout the study. Despite a reduction in systemic VEGF levels was observed after 3 months treatment, DFUs exhibited a tendency towards increased median number of vessels in the HBOT group one month upon treatment initiation, while the opposite tendency occurred in the non-HBOT DFUs. These findings imply that locally, at the DFU tissue, HBOT improves vascularization. These conflicting results (reduced VEGF serum levels and increased microvessel density in DFU of HBOT subjects), can be explained by the fact that local environment plays a crucial role in the angiogenic process in diabetic patients. This assumption comes in line with the well-established angiogenic paradox hupothesis, which highlights that the same patient may present exacerbated angiogenesis in organs like retina and kidney, and simultaneously angiogenesis impairment in others (e.g. limbs and myocardial ischemia) (44)

Infection and PAD are considered by several classifications as the most important factors along with wound depth, for DFU prognostic assessment (45). HBOT improves circulation by stimulating micro-vascularization and optimizes oxygen delivery ameliorating leucocytes function and anti-bacterial effect. We believe that such mechanisms are responsible for such good clinical outcomes and are evident in our results.

Remarkably, only one subject suffered a minor side effect due to HBOT (ear barotrauma), that responded to treatment. Thus, these findings reinforce the role of this treatment in diabetic patients exhibiting aggressive DFU.

According to our non-randomized trial study, HBOT represents a more feasible methodology in clinical practice for DFU patients. However, it presents several limitations. DFU control group (non-HBOT subjects), was selected by refusal or contra-indication, instead of using randomization techniques. This may lead to a selection bias. Comparing baseline characteristics, we observed that the main difference lies in the fact that there was significantly more history of

previous DFU in the non-HBOT DFU individuals than in the HBOT DFU. This might be one of the possible reasons for them to refuse HBOT as they might be depressed (46) and tired of long-term unsuccessful therapies. Moreover, a small sample size was achieved, due to limited selection of adequate patients and financial constraints. Nevertheless, significant results were obtained for several measures.

Due to ethical reasons, we were unable to perform DFU biopsy. Small fragments were collected from wound debridement. Therefore, not all collected samples were representative of DFU bed tissue, limiting the evaluation of microvessel density. Despite this, it appears that HBOT DFU subjects tend to have an increase in vessel number after 1 month of therapy, with the opposite occurring in the non-HBOT group.

In order to overcome the described weaknesses of the study, the selection of DFU individuals for HBOT was performed by an independent team, including only patients with optimized standard care and still no DFU improvement; further, laboratory/molecular and microvessel density evaluation analysis were performed by blinded researchers; clinical end-points were objective (percentage of ulcer healing, amputation and death) and all DFU measurements were performed using a digital laser measuring device.

Due to defined criteria, our diabetic foot clinic team referred for HBOT mainly DFUs that reached the bone, were infected, ischemic (grade III and stage D in the UTC) and after minor LEA has occurred. This fact may limit the results' generalizability and it may diminish the magnitude of effect. Accordingly, if such clinical results were observed, an even better outcome in a less severely affected population is expected.

In conclusion, our data reinforces the potential molecular and clinical efficacy and benefit of HBOT when added to current standard treatment of DFU. But further studies are required, particularly, increase the total number of patients in whom angiogenic, vasculogenic and inflammatory markers are studied not only in serum but also in the DFU bed; and evaluation of the HBOT modulator effect on DFU bed tissue using an animal model. These would highlight the molecular mechanisms involved.

It is also crucial to define the optimal HBOT timing after revascularization for DFU treatment, since revascularization improves macrovascular disease and HBOT seems to ameliorate microvasculature.

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LIST OF ABBREVIATIONS

ABI	Ankle–brachial Index
ATA	Atmospheres Absolute
CRP	C-reactive Protein
DFU	Diabetic Foot Ulcer
DM	*Diabetes mellitus*
DPN	Diabetic Peripheral Neuropathy
EPC	Endothelial Progenitor Cells
ESR	Erythrocyte Sedimentation Rate
HBOT	Hyperbaric Oxygen Therapy
H&E	Haematoxilin-eosin
HIF-1	Hypoxia Inducible Factor 1
kPa	Kilopascal
LEA	Lower Extremity Amputation
non-HBOT	Non-Hyperbaric Oxygen Therapy
PlGF	Placental Growth Factor
PAD	Peripheral Arterial Disease
pO_2	Tissue Oxygen Pressure
SDF1-α	Stromal Derived Factor-alfa
$TcPO_2$	Transcutaneous Partial Pressure of Oxygen
VEGF	Vascular Endothelial Growth Factor

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Table 1. Participants baseline characteristics

Variables	Global (n=25)	Non-DFU (n=5)	NHBOT DFU (n=6)	HBOT DFU (n=14)	p value
SUBJECT CHARACTERIZATION					
Age (mean (SD))	62 (12)	68 (10)	63 (11)	61 (13)	0.6*,a
Male gender (n (%))	18 (72)	1 (20)	6 (100)	11 (79)	**0.009†/0.006¥**
Visual impairment (n (%))	19 (76)	3 (60)	5 (83)	11 (79)	0.6†/0.5¥
Physical impairment (n (%))	16 (64)	3 (60)	4 (67)	9 (64)	0.7†/0.9¥
Past or present smoker (n (%))	13 (52)	1 (20)	4 (67)	8 (57)	0.3†/0.3¥
DM AND ITS COMPLICATIONS					
Type 2 (n (%))	23 (92)	4 (80)	6 (100)	13 (93)	0.5†/0.5¥
Duration (in years) (mean (SD))	18 (9)	26 (19)	15 (7)	20 (10)	0.3*, a
Insulin use (n (%))	17 (68)	3 (60)	5 (83)	9 (64)	0.6†/0.9¥
Cardiovascular complications history (n (%))	7 (28)	1 (20)	1 (17)	5 (36)	0.6†/0.4¥
Retinopathy complications history (n (%))	21 (84)	3 (60)	6 (100)	12 (86)	0.2†/0.3¥
Nephropathy complications history (n (%))	12 (48)	1 (20)	5 (83)	6 (43)	*0.1†*/0.7¥
Cerebrovascular complications history (n (%))	3 (12)	0 (0)	0 (0)	3 (21)	0.3†/0.1¥
PAD complications history (n (%))	18 (72)	1 (20)	6 (100)	13 (93)	**0.001†/0.003¥**
Neuropathy complications history (n (%))	22 (88)	5 (100)	4 (67)	13 (93)	0.2†/1.0¥
Metabolic complications history (n (%))	11 (44)	2 (40)	2 (33)	7 (50)	0.8/†/0.6¥
Complications count (mean (SD))	4 (1)	3 (1)	4 (2)	4 (2)	0.1*, a
DFU FOOT CHARACTERIZATION					
Foot deformity (n (%)) [b]	14 (61)	3 (60)	2 (14)	9 (64)	0.7†/0.8¥
Total foot pulses ≤ 1 (n (%))	20 (80)	1 (20)	6 (100)	13 (93)	**0.001†/0.003¥**
ABI < 0.8 (n (%))	5 (75)	NA	2 (33)	3 (21)	0.6ᵞ
$TcPO_2$ (median (range))	18 (67)	NA	24 (34)	16 (67)	0.6 α
Intermittent claudication (n (%))	12 (48)	0 (0)	5 (83)	7 (50)	**0.02†**/0.2¥
DPN symptoms (n (%))	23 (92)	5 (100)	5 (83)	13 (93)	0.6†/0.8¥
Altered SWM sensation (n (%)) [c]	17 (81)	3 (60)	3 (60)	11 (100)	*0.07†/**0.04¥***

Previous DFU (n (%))	15 (60)	1 (20)	6 (100)	8 (57)	**0.03**[†]/0.4[¥]
Previous LEA (n (%))	7 (28)	0 (0)	3 (50)	4 (29)	0.2[†]/0.4[¥]
DFU CHARACTERIZATION					
Texas grade					
III (Bone or joint) (n (%))	25 (100)	NA	6 (100)	14 (100)	1.0[γ]
Texas stage					
B (Infection) (n (%))	1 (5)	NA	0 (0)	1 (7)	1.0[γ]
D (Infection plus ischemia) (n (%))	19 (95)	NA	6 (100)	13 (93)	
Located at toes (n (%))	5 (25)	NA	3 (50)	2 (14)	0.1[γ]

*: One-Way ANOVA test with Bonferroni correction, [†]: Chi-square test for association, [¥]: Chi-square test for tendency, [α]: Mann-Whitney U test, [γ]: Fisher's exact test, [a]: No statistical difference between groups, [b]: in 2 subjects it was not applicable, [c]: in 4 subjects it was not possible to conduct, ABI: Ankle-brachial index, DFU: Diabetic foot ulcer, DPN: Diabetic peripheral neuropathy, HbA_{1c}: Glycated haemoglobin, HBOT: Hyperbaric oxygen therapy, LEA: Lower extremity amputation, NA: Not applicable, NHOTB: No hyperbaric oxygen therapy, PAD: Peripheral arterial disease, SD: Standard deviation, SWM: Semmes-Weistein monofilament, $TcPO_2$: Transcutaneous Partial Pressure of Oxygen

Table 2. Laboratory markers

Variables	Global			Non DFU (n=5)	NHBOT DFU patients (n=6)		Paired samples tests	HBOT DFU patients (n=14)			Paired samples tests	Independent samples tests p value	
	M0 (n=25)	M3 (n=20)	M6 (n=14)	M0	M0	M3	p value	M0	M3	M6	p value	M0	M3
Glucose (mg/dl) (mean (SD))	172 (75)	213 (82)[a]	211 (75)	165 (60)	194 (110)	236[a] (109)	0.3[ε]	165 (64)	200 (68)	211 (75)	0.2*,[b]	0.5[ж]	0.4[ж]
HbA_{1C} (in %) (mean (SD))	8.1 (1.7)	8.3 (1.8)[a]	8.2 (1.6)	8.5 (1.5)	8.3 (2.1)	9.5 (2.9)[a]	0.2[ε]	7.9 (1.7)	7.8 (1.1)	8.2 (1.6)	0.8*,[b]	0.6[ж]	0.3[ж]
Haemoglobin (g/dl) (mean (SD))	12 (1)	12 (2)[a]	12 (2)	13 (2)	12 (1)	11 (2)[a]	0.3[ε]	12 (1)	12 (2)	12 (2)	0.8*,[b]	0.8[ж]	0.6[ж]
Leukocytes ($x10^3$/dl) (mean (SD))	9.5 (3.4)	10.0 (3.7)[a]	7.9 (1.8)	6.6 (0.9)	11.5 (4.1)	12.5 (4.4)	0.4[ε]	9.4 (3.0)	8.7 (2.7)	7.9 (1.8)	0.3*,[b]	0.2[ж]	**0.03[ж]**
Platelets ($x10^3$/dl) (mean (SD))	267 (71)	265 (79)[a]	229 (76)	236 (34)	256 (57)	305 (90)[a]	0.2[ε]	277 (81)	244 (67)	229 (76)	0.2*,[b]	0.6[ж]	0.1[ж]
C-reactive protein (mg/dl) (median (range))	0.8 (15.7)	0.3 (22.5)[a]	0.3 (11.2)	0.7 (0.6)	2.1 (11)	11.8 (22.4)[a]	0.1[*]	0.7 (15.7)	0.3 (5.4)	0.3 (11.2)	0.3[ⱷ]	0.2[α]	**0.03[α]**
Total cholesterol (mg/dl) (mean (SD))	170 (54)	153 (37)[a]	151 (24)	180 (68)	163 (68)	168 (57)[a]	0.5[ε]	170 (46)	147 (26)	151 (24)	0.2*,[b]	0.8[ж]	0.3[ж]
LDL cholesterol (mg/dl) (mean (SD))	101 (47)	84 (32)[a]	83 (24)	98 (56)	97 (57)	97 (53)[a]	0.5[ε]	104 (43)	79 (20)	83 (24)	0.1*,[b]	0.8[ж]	0.3[ж]
HDL cholesterol (mg/dl) (mean (SD))	44 (10)	41 (12)[a]	42 (17)	55 (10)	43 (10)	36 (13)[a]	0.1[ε]	41 (9)	43 (11)	42 (17)	0.9*,[b]	0.7[ж]	0.3[ж]
Triglycerides (mg/dl) (mean (SD))	130 (70)	143 (85)[a]	131 (70)	146 (119)	112 (44)	177 (82)[a]	0.1[ε]	133 (60)	130 (86)	131 (70)	0.8*,[b]	0.5[ж]	0.3[ж]

Creatinine (mg/dl) (median (range))	0.8 (7.3)	0.9 (8.2)[a]	0.9 (2.2)	0.6 (0.8)	1.3 (7.3)	1.6 (8.2)[a]	0.2[*]	1.0 (1.8)	0.9 (2.9)	0.9 (2.3)	0.8[ϫ]	0.7[α]	1.0[α]
Urea (mg/dl) (mean (SD))	55 (23)	69 (47)[a]	63 (34)	42 (7)	66 (33)	92 (65)[a]	0.1[ε]	54 (21)	57 (32)	63 (34)		0.5[ж]	0.3[ж]
Microalbuminuria (mg/dl) (n (%))	12 (48)	12 (63)[a]	7 (64)	0 (0)	5 (83)	4 (80)[a]	NA	7 (50)	8 (57)	7 (64)	NA	**0.02**[†]/0.2[¥]	0.6[γ]
VEGF (pg/ml) (median (range))	70 (268)	66 (355)[a]	40 (131)	37 (101)	189 (236)	99 (344)[a]	0.9[*]	72 (268)	56 (129)	40 (131)	0.6[ϫ]	0.6[α]	*0.07*[α]
PlGF (pg/ml) (median (range))	12.6 (66.5)	8.5 (65.8)[a]	10.5 (51.2)	6.8 (8.3)	3.1 (14.7))	7.4 (12.1)[a]	0.7[*]	14.8 (65.7)	9.6 (64.0))	10.5 (51.2)	0.4[ϫ]	0.2[α]	0.4[α]
SDF1- α (pg/ml) (mean (SD))	1911 (642)	2030 (713)[a]	1739 (2136)	1992 (470)	2297 (514)	2062 (1054)[a]	0.5[ε]	1716 (691)	2017 (562)	1739 (2136)	0.8*[,b]	*0.08*[ж]	0.9[ж]

[ε]: Student's t test for paired samples, [ж]: Student's t test for independent samples, [α]: Mann-Whitney U test, [*]: Wilkoxon signed ranks test, *: One-Way ANOVA test with Bonferroni correction, [ϫ]: Friedman test, [†]: Chi-square test for association, [¥]: Chi-square test for tendency, [γ]: Fisher's exact test, [a]: 1 missing value, [b]: No statistical difference between groups, %: Percentage, DFU: Diabetic Foot Ulcer, dl: Deciliter, g: Gram, HbA_{1c}: Glycated Hemoglobin, HBOT: Hyperbaric Oxygen Therapy, HDL: High Density Lipoprotein, LDL: Low Density Lipoprotein, M0: Month 0, M3: Month 3, M6: Month 6, mg: Milligram, ml: Millilitre, NA: Not Applicable, NHOTB: No Hyperbaric Oxygen Therapy, pg: Pico gram; PlGF: Placental Growth Factor, SD: Standard Deviation, SDF1- α: Stromal Cell-derived Factor 1 Alpha, VEGF: Vascular Endothelial Growth Factor

Table 3. Clinical outcome

Variables	Global (n=20)			NHBOT DFU patients (n=6)			HBOT DFU patients (n=14)			Comparison between groups Fisher's exact test p value		
	M3	**M6**	**M12**	**M3**	**M6**	**M12**	**M3**	**M6**	**M12**	**M3**	**M6**	**M12**
Complete healing / improvement (n (%))	15 (75)	15 (75)	12 (60)	1 (17)	1 (17)	1 (17)	14 (100)	14 (100)	11 (79)	**<0.001**	**<0.001**	**0.02**
LEA or death (n (%))	5 (25)	5 (25)	8 (40)	5 (83)	5 (83)	5 (83)	0 (0)	0 (0)	3 (21)			

HBOT: Hyperbaric oxygen therapy, LEA: Lower extremity amputation, M0: Month 0, M6: Month 6, M12: Month 12, NHBOT: No hyperbaric oxygen therapy

Table 4. Diabetic foot ulcer characteristics at baseline and third month of follow-up

Variables	Global (n=20)		NHBOT DFU patients (n=6)		Paired samples tests p value	HBOT DFU patients (n=14)		Paired samples tests p value	Independent samples tests p value	
	M0	M3	M0	M3		M0	M3		M0	M3
Baseline ulcer area (in cm^2) (median (range))	11.0 (31.1)[a]	2.7 (24.8)[b]	12.1 (16.1)	13.1 (23.5)[b]	NP	7.3 (31.1)[a]	2.7 (16.3)	**0.001**[*]	0.4[α]	NP
Mean depth (in mm) (median (range))	2.3 (11.6)[c]	1.7 (6.5)[d]	1.8 (4.7)	3.5 (3.6)[b]	NP	2.3 (11.6)[c]	1.4 (6.5)[a]	*0.1*[*]	0.6[α]	NP
Maximum depth (in mm) (median (range))	4.6 (18.3)[c]	3.3 (10.7)[d]	3.6 (8.2)	7.9 (5.2)[b]	NP	4.6 (18.1)[c]	3.2 (10.7)[a]	**0.03**[*]	0.3[α]	NP
Volume (in cm^3) (median (range))	1.2 (24.8)[c]	0.4 (13.8)[d]	1.5 (10.4)	7.0 (13.6)[b]	NP	1.2 (24.5)[c]	0.4 (10.7)[a]	**0.006**[*]	0.8[α]	NP

[*]: Wilkoxon signed ranks test, [α]: Mann-Whitney U test, [a]: 1 missing values, [b]: 4 missing values, [c]: 2 missing values, [d]: 5 missing values, cm^2: Squared Centimetre, cm^3: Cubic Centimetre, DFU: Diabetic Foot Ulcer, DFU: Diabetic Foot Ulcer, HBOT: Hyperbaric Oxygen Therapy, M0: Month 0, M3: Month 3, mm: Millimetre, NHBOT: No Hyperbaric Oxygen Therapy, NP: Not Possible

Table 5. Diabetic foot ulcer characteristics at baseline, 6th and 12th month of follow-up

Variables	Global (n=20)			NHBOT DFU patients (n=6)			Paired samples tests	HBOT DFU patients (n=14)			Paired samples tests
	M0	M6	M12	M0	M6	M12	p value	M0	M6	M12	p value
Baseline ulcer area (in cm^2) (median (range))	11.0 (31.1)[a]	0.4 (6.3)[b]	0.0 (1.9)[c]	12.1 (16.1)	0.0 (0.0)[b]	0.0 (0.0)[b]	NP	7.3 (31.1)[a]	0.4 (6.3)	0.0 (1.9)[d]	**<0.001** [χ]
Mean depth (in mm) (median (range))	2.3 (11.6)[e]	0.7 (2.2)[f]	0.0 (2.7)[g]	1.8 (4.7)	0.0 (0.0)[b]	0.0 (0.0)[b]	NP	2.3 (11.6)[e]	0.9 (2.2)[e]	0.4 (2.7)[f]	**0.001** [χ]
Maximum depth (in mm) (median (range))	4.6 (18.3)[c]	1.3 (4.3)[g]	0.0 (4.7)[h]	3.6 (8.2)	0.0 (0.0)[b]	0.0 (0.0)[b]	NP	4.6 (18.1)[e]	1.7 (4.3)[e]	1.3 (4.7)[f]	**0.006** [χ]
Volume (in cm^3) (median (range))	1.2 (24.8)[e]	0.0 (6.2)[g]	0.0 (0.3)[h]	1.5 (10.4)	0.0 (0.0)[b]	0.0 (0.0)[b]	NP	1.2 (24.5)[e]	0.0 (6.2)[e]	0.0 (0.3)[f]	**0.001** [χ]

[χ]: Friedman test, [α]: Mann-Whitney U test, [a]: 1 missing values, [b]: 5 missing values, [c]: 8 missing values, [d]: 3 missing values, [e]: 2 missing values, [f]: 4 missing values, [g]: 7 missing values, [h]: 9 missing values, cm^2: Squared Centimetre, cm^3: Cubic Centimetre, DFU: Diabetic Foot ulcer, HBOT: Hyperbaric Oxygen Therapy, M0: Month 0, M3: Month 3, mm: Millimetre, NP: Not Possible, NHOTB: No Hyperbaric Oxygen Therapy

FIGURE LEGEND

Figure 1. Boxplot of number of vessels at baseline and 1 month in patients undergoing or not Hyperbaric Oxygen therapy.

Figure 2. Microvessel density as evaluated by CD31 immunostaining at baseline and one month after HBOT in patients with active DFU. (A) Patient treated with HBOT, at baseline; (B) Non-HBOT patient at baseline; (C) Patient treated with HBOT at one month after treatment initiation; (D) Non-HBOT patient one month after treatment initiation. Magnification 200x.

Figure 1.

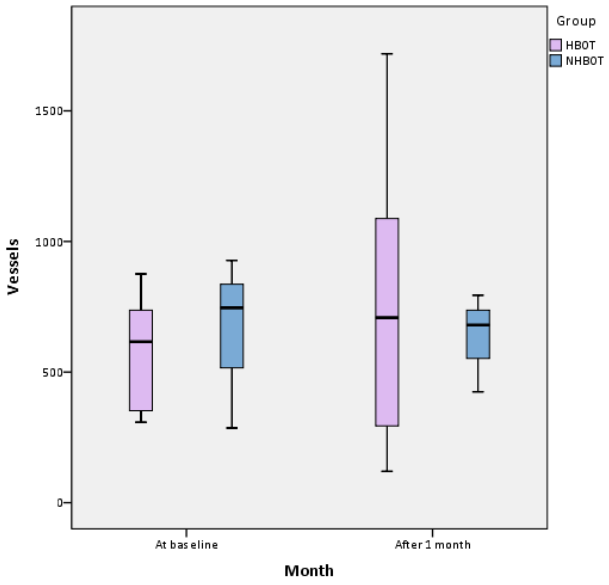
Group
HBOT
NHBOT
1500
1000
500
0
Vessels
At baseline
After 1 month
Month

Figure 2.