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THE EXPRESSION PROFILE OF ANGIOGENIC GENES IN CRITICAL LIMB ISCHEMIA POPLITEAL ARTERIES

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Critical limb ischemia (CLI) represents the most severe form of peripheral arterial disease (PAD) and is the leading cause of non-traumatic amputations in western populations. In recent years, therapeutic angiogenesis has been considered to be a potential treatment option for CLI patients, however the molecular mechanism of ischemia-induced vascularization is still not fully understood. The identification of genetic factors underlying vascular responses to ischemia will improve our understanding of the biological causes of the disease and enhance personalized therapies in the future. In this work, we determined, for the first time, the expression profile of angiogenesis-related genes utilizing unique human material: the popliteal arteries retrieved during lower limb amputation from patients with CLI. Using custom-designed TaqMan Low-Density Array (TLDA) cards we investigated the mRNA level of 90 genes on CLI samples compared to healthy donors. We identified three significantly up-regulated genes in CLI group: matrix metalloproteinase 9 (*MMP-9*), VE-cadherin (*CDH5*) and integrin alpha 4 (*ITGA4*). However, among all investigated genes, only lymphatic vessel endothelial hyaluronan receptor 1 (*LYVE1*) was significantly reduced. In order to verify whether hypoxic conditions occur in popliteal arteries of CLI patients, we validated the transcription level of selected proangiogenic genes by real-time PCR on a larger number of samples. These results showed that the expression of key genes involved in angiogenesis, such as *MMP9*, *HGF*, *HIF1A*, *VEGF-A* and *FLT1* were elevated in patients with CLI. Moreover, the study revealed that the expression of *VEGF-A* and *FLT1* was associated with activation of *HIF1A* transcription. In conclusion, our data revealed the alteration in the mRNA level of genes involved in matrix remodelling, cell-cell adhesion as well as endothelial cell migration and proliferation in human popliteal arteries.

Key words: *angiogenesis, arteriogenesis, critical limb ischemia, gene expression, vascular endothelial growth factor, metalloproteinase 9, hypoxia inducible factor-1*

INTRODUCTION

Peripheral arterial disease (PAD) results from a significant narrowing of arteries, most often due to athero-thrombosis. Despite improvements in medical management, PAD remains a significant clinical problem in industrialized countries. The number of people living with PAD worldwide increased by 23.5%, from about 164 million in 2000 to 202 million in 2010 (1). Critical limb ischemia (CLI) represents the most advanced clinical stage of PAD manifested by rest pain, non-healing wounds, ulcers, necrosis and/or gangrene of affected limb. Patients with CLI are considered candidates for vascular and endovascular procedures, however, amputation still remains the only surgical option for approximately 20 – 30% of them (2). It is known that CLI is a complex process that affects microvascular and macrovascular systems and surrounding tissues as well (3). A number of novel approaches to improve angiogenesis and/or arteriogenesis are currently being investigated including gene- and cell-based therapies, however, the results are not ready for widespread clinical use. Recent preclinical and clinical data have indicated that the application of stem cell therapy for patients with CLI promoted adult vasculogenesis, nevertheless the capillaries are too small and cannot restore limb circulation (4, 5).

One of the most potent angiogenesis regulators is hypoxia inducible factor-1 (HIF subunit *-1*α and *-1*β) which stimulates the expression of pro- and anti-angiogenic genes (6). Under hypoxic conditions, the degradation of the *HIF-1*α subunit is inhibited and its half-life and transcriptional activity increased (7). The HIF pathway triggers the transcription of multiple proangiogenic growth factors including vascular endothelial growth factor (*VEGF-A*), angiopoetin-1 and -2 (*ANGPT1* and *ANGPT2*), placental growth factor (PIGF) and platelet-derived growth factor-B (*PDGFB*) (8). Intriguingly, recent findings suggested that polyphenols from grape stem extract can influence VEGF-A expression in endothelial cells (ECs) by HIF-1 independent mechanism (9). VEGF-A is considered as the master regulator of angiogenesis, that participates in ECs migration and proliferation *via* surface receptor VEGFR-2 (KDR/Flk-1) (10). In addition, VEGF-A initiates vascular permeability and mediates in extracellular matrix degradation by proteases, mostly matrix metalloproteinases (MMPs) (11). Interestingly, Choksy and

coworkers demonstrated that ischemic conditions were able to elevate the level of *VEGF-A* mRNA and protein in distal skin and muscle biopsies compared with proximal CLI samples (12). In contrast, van Weel *et al*. reported that *VEGF-A* mRNA level decreased in the ischemic muscle (13) while a recent study has revealed that the level of the *VEGF-A* transcript was higher in ischemic skin of CLI patients than in healthy controls (14). Unlike previous reports, Palmer-Kazen *et al*. found no association between VEGF-A protein concentration and the ischemic muscle areas of the lower limbs (15). These contradictory results, however, do not indicate unequivocally that ischemia increased *VEGF-A* expression. Furthermore, a more recent study highlighted that VEGF expression decreased in aging human dermal microvascular ECs and aging individuals have impaired angiogenesis (16). Thus age could be another important coexisting factor, moreover, the formation of stable and functional new vessels can result of many different factors. Due to the fact that CLI is caused by arterial occlusion it seems that angiogenesis is not sufficient to replace a conducting artery. Therefore, arteriogenesis of collateral vessels (transformation of a small arteriole into a larger artery) by increasing their lumen can improve blood flow in the lower limbs (17). Surprisingly, stenosis of a large artery results in increased blood flow and shear stress in the collaterals that can enlarge vessels more than 20 times (18, 19). The exposure to chronic shear stress leads to ECs activation, switches on gene expression, especially chemokines (monocyte chemoattractant protein-1, MCP-1) and adhesion molecules (intracellular adhesion molecule-1, ICAM-1) (17). Although some studies revealed weak arteriogenic response after VEGF-A infusion (17), others reported increased microvasculature in an animal model of CLI (20) and in large mammalian models of myocardial ischemia (21, 22). Recent *in vitro* study has revealed that the laser radiation increased significantly ECs proliferation and decreased VEGF concentration at the same time (23). It is plausible that the reason for these discrepancies may result from VEGF pleiotropic effect (24).

To conclude, angiogenesis and arteriogenesis are very complex and require dynamic interactions of various types of molecules and cells. Although, the mechanisms of arteriogenesis have been clarified in animal models of limb ischemia, there are limited data regarding CLI patients. The most widely used clinical models of new blood vessels formation in CLI are human skin and muscle biopsies.

In this study, we evaluated for the first time the expression profile of angiogenesis-related genes in popliteal arteries obtained after lower limb amputation from patients with CLI. Our results demonstrated changes in *HIF-1α*, *VEGF-A* and *FLT1* mRNA levels as well as shear stress-induced genes.

Table 1. Demographics and clinical data of patients with critical limb ischemia.

Variable	Mean (range) or number of patients
Age (years)	63 (42 – 88)
Gender (male/female)	23/11
BMI (kg/m^2)	26.9 (19.5 – 39.1)
Underlying disease	
Hypertension	22
Diabetes mellitus	3
MI	4
Stroke	4
Kidney disease	5
Active smoking	8
Laboratory tests	
White blood cells (K/uL)	11.6 (5.25 – 20.01)
Red blood cells count (million/µl)	4.0 (2.78 – 6.26)
PLT (K/ µl)	281.8 (94 – 638)
Creatinine (mg/dl)	0.89 (0.45 – 1.58)
Glucose (mg/dl)	122.6 (82 – 293)
APTT (sek)	34.7 (25.7 – 48.3)
INR	1.11 (0.90 – 1.60)
Prothrombin index (%)	93.2 (66.7 – 109.6)

BMI, body mass index; MI, myocardial infarction; PLT, platelet count; APTT, activated partial thromboplastin time; INR, international normalized ratio, n = 34.

MATERIALS AND METHODS

Clinical samples

The study was conducted in accordance with the ethical standards of the latest revision of the Helsinki Declaration and approved by the Bioethical Committee of Wroclaw Medical University (approval No. KB-347/2008). Written informed consent was obtained from all patients before enrolment in the study. Patients enrolled in this study were hospitalized in the Department and Clinic of General, Vascular and Transplantation Surgery, Wroclaw Medical University. All tissue samples were collected and immediately stabilized in RNAlater® (Sigma-Aldrich) and then stored at –80°C until use.

Samples of popliteal arteries were obtained during lower limb amputation from 34 patients with CLI (23 men and 11 women; median age, 63 years; range 42 – 88 years). All CLI patients underwent an above-the-knee amputation. The arteries were occluded by thrombus or atherosclerotic lesions in all patients enrolled into the study. Detailed description of patients is presented in *Table 1*. The control group consisted of 13 multi-organ transplant donors.

RNA isolation

Total RNA was isolated from 100 mg of the whole wall of the popliteal artery by a combination of TRI Reagent® (Sigma-Aldrich) method with silica-based purification technology using E.Z.N.A Total RNA kit (Omega Bio-Tek). Briefly, disruption and homogenization steps with MagNA Lyser Green Beads were performed in 1 ml of TRI Reagent® using the MagNA Lyser System (Roche Applied Science) (3 × 30 s at 7000 rpm). The homogenized samples were incubated at room temperature for 5 min and then 0.2 ml of chloroform was added. The mixture was thoroughly shaken by vortexing for 15 s and incubated at room temperature for an additional 15 min. Phase separation was carried out by centrifugation at 4°C and 12,000 × g for 15 min. The aqueous phase was collected and RNA precipitation was done by adding an equal volume of 70% ethanol. The RNA samples were further purified using spin columns according to manufacturer's recommendation.

The concentration and purity of RNA were determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and then samples were stored at –80°C.

cDNA synthesis

cDNA synthesis was performed using total RNA (1 µg) and random hexamer primers with the High Capacity cDNA Reverse

Table 2. Genes statistically significant up- and downregulated and a trend toward significance determined by TLDA.

Gene symbol	Assay ID	Mean FC ± SD CLI	P-value
MMP9	Hs00957562_m1	35.5±61.8	0.014
MDK	Hs00171064_m1	6.74±6.31	0.108
CDH5	Hs00174344_m1	3.62±2.11	0.021
TIE1	Hs00178500_m1	3.39±2.35	0.108
PECAM1	Hs00169777_m1	2.37±1.35	0.108
ITGA4	Hs00168433_m1	3.09±2.37	0.030
KDR	Hs00176676_m1	3.02±1.85	0.087
VEGFC	Hs00153458_m1	2.25±1.42	0.087
VEGFA	Hs00900054_m1	1.98±1.05	0.087
FLT1	Hs00176573_m1	1.59±1.07	0.523
HGF	Hs00300159_m1	1.38±0.98	0.787
FGF-1	Hs00265254_m1	1.24±1.48	0.586
HIF1A	Hs00936368_m1	1.23±1.20	0.650
TIMP1	Hs00171558_m1	0.89±0.57	0.651
FGF2	Hs00266645_m1	0.44±0.35	0.408
LYVE1	Hs00272659_m1	0.41±0.34	0.040
FBLN5	Hs00197064_m1	0.36±0.25	0.160

Assay ID indicates primers and TaqMan probe sets for the target gene; Control - healthy donors (n = 3); CLI - patients with Critical Limb Ischemia (n = 21); FC - fold change, calculated as $2^{-\Delta\Delta Ct}$, SD - standard deviation; P-value - significant at < 0.05, Mann-Whitney U test.

Table 3. The relative expression of selected proangiogenic genes verified by real-time PCR.

Gene symbol	Assay ID	Mean FC ± S.D. CLI	P-value
MMP9	Hs00957562_m1	**35.35 ± 75.11**	**0.007**
TIMP1	Hs00171558_m1	1.99 ± 1.37	0.117
HIF1A	Hs00936368_m1	**2.17 ± 2.13**	**0.041**
HIF2A	Hs01026149_m1	0.97 ± 0.68	0.518
VEGFA	Hs00900054_m1	**2.51 ± 1.81**	**0.023**
FLT1	Hs00176573_m1	**3.46 ± 2.43**	**0.003**
HGF	Hs00300159_m1	**1.78 ± 1.10**	**0.043**
MET	Hs01565584_m1	2.27 ± 1.71	0.132
FGF2	Hs00266645_m1	1.10 ± 1.10	0.214
FGFR2	Hs01552926_m1	1.01 ± 1.16	0.242

Assay ID indicates primers and TaqMan probe sets for the target gene; Control - healthy donors (n = 13); CLI - patients with Critical Limb Ischemia (n = 34); FC - fold change, calculated as $2^{-\Delta\Delta Ct}$, S.D. - standard deviation; P-value - significant at < 0.05 (in bold), Mann-Whitney U test.

Transcription Kit (Life Technologies) in a final volume of 40 µl according to the manufacturer's guidelines.

TaqMan Low Density Array (TLDA) profiling

Custom-designed TaqMan Low Density Array (TLDA) cards (Life Technologies) were used to determine the expression level of 90 angiogenesis-related genes and 4 endogenous control (i.e., *GAPDH*, *HPRT1*, *GUSB* and 18S rRNA). The 96a format (4 samples/card) was chosen and selected genes were placed on the 384-well TaqMan® Array Micro Fluidic Card. See *Table 1S* for a list of gene and assay ID. Profiling studies were performed on 21 and 3 samples from CLI patients and healthy donors, respectively. The procedure was carried out according to the manufacturer's instructions. Briefly, RT product (diluted 1:4 in nuclease-free water) was mixed with TaqMan Universal PCR Master Mix® (Life Technologies) and each sample was loaded into two ports (100 µl/port) of the card. The PCR reactions were performed using the 7900HT Fast Real Time PCR System (Life Technologies) under the following thermal cycling conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles, 30 s at 97°C and 1 min at 60°C.

Validation of the expression of selected proangiogenic genes

In order to investigate whether hypoxic conditions can regulate gene expression in popliteal arteries in CLI patients, real-time PCR for 10 selected genes (*MMP9, TIMP1, HIF1A, HIF2A, VEGF-A, FLT-1, HGF, MET, FGF2* and *FGFR2*), and one endogenous control (*GAPDH*) was carried out on a larger number of samples: 34 and 13 samples from patients and controls, respectively. Diluted cDNA (4 times) in nuclease-free water was amplified with TaqMan Fast Universal PCR Master Mix (Life Technologies) and TaqMan® Gene Expression Assays (Life Technologies). The gene symbol and assays ID are shown in *Table 3*.

All reactions were run in triplicate on the 7900HT Fast Real Time PCR System (Life Technologies). The following cycling conditions were used: 20 s at 95°C followed by 40 cycles, 1 s at 95°C, 20 s at 60°C.

Data analysis

Data analyses were done with the SDS software v2.2.2 and gene expression levels were determined by the comparative Ct method ($2^{-\Delta\Delta Ct}$) and converted to fold change (FC). The expression of target genes were normalized to *GAPDH* (the most stable reference gene in our study) and calibrated against mean delta Ct of the control group. Samples with undetermined as well as low and high Ct value (<15 or >35) were excluded from further analysis. The fold change cut-offs of >2 or <0.5 were used to identify up-regulated or down-regulated genes, respectively.

Statistical analysis

Statistical analysis was performed using R Statistical Software (R for Windows, version 3.0.2, The R Foundation for Statistical Computing, Vienna, Austria). Results were expressed as means ± S.D., and statistical significance was assessed with the nonparametric Mann-Whitney test. P-value of < 0.05 was considered statistically significant.

RESULTS

TaqMan Low Density Array gene expression screening in popliteal arteries of critical limb ischemia patients

The TLDA screening technique was used to assess the expression levels of 90 angiogenesis-related genes in popliteal arteries of 21 patients with CLI and 3 healthy controls. The expression results of all analyzed transcripts in CLI and control groups are presented in *Table 1S*. The transcripts corresponding to 20 out of 90 genes were undetectable in most CLI and control samples and excluded from further analysis. We found that 33 genes tended to be up-regulated in CLI patients vs. healthy

controls, however, only 3 of them were statistically significant (*Table 1S*). The highest mean expression level was observed for matrix metalloproteinase 9 *(MMP9)* (FC = 35.57). Over 3-fold significant up-regulation was noticed for mRNAs of VE-cadherin (*CDH5*) and integrin alpha 4 (*ITGA4*) (FC = 3.62, FC = 3.09, respectively). In contrast, 4 genes were down-regulated more than 2 times in CLI patients compared to the control group, but only Lymphatic Vessel Endothelial Hyaluronan Receptor 1 (*LYVE1*) was significantly reduced (FC = 0.41).

In order to visualize the global gene expression level in all patients and healthy donors, a heat map analysis was done (*Fig. 1*). We observed intra-patient variability in the expression level of the analyzed genes. For example, most genes were apparently down-regulated in patient No. 17 and 20, and up-regulated in patient No. 10. The mRNA level of metalloproteinases was increased in more than 50% of CLI samples (71% for *MMP9*, 52% for *MMP14*) while only *MMP2* was up-regulated in 43% of CLI. Interestingly, elevated levels of *MMPs* were accompanied by a decrease in *TIMP1*, *TIMP2*, and TIPM3 mRNAs in 33% of patients. The amount of mRNA of adhesion molecules, including-cadherin, integrin α4 and α5 subunits as well as CD31 endothelial marker (*PECAM1*), were elevated in more than 50% of CLI samples.

The expression level of the main angiogenesis inducers such as *VEGF* family genes showed an upward trend in CLI group (*Table 1S*). The mean of FC for *VEGF-C* and *VEGF-B* was increased more than 2-fold and their elevated level was observed in more than 50% of CLI samples (*Fig. 1*). Despite the fact, that the mean expression value of *VEGF-A* was slightly below the accepted level of FC cut-offs (FC = 1.98), the up-regulation of this gene was observed in more than 38% of CLI patients (*Fig. 1*).

Interestingly, we observed changes in the expression level of genes triggered under hypoxic conditions. The mRNA levels of *HIF1A* and its downstream targets, such as interleukin 8 *(IL8)*,

Table 1S. The expression profile of genes analyzed in popliteal arteries by TaqMan Low Density Array (TLDA).

Gene symbol	Assay ID	Mean ΔCt ± S.D. Controls	Mean ΔCt ± S.D. CLI	Mean FC ± S.D. CLI	P-value
Genes with higher mean expression in CLI compared with healthy donors					
MMP9	Hs00957562_m1	8.13±1.13	4.89±2.39	35.57±61.86	0.014
ROBO1	Hs00268049_m1	6.71±0.68	5.05±2.23	10.47±20.43	0.160
MDK	Hs00171064_m1	7.10±0.76	5.19±1.86	6.74±6.31	0.108
NOS3	Hs01574659_m1	7.09±1.21	6.82±2.36	6.58±15.02	0.586
TLR5	Hs00152825_m1	8.43±0.37	7.25±1.96	4.99±7.33	0.308
TLR3	Hs00152933_m1	7.13±1.17	6.62±2.16	4.46±10.07	0.534
PDGFB	Hs00234042_m1	5.67±0.08	4.15±1.46	4.43±4.28	0.160
FIGF	Hs00189521_m1	8.92±1.64	7.426±1.888	4.25±9.34	0.262
SFRP2	Hs00293258_m1	1.97±0.46	0.83±1.80	4.16±1.18	0.356
IL8	Hs00174103_m1	4.02±2.51	3.90±2.04	3.68±9.84	0.787
ANGPT1	Hs00181613_m1	2.85±0.26	4.77±1.95	3.64±3.31	0.408
CDH5	Hs00174344_m1	4.91±0.87	3.32±0.94	3.62±2.12	0.021
ANGPTL1	Hs00559786_m1	5.73±1.16	5.01±1.96	3.59±6.04	0.464
SEMA3F	Hs00188273_m1	7.81±0.51	7.79±1.83	3.56±10.63	1.000
TIE1	Hs00178500_m1	7.26±1.16	5.88±1.15	3.40±2.35	0.108
PECAM1	Hs00169777_m1	2.82±0.84	1.83±092	2.37±1.35	0.108
SERPINF1	Hs00171467_m1	2.11±1.30	0.92±1.42	3.35±2.62	0.160
LGALS3	Hs00173587_m1	4.00±1.20	2.88±1.33	3.31±3.26	0.160
MMP14	Hs01037009_g1	6.24±1.33	5.24±1.52	3.14±2.90	0.256
ITGA4	Hs00168433_m1	6.14±0.12	4.85±1.03	3.09±2.37	0.030
KDR	Hs00176676_m1	5.92±1.37	4.69±1.17	3.02±1.85	0.087
TLR1	Hs00413978_m1	7.27±1.28	6.26±1.32	2.96±2.82	0.265
ROBO3	Hs00223636_m1	7.66±0.72	6.67±1.35	2.85±2.29	0.226
TNFSF15	Hs00270802_s1	7.57±2.28	7.29±1.52	2.61±5.87	0.975
CXCL12	Hs00171022_m1	0.84±0.73	0.33±1.86	2.59±2.91	0.408
ITGA5	Hs00233743_m1	3.77±0.35	2.95±1.41	2.53±2.22	0.087
CTNNB1	Hs00355045_m1	2.51±0.39	1.76±1.44	2.52±2.17	0.356
VEGFC	Hs00153458_m1	7.55±0.25	6.71±.1.09	2.25±1.42	0.087
MMP2	Hs00234422_m1	0.06±1.01	-0.47±1.66	2.39±2.25	0.586
VEGFB	Hs00173634_m1	3.99±0.26	3.29±1.31	2.21±1.51	0.308
ANGPT2	Hs00169867_m1	5.79±1.03	5.02±1.20	2.18±1.36	0.308
ANG;RNASE4	Hs02379000_s1	8.56±1.67	8.77±1.71	2.17±4.48	1.000
DNMT1	Hs00945899_m1	6.25±0.73	5.67±1.22	2.03±1.50	0.523
Genes with lower mean expression in CLI compared with healthy donors					
FGF2	Hs00266645_m1	3.60±3.22	5.13±0.99	0.44±0.35	0.408
LYVE1	Hs00272659_m1	1.99±0.73	3.93±1.55	0.41±0.34	0.040
FBLN5	Hs00197064_m1	0.72±2.31	2.71±1.50	0.36±0.25	0.160
DNMT3A	Hs01027166_m1	3.46±2.23	5.44±0.93	0.30±0.90	0.132

Gene symbol	Assay ID	Mean ΔCt ± S.D. Controls	Mean ΔCt ± S.D. CLI	Mean FC ± S.D. CLI	P-value
No difference in gene expression between CLI and healthy donors					
VEGFA	Hs00900054_m1	4.91±0.49	4.12±0.79	1.98±1.05	0.087
TLR2	Hs00610101_m1	4.99±2.82	5.04±1.22	1.81±2.47	0.718
ENPP2	Hs00196470_m1	4.62±0.65	4.43±1.49	1.78±1.61	0.928
FST	Hs00246256_m1	7.13±0.59	6.64±1.02	1.74±1.11	0.408
TYMP	Hs00157317_m1	6.71±0.81	6.24±0.97	1.66±0.94	0.308
FLT1	Hs00176573_m1	5.39±0.21	4.98±0.87	1.59±1.07	0.523
AMOT	Hs00611096_m1	5.56±1.73	5.69±1.68	1.50±1.53	0.928
HSPG2	Hs00194179_m1	1.30±0.92	1.04±0.94	1.44±0.85	0.718
PDGFRB	Hs00387364_m1	1.50±0.52	1.38±1.13	1.39±1.04	0.651
HGF	Hs00300159_m1	5.00±0.99	4.89±1.10	1.38±0.98	0.787
ANGPTL2	Hs00765775_m1	3.55±1.27	3.69±1.31	1.37±1.53	0.928
PDGFRA	Hs00998026_m1	1.84±0.97	1.99±1.35	1.34±1.36	0.929
FGF1	Hs00265254_m1	5.69±2.84	6.31±1.74	1.24±1.48	0.586
HIF1A	Hs00936368_m1	1.89±0.30	2.15±1.33	1.23±1.20	0.650
TEK	Hs00176096_m1	5.34±0.84	5.44±1.09	1.20±0.91	1.000
TIMP3	Hs00165949_m1	0.93±0.37	1.08±1.16	1.18±0.78	0.929
FZD2	Hs00361432_s1	7.35±2.91	7.87±1.61	1.17±1.23	0.523
ITGAV	Hs00233808_m1	2.39±0.89	2.90±1.42	1.15±1.25	0.523
TLR4	Hs00152939_m1	5.43±1.21	5.76±1.32	1.12±0.88	0.857
CXCL10	Hs00171042_m1	4.47±1.79	7.05±3.22	1.10±2.21	0.191
CXCL2	Hs00601975_m1	3.63±0.43	4.02±1.34	1.09±0.89	0.857
ADAMTS1	Hs00199608_m1	1.98±0.56	2.06±0.71	1.05±0.49	1.000
TGFB1	Hs99999918_m1	3.98±0.81	4.28±1.09	1.05±0.72	0.226
TIMP2	Hs00234278_m1	0.45±0.28	0.91±1.23	0.96±0.62	0.718
CD44	Hs00153304_m1	1.21±1.07	1.51±0.80	0.95±0.53	0.651
PTGIS	Hs00168766_m1	3.51±0.62	3.98±1.11	0.91±0.60	0.586
SFRP1	Hs00610060_m1	2.33±0.44	3.32±1.72	0.90±0.96	0.308
TIMP1	Hs00171558_m1	-1.00±0.36	-0.44±1.22	0.89±0.57	0.651
MCAM	Hs00174838_m1	2.66±2.09	3.56±1.73	0.89±0.80	0.464
CTGF	Hs00170014_m1	0.06±1.58	0.85±1.06	0.72±0.44	0.226
LOXL1	Hs00173746_m1	3.56±1.43	5.01±2.11	0.65±0.50	0.191
EDIL3	Hs00174781_m1	2.49±1.37	3.88±1.92	0.64±0.55	0.265
ITGB3	Hs01001469_m1	2.41±1.65	3.65±1.01	0.52±0.32	0.191
Undetermined genes in CLI and healthy donors					
ANGPT4	Hs00211115_m1				
CSF3	Hs99999083_m1				
IFNB1	Hs01077958_s1				
ANGPTL3	Hs00205581_m1				
LEP	Hs00174877_m1				
SERPINC1	Hs00166654_m1				
SERPINB5	Hs00184728_m1				
CEACAM1	Hs00236077_m1				
TNF	Hs00174128_m1				
FGF4	Hs00173564_m1				
IL12A	Hs00168405_m1				
PRL	Hs00168730_m1				
IFNG	Hs00174143_m1				
TGFA	Hs00608187_m1				
DNMT3B	Hs01003405_m1				
MMP7	Hs01042795_m1				
ROBO2	Hs00326067_m1				
TP73	Hs01065727_m1				
FLT4	Hs01047677_m1				
HEY1	Hs00232618_m1				

Assay ID - indicates primers and TaqMan probe sets for the target gene; Control - healthy donors (n = 3); CLI - patients with Critical Limb Ischemia (n = 21); FC - fold change, calculated as $2^{-\Delta\Delta C_t}$; S.D. - standard deviation; P-value, significant at < 0.05, Mann–Whitney U test.

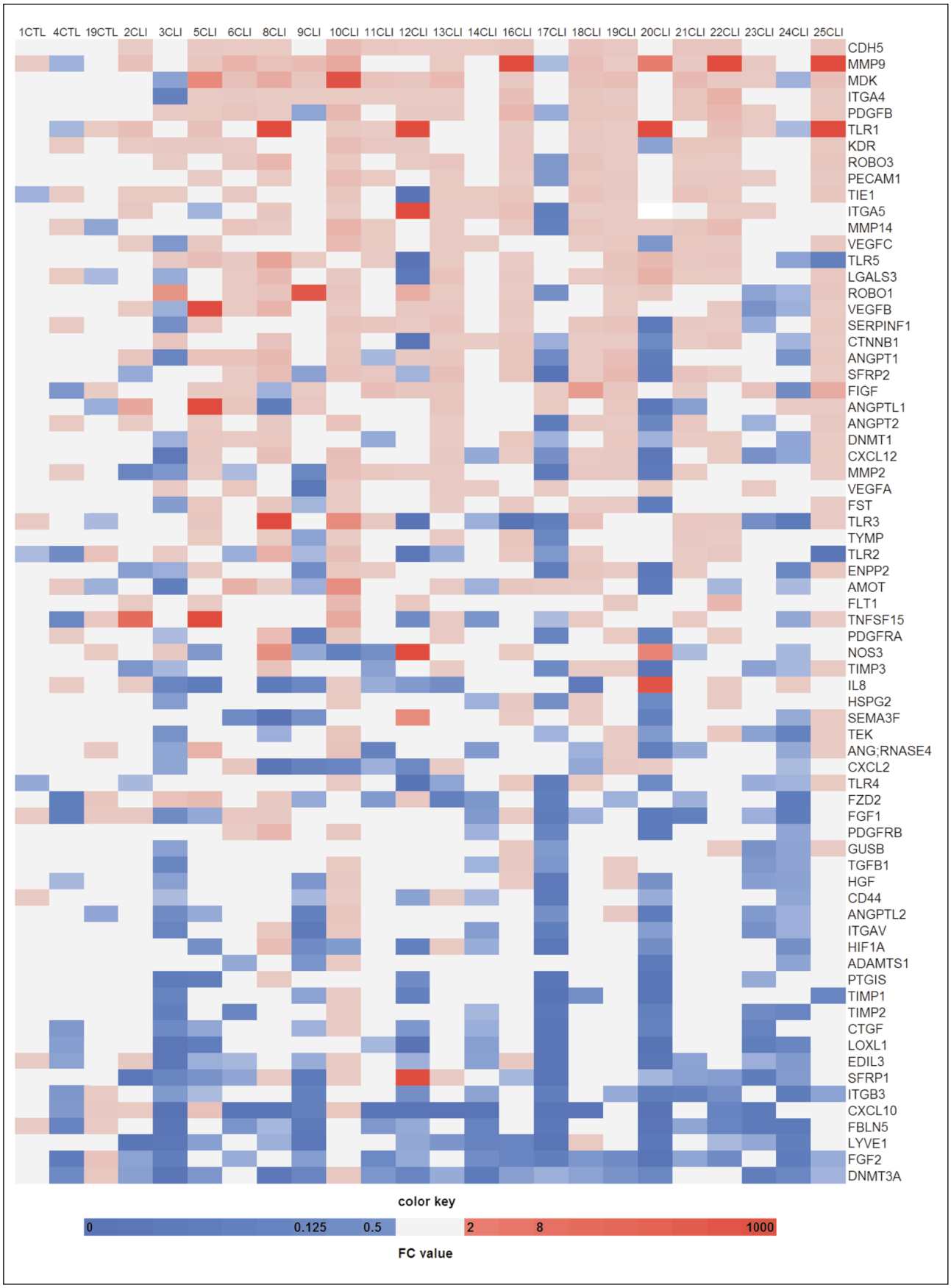

Fig. 1. Heat map representation of TaqMan Low Density Array results. Heat map shows the global gene expression pattern in 21 critical limb ischemia (CLI) samples and 3 healthy controls (CTL). Shades of red indicate upregulation (FC > 2) while shades of blue indicate downregulation (FC < 0.5). White color indicates unchanged expression (FC value between 0.5 and 2). Color intensity reflect the higher FC value. A gene was considered upregulated if FC was > 2 and downregulated if FC was < 0.5.

nitric oxide synthase 3 *(NOS3*), *FLT1* as well as *VEGF-A* were elevated in some CLI samples (*Table 1S*). Moreover, the expression of hypoxia-sensitive *PDGFB* and midkine (*MDK)* were elevated in over 60% of CLI samples (*Fig. 1*).

Genes that show a statistical significance and trend towards significance in expression as well as genes from hypoxia-induced angiogenesis pathway are presented in *Table 2*.

The expression of selected proangiogenic genes in critical limb ischemia patients

In order to confirm whether hypoxic conditions can regulate gene expression in popliteal arteries in CLI patients, we validated the mRNA level of selected genes by real-time PCR method on a larger number of samples (34 CLI patients and 13 healthy donors).

Similar to TLDA profiling results, a statistically significant increase in the level of *MMP9* transcript was noticed (FC = 35.35) (*Table 3*). Although, trends to increased expression of metalloproteinase inhibitor *TIMP1* were observed, the results were not statistically significant. Surprisingly, the mean FC value of *HIF1A* was up-regulated, but *HIF2A* transcript was unchanged in patients with CLI. Furthermore, the expression of *VEGF-A* and its receptor *FLT1* (both regulated by HIF1) was increased significantly in CLI patients compared to the control group (FC = 2.51 and FC = 3.46, respectively) (*Table 3*). The mRNA level of *FGF2* and its receptor *FGFR2* was unchanged, whereas we observed upward trends in the expression of *MET* gene (FC = 2.27), however the difference was not statistically significant (P = 0.132) (*Table 3*). To recapitulate, our results indicated that the expression of key genes involved in angiogenesis, such as *HGF*, *HIF1A*, *VEGF-A* and *FLT1*, was indeed elevated in patients with CLI.

To assess whether activation of hypoxia-mediated signalling pathways occurred, CLI patients were divided into two groups based on the FC value of the *HIF1A* gene: the first group (called 'hypoxic group') with FC ≥ 2 accounted for 38% (n = 13) and the second one (called 'normoxic group') with FC <2 accounted for 62% (n = 21). Our results revealed statistically significant differences in the expression level of *VEGF-A*, *FLT1* and *HGF* between the hypoxic and normoxic group (*Fig. 2*). It is noteworthy that the *VEGF-A* and *HGF* transcripts increased more than 2-fold in the hypoxic group only, but mRNA level of *FLT1* exceeded this value in both analyzed groups. However, the expression of *FLT1* gene was remarkably higher in the hypoxic than the normoxic group (more than 5-fold vs. 2.4-fold).

DISCUSSION

Critical limb ischemia is an advanced manifestation of PAD, occurring after a chronic lack of blood supply that ultimately leads to long-term hypoxic conditions. Paradoxically, hypoxia is a strong stimulus for proangiogenic molecules including VEGF-A, FLT1, HIF1A and HIF2A. So far, molecular studies have focused mainly on deregulation of angiogenic factors in the ischemic areas of skin and muscles (12, 14).

The present study aimed to analyze the expression profile of 90 genes related to arterio- and/or angiogenesis within the human popliteal arterial wall. We found that only 3 genes were statistically significantly up-regulated: *MMP9*, *CDH5* and *ITGA4* (*Table 1S*). We observed that the expression of *MMP9* gene increased more than 35-fold (P = 0.014). MMP9, also known as type IV collagenase, is an important factor promoting angiogenesis through the degradation of vascular basement membrane and the release of extracellular matrix (ECM)-bound VEGF (25). It is therefore very likely that MMP-dependent proteolysis of the ECM is potentiated and may be crucial for growth of collateral vessels in CLI patients. Interestingly, a previous study showed that an elevated level of mRNA and protein of MMP9 occurred in skeletal muscles of CLI and ALI (acute limb ischemia) patients (26). Surprisingly, the mRNA level of *TIMP1* and *TIMP2* (inhibitors of MMPs) was increased only in ALI but was unchanged in CLI ischemic muscles (26). Similarly to previous findings, we also did not observe any differences in the transcript level of *TIMP1* between CLI patients and healthy controls (*Table 1S*). Other research studies indicated that shear stress can increase the level of MMP9 in endothelial and smooth muscle cells (27, 28) and is also considered the main inducer for the expression of some adhesion

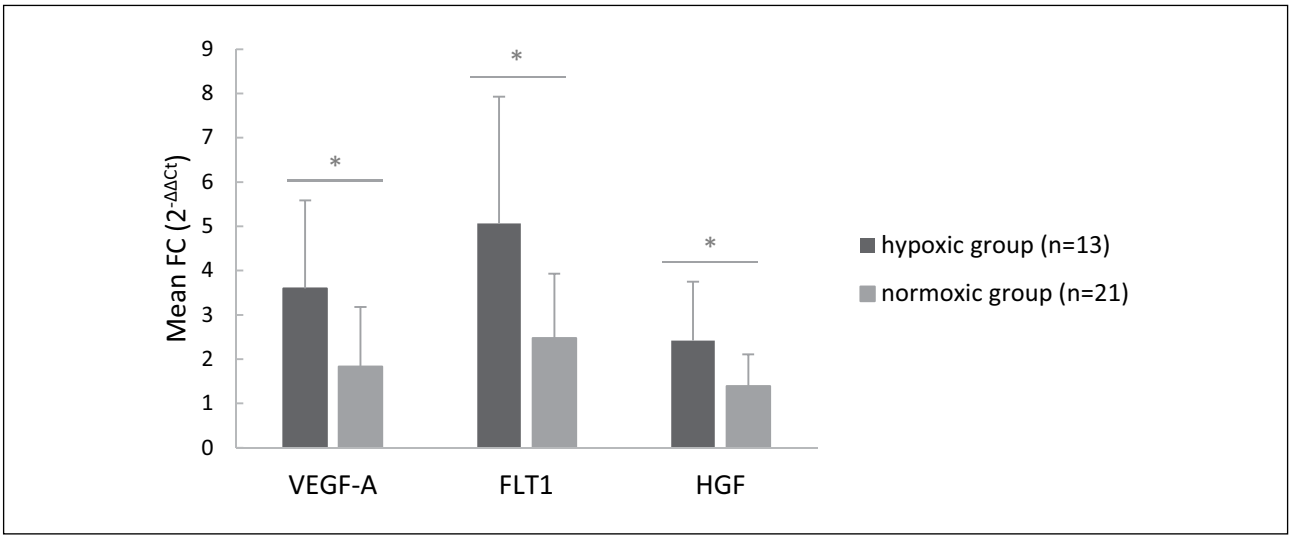

Fig. 2. The relative expression level of proangiogenic genes in hypoxic and normoxic groups of CLI patients. Patients were divided into two groups according to FC value of *HIF1A* gene (hypoxic group – FC ≥ 2 and normoxic group – FC < 2). The mean FC and P values of genes (hypoxic vs. normoxic): *VEGF-A* (3.59 ± 1.98 vs. 1.54 ± 1.08, P = 0.005), *FLT1* (5.06 ± 2.86 vs. 2.49 ± 1.45, P = 0.014) and *HGF* (2.42 ± 1.33 vs. 1.45 ± 0.59, P = 0.023).

molecules, such as VE-cadherin (coding by *CDH5* gene) and integrins (i.a., ITGA4) (29, 30). Additional investigations are required to elucidate the reasons of the increased level of mRNA *CDH5* as well - an immunohistochemistry study should be carried out to assess its impact on arteriogenesis.

Our TLDA analysis indicated a trend towards an increased expression level of many genes including those induced by hypoxia (*MDK*, *VEGF-A*, *FLT1*, *IL8*, *NOS* and *PDGFB*) (*Table 2*). We also noticed the tendency of the expression of four genes to decline (*Table 2*), but only one gene, *LYVE-1*, was significantly down-regulated (FC = 0.4; P = 0.04). *LYVE-1* is known as a marker of lymphatic endothelial cells, while, its expression was also detected in other cells including sinusoidal endothelial cells of lymph nodes, liver and spleen as well as embryonic blood vessels (31, 32). It is proposed that LYVE-1-mediated signalling pathways, triggered by interactions with hyaluronan and FGF2, are engaged in lymphangiogenesis (33, 34), however, its physiological function is still being uncovered. Our mRNA profiling approach, for the first time, identified *LYVE-1* transcript in adult popliteal arteries and similarly to a previous study, we have shown that the expression of *LYVE-1* is not only restricted to lymphatic vasculature.

It is well known that transcription factors HIF1A and HIF2A activate ischemia-driven angiogenesis by influencing their various target genes (35). In order to verify whether hypoxia-inducible genes can be activated in popliteal arteries of CLI patients, we assessed the mRNA level of *HIF1A*, *HIF2A*, *VEGF-A*, and *FLT1*. Our study revealed that the expression of *HIF1A* was significantly increased in CLI arteries in contrast to *HIF2A* (2.2-fold vs. 1.0-fold) (*Table 3*). We found that the mRNA level of *HIF1A* increased in almost 40% of CLI patients ('hypoxic group'). These results are consistent with a previous study that showed an elevated level of *HIF1A* in CLI ischemic muscles (36). However, Vasuri *et al*. have found a loss of stabilization of HIF1α protein in CLI skin, despite a 3-fold increase in mRNA level (37). We expect that strong hypoxia stimuli existed in some CLI patients, however, it is also important to emphasize that an unchanged level of *HIF-1A* transcripts was detected in ~60% of clinical samples. Further studies are needed to explain the reason for these differences.

Our data has also demonstrated that the expression of *VEGF-A* and its receptor *FLT1*, both belonging to the hypoxia-responsive element-regulated genes (HRE), were significantly higher in the hypoxic group (*Fig. 2*). The obtained results strongly suggest that transcription of *VEGF-A* can be mediated by the HIF-1 pathway and popliteal arteries seem to be an important source of circulating VEGF-A. Interestingly, Findley and coworkers revealed elevated plasma levels of VEGF-A in CLI patients, thus, these findings may indirectly support our hypothesis (38).

Importantly, increased expression of VEGF-A may exert positive or negative effect on the course of PAD. On the one hand, it promotes collateral blood flow, regulates systemic blood pressure and attenuates endothelial lesions (39). On the other hand, VEGF-A stimulates instability of plaque and induces pro-apoptotic effect *via* NO-dependent manner (39). VEGF-A proangiogenic and proarteriogenic activity is determined by its receptors FLT1 (VEGFR1) and KDR (VEGFR2). An *in vitro* study revealed that the transcription of *FLT1* receptor was extremely activated by HIF-1 molecules in endothelial cells (40). In line with this study, our results also showed increased mRNA level of *FLT1* (more than 5-fold, *Fig. 2*) as a result of the activation of *HIF1A* gene in the hypoxic group. It is, therefore, very likely that the expression of *FLT1* is activated by hypoxic conditions in CLI popliteal arteries. Although, it is noteworthy that the mean expression level of *FLT1* was increased in both the hypoxic (FC > 5.0) and normoxic group (FC > 2.4), it was significantly higher in hypoxic patients (P = 0.013). We assume that impairment of arteriogenesis in CLI patients could be explained, at least in part, by VEGF interaction with its two membrane receptors. FLT1 has a high affinity to VEGF-A (10-times higher than KDR - the major signal transducer for angiogenesis), but shows weak tyrosine activity, so it can exert a suppressive role in VEGF signalling (41, 42). It seems that FLT1 can compete with the KDR receptor and thus inhibits a VEGF-stimulated pro-arteriogenic signal.

Our next important findings include statistically significant alterations in the transcript level of *HGF* (FC = 2.42, P = 0.023) in the 'hypoxic group' only. Although, Vasuri *et al*. reported that serum levels of HGF did not change significantly in CLI patients compared to healthy subjects (37), an *in vitro* study demonstrated that hypoxia increased expression of hepatocyte growth factor activator (HGFA) through HIF1A-dependent transcription (43). Interestingly, HGFA plays an important role in processing pro-HGF to mature HGF. Next, HGF binds to the specific receptor, Met, which triggers a signal transduction cascade involving PI3K and ultimately activates HIF-1 protein (44). Taken together, HGFA/HGF/Met may act as an alternative angiogenic pathway under hypoxic conditions enhancing HRE-mediated transcription. With regard to these findings, further studies are required to determine whether the level of *HGFA* transcript is elevated in CLI patient and whether the HGFA-dependent pathway is involved in *in vivo* pro-HGF activation.

This study has some limitations, which need to be pointed out. Firstly, the statistical analysis of TLDA results from an unequal number of participants among the groups may be flawed, however to eliminate that problem we validated mRNA levels of interesting genes for a larger number of participants in the control group. Secondly, the heterogeneity of samples associated with different types of cells as well as mRNA results have not been confirmed at the protein level. The only way to eliminate such obstacles and confirm our observations is to use microdissection techniques, which allows for analyzing selected vascular cells.

In conclusion, to our knowledge this is the first expression profiling analysis of human proangiogenic genes in the popliteal arterial wall. Results from our group revealed that the level of mRNA of shear stress-induced genes (*MMP9*, *CDH5* and *ITGA4*) increased significantly in CLI patients. Additionally, we also observed the elevated level of hypoxia-inducible transcription factor *HIF1A* and up-regulation of its downstream target genes (*VEGF-A* and *FLT1*). Moreover, another interesting fact is that the expression level of *LYVE-1* was significantly decreased in the majority of patients with CLI and *HGF* transcript increased only in the hypoxic group. Our findings shed new light on the molecular alterations within the popliteal arterial wall in patients with CLI. However, further studies are needed to confirm whether shear stress and hypoxia are able to induce HIF1 signalling as well as arterial wall remodelling and whether the gene expression is cell type-dependent. At what stage of arteriogenesis will disruptions appear despite the presence of high levels of angio- and arteriogenic molecules identified in CLI patients? This is another question awaiting further investigation.

Author contributions: Conceived the idea - DB, JS, ASC; sample collection - PB, JS; RNA isolation - DM; TLDA - DM; gene validation - DB; research design - DB, DM; statistical analysis - MK, DB; writing the manuscript - DB, DM, ASC.

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