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Molecular characterization of peripheral arterial disease in proximal extremity arteries

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ABSTRACT

Purpose: Although risk factors for atherosclerosis in peripheral arterial disease (PAD) are well defined, the underlying mechanisms are poorly understood and no medication exists for causal therapy. Molecular pathways that could be targeted have not been identified so far. To address this issue, we compared the molecular profiles of healthy *versus* PAD proximal femoral arteries.
Methods: Gene expression profiles from proximal femoral arteries of patients with PAD (Fontaine stage IIb–IV; $n = 20$) and femoral arteries from healthy controls (CO) ($n = 3$) were compared by microarray technology. We evaluated all samples by histopathology and performed microdissection on the CO tissue before molecular analysis. We analyzed genes regarding their cellular localization, molecular function, and risk factors such as hypercholesterolemia, smoking, and diabetes. We used a selected panel of genes for polymerase chain reaction validation of microarray results and compared the data with previously published studies.
Results: Most genes overexpressed in PAD *versus* CO were located in the cytoplasm, membrane, and nucleus. Functionally, they had binding activity to nucleotides, cytoskeletal proteins, and transcription factors. They were mainly involved in immune regulation (e.g., interleukin-8, chemokine ligand 18, and allograft-inflammatory factor-1) ($P < 0.01$). Down-regulated genes in PAD *versus* CO were located in the extracellular region. They had transporter and G-protein receptor activity. They were associated with signaling, cell growth, and tissue formation (e.g., myosin VB, marker for differentiated aortic smooth muscle, myosin 11) ($P < 0.01$). Polymerase chain reaction successfully validated the expression of the differences among 10 selected genes (e.g., chemokine ligand 18, common leukocyte antigen, killer cell lectin-like receptor subfamily B, member 1, and interleukin-8).
Conclusions: Genes enrolled in immune regulation and inflammatory response were identified as key players in PAD. Various membrane-bound molecules with binding activity are hereunder. Identification of such molecules may elucidate relevant players that act as candidates for therapeutic targets or prognostic markers in the future.

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1. Introduction

Epidemiological studies indicate that 20% of people beyond 70 y of age are affected by atherosclerosis. Changes in the lower-extremity arteries become clinical relevant as peripheral artery disease (PAD). Men and women experience these changes equally, which result in immobility and reduced quality of life [1,2]. Risk factors such as smoking, diabetes mellitus, arterial hypertension, and hypercholesterolemia are well defined [1,3,4]. The most important risk factor in men above 40 y of age is smoking and diabetes mellitus, whereas in postmenopausal women, reduced levels of estrogen accelerate the progress of the disease [1,5]. Patients with diabetes mellitus have a fourfold increased risk to develop PAD [2,6,7]. Based on this knowledge, treatment guidelines for PAD recommend the reduction of risk factors. Medicaments such as thrombocyte inhibitors are recommended to reduce the incidence of acute ischemia [2]. To date, a specific targeted therapy that addresses the disease at its roots to stop the progression does not exist. One reason is the limited knowledge of underlying molecular mechanisms that induce and promote PAD.

Nevertheless, the nicotinic acetylcholine receptor was identified as an important target for nicotine that is released from cigarette smoke. The downstream activation of transcription factors such as **cAMP response element-binding** or nuclear factor-κB induce the expression of plasminogen activator inhibitor-1, tissue plasminogen activator, vascular cell adhesion molecule-1, angiotensin converting enzyme I, and von Willebrand factor that may contribute to PAD [8]. Other hypotheses prefer the reactive interaction of endothelial and muscle cells based on the release of endothelin-1, angiotensin II, and homocysteine within the vessel wall [8]. In patients with coronary sclerosis, interleukin (IL)-1, -2, -4, -6, and -10, tumor necrosis factor-α, insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor were identified as highly expressed molecules by microarray analysis [9]. Their involvement during the course of PAD remains unclear. In PAD, comparison of affected vessels with healthy tissue is limited. Healthy vessels from peripheral arteries cannot be harvested easily, because patients with healthy arteries usually undergo no surgery and specimens cannot be collected. We solved this problem by using healthy proximal femoral arteries from organ donors after histopathologic exclusion of PAD. We compared these with PAD affected vessels by microarray analysis.

2. Materials and methods

2.1. Patients

After we obtained informed consent, we harvested thromboendarterectomy specimens of the common femoral artery from 20 patients with PAD (Fontaine stage IIb–IV) immediately after surgery. We excluded from the study patients with a history of hemodialysis or former malignant disease. We collected 10 samples of common femoral arteries and biopsies of the abdominal aorta from organ donors (controls: CO) after informed consent. We included as CO only patients with no signs of atherosclerosis in the common femoral arteries or aorta during histopathologic investigation ($n = 3$) for this study. Table 1 lists patient characteristics. The Ethical Commission of the University Erlangen–Nuremberg approved this study, which we carried out in concordance with the Guidelines of Helsinki.

2.2. Sample workup

We harvested tissue samples of CO and PAD in RNlater (Qiagen, Hilden, Germany) and stored them at −80°C until further workup. We investigated aorta and common femoral artery specimens of CO histopathologically for early signs of atherosclerosis, which was an exclusion criterion for the study (Fig. 1). Femoral arteries from CO underwent microscopically assisted manual microdissection (MAMD) to dissect adventitia and parts of the muscular layer from the vascular wall [10]. This procedure guaranteed comparability between CO and PAD, because during thromboendarterectomy, adventitia and parts of the muscular layer do not adhere at the specimens. We isolated RNA using commercial kits (RNeasy-Kit; Qiagen), following the manufacturer's protocol. We included DNAse (Qiagen) digestion in this protocol. RNA quality and quantity were determined by the Lab-on-a-Chip method (Bioanalyzer 2100; Agilent Technologies, Palo Alto, CA), following the manufacturer's instructions [11]. We used the 3′/5′-ratios for the housekeeping genes glycerinaldehyde-3-phosphatase and ß-actin supplied by the microarrays as further measures of RNA quality to exclude partial degradation. A 3′/5′-ratio below 3 was regarded as an indicator of good RNA quality according to the manufacturer's protocol (Affymetrix, Santa Clara, CA) [12].

2.3. Microarray analysis

We examined gene expression using GeneChip technology (Affymetrix). Biotin-labeled cRNA was generated by *in vitro*

Table 1 – Patient characteristics.

	Age (mean [range])	M	F	Smoking	Diabetes mellitus	Hypercholesterolemia	Arterial hypertension
CO	53 (27–82)	2	1	0	0	0	0
PAD	67 (51–86)	13	7	11	5	6	8

PAD = peripheral artery disease Fontaine stage IIb–IV.

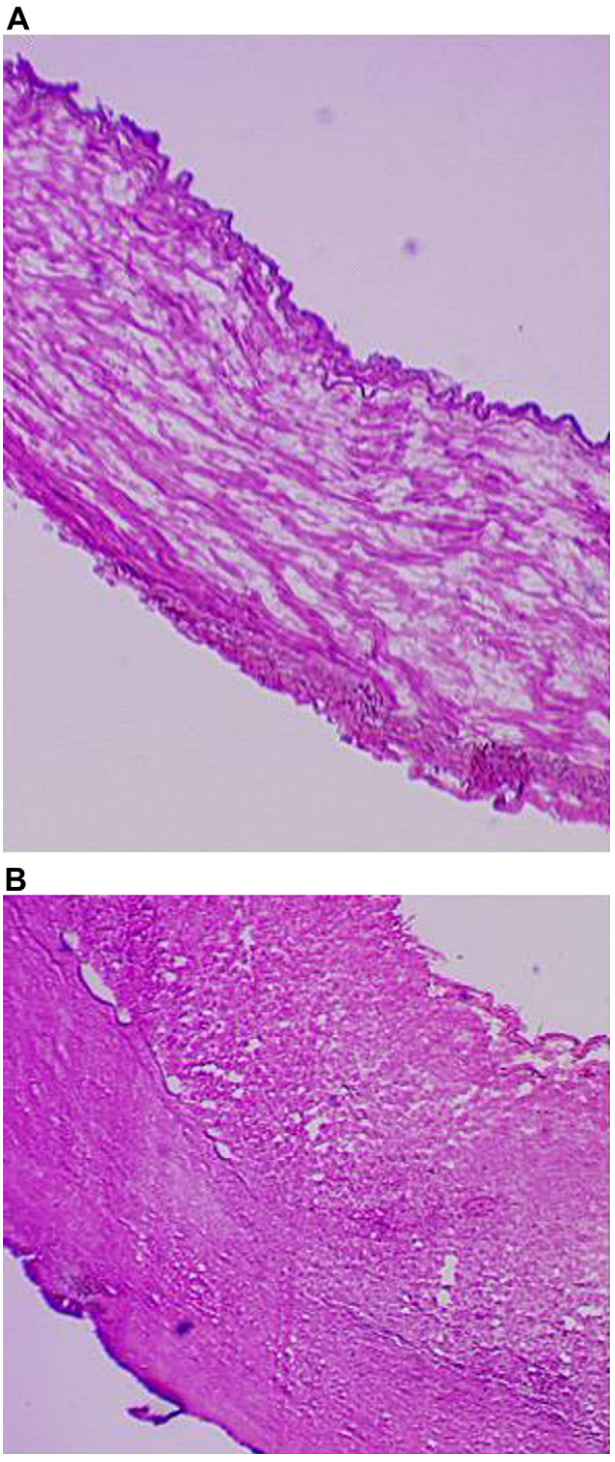

Fig. 1 – Hematoxylin and eosin stain of healthy femoral artery beforehand (control) (A) and vessel with peripheral artery disease (PAD) (B).

transcription, as described previously, and hybridized to the GeneChips (HG-U133A Plus 2) following the manufacturer's instructions [13]. For first-strand cDNA synthesis, 9 μL (13.5 μg) of total RNA was mixed with 1 μL of a mixture of three polyadenylated control RNAs. First was 1 μL 100 μmol/L T7-oligo-d(T)21-V primer (5′-GCATTAGCGGCCGCGAAATTAATACGACTC-ACTATAGGGAGA(T)21V-3′), incubated at 70°C for 10 min and put on ice. Next, we added 4 μL of 5× first-strand buffer, 2 μL 0.1 MDTT, and 1 μL 10 mmol/L dNTPs and preincubated the reaction at 42°C for 2 min. Then, we added 2 μL (200 U) Superscript II (Life Technologies, Karlsruhe, Germany) and continued incubation at 42°C for 1 h. For second-strand synthesis, we added 30 μL 5× second-strand buffer, 91 μL RNAse-free water, 3 mL 10 mmol/L dNTPs, 4 μL (40 U) *Escherichia coli* DNA polymerase I (Life Technologies), 1 μL (12 U) *E. coli* ligase (TaKaRa Biomedical Europe, Gennevilliers, France), and 1 μL (2 U) RNAse H (TaKaRa), and incubated the reaction at 16°C for 2 h. Afterward, we added 2.5 mL (10 U) T4 DNA polymerase I (TaKaRa) at 16°C for 5 min. The reaction was stopped by adding 10 μL 0.5 mol/L ethylenediaminetetra acetic acid, extracted the double-stranded cDNA with phenol/chloroform, and recovered the aqueous phase by phase lock gel separation (Eppendorf, Hamburg, Germany). After precipitation, we restored the cDNA in 12 μL RNAse-free water. We used 5 μL double-stranded cDNA to synthesize biotinylated cRNA using the BioArray High Yield Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY). We purified labeled cRNA using the RNeasy mini kit (Qiagen). We performed fragmentation of cRNA, hybridization to GeneChips, washing, staining, and scanning of the arrays in the GeneArray scanner (Agilent) as recommended by the Affymetrix Gene Expression Analysis Technical Manual.

2.4. *Polymerase chain reaction validation of selected microarray results*

We used the following primers for polymerase chain reaction (PCR) validation of selected genes: chemokine ligand 18 (CCL 18) forward: 3′-tacctcctggcagattccac-5′, reversed: 5′-caggcattcagcttcaggtc-3′; CD45 forward: 3′-ctccaacaccaccatcacag-5′, reversed: 5′-ctcatgttcgggttcaaggt-3′; killer cell lectin-like receptor subfamily B (KLRB1) forward: 3′-gccctgaaacttagctgtgc-5′, reversed: 5′-ttg gcagatccatctgatttc-3′; IL8 forward: 3′-gtgcagttttgccaaggagt-5′, reversed: 5′-ctctgcacccagttttcctt-3′; IL-23A forward: 3′-gaagtc cccaatggctacaa-5′, reversed: 5′-gacgatctgggtgacaggtt-3′; IL-7R forward: 3′-tcgctctgttggtcatcttg-5′, reversed: 5′-cctgagcaactgggtt caat-3′; TPSAB1 forward: 3′-cgggagcagcacctctacta-5′, reversed: 5′-agtggtggatccagtccaag-3′; allograft-inflammatory factor-1 (AIF-1) forward: 3′-aaaagctttcggactgctga-5′, reversed: 5′-atctcttgcccagcatcatc-3′; HMOX1 forward: 3′-acatctatgtggccctggag-5′, reversed: 5′-gctctggtccttggtgtcat-3′; Toll-like receptor-4 (TLR4) forward: 3′-cctgtgcaatttgaccattg-5′, reversed: 5′-tgccattgaaagcaactctg-3′. For cDNA synthesis, we mixed 0.5μg total RNA from CO or PAD samples with 1 μL oligo $(dT)_{18}$ primer (541 ng/μL) and 1 μL dNTP mix (10 mmol/L each). We added diethylpyrocarbonate water, 2 μL 0.1 mol/L dithiothreitol, 1 μL RNAsin (40 U/μL), and 1 μL Superscript reverse-transcriptase (RT). For PCR, we mixed 2.5 μL PCR puffer, 0.7 μL $MgCl_2$, 0.2 μL dNTPs, forward and reversed primers, and Taq polymerase. We carried out 35 cycles of PCR for 5 min and 3 × 30 s at 94°C, 94°C, 60°C, and 72°C each. We separated PCR products and visualized them on 1.5% agarose gel.

2.5. *Statistics*

We processed the CEL files in Partek Genomics Suite (v6.5, Partek Inc, MO) using default RMA import settings. We included

pre-background adjustment for GC-content, background correction, and quantile normalization. Differentially expressed probe sets were identified by analysis of variance using the false discovery rate q-value approach to correct Type I errors for multiple tests [14]. We subjected target gene lists to overrepresentation analysis in Genetrail using the entire HG-U133Plus_2.0 array as a reference [15]. We addressed genes to their cellular location and molecular function (Tables 2 and 3). To exclude age-dependent gene expression bias, we performed analysis between patients ≤60 *versus* >60 y of age and excluded differentially expressed genes from further analysis. Furthermore, we analyzed the data regarding risk factors for atherosclerosis: smoking, diabetes, and hypercholesterolemia (Supplementary Tables 1–3). Four previously published studies presenting molecular differences between healthy and arteriosclerotic vessels identified by microarray technology have been selected from the literature [16–19]. We compared the genes identified in these studies with our findings and calculated the relative expression differences between healthy and arteriosclerotic tissue as fold change (FC).

3. Results

3.1. *Patients and histopathology*

The mean age of CO patients was 53 y, compared with 67 y of age for PAD patients. No patient of the CO group smoked or had diabetes mellitus or hypercholesterolemia. In the PAD group we identified 11 smokers, five patients with diabetes mellitus, and six with hypercholesterolemia; 11 patients had more than one risk factor for PAD. Five patients were smokers and had diabetes mellitus; two were smokers who had diabetes mellitus and arterial hypertension, one was a smoker with arterial hypertension, and three had both arterial hypertension and hypercholesterolemia, (Table 1). No patient underwent hemodialysis or experienced malignancies. Histopathological control of the PAD femoral artery specimens showed advanced atherosclerotic disease in all cases. The histopathological workup of the CO femoral arteries showed no atherosclerosis (Fig. 1). Furthermore, we detected no signs of early atherosclerotic features in the histopathologic investigation of the CO aorta specimens.

Table 2 – Cellular localization of up-regulated and down-regulated genes in PAD.

Cellular component	Genes (*n*)	*P* value
Up-regulated		
Cytoplasm	1.640	<0.001
Membrane	1.523	0.033
Nucleus	1.095	0.04
Non–membrane-bound organelle	439	0.028
Cytoskeleton	287	0.003
Nucleoplasm	182	0.045
Actin cytoskeleton	78	0.005
Endosome	71	0.04
Vacuole	70	0.003
Lytic vacuole	67	0.002
Lysosome	67	0.002
Cell surface	56	0.04
Receptor complex	35	0.004
Integrin complex	14	0.029
Major histocompatibility protein complex	17	0.013
Immunological synapse	13	0.008
Major histocompatibility class II protein complex	9	0.04
T-cell receptor complex	9	0.006
Down-regulated		
Extracellular region	330	<0.001
Ion channel complex	16	0.014
Intermediate filament cytoskeleton	12	0.003

Table 3 – Molecular function of up-regulated and down-regulated genes in PAD.

Molecular function	Genes (*n*)	*P* value
Up-regulated		
Binding activity	2.797	<0.001
Nucleotide binding	526	0.007
Cytoskeletal protein binding	142	<0.001
Transcription factor binding	121	0.01
Enzyme binding	93	0.001
Phospholipid binding	47	0.04
Antigen binding	16	0.04
Ig binding	11	0.005
IgG binding	9	0.002
Interferon binding	4	0.041
Signal transducer activity	470	0.02
Transferase activity	247	0.017
Enzyme regulator activity	212	0.03
Kinase activity	177	0.03
Protein dimerization activity	116	0.001
GTPase regulator activity	118	0.009
Hsp70 protein regulator activity	4	0.04
Down-regulated		
Transmembrane transporter activity	227	0.03
Ion transmembrane transporter activity	115	0.005
G-protein–coupled receptor activity	83	0.002
Substrate-specific channel activity	55	0.004
Gated channel activity	42	0.005
Cytokine receptor binding	3	0.005
Neurotransmitter binding	5	0.002

3.2. *Microarray results*

3.2.1. *Control* versus *atherosclerosis*

During cluster analysis, the CO samples clustered together into one distinct group. Six PAD samples clustered close to the CO group, whereas all other PAD samples were clearly separated from the CO patients. The PAD samples did not cluster regarding patients' risk factors for atherosclerosis (Fig. 2). We identified 6.537 differentially expressed genes between CO *versus* PAD regarding our selection criteria ($P < 0.01$).

Up-regulated genes in PAD were mainly located in the cytoplasm (1.640), cellular membrane (1.523), or nucleus (1.095). Down-regulated genes were addressed to the extracellular region (330) (Table 2). Most high-expressed genes in the PAD group had binding activity (2.797) to nucleotides

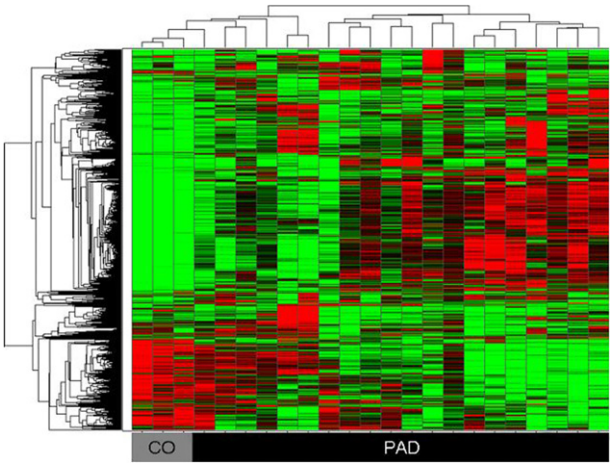

Fig. 2 – Hierarchical cluster analysis of microarray results between healthy CO and those with PAD. Red = up-regulated genes; green = down-regulated genes.

(526), the cytoskeleton (526) or transcription factors (121). Down-regulated genes in the PAD group had transmembrane transporter (227) or ion transmembrane transporter activity (115). Others were G-protein–coupled receptors (83) (Table 3).

Five genes were up-regulated more than 100-fold for PAD compared with CO. Among these genes exons coding for immunoglobulins, matrix metalloproteinase 13 (MMP13) and step II splicing factor were identified. In the overexpressed proportion of genes within the PAD group, many players were associated with the immune response. Cytokines such as IL8, CCL18, and AIF-1, and alternative activated macrophage-specific CC chemokine 1 or receptor types for cellular compartments of the immune system such as KLRB1, macrophage receptor with collagenous structure, or the T cell receptor beta chain; and other receptor types such as IL7R, TLR4, tumor necrosis factor receptor superfamily, member 17, or prostaglandin E receptor 2 (subtype EP2) were up-regulated in the PAD group. Furthermore, we identified matrix metalloproteinases (MMPs) such as MMP1, MMP9, and MMP12 or structure proteins such as alpha-1 type XI collagen as up-regulated genes in the PAD *versus* CO group (Table 4). Down-regulated genes in the PAD *versus* CO group were more heterogeneously spread. Transcription factor (p38 interacting protein) and Purkinje cell protein 4, which were the main underexpressed genes, were 12-fold down-regulated in the PAD *versus* CO samples and contactin 3 (plasmacytoma associated) was 11-fold underexpressed in the PAD *versus* CO group. The marker for differentiated aortic smooth muscle and down-regulated with vascular injury and vasoactive intestinal peptide receptor 2 was fivefold down-regulated in the PAD samples. Muscle-associated genes such as myosin VB, actin, alpha, cardiac muscle precursor, and myosin, heavy polypeptide 11 were underexpressed in the PAD *versus* CO group (Table 5). Tables 4 and 5 present a detailed list of the main 200 differentially expressed genes between PAD and CO.

3.2.2. *Risk factor of hypercholesterolemia*

Comparing the gene expression values between patients with and without hypercholesterolemia, we identified 10 differentially regulated genes ($P < 0.01$; FC > 3). LOC440416 and IGF1R were up-regulated, whereas CEBPD, HIST2H2AA3, LIF, IGFBP1, DKK2, KIAA0146, and SOD2 were down-regulated in the hypercholesterolemia *versus* non-hypercholesterolemia group (Supplementary Table 1).

3.2.3. *Risk factor of smoking*

Comparing the gene expression values between smokers and nonsmokers, we found 330 differentially expressed genes in the investigated specimens ($P < 0.01$; FC > 3). ENO1, C1S, MYH11, and P4HB were up-regulated > FC10 in the smoker group, whereas S100P, TUBB1, AHSP, HBM, S100A12,PF4, CA1, SLC4A1, HBD, and PPBP were down-regulated < FC10 in the smoker *versus* nonsmoker group (Supplementary Table 2).

3.2.4. *Risk factor of diabetes*

Comparing the gene expression values between patients with diabetes mellitus and those without diabetes mellitus, only four differentially expressed genes could be identified ($P < 0.01$; FC > 3). IGLV3-19 and RASSF6 were up-regulated and CLU and SLC25A37 were down-regulated in the diabetes *versus* non-diabetes group (Supplementary Table 3).

3.3. *PCR validation*

We subjected 10 selected overexpressed genes in the PAD *versus* CO group to validation by PCR analysis: CCL18, CD45, KLRB1, IL8, IL23A, IL-7R, TPSAB, AIF-1, HMOX 1, and TLR4. These genes have been previously described as being involved in the occurrence of atherosclerosis; we therefore selected them as validation panel [13,20–23]. They were identified as 14- to 73-fold overexpressed in the PAD *versus* CO group. Polymerase chain reaction analysis in 10 PAD samples showed a clear up-regulation compared with three CO samples (Fig. 3). Comparison of the PCR results with the microarray signals underlines the validity of the identified differentially expressed genes for PAD *versus* CO.

3.4. *Data comparison with the literature*

We compared our results with those of four previously published studies investigating gene expression differences between healthy and arteriosclerotic tissue with microarray technology [16–19]. Those studies used various methods of samples, tissue workup, microarray technology, and statistical analysis (Table 6). The signature of 55 genes could be validated in our study. Of these genes, 15 were up-regulated or down-regulated ≤ FC 1, compared with our results (endothelial cell–specific molecule 1, tumor necrosis factor, α-induced protein 3, major histocompatibility complex class IE, proto-oncogene CRK-II, fibroblast activation protein, estradiol 17-ß dehydrogenase 2, Janus kinase 1, Proto-oncogene c-*fes*, thymosin ß-4, complement component 7, glutathione-S-transferase homolog, proliferation cyclic nuclear antigen, colony-stimulating factor 3, integrin ß2, p95, and IL3Rα). Only

Table 4 – Main up-regulated genes in PAD *versus* healthy CO.

Probeset ID	CO mean	PAD mean	FC PAD/CO	P value	GenBank ID	Description
						Immune response
217378_x_at	24	4687	198	0.006	X51887	V108 gene encoding an immunoglobulin kappa orphon
217022_s_at	257	27718	108	0.006	S55735	Immunoglobulin A1-A2 lambda hybrid GAU heavy chain
216984_x_at	40	3904	97	0.008	D84143	Immunoglobulin (mAb59) light chain V region
214777_at	22	2163	97	0.006	BG482805	Rearranged gene for kappa immunoglobulin subgroup V kappa IV
215379_x_at	232	21440	92	0.006	AV698647	Immunoglobulin lambda joining 3
211645_x_at	46	4022	88	0.006	M85256	Immunoglobulin kappa-chain VK-1 (IgK)
208168_s_at	24	2146	88	0.014	NM_003465	Chitinase 1 (chitotriosidase)
215176_x_at	120	9898	83	0.006	AW404894	Partial IGKV gene for immunoglobulin kappa chain variable region, clone 30
214677_x_at	424	33886	80	0.006	X57812	Rearranged immunoglobulin lambda light chain
216401_x_at	65	5088	78	0.006	AJ408433	Partial IGKV gene for immunoglobulin kappa chain variable region, clone 38
211430_s_at	465	35501	76	0.006	M87789	(Hybridoma H210) anti-hepatitis A IgG variable region
214470_at	**13**	**914**	**73**	**0.006**	**NM_002258**	***KLRB1***
209138_x_at	511	35739	70	0.006	M87790	(Hybridoma H210) anti-hepatitis A immunoglobulin lambda chain variable region
215121_x_at	574	39373	69	0.006	AA680302	Immunoglobulin lambda locus
214146_s_at	53	3533	67	0.011	R64130	Pro-platelet basic protein
209924_at	***221***	***13857***	***63***	***0.008***	**AB000221**	***Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated) CCL18***
223565_at	6	339	56	0.006	AF151024	HSPC190
202859_x_at	**124**	***7302***	**59**	***0.006***	**NM_000584**	***IL8***
216576_x_at	83	4334	52	0.006	AF103529	Isolate donor N clone N88K immunoglobulin kappa light chain variable region
221671_x_at	802	41601	52	0.006	M63438	Ig rearranged gamma chain mRNA, V-J-C region and complete cds.
217148_x_at	107	5376	50	0.006	AJ249377	Partial mRNA for human Ig lambda light chain variable region, clone MB91 (331 bp)
221651_x_at	930	42354	46	0.006	BC005332	Similar to immunoglobulin kappa constant, clone MGC:12418
205819_at	35	1589	45	0.014	NM_006770	Macrophage receptor with collagenous structure (MARCO)
234764_x_at	73	3285	45	0.006	U96394	anti-streptococcal anti-myosin immunoglobulin lambda light chain variable region
211796_s_at	37	1628	44	0.006	AF043179	T-cell receptor beta chain (TCRBV13S1-TCRBJ2S1)
217179_x_at	31	1320	42	0.006	X79782	mRNA for Ig lambda light chain
1552798_a_at	**21**	**852**	**41**	**0.006**	**NM_138557**	***Toll-like receptor 4 (TLR4), transcript variant 4***
1555745_a_at	191	7491	39	0.011	U25677	Lysozyme precursor
217388_s_at	80	3025	38	0.006	D55639	Monocyte PABL (pseudoautosomal boundary-like sequence)
222838_at	38	1400	37	0.011	AL121985	SLAM gene for signaling lymphocytic activation molecule
211644_x_at	116	3919	34	0.018	L14458	Ig rearranged kappa-chain gene V-J-region
202902_s_at	323	11007	34	0.006	NM_004079	Cathepsin S (CTSS)
221491_x_at	180	5800	32	0.011	AA807056	MHC class II, DR beta 3
205798_at	**58**	***1736***	**30**	***0.006***	**NM_002185**	***IL7R***
214669_x_at	376	11230	30	0.006	BG485135	Immunoglobulin kappa variable 3D-15
224795_x_at	1386	41396	30	0.006	AW575927	Immunoglobulin kappa constant
210084_x_at	**38**	***1122***	**29**	***0.006***	**AF206665**	***Mast cell alpha II tryptase (TPSAB1)***
226218_at	82	2376	29	0.006	BE217880	Interleukin 7 receptor
211372_s_at	12	341	29	0.006	U64094	Soluble type II interleukin-1 receptor
211478_s_at	14	407	29	0.006	M74777	Dipeptidyl peptidase IV (CD26)
205495_s_at	13	366	27	0.011	NM_006433	Granulysin (GNLY), transcript variant NKG5
210972_x_at	54	1343	25	0.006	M15565	T-cell receptor rearranged alpha-chain V-region (V-D-J)
209901_x_at	***296***	***7227***	**24**	***0.006***	**U19713**	***Allograft-inflammatory factor-1***
206641_at	9	221	24	0.018	NM_001192	Tumor necrosis factor receptor superfamily, member 17 (TNFRSF17)
216207_x_at	286	6790	24	0.006	AW408194	Immunoglobulin kappa variable 1-13
215946_x_at	366	8632	24	0.006	AL022324	Immunoglobulin lambda-like polypeptide 3
32128_at	591	13425	23	0.011	Y13710	Alternative activated macrophage specific CC chemokine 1
217147_s_at	10	237	23	0.006	AJ240085	T-cell receptor interacting molecule protein, splice variant (TRIM gene)
210031_at	26	575	23	0.006	J04132	T cell receptor zeta-chain
221286_s_at	20	460	23	0.014	NM_016459	Hypothetical protein (LOC51237)

(*continued on next page*)

Table 4 – (continued)

Probeset ID	CO mean	PAD mean	FC PAD/CO	P value	GenBank ID	Description
205159_at	173	3875	22	0.006	AV756141	Colony-stimulating factor 2 receptor beta, low-affinity (granulocyte-macrophage)
205114_s_at	268	5970	22	0.008	NM_002983	Small inducible cytokine A3 (homologous to mouse Mip-1a) (SCYA3)
204006_s_at	194	4083	21	0.006	NM_000570	Fc fragment of IgG, low-affinity IIIb, receptor for (CD16) (FCGR3B)
212588_at	***644***	***9237***	***14***	***0.006***	***Y00062***	***T200 leukocyte common antigen (CD45, LC-A)***
						Metabolism
203548_s_at	72	3730	52	0.006	BF672975	Lipoprotein lipase
204638_at	208	9491	46	0.011	NM_001611	Acid phosphatase 5, tartrate resistant (ACP5)
206496_at	20	868	44	0.006	NM_006894	Flavin-containing monooxygenase 3 (FMO3)
221210_s_at	97	3478	36	0.006	NM_030769	Hypothetical protein similar to swine acylneuraminate lyase (C1ORF13)
209301_at	40	1337	34	0.008	M36532	Carbonic anhydrase II
204561_x_at	37	1047	28	0.018	NM_000483	Apolipoprotein C-II (APOC2)
203381_s_at	303	8384	28	0.006	N33009	Apolipoprotein E
202345_s_at	611	15624	26	0.006	NM_001444	Fatty acid binding protein 5 (psoriasis-associated) (FABP5)
213888_s_at	61	1509	25	0.006	AL022398	HSD11B1 gene for hydroxysteroid (11-beta) dehydrogenase 1
203980_at	507	12465	25	0.018	NM_001442	Fatty acid binding protein 4, adipocyte (FABP4)
213592_at	72	1722	24	0.014	X89271	HG11 orphan receptor
203665_at	***568***	***12713***	***22***	***0.008***	***NM_002133***	***Heme oxygenase (decycling) 1 (HMOX1)***
						Transportation and signaling
204848_x_at	47	1956	41	0.006	NM_000559	Hemoglobin, gamma A (HBG1)
209116_x_at	2608	82518	32	0.006	M25079	Sickle cell beta-globin
217232_x_at	2777	73663	27	0.006	AF059180	Mutant beta-globin (HBB) gene
214390_s_at	21	473	23	0.018	AI652662	Branched chain aminotransferase 1, cytosolic
203388_at	64	1741	27	0.006	NM_004313	Arrestin, beta 2 (ARRB2)
231804_at	18	462	26	0.006	AI805323	Leucine-rich repeat-containing G protein–coupled receptor 7
209732_at	316	6965	22	0.006	BC005254	C-type (calcium-dependent, carbohydrate-recognition domain) lectin, member 2
206343_s_at	8	169	22	0.006	NM_013959	Neuregulin 1 (NRG1), transcript variant SMDF
223344_s_at	199	4338	22	0.006	AB026043	MS4A7
206631_at	23	493	21	0.006	NM_000956	Prostaglandin E receptor 2 (subtype EP2)
						Cell adhesion and migration
205959_at	17	2052	120	0.018	NM_002427	MMP13 (collagenase 3)
204580_at	149	10092	68	0.011	NM_002426	MMP12 (macrophage elastase)
203936_s_at	437	22758	52	0.008	NM_004994	MMP9
206488_s_at	209	7335	35	0.011	NM_000072	CD36 antigen (collagen type I receptor, thrombospondin receptor) (CD36)
204475_at	57	1905	33	0.011	NM_002421	MMP1 (interstitial collagenase)
205885_s_at	20	542	28	0.006	L12002	Integrin alpha 4 subunit
37892_at	105	2478	24	0.011	J04177	Alpha-1 type XI collagen (COL11A1)
205997_at	47	1129	24	0.006	NM_021778	Disintegrin and metalloproteinase domain 28 (ADAM28), transcript variant 2
						Cell growth and development
207238_s_at	46	3701	80	0.006	NM_002838	Protein tyrosine phosphatase, receptor type, C (PTPRC)
204661_at	90	5568	62	0.006	NM_001803	CDW52 antigen (CAMPATH-1 antigen) (CDW52)
209555_s_at	176	7888	45	0.006	M98399	Antigen CD36
202005_at	55	1157	21	0.011	NM_021978	Suppression of tumorigenicity 14 (colon carcinoma, matriptase, epithin) (ST14)
						Clotting
206214_at	106	3315	31	0.014	NM_005084	Phospholipase A2, group VII (PLA2G7, plasma)
227361_at	11	350	31	0.006	AA780067	Heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1
						Miscellaneous
212592_at	92	9358	102	0.006	AV733266	Step II splicing factor SLU7
218876_at	28	1094	39	0.006	NM_016140	Brain-specific protein (LOC51673)
1555728_a_at	186	6071	33	0.006	AF354928	MS4A4A protein

FC = fold change.
Bold and cursive letters are genes that underwent PCR validation.

glyceraldehyde-3-phosphatase dehydrogenase could be identified as differentially expressed in three studies, with various results (FC −2 to 2). We could not detect 16 previously published genes in our list. A total of 18 genes showed an underexpression in PAD in our study, whereas they were published as up-regulated elsewhere (Table 7).

Table 5 – Main down-regulated genes in PAD *versus* healthy CO.

Probeset ID	CO mean	PAD mean	FC PAD/CO	P value	GenBank ID	Description
						Signaling
217066_s_at	1856	505	−5	0.008	M87313	Myotonin protein kinase (DM)
211489_at	443	120	−5	0.011	D32201	Alpha 1C adrenergic receptor isoform 3
214510_at	1078	289	−5	0.011	NM_005293	G protein–coupled receptor 20
204916_at	8707	2344	−5	0.006	NM_005855	Receptor (calcitonin) activity modifying protein 1 precursor
204648_at	3418	909	−5	0.006	NM_000906	Natriuretic peptide receptor A guanylate cyclase A (atrio natriuretic peptide receptor A)
203951_at	80569	21011	−5	0.006	NM_001299	Calponin 1, basic, smooth muscle
212669_at	10179	2629	−5	0.006	AI093569	Calcium calmodulin-dependent protein kinase (CaM kinase) II gamma
216134_at	197	51	−5	0.008	AK000244	KIAA1013 protein
208481_at	157	40	−5	0.018	NM_016116	ASB-4 protein
215807_s_at	1414	361	−5	0.014	AV693216	Plexin B1
32625_at	7323	1820	−5	0.006	X15357	Natriuretic peptide receptor (ANP-A receptor)
205947_s_at	418	103	−5	0.014	NM_003382	Vasoactive intestinal peptide receptor 2
211598_x_at	1097	269	−5	0.006	U18810	PACAP type-3VIP type-2 receptor
235649_at	10490	2553	−5	0.014	AW207389	Zinc metalloendopeptidase
215070_x_at	892	205	−5	0.006	AK022408	Rab6 GTPase activating protein
208314_at	174	40	−5	0.006	NM_006583	Retinal pigment epithelium-derived rhodopsin homolog
205478_at	1224	279	−5	0.008	NM_006741	Protein phosphatase 1, regulatory (inhibitor) subunit 1A
218266_s_at	1411	312	−6	0.006	NM_014286	Frequenin (*Drosophila*) homolog (FREQ)
1556096_s_at	630	139	−6	0.014	AL834407	Munc13-3 ; cDNA DKFZp547H074
206169_x_at	4974	1077	−6	0.006	NM_025013	KIAA1031 protein (KIAA1031)
214369_s_at	3990	832	−6	0.006	AI688812	RAS guanyl releasing protein 2 (calcium and DAG-regulated)
214966_at	517	105	−6	0.006	S40369	Kainite receptor subunit (human, hippocampus, mRNA, 2943 nt)
227819_at	4875	938	−6	0.006	AA524536	VTS20631 mRNA, g-protein coupled receptor family
211909_x_at	263	49	−6	0.011	L32662	prostaglandin E2 receptor EP3 subtype isoform IV
210401_at	6606	1046	−8	0.008	U45448	P2X1 receptor
205549_at	2576	242	−12	0.006	NM_006198	Purkinje cell protein 4
						Cell growth and development
214285_at	3342	919	−4	0.008	AI041520	Fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
225301_s_at	940	259	−4	0.014	AI991160	Myosin VB
205833_s_at	1578	427	−5	0.008	AI770098	Prostate androgen-regulated transcript 1
205104_at	1107	294	−5	0.006	NM_014723	Syntaphilin (KIAA0374)
222570_at	2438	627	−5	0.006	AA045247	Frequenin (*Drosophila*) homolog
205278_at	1536	376	−5	0.006	NM_000817	Glutamate decarboxylase 1 (brain, 67kD) (GAD1), transcript variant GAD67
1553613_s_at	22864	5474	−5	0.006	NM_001453	Forkhead box C1
209959_at	2197	522	−5	0.008	U12767	Mitogen-induced nuclear orphan receptor
227915_at	3044	725	−5	0.006	AI872284	Ankyrin repeat-containing protein ASB-2
229032_at	2869	638	−6	0.006	BE962770	Proliferating cell nuclear antigen
218934_s_at	17204	3814	−6	0.006	NM_014424	Heat-shock 27kD protein family, member 7 (cardiovascular)
206349_at	543	93	−8	0.018	NM_005097	Leucine-rich, glioma inactivated 1 precursor
						Transcription
227347_x_at	3255	876	−5	0.011	NM_021170	bHLH factor Hes4
228854_at	48337	12838	−5	0.018	AI492388	Poly(A)-binding protein, nuclear 1
209781_s_at	6950	1812	−5	0.008	AF069681	T-Star
226523_at	30167	7620	−5	0.006	AI082237	Proprotein convertase subtilisinkexin type 7
207837_at	2671	643	−5	0.008	NM_006867	RNA-binding protein gene with multiple splicing
1555352_at	667	155	−5	0.011	AF467257	Forkheadwinged helix transcription factor
1554776_at	174	32	−6	0.006	AF450454	Zinc finger protein 42
205727_at	737	132	−6	0.011	NM_007110	Telomerase-associated protein 1
1555318_at	1131	178	−8	0.018	BC026308	Hypoxia inducible factor 3, alpha subunit
213931_at	15823	2466	−8	0.008	AI819238	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein
213906_at	9046	1348	−9	0.006	AW592266	v-myb avian myeloblastosis viral oncogene homolog-like 1
214027_x_at	22661	1842	−12	0.006	AA889653	Transcription factor (p38 interacting protein)
						Immune response
231829_at	1840	489	−5	0.008	AB033097	KIAA1271 protein
1558924_s_at	439	110	−5	0.008	BF673049	Cytoplasmic linker protein CLIP-170

(*continued on next page*)

Table 5 – (continued)

Probeset ID	CO mean	PAD mean	FC PAD/CO	P value	GenBank ID	Description
230087_at	4089	957	−5	0.008	AI823645	Lymphocyte antigen 6 complex, locus E
220273_at	1499	229	−8	0.006	NM_014443	Interleukin 17B
206595_at	5740	1130	−6	0.008	NM_001323	Cystatin EM (CST6)
215255_at	1771	306	−7	0.006	AB028953	KIAA1030 protein
						Adhesion, cytoskeletal development, and migration
213371_at	14880	3798	−5	0.006	AI803302	Z-band alternatively spliced PDZ-motif
203861_s_at	722	176	−5	0.018	AU146889	Actinin, alpha 2
207876_s_at	9348	2276	−5	0.006	NM_001458	Filamin C, gamma (actin-binding protein-280) (FLNC)
205265_s_at	7400	2010	−5	0.008	NM_005876	Marker for differentiated aortic smooth muscle and down-regulated with vascular injury
205132_at	84736	21663	−5	0.011	NM_005159	Actin, alpha, cardiac muscle precursor
210632_s_at	4468	973	−6	0.006	L35853	adhalin-35
232054_at	1972	427	−6	0.006	AA040057	Proto cadherin 20
214961_at	412	88	−6	0.008	AI818409	KIAA0774 protein
207390_s_at	21238	4311	−6	0.008	NM_006932	Smoothelin (SMTN)
218744_s_at	416	83	−6	0.006	NM_016223	SH3 domain-containing protein 6511 (LOC51165)
227209_at	852	168	−6	0.008	AI091445	Contactin 1
1568760_at	9127	2285	−5	0.006	BF510409	Myosin, heavy polypeptide 11
229831_at	1926	237	−11	0.006	BE221817	Contactin 3 (plasmacytoma associated)
						Metabolism
208491_s_at	860	203	−5	0.006	NM_021965	Phosphoglucomutase 5
209646_x_at	7984	1884	−5	0.006	BC001619	Aldehyde dehydrogenase 5
1563933_a_at	1787	310	−7	0.006	AK091691	Schwannoma-associated protein (SAM9)

4. Discussion

We compared the differential gene expression between atherosclerotic and healthy common femoral arteries. This is the first time that healthy specimens of proximal extremity arteries from controlled vascular healthy donors were compared with atherosclerotic vessels. The main challenge for studies analyzing the gene expression in proximal extremity arteries is the limited amount of healthy control samples, because few patients are available as donors. We used vascular healthy organ donors for our investigations. In this special cohort, we were able to recruit three patients. One reason for this small number is the low agreement to donate vessel tissue for study purpose. The other reason is that many organ donors are already affected with early signs of atherosclerosis, which was an exclusion criterion for our study. We recruited patients with various risk factors for atherosclerosis (Table 1). During hierarchical cluster analysis, the PAD group could be sufficiently separated from the CO samples (Fig. 2), but the patients could not be clustered regarding their risk factors or the stage of PAD. We analyzed only patients Fontaine stage II b–IV because surgery is feasible only in symptomatic cases. These patients are already in an advanced stage of the disease. The identified differentially expressed genes respect this clinical aspect, because PAD that has already progressed seems to be characterized by a similar molecular profile which can not be divided in clinical stages by molecular patterns. This means that if PAD is initiated for any reason and narrowing proliferation processes of the vascular wall have been initiated, the ongoing disease seems to be homogeneously on a molecular level irrespective of the discrimination of the clinical stages. Thus, there is a kind of common molecular road in PAD after it has started. During microarray analysis regarding the risk factors for atherosclerosis, we identified most differences between the smoking and nonsmoking groups. This finding underlines the important role of smoking as an extreme risk factor for atherosclerosis, which induces the most gene expression changes within the vessels compared with other etiological factors.

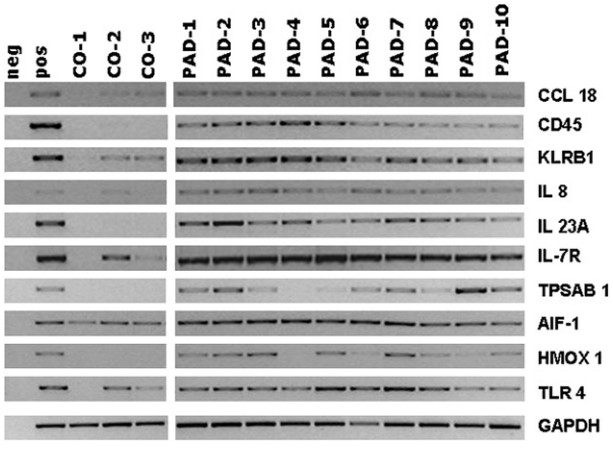

Fig. 3 – Polymerase chain reaction validation of microarray results from differentially expressed genes between healthy controls (CO) and those with PAD. neg = negative control; pos = positive control.

All samples underwent histopathological examination. In the CO group, specimens of the aorta that are the first regions for occurrence of early atherosclerosis were controlled by histopathology [24]. Therefore, we carefully characterized the CO group to exclude early disease patterns. In contrast, all PAD samples were clearly affected by atherosclerosis, as

Table 6 – Methods of microarray studies comparing peripheral arterial disease with controls.

	Hiltunen [17]	Martinet [18]	Tuomisto [19]	Burton [16]	Croner [current study]
Tissue type	Arteriosclerotic arteries (aorta, iliac, crural)	A. carotis (endarterectomy)	Arteriosclerotic arteries (aorta, iliac, coronary)	Smooth muscle cells vascular wall	Arteria femoralis communis (endarterectomy)
Tissue workup	NR	Laser microdissection	Laser microdissection	Cell cultures	Manual microdissection
RNA-isolation	Micro-FastTrack 2.0 (Invitrogen)	Trizol (Invitrogen)	RNA microprep (Strategene)	Trizol (Invitrogen)	RNeasy (Quiagen)
RNA-control	NR	SMART PCR (ClonTech)	RiboGreen RNA kit	2100 Bioanalyser (Agilant)	2100 Bioanalyser (Agilant)
Microarray	cDNA (Gene Discovery Array 1.3, Incyte Genomics)	cDNA (Human Apoptosis Array, ClonTech)	cDNA (Sanger center Hver1.2.1)	cRNA (HG-U133A, Affymetrix)	cRNA (HG-U133 plus 2, Affymetrix)
Statistics	ArrayVision (Imaging Research)	Student's t-test	GeneSpring (Silicon Genetics)	MAS 5, Bioconductor	Partek Genomics Suite (v6.5), Genetrail
Validation	RT-PCR, in situ Hybridization	RT-PCR, Western blot	RT-PCR	RT-PCR, Western blot	PCR
Identified genes	92	17	72	327	6.537
P value	NR	<0.05	NR	NR	<0.01

NR = not reported.

identified by histopathology (Fig. 1). During thromboendarterectomy of the femoral arteries, the plaques were mobilized between the tunica elastica interna and externa, resulting in loss of the adventitia and parts of the media. To make the CO specimens comparable with the PAD samples, all CO underwent MAMD to remove the adventitia and parts of the media. This tissue workup enabled us to investigate the endothelial wall and the media, which are the main affected regions by atherosclerosis. Microscopically assisted manual microdissection is a sufficient method for tissue workup before microarray analysis that was successfully used previously [10]. Although only limited amounts of CO were available, the accurate characterization of the tissue by histopathologic control and the specific tissue workup guaranteed sufficient results of microarray analysis. The extreme high data reproducibility during PCR validation of selected markers underlines this assumption (Fig. 3). Comparison of our data with previous studies resulted in good overlap [16–19]. Nevertheless, among these studies, only one gene was frequently described (Table 7). This finding further indicates the reliability of the data we evaluated. But there is an ongoing problem in that various methods during microarray procedures produce different results, which shows limited comparability (Table 6). This was already described for other types of disease and could even be detected in PAD [25].

The genes identified as up-regulated in PAD were mainly located in the cytoplasm, membrane, and nucleus of the cells (Table 2). Their function is binding activity for nucleotides, proteins, and transcription factors (Table 3). This makes them ideal targets for therapeutic strategies. They are related to the immunological system, which demonstrates the high value of this compartment in PAD. The high expression of macrophage and T-cell receptors (e.g., macrophage receptor with collagenous structure, T-cell receptor rearranged α-chain, T-cell receptor interacting molecule protein, splice variant, T cell receptor ζ-chain, or immune cell proliferation stimulators (colony-stimulating factor 2Rß and granulocyte-macrophage) underline the necessity of inflammatory cellular activity in PAD. Elevated levels of expressed cytokines and chemokines in PAD (e.g., IL8, CCL18) are necessary for local immune cell attraction and recruitment. High circulating levels of IL8 were recently identified as a risk factor for cerebrovascular and cardiovascular events in patients with PAD [26]. Furthermore, we identified several MMPs (e.g., MMP13, MMP 12, MMP 9, and MMP1) that initiate invasion and proliferation as highly up-regulated in PAD. In particular, increased plasma levels of MMP9 were recently identified as an indicator for the development and progression of PAD [27]. These findings are obvious indicators for ongoing severe inflammatory processes during PAD in extremity vessels. Interestingly, CD52, which was highly expressed in PAD, is associated with chronic lymphoproliferative disease [28]. The efficacy of targeted therapy (alemtuzumab) in CLL, for instance, depends on the CD52 expression level [29]. Therefore, such overexpressed molecules identified in our study may act as new therapeutic targets for PAD. Although chemotherapy is considered to be an extreme treatment for PAD, it might be indicated in severe clinical cases, because to date, no effective causal medication of atherosclerosis exists. Other genes such as NRG1 or ST14

Table 7 – Comparison of recently published lists of arteriosclerosis-associated up-regulated genes identified by microarray technique [12–15].

Accession No. GenBank	Gen	Symbol	Hiltunen 2002 [17]	Martinet 2002 [18]	Tuomitso 2003 [19]	Burton 2009 [16]	Croner [current study]
NM_000900	MatrixG1a protein	MGB	–	–	–	23.4	−1.8
M15330	Interleukin 1ß	IL1B	–	–	–	9.4	4.1
NM_000201	Intercellular adhesion molecule 1 (CD54)	ICAM1	–	–	–	7.6	3.45
NM_002546	Osteoprotegerin	TNFRSF11B	–	–	–	5.5	−1.4
NM_007036	Endothelial cell-specific molecule 1	ESM1	–	–	–	5.5	4.5
AF138302	Decortin	DCN	–	–	–	4.7	3.3
AU149305	Matrix metallopeptidase 14	MMP14	–	–	–	4	1.3
AF043337	Interleukin 8	IL 8	–	–	–	3.8	28
NM_001200	Bone morphogenetic protein 2	BMP2	–	–	–	3	1.1
AF091352	Vascular endothelial growth factor	VEGF	–	–	–	2.1	3.4
AI738896	Tumor necrosis factor-α induced protein 3	TNFAIP3	–	–	–	2	2.5
NM_003377	Vascular endothelial growth factor B	VEGFB	–	–	–	2.4	−1.1
NM_001124	Adrenomedullin	ADM	–	–	–	1.9	4.1
S69738	Chemokine (C-C motif) ligand 2	CCL2	–	–	–	1.9	3.2
M83248	Secreted phosphoprotein 1	SPP 1	–	–	–	1.4	6.5
R79246	Melanoma adhesion molecule	MCAM	56.5	–	–	–	1.4
R78870	Neuronal PAS domain protein	NPAS	49.8	–	–	–	−1.7
R68089	HS solute carrier family 31 member 2	SLC31A2	44.8	–	–	–	6.2
H12633	Proteasome subunit, α type 2	PSMC2	37.6	–	–	–	1.2
R81942	Oligopherin 1	OPHN1L	34.4	–	–	–	1.3
R33252	Plated endothelial cell adhesion molecule-1 (CD31)	PECAM-1	29.5	–	–	–	6
H12682	Ubiquitin-conjugated enzyme E2D1	E2D1	21.3	–	–	–	1.6
R67754	NADH-ubiquinone oxidoreductase	NDUFB2	11.6	–	–	–	1.3
R70035	Human non-muscle myosin alkali light chain	MYL6	10.1	–	–	–	–
AA005168	Zinc finger protein 7	ZNF7	6	–	–	–	3.1
R80719	Proteasome, chain 7	PSMB7	4.5	–	–	–	–
R62231	Ribonuclease, pancreatic	RNASE1	4.1	–	–	–	7.8
H01482	Pleckstrin p47	PLEK	3.2	–	–	–	–
AA114835	Cytochrome P450 c21B	CYP21A2	3.1	–	–	–	–
N90788	Annexin IV	ANXA4	2.8	–	–	–	1.5
N90527	Proto-oncogene pim-1	PIM1	2.7	–	–	–	–
T87872	Mitogen-activated protein kinase activator 1	MAPK1	2.6	–	–	–	–
N21289	Glycoprotein MUC 18	MCAM	2.5	–	–	–	–
N44748	Epidermal growth factor receptor HER3	HER3	2.4	–	–	–	1
H14567	Immunoglobulin lambda light chain, VDJ regions	IGHM	2.3	–	–	–	–
R74335	Major histocompatibility complex, class I, E	HLA-E	2.3	–	–	–	2.9
R72217	Acetyl-coenzyme A carboxylase	ACACA	2.2	–	–	–	–
N89746	Myosin, light chain, smooth muscle		2.2	–	–	–	–
H75531	Proto-oncogene CRK-II	CRK-II	2.2	–	–	–	1.2
W42634	Fibroblast activation protein	FAP	2.1	–	–	–	2.7
W03282	Dihydrofolate reductase	DHFR	2	–	–	–	3.8
R10875	Estradiol 17ß dehydrogenase 2	HSD17B	2	–	–	–	1.6
R99810	Protein kinase plk-1	PLK1	2	–	–	–	−1.1
R46266	Carbonic anhydrase	CAII	1.9	–	–	–	33.7
H18190	Janus kinase 1	JAK-1	1.9	–	–	–	1.4
T95816	Insulin-like growth factor binding protein 5	IGFL5	1.8	–	–	–	–
N32567	Proto-oncogene c-*fes*	FES	1.6	–	–	–	1.5
N36818	Thymosin ß4	TMSB4	1.8	–	–	–	1
AA054271	Glyceraldehyde-3-phosphatase dehydrogenase	G3PT	1.7	2.4	–	–	−2
W31678	UDP-glucose pyrophosphorylase	UGP2	1.7	–	–	–	–

(*continued on next page*)

Table 7 – (*continued*)

Accession No. GenBank	Gen	Symbol	Hiltunen 2002 [17]	Martinet 2002 [18]	Tuomitso 2003 [19]	Burton 2009 [16]	Croner [current study]
R35713	VEGF receptor 2	VEGFR-2	1.6	–	–	–	–
R23778	Complement component 7	C7	1.5	–	–	–	1.5
X76104	Death-associated protein kinase	DAP kinase	–	5	–	–	–
M11886	HLA class I histocompatibility antigen C4	HLAC	–	3.6	–	–	1.9
M32315	Tumor necrosis factor receptor 2 precursor	TNF-R2	–	3.5	–	–	1.8
U13699	Caspase-1 precursor	CASP1	–	3.4	–	–	8.3
L22474	Apoptosis regulator BAX	TMBIM1	–	3	–	–	−1.3
L29511	Growth factor receptor-bound protein 2	GRB2	–	2.9	–	–	−2.2
U90313	Glutathione-S-transferase homolog	GSTO1	–	2.8	–	–	1.9
M15796	Proliferation cyclic nuclear antigen	PCNA	–	2	–	–	2.1
NM_001172	Arginase type II	AEG2	–	–	8	–	−1.1
BC068023	ATPase H+ transporting, lysosomal	ATP6V1G2	–	–	25.9	–	1
NM_001911	Cathepsin G	CTSG	–	–	5.2	–	8
BC038398	Chemokine (C-C motif) receptor 5	CCR5	–	–	4.1	–	6.4
AK225764	Chemokine-like receptor 1	CXC3CR1	–	–	2.1	–	3.6
NM_005211	Colony stimulating factor 1 receptor	CSF1R	–	–	2.1	–	5.3
NM_006140	Colony-stimulating factor 2 receptor	CSF2RA	–	–	19.9	–	8.9
NM_172313	Colony-stimulating factor 3	CSF3R	–	–	4.4	–	4.3
NM_004010	Dystrophin	DMD	–	–	2.6	–	−1.4
NM_004757	Endothelial monocyte-activating polypeptide	SCYE1	–	–	12.4	–	−1.1
NP_006030	Endocyte receptor	MRC2	–	–	2.1	–	–
NM_005797	Epithelial V-like antigen 1	EVA1	–	–	9.6	–	6.8
U63917	G protein–coupled receptor	GPCR	–	–	3	–	−2.1
AC002310	Integrin alpha L	CD11a	–	–	2.2	–	6.4
NM_000632	Integrin αM	CD11b	–	–	2	–	7.1
NM_000211	Integrin ß2, p95	CD18	–	–	10.6	–	10.9
BE563442	Interleukin 1 receptor, type I	IL1RN	–	–	4	–	−2.4
NM_014339	Interleukin 17 receptor	IL17R	–	–	3.4	–	1.3
AK290568	Interleukin 3 receptor. Alpha	IL3RA	–	–	2.1	–	1.7
BC037961	Interleukin 8 receptor, ß	IL8RB	–	–	4.7	–	5.4
NC_000019	Leukocyte immunoglobulin-like receptor, subfamily B, member 4	LILRB4	–	–	18.2	–	–
BC037961	Macrophage stimulating 1 receptor	MST1R	–	–	10.5	–	1
BC006390	Mannosyl (α-1,6)-glycoprotein	MGAT2	–	–	3.5	–	1.9
NC_000006	Mitogen-activated protein kinase 4	MAP3K4	–	–	6.4	–	–
NT_025741	Mitogen-activated protein kinase 5	MAP3K5	–	–	2.3	–	–
AF049656	Nitric oxide synthase	iNOS	–	–	2.6	–	−1.7
NM_004794	Member RAS oncogene family	RAB33A	–	–	4.7	–	−1.1
NM_003123	Sialophorin	CD43	–	–	2.2	–	−1.1
NM_003264	Toll-like receptor 2	TLR2	–	–	2.2	–	4.7
NM_003347	Ubiquitin.conjugating enzyme E2L3	UBE2L3	–	–	2.8	–	−1,9
AK292144	Zinc finger protein 272	ZNF272	–	–	2.4	–	−1.7

Values show the relative overexpression (fold change) of arteriosclerosis *versus* controls. Minus signs show genes not reported or identified in the study.

that were highly up-regulated in PAD are related to cell–cell interaction and growth or development of several organ systems. They have been previously described as being deregulated in several cancer types (e.g., breast, colon, and ovarian) and metastasis. Their role in atherosclerosis is unclear and has to be further clarified. Many genes involved in cell growth and development (e.g., myosin VB, marker for differentiated aortic smooth muscle, myosin, and heavy polypeptide-11) were down-regulated in PAD. Loss of structural proteins during vascular wall remodeling in atherosclerosis weakens the vessel wall. Clinical manifestations of this finding may be aneurysms for what arteriosclerosis is known to act as a risk factor.

We identified a broad spectrum of inflammatory-associated genes in PAD *versus* CO that characterizes atherosclerosis in peripheral extremities as extreme inflammation. In this way, some targets may be defined that may act as future causal treatment targets for PAD. The remodeling with weakening of the vascular wall that becomes obvious during the clinical course of the disease could be defined on a molecular base. These findings lead to a more detailed understanding of ongoing molecular processes in peripheral extremity arteries evaluated in extremely detailed characterized and comparable specimens. A prognostic molecular risk profiling of PAD may be possible in the future.

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Supplementary data.

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jss.2012.07.024.

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