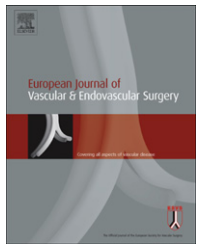
Eur J Vasc Endovasc Surg (2010) **40**, 549–558



Carotid Artery Disease: Novel Pathophysiological Mechanisms Identified by Gene-expression Profiling of Peripheral Blood

L. Rossi [a], I. Lapini [a], A. Magi [a], G. Pratesi [b], M. Lavitrano [c], G.M. Biasi [c], R. Pulli [d], C. Pratesi [d], R. Abbate [a], B. Giusti [a,*]

[a] *Department of Medical and Surgical Critical Care and DENOTHE Center, University of Florence, Florence, Italy*
[b] *Vascular Surgery Unit, Department of Surgery, University of Rome 'Tor Vergata', Rome, Italy*
[c] *Department of Surgical Sciences, University of Milano-Bicocca, Milan, Italy*
[d] *Department of Vascular Surgery, University of Florence, Florence, Italy*

Submitted 13 April 2010; accepted 9 July 2010
Available online 14 August 2010

KEYWORDS
Carotid artery stenosis;
Abdominal aortic aneurysm;
Gene-expression;
Immune response;
Whole blood

Abstract *Object:* The pathogenesis of carotid artery stenosis (CAS) as well as the mechanisms underlying the different localisation of the atherosclerotic lesions remains poorly understood. We used microarray technology to identify novel systemic mediators that could contribute to CAS pathogenesis.

Moreover, we compared gene-expression profile of CAS with that of patients affected by abdominal aortic aneurysm (AAA), previously published by our group.

Methods and results: By global gene-expression profiling in a pool of 10 CAS patients and 10 matched controls, we found 82 genes differentially expressed. Validation study in pools used for profiling and replication study in larger numbers of CAS patients ($n = 40$) and controls ($n = 40$) of 14 genes by real-time polymerase chain reaction (RT-PCR) confirmed microarray results. Fourteen out of 82 genes were similarly expressed in AAA patients. Gene ontology analysis identified a statistically significant enrichment in CAS of differentially expressed transcripts involved in immune response and oxygen transport. Whereas alteration of oxygen transport is a common tract of the two localisations, alteration of immune response in CAS and of lipid metabolic process in AAA represents distinctive tracts of the two atherosclerotic diseases.

Conclusions: We describe the systemic gene-expression profile of CAS, which provides an extensive list of potential molecular markers.

* Corresponding author at: Department of Medical and Surgical Critical Care, University of Florence, Viale Morgagni 85, 50134 Florence, Italy. Tel.: +39 0557949420; fax: +39 0557949514.
E-mail address: betti.giusti@unifi.it (B. Giusti).

1078-5884/$36
doi:10.1016/j.ejvs.2010.07.006

Carotid artery disease is a major cause of ischaemic stroke,[1] and cerebrovascular disease is one of the main causes of death and disability in developed countries.[2] Atherosclerosis is a chronic immune-inflammatory disease of the arterial wall, where both innate and adaptive immunologic and inflammatory mechanisms are involved.[3] The fine mechanisms underlying carotid artery stenosis (CAS) pathogenesis as well as the localisation of the atherosclerotic lesions in different districts of the arterial tree remain poorly understood.

Several genomic approaches have been applied to understand the multifactorial nature and different localisation of atherosclerosis: these include genome-wide and candidate gene association, as well as transcriptome and proteome studies.[4] Besides traditional cardiovascular risk factors, there is a current interest in the identification of novel molecular risk markers of atherogenesis and atherothrombosis.

A large number of expression profiling studies has been conducted over the past few years to investigate the molecular pathways involved in the onset and progression of atherosclerosis in CAS as well as other arteries' diseases.[5] Most studies compared whole atherosclerotic plaques or specific plaque regions to non-diseased arteries/regions. Transcript levels were analysed to study either the early phases of atherosclerosis or the progression from stable to ruptured lesions.[5] Few data are available on the global transcriptomic profile at systemic level.

In this study, we used a gene-expression profiling approach by using a two-colour microarray technology on peripheral blood to find possible mediators/markers, at systemic level, of CAS. In fact, blood is an easy specimen to obtain that could represent a valuable resource to translate results in diagnosis and prognosis processes. Moreover, blood is a fitting surrogate for atherosclerotic tissue, because it contains the inflammatory cells that are involved in its pathogenesis.[6]

Therefore, our study aimed to: (1) identify novel mediators/markers of CAS at the systemic level by studying the gene-expression profile of peripheral blood of patients with respect to control subjects; (2) characterise common and peculiar alterations of the gene-expression profile of CAS with respect to another atherosclerotic disease, with a different localisation of the lesions, abdominal aortic aneurysm (AAA), previously studied by our group.[7]

Materials and Methods

Subjects

The study group included 50 patients, undergoing carotid endarterectomy for severe stenosis (>70%) of the extracranial carotid artery, referred to the Vascular Surgery Department (University of Florence). All the patients were asymptomatic. Carotid stenosis was assessed by duplex scanning and confirmed by angiographic computed tomography and the degree of the stenosis was defined according to the North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria.[8] To assess for the presence of additional atherosclerotic disease, all subjects underwent clinical and diagnostic examinations, clinical cardiology evaluation, electrocardiogram (ECG), echocardiogram and peripheral duplex scanning.

A control group of 50 healthy subjects matched for age and gender were recruited in the same period. The control group had a negative history of vascular diseases. Controls were screened for carotid artery disease.

We used a structured questionnaire to identify symptom-free controls and to exclude subjects, who were suspected of having any form of arterial or venous vascular disease.

In patients and controls, blood was collected after an overnight fast. To avoid stress or conditions that may alter the expression profiles, patients were enrolled during the presurgical visit (1 month/15 days before surgery).

Hypertension,[9] dyslipidaemia[10] and diabetes[11] were defined according to international guidelines (additional information in Supplementary Data). The Local Ethics Committee approved the study, and written informed consent was obtained from all participants.

To compare CAS gene-expression profiles obtained in the present study with those of AAA patients obtained in our previous study,[7] we refer to materials and methods reported in the previous article. To facilitate the comparison of demographic and clinical characteristics, data on CAS patients, AAA patients (previous paper)[7] and healthy subjects (present and previous[7] paper) are reported in Table 1.

Haematological parameters

Blood samples were taken from all subjects for blood counts (red blood cells (RBC), white blood cells (WBC), haemoglobin (Hb), haematocrit (Hct) and reticulocytes) and for serum erythropoietin (EPO) level measurement. The RBC, WBC and reticulocyte counts, Hb and Hct were evaluated using an automated counter, while EPO was determined by DSL1100 Erythropoietin RIA (Diagnostic Systems Laboratories, TX, USA).

Total RNA preparation

Total RNA was extracted from venous peripheral blood using PAXgene Blood RNA Kit (Qiagen, Germany), and RNA quality and integrity were evaluated by capillary electrophoresis with 2100 Bioanalyzer (Agilent, CA, USA) (additional information in Supplementary Data). Equal aliquots of RNA from 10 CAS patients and 10 age- and gender-matched controls were pooled in four different pools, according to the experimental design reported below.

Experimental design and microarray gene-expression analysis

We performed two different pools for 10 CAS patients ($n = 5$ patients in pool A and $n = 5$ patients in pool B) and two different pools for 10 healthy subjects ($n = 5$ controls in pool C and $n = 5$ controls in pool D). Two replicates (dye swap) of the two microarray experiments were made.

Microarrays experiments were performed as described in our previous articles.[7,12] We used arrays representing 14 000 genes (70mer oligonucleotides; Human AROS v1.1, Operon Technologies, CA, USA) (additional information in Supplementary Data).

Table 1 Demographic and clinical characteristics of CAS and AAA patients and controls used in pools (gene-expression profiling) and individually (RT-PCR validation analyses on a second larger population).

CAS	CAS in pool ($N = 10$)	CTR in pool ($N = 10$)	p	CAS patients ($N = 40$)	CTR subjects ($N = 40$)	p
Age	68 (45–81)	68 (44–83)	–	69 (45–86)	65 (43–87)	–
Sex (male) N (%)	6 (60.0)	6 (60.0)	–	27 (67.5)[b]	23 (57.5)	0.488
Smoking N (%)	7 (70.0)	3 (30.0)	0.179	29 (72.5)	15 (37.5)	0.003
Diabetes N (%)	2 (20.0)	1 (10.0)	1	9 (22.5)	1 (2.5)	0.014
Hypertension N (%)	6 (60.0)	3 (30.0)	0.369	28 (70.0)	10 (25.0)	0.0001
Dyslipidemia N (%)	6 (60.0)	2 (20.0)	0.169	24 (60.0)	4 (10.0)	4.23×10^{-6}
CAD N (%)	3 (30.0)	0	0.210	8 (20.0)	0	0.053
PAD N (%)	1 (10.0)	0	1	5 (12.5)	0	0.054
COPD N (%)	3 (30.0)	0	0.210	7 (17.5)[c]	0	0.0117
AAA[a]	AAA in pool ($N = 10$)	CTR in pool ($N = 10$)	p	AAA patients ($N = 36$)	CTR subjects ($N = 36$)	p
Age	68 (60–82)	66 (52–85)	–	69 (55–84)	70 (52–86)	–
Sex (male) N (%)	8 (80.0)	8 (80.0)	–	32 (88.9)	32 (88.9)	–
Smoking N (%)	8 (80.0)	2 (20.0)	0.012	24 (66.6)	12 (33.3)	0.005
Diabetes N (%)	1 (10.0)	0	0.499	3 (8.3)	0	0.119
Hypertension N (%)	6 (60.0)	2 (20.0)	0.085	27 (75.0)	8 (22.2)	7.46×10^{-6}
Dyslipidemia N (%)	7 (70.0)	1 (10.0)	0.009	25 (69.4)	2 (5.5)	2.14×10^{-8}
CAD N (%)	1 (10.0)	0	0.499	13 (36.1)	0	6.80×10^{-5}
PAD N (%)	1 (10.0)	0	0.499	8 (22.2)	0	0.003
COPD N (%)	6 (60.0)	0	0.005	21 (58.3)	0	5.18×10^{-8}

CAS = carotid artery stenosis patients; CTR = control subjects; AAA = abdominal aortic aneurysm; CAD = coronary artery disease; PAD = Peripheral arterial occlusive disease; COPD = chronic obstructive pulmonary disease.
[a] previous paper.[7]
[b] CAS vs AAA $p = 0.03$.
[c] CAS vs AAA $p = 0.0003$.

Image processing and statistical analysis

Scanned images were processed using the GenePix Pro 4.1 software (Axon Instruments, CA, USA). For each microarray, we performed a local intensity-dependent normalisation.[13]

Data matrices obtained from the pre-processing procedure were analysed in R environment (http://www.r-project.org). Normalisation procedure was done by Statistical Microarray Analysis (sma) package (http://stat-www.berkeley.edu/users/terry/zarray/Software/smacode.html). Due to the Significance Analysis of Microarray (SAM)-method requirements, we analysed all the transcripts that had at least two values out of four for control and CAS patient pools (analysable transcripts).

Data were then analysed by the SAM algorithm.[14] In SAM analysis, we chose a delta value of 0.8 that allowed us to identify differentially expressed genes with a false discovery rate (FDR) of 2.2%. The full data set is available at ArrayExpress (E-MEXP-2113 in http://www.ebi.ac.uk/microarray-as/ae/browse.html) (additional information in Supplementary Data).

Gene ontology analysis

We used the biological process ontology by the Gene Ontology (GO) consortium.[15] The mappings were downloaded from ftp://ftp.ncbi.nlm.nih.gov/gene/DATA/gene2go.gz. Details on GO analysis were reported in our previous articles.[7,12]

Real-time PCR based gene-expression analysis

Fourteen differentially expressed genes were selected for validation of microarray analysis using RT-PCR in the subjects included in the pools or in the 40 patients and 40 controls used individually to replicate data obtained by microarray experiments. In the selection, we took care to validate genes with increased and decreased expression in CAS. We performed TaqMan RT-PCR using TaqMan pre-developed assays on an ABI Prism 7900 instrument (Applied Biosystems, CA, USA) to quantify the following genes: haemoglobin alpha 2 (*HBA2*), haemoglobin epsilon 1 (*HBE1*), haemoglobin theta 1 (*HBQ1*), dematin (*EPB49*), glycophorin c (*GYPC*), basigin (*BSG*), regulator of G-protein signalling 2 (*RGS2*), major histocompatibility complex, class II, DQ beta 1 (*HLA-DQB1*), major histocompatibility complex, class II, DP alpha 1 (*HLA-DPA1*), major histocompatibility complex, class II, DP beta 1 (*HLA-DPB1*), major histocompatibility complex, class I, B (*HLA-B*) and low-density lipoprotein receptor-related protein 5 (*LRP5*). The 'delta–delta Ct method' was used for comparing relative gene-expression results (PE Applied Biosystems, Perkin Elmer, CA). Expression of target genes was standardized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and displayed as fold-change relative to control RNA used as the calibrator.

Statistical analysis

Statistical analyses were carried out using statistical tests as implemented in SPSS software (version 11.5). All values

were expressed as median and range. When comparing groups, statistical significance was determined by using non-parametric Mann–Whitney test.

Results

Gene-expression profile by microarray technology of peripheral venous whole blood

Of the 14 000 transcripts represented on our arrays, after data processing and application of the filtering criteria, the analysable transcripts numbered 5084.

SAM analysis showed 82 genes differentially expressed in peripheral whole blood of CAS patients compared with controls: 61/82 genes had increased expression and 21/82 genes showed decreased expression in CAS patients (Table 2).

Gene ontology analysis of differentially expressed genes in CAS patients

According to GO analysis, we observed four statistically significant enriched biological processes associated with CAS: 'Major Histocompatibility Complex (MHC) class II (antigen processing and presentation)', 'Oxygen Transport', 'MHC class I (antigen processing and presentation)' and 'Immune Response' (Table 3). In Table 3, differentially expressed genes in CAS patients linked to each biological process term are also reported.

Comparison of gene-expression profiles of CAS and AAA patients

Comparing gene-expression profiles obtained in CAS patients (this article) and in AAA patients (previous article),[7] we found 14 genes differentially expressed in both CAS and AAA (Table 2 and Fig. 1). Of these 14 genes, 10 showed an increased expression and four a reduced expression in CAS and AAA patients with respect to the respective controls (Table 2 and Fig. 1).

By contrast, a larger number of genes showed a distinctive expression signature in CAS or AAA patients, which may therefore serve as specific signatures of disease.

Comparing the statistically significant enriched GO biological processes of CAS (Table 3) and AAA patients[7] ('oxygen transport': *HBA2*, *HBD*, *HBE1*, *HBQ1*; 'positive regulation of protein kinase activity': *MAP2K3*, *PTPRC*; and 'lipid metabolic process': *ACADS*, *CIDEA*, *LRP5*, *MGLL*, *ADIPOR1*, *HSD17B14*), we found that the alteration of the 'oxygen transport' is a common feature of the two diseases. On the contrary, the alteration of 'immune response' as well as that of the 'lipid metabolism' seems to be a distinctive signature of the CAS and AAA disease, respectively.

Microarray data validation by RT-PCR

To validate and replicate microarray experimental results, the messengerRNA (mRNA) expression of 14 out of 82 differentially expressed genes in CAS was investigated by RT-PCR. We evaluated these genes in the same pools of patients and controls used for microarray experiments (validation study, Table 4). Furthermore, we extended the RT-PCR analysis of these 14 genes in two independent larger populations of CAS patients ($n = 40$) and controls ($n = 40$) (replication study, Table 4). We selected five genes that were associated with GO terms altered only in CAS patients: three transcripts associated to the 'MHC class II (antigen processing and presentation)' and 'immune response' (*HLA-DPA1*, *HLA-DPB1*, *HLA-DQB1*); one transcript associated with 'MHC class I (antigen processing and presentation)' and 'immune response' (*HLA-B*); and one transcript associated to the 'immune response' (*IFIT1*).

Moreover, we validated nine genes selected among the 14 genes similarly altered in CAS and AAA patients (previous article)[7] (Table 2 and Fig. 1). These nine genes are involved in 'oxygen transport' (*HBA2*, *HBE1*, *HBQ1*), erythrocyte membrane (*EPB49* and *GYPC*), B-cell development and function as well as signalling in other white cells and platelets (*BTK*), induction of metalloproteinases (*BSG*), regulation of G-protein signalling (*RGS2*) and lipid metabolic process (*LRP5*).

All the mRNA levels of genes detected by RT-PCR on the pools were consistent with the microarray experiment (Table 4).

Concerning immune response genes, the replication of microarray data on the larger number of CAS patients and controls confirmed the statistically significant increased expression of *HLA-DPA1*, *HLA-DPB1* and *HLA-DQB1* and the significant decreased expression of *HLA-B* (Table 4).

RT-PCR analysis showed the significant higher expression of *HBQ1*, *HBE1*, *EPB49*, *GYPC* and *BSG* in CAS patients compared with controls. Only the *HBA2* gene failed to reach the statistical significance, even if it showed a trend to an increased expression in patients. *RGS2* and *LRP5* were confirmed to have a significant decreased expression in patients with respect to controls (Table 4). In Fig. 2, we showed the scatter plots of the individual ΔCt values of the three most significant genes (*HLA-B*, *BSG* and *RGS2*).

Haematological parameters in CAS patients and controls

To investigate the possible mechanisms and/or effects of expression changes in erythrocyte genes, we evaluated some haematological parameters (Table 5). CAS patients had significantly higher values of WBC and RBC counts, Hct and EPO plasma levels with respect to controls.

Discussion

In this article, we reported the peripheral blood gene-expression profile of CAS patients. Moreover, we identified common molecular pathways sustaining similar pathophysiological processes or novel molecular mechanisms of two important atherosclerotic diseases, CAS and AAA, by integrating systemic gene-expression data of CAS obtained in this study with those of AAA obtained in our previous article.[7]

Based on GO classification, we found that CAS patients differentially express a large variety of transcripts involved in immune response, antigen processing and presentation

Table 2 Genes differentially expressed in the blood of CAS patients.

Gene name	Symbol	Accession Number	Gene ID	*d*	*d*§
Genes with increased expression in CAS patients					
PDZK1 interacting protein 1	PDZK1IP1	NM_005764	10158	6.52	
haemoglobin, alpha 2[a]	HBA2	V00488	3040	4.24	5.30
Tescalcin	TESC	AK000614	54997	4.22	
solute carrier family 25, member 39	SLC25A39	NM_016016	51629	4.20	2.88
F-box protein 7	FBXO7	AL050254	25793	4.04	
guanylate kinase 1	GUK1	L76200	2987	3.58	3.52
calcium channel,voltage-dependent,gamma subunit 5	CACNG5	NM_014404	27091	3.45	
haemoglobin, theta 1[a]	HBQ1	NM_005331	3049	3.37	2.44
erythrocyte membrane protein band 4.9 (dematin)[a]	EPB49	U28389	2039	3.31	1.93
interferon-induced protein with tetratricopeptide repeats 1	IFIT1	X03557	3434	3.19	
ATPase, H+ transporting, lysosomal 16 kDa, V0 subunit c	ATP6V0C	M62762	527	3.18	
haemoglobin, epsilon 1[a]	HBE1	NM_005330	3046	3.18	3.49
minichromosome maintenance complex component 3	MCM3	AL034343	4172	3.09	
ubiquitin B pseudogene 1	UBBP1	X04801	7315	3.01	
family with sequence similarity 89, member B	FAM89B	AF052151	23625	2.99	
glutathione peroxidase 1	GPX1	Y00483	2876	2.93	
mitogen-activated protein kinase 3	MAP2K3	D87116	5606	2.92	1.71
ubiquitin B pseudogene 2	UBBP2	X04802	23668	2.70	
immunoglobulin kappa constant	IGKC	M63438	3514	2.69	1.73
eukaryotic translation elongation factor 2	EEF2	Z11692	1938	2.68	
ring finger protein 10	RNF10	D87451	9921	2.67	
RecQ protein like 4	RECQL4	AB006532	9401	2.62	
major histocompatibility complex, class II, DP beta[a]	HLA-DPB1	X02228	3115	2.54	
polymerase (RNA) III (DNA directed) (32kD)	POLR3G	U93868	10622	2.48	
H3 histone family, member C	HIST1H3C	X57128	8352	2.47	
BCL2-like 1	BCL2L1	Z23115	598	2.40	
cyclin I	CCNI	AF135162	10983	2.36	
sorbin and SH3 domain containing 3	SORBS3	AF037261	10174	2.31	
capping protein (actin filament), gelsolin-like	CAPG	M94345	822	2.28	
glycophorin C (Gerbich blood group)[a]	GYPC	NM_002101	2995	2.28	2.16
Basigin[a]	BSG	X64364	682	2.25	1.64
procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide	P4HB	J02783	5034	2.23	
T-cell receptor beta joining 2-1	TRBJ2-1	X00437	28629	2.23	
GA-binding protein transcription factor, alpha subunit (60kD)	GABPA	U13044	2551	2.19	
ribosomal protein L18	RPL18	L11566	6141	2.17	
transducin (beta)-like 3	TBL3	U02609	10607	2.16	
Albumin	ALB	M12523	213	2.12	
S100 calcium-binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	S100A4	M80563	6275	2.11	
solute carrier family 25, member 37	SLC25A37	AF223466	51312	2.09	
asparagine-linked glycosylation 3 homolog (*S. cerevisiae*, alpha-1,3-mannosyltransferase)	ALG3	Y09022	10195	2.09	
guanine nucleotide binding protein (G-protein), beta polypeptide 2-like 1	GNB2L1	M24194	10399	2.07	
karyopherin alpha 4 (importin alpha 3)	KPNA4	AB002533	3840	2.06	
erythroid associated factor	ERAF	AF208865	51327	2.06	
a disintegrin and metalloproteinase domain 7	ADAM7	AF090327	8756	2.05	
major histocompatibility complex, class II, DR beta 3	HLA-DRB1	V00522	3123	2.04	
	HLA-DRB3		3125		
	HLA-DRB4		3126		
N-myc downstream regulated	NDRG1	D87953	10397	2.03	
drebrin-like	DBNL	NM_014063	28988	2.03	
enoyl Coenzyme A hydratase, short chain, 1, mitochondrial	ECHS1	D13900	1892	2.01	
major histocompatibility complex, class II, DQ beta1[a]	HLA-DQB1	M81141	3119	2.01	

(*continued on next page*)

Table 2 (*continued*)

Gene name	Symbol	Accession Number	Gene ID	*d*	*d*§
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5	SMARCA5	AB010882	8467	2.01	
RNA-binding protein S1, serine-rich domain	RNPS1	L37368	10921	2.01	
apolipoprotein L, 2	APOL2	Z95114	23780	2.00	
SH3-binding domain glutamic acid-rich protein like	SH3BGRL	AF042081	6451	2.00	
neurogranin (protein kinase C substrate, RC3)	NRGN	X99076	4900	1.99	
trans-golgi network protein 2	TGOLN2	AF027516	10618	1.97	
RBM38 RNA-binding motif protein 38	RBM38	X75315	55544	1.97	
LIM homeobox protein 3	LHX3	AF156889	8022	1.96	
major histocompatibility complex, class II, DP beta1[a]	HLA-DPA1	X03100	3113	1.93	
glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)	GOT2	M22632	2806	1.93	
origin recognition complex, subunit 5 (yeast homolog)-like	ORC5L	U92538	5001	1.89	
ectonucleoside triphosphate diphosphohydrolase 5	ENTPD5	AF039918	957	1.89	
Genes with decreased expression in CAS patients					
neuroblastoma breakpoint family, member 12	NBPF12	AF131738	440675	−6.28	
AHNAK nucleoprotein (desmoyokin)	AHNAK	M80899	79026	−5.42	
cytokine receptor-like factor 1	CRLF1	AF059293	9244	−5.13	
suppressor of cytokine signalling 6	SOCS6	AF161545	9306	−4.69	
S100 calcium-binding protein A8 (calgranulin A)	S100A8	X06234	6279	−4.44	
complement factor H-related 4	CFHL4	X98337	10877	−3.86	
colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	CSF2RB	M59941	1439	−3.84	
coiled-coil domain containing 72	CCDC72	NM_015933	51372	−3.74	
low-density lipoprotein receptor-related protein 5[a]	LRP5	AF077820	4041	−3.70	−3.46
early growth response 1	EGR1	AJ243425	1958	−3.61	
Homo sapiens Bruton's tyrosine kinase (BTK), alpha-D-galactosidase A (GLA), L44-like ribosomal protein	BTK	U78027	695	−3.38	−2.76
guanine nucleotide binding protein (G-protein), alpha13	GNA13	AF493902	10672	−3.35	
receptor-associated protein of the synapse, 43kD	RAPSN	Z33905	5913	−3.34	−2.46
Homo sapiens clone R2 ErbB-3 R2 (c-erbB-3) mRNA, partial cds	ERBB3	U88358	2065	−3.30	
CDC-like kinase 1	CLK1	L29219	1195	−3.21	
chromosome 12 open reading frame 35	C12orf35	AK000703	55196	−3.21	
guanine nucleotide binding protein 10	GNG10	U31383	2790	−3.11	
major histocompatibility complex, class I, B[a]	HLA-B	AJ250917	3106	−3.11	
regulator of G-protein signalling 2, 24kD[a]	RGS2	L13463	5997	−2.65	−1.97
wingless-type MMTV integration site family member 2	WNT2	X07876	7472	−2.52	
ribosomal protein L7	RPL7	X57958	6129	−2.45	

d-value = significance analysis of microarrays (SAM) t-statistic for CAS patients vs controls.
d§-value = significance analysis of microarrays (SAM) t-statistic for AAA patients vs controls [8].
[a] Genes validated by real-time PCR.

and oxygen transport. Whereas the differential expression of genes involved in oxygen transport and of erythrocyte genes, such as *GYPC* and *EPB49*, represents a common feature between CAS and AAA patients, the differential expression of genes involved in immune response and antigen processing and presentation represents a distinctive signature of CAS disease. CAS and AAA patients have common risk factors, but they develop one of these two disorders or both. Gene alterations described in this article could explain, at least in part, this paradox.

In CAS, we found an increased expression of genes coding components of the MHC class II antigen processing and presentation process (*HLA-DPA1*, *HLA-DPB1*, *HLA-DQB1*, *HLA-DRB1/3/5*) and a decreased expression of genes coding components of the MHC class I antigen processing and presentation process (*HLA-B*, *IGKC*). These data confirm an activation of MHC class II molecules involved in the development and activation of CD4+ T helper cell-dependent immune response, and indicate a decreased expression of MHC class I molecules involved in activation of CD8+ T cell-dependent immune response. The increased expression of *DBLN*, a gene coding an important adaptor protein that regulates the T-cell activation and immune responses,[16] and the altered expression of the two interferon-

Table 3 List of all significantly enriched GO terms among genes differentially expressed in CAS.

GO ID	*p*	N	n	GO Term	Genes
0002504	8.02×10^{-7}	11	4	MHC class II (antigen processing and presentation)	*HLA-DPA1*(↑), *HLA-DPB1*(↑), *HLA-DQB1*(↑), *HLA-DRB1/3/5*(↑)
0015671	2.97×10^{-9}	9	3	Oxygen Transport	*HBA2*(↑), *HBE1*(↑), *HBQ1*(↑)
0002474	0.0045	14	2	MHC class I (antigen processing and presentation)	*HLA-B*(↓), *IGKC*(↓)
0006955	0.0047	366	8	Immune Response	*HLA-B*(↓), *HLA-DPA1*(↑), *HLA-DPB1*(↑),*HLA-DQB1*(↑), *HLA-DRB1/3/5*(↑),*IFIT1*(↑), *IGKC* (↓), *DBNL* (↑)

n, number of differentially expressed genes annotated to the GO term; N, number of genes represented on the array annotated to the GO term (the reference gene set is the total number of transcripts spotted in the array); *p* values were adjusted by using the false discovery rate (FDR) multiple testing correction.

regulated genes, *IFIT1* (increased) and *SOCS6* (decreased), suggests further cytokine signalling pathways to be investigated in CAS.

The increased expression of *HLA-DPB1*, *HLA-DQB1* and *HLA-DRB1* was consistent with the gene-expression profiling data obtained in the femoral atherosclerotic lesions of patients with peripheral arterial occlusive disease (PAD).[17] The predominant alteration of the immune system-related genes and pathways observed in CAS and PAD patients, but not in AAA patients, might suggest that up-regulation of immune genes may be a critical signature of the atherosclerotic disease in femoral and carotid arteries. Moreover, several literature data on carotid artery lesions suggest that immune reactions with activation of T lymphocytes are a feature of the disease.[18,19]

Several studies have discussed the role of inflammation as the first step in promoting endothelial dysfunction and progression of atherosclerotic processes. Moreover, the auto-immune response against antigens expressed in the endothelium and the greater prevalence of atherosclerosis in immune-mediated rheumatic diseases such as rheumatoid arthritis and systemic lupus erythematosus strongly suggest the involvement of auto-immunity in the atherosclerotic process.[3] Several pieces of evidence suggested that atherosclerosis could be caused by an immune reaction against autoantigens such as oxidised low-density lipoprotein (LDL) and heat shock proteins (HSP).[3] Auto-immune

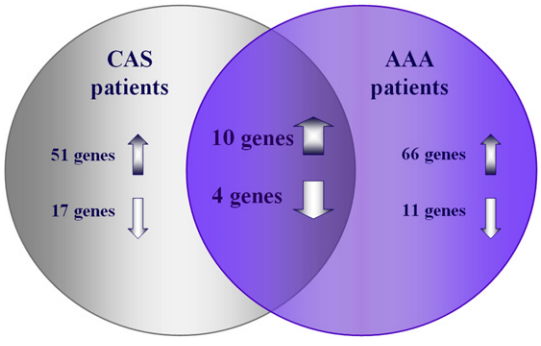


Figure 1 Venn diagram showing the comparison between differentially expressed genes in CAS (present paper) and AAA patients.[7]

mechanisms could also play a significant role in diabetes-related atherosclerosis.[20] Moreover, numerous studies have demonstrated a pathogenetic relationship between atherosclerosis and micro-organisms (bacteria and viruses) causing chronic infections.[20]

Interestingly, the microarray profile highlights 14 genes that might sustain common molecular alterations in CAS and AAA. A large part of these genes, although not all, was also validated by RT-PCR analysis in independent larger population of patients and controls.

In particular, we found the decreased expression of the *RGS2* gene, coding one of the regulator proteins of the G-protein signalling pathway, that could be responsible for, at least in part, the high prevalence of hypertension in AAA and CAS patients. *RGS2* was shown to be crucial in the regulation of vascular tone as *RGS2* deficiency leads to severe hypertension and prolonged vasoconstriction in animal models.[21] Decrease in mRNA and protein *RGS2* levels was shown in cells from hypertensive patients.[22]

BSG gene coding basigin or *EMMPRIN*, a 58-kDa membrane glycoprotein member of the immunoglobulin superfamily, showed an increased expression. This datum demonstrates the increased expression in circulating cells of this inductor of several metalloproteinases (MMPs) and, in particular, of MMP9. This finding suggests that *EMMPRIN* activation could be a mechanism of monocytes/macrophage MMPs expression induction either at the level of the atherosclerotic lesion or at the systemic level. In fact, previous data showed the up-regulation of *EMMPRIN* in monocytes isolated from patients with acute myocardial infarction, in atherosclerotic lesions from patients with CAS and in ventricles of heart failure patients.[23,24]

Our data in CAS confirmed the important role of decreased expression of *LRP5* previously observed in AAA[7] in which we demonstrated an association between decreased expression levels of *LRP5* gene and increased levels of Lp(a), suggesting the potential role of *LRP5* in Lp(a) catabolism. Even if we observed decreased expression of *LRP5* in both CAS and AAA patients, the alteration of the lipid metabolic process was not observed in CAS patients and seems to be a distinctive signature of the aneurysmal disease.[7]

Among common features in CAS and AAA patients, our data strongly indicated the role of the alteration of a large number of erythroblast genes involved in oxygen transport

Table 4 Fold-Change of increase or decrease in expression of genes analysed by RT-PCR in CAS patients versus controls and *d* values of microarray experiments.

Gene	Pooled samples from CAS patients and controls			CAS patients ($N = 40$) and controls ($N = 40$)	
	Fold-Change (range)	*p*	*d*	Fold-Change (range)	*p*
HLA-DPA1	2.71 (2.40–3.10)	0.0009	1.93	1.51(0.70–3.23)	0.044
HLA-DPB1	3.08 (2.80–3.39)	0.0003	2.54	1.89 (0.86–4.14)	0.010
HLA-DQB1	1.95 (1.81–2.11)	0.0005	2.01	1.69 (0.80–3.63)	0.008
HLA-B	0.52 (0.49–0.55)	0.0002	−3.11	0.47(0.27–0.82)	3.77×10^{-7}
IFIT1	2.92(2.15–3.97)	0.001	3.19	2.10(1.45–3.03)	0.003
LRP5	0.49 (0.45–0.54)	0.001	−3.70	0.51(0.25–1.04)	0.001
HBA2	2.51 (2.37–2.67)	0.001	4.24	1.78 (0.69–4.56)	0.294
HBQ1	4.01 (3.02–5.33)	0.0003	3.37	2.93 (1.36–6.15)	0.001
HBE1	2.55 (1.79–3.63)	0.0005	3.18	2.46 (0.91–6.63)	0.001
GYPC	3.33 (2.78–3.99)	0.001	2.28	2.60 (0.89–7.61)	0.046
EPB49	4.02 (3.64–4.43)	0.0008	3.31	2.69 (1.27–5.70)	0.042
BSG	4.34 (2.86–6.58)	0.001	2.25	2.69(0.99–7.31)	6.38×10^{-6}
RGS2	0.44 (0.28–0.70)	0.0004	−2.65	0.51(0.25–1.08)	0.0001
BTK	0.56(0.36–0.88)	0.0009	−3.38	0.55(0.38–0.80)	0.015

RT-PCR = Real-Time Polymerase Chain Reaction, d-value = significance analysis of microarrays (SAM) t-statistic; non-parametric Mann–Whitney test was used for determining statistical significance; *p* values were adjusted by using the false discovery rate (FDR) multiple testing correction.

and red cell structure stability. Of particular interest were the increased expressions of *HBQ1* and *HBE1*, which encode the theta and epsilon embryonic haemoglobin chains, respectively, suggesting a switch toward the reactivation of genes not normally expressed in adult humans. The increased expression of haemoglobin genes could represent a secondary response to chronic hypoxia and oxidative stress. In addition, the increased expression of structural erythrocyte genes, such as *GYPC*, might play a role in the adaptative processes of the red cells to chronic hypoxia and haemodynamic stress. Haematological data are consistent with those obtained by other groups concerning the strong association of high haematocrit and blood viscosity with the severity of carotid atherosclerosis.[25,26] Due to the evaluation of gene-expression in whole blood, one of the limitations of our study was the variation in number and types of the different cell populations. However, this approach allowed us to search for markers of disease in an easily available specimen for translational studies. Moreover, due to the higher prevalence of traditional cardiovascular risk

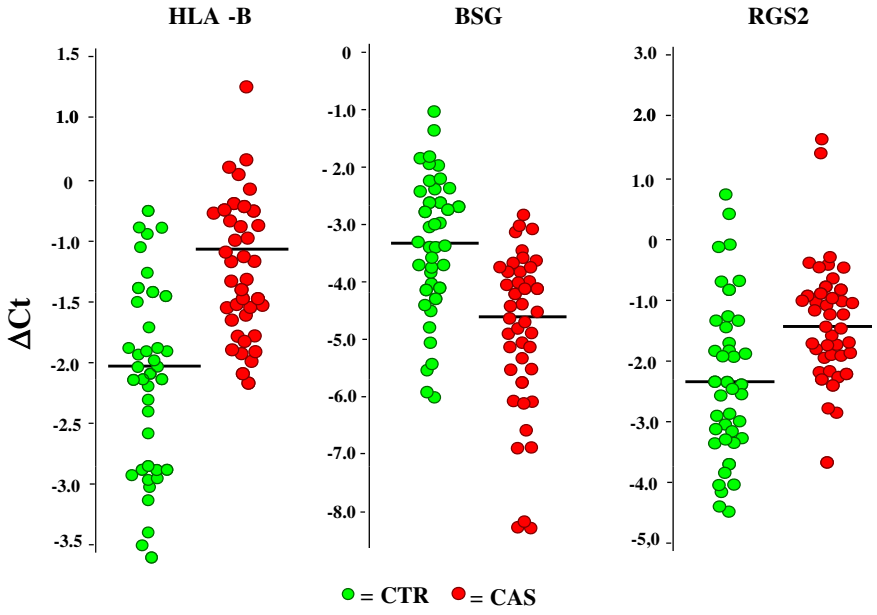


Figure 2 Scatter plot of the individual ΔCt values of the 3 most significant genes in the replication of microarray data by RT-PCR on the larger number of CAS patients ($n = 40$) and controls ($n = 40$). HLA-B = major histocompatibility complex, class I, B; BSG = basigin; RGS2 = regulator of G-protein signalling 2; ΔCt = Expression of target gene standardized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Table 5 Haematological parameters in CAS patients and controls.

Haematological parameters	Controls ($N = 40$)	Patients ($N = 40$)	p
White Blood Cells ($10^3/\mu L$)	5.4 (3.0—8.3)	6.8 (4.9—13.2)	1.27×10^{-6}
Red Blood Cells ($10^6/\mu L$)	4.5 (3.9—5.3)	4.7 (3.8—5.5)	0.036
Haemoglobin (g/dL)	13.5 (11.7—15.2)	14.2 (10.5—16.2)	0.089
Hematocrit (%)	39.4 (34.6—44.6)	43.4 (31.3—49.5)	7.60×10^{-5}
Reticulocytes (%)	0.9 (0.5—1.5)	0.9 (0.1—1.5)	0.881
Erythropoietin (mU/mL)	11.5 (7.6—24.1)	14.5 (5.0—36.5)	0.014

Values of the different haematological parameters are expressed as median and range. Non-parametric Mann—Whitney test was used for determining statistical significance. A p value less than 0.05 was considered statistically significant.

factors and other clinical manifestations of atherosclerosis in patients (coronary artery disease (CAD), peripheral artery disease (PAD) and chronic obstructive pulmonary disease (COPD)), we cannot confirm that the differentially expressed genes identify biomarkers of CAS versus atherosclerosis. Nevertheless, the comparison of CAS and AAA gene-expression signatures represents an indirect response to this issue.

For a large part of genes differentially expressed in whole blood (beyond those commented upon), few data are available on their expression in CAS patients' arterial tissue. Further studies are needed to evaluate this issue and analyse whether these findings reflect the biological changes in the artery itself.

Conclusion

In conclusion, our data identify novel molecular alterations possibly involved in the pathogenesis of CAS. GO analysis identified a statistically significant enrichment in CAS of differentially expressed transcripts involved in immune response and oxygen transport. Whereas alteration of oxygen transport is a common tract of the two localisations (CAS and AAA), alteration of immune response in CAS and of lipid metabolic process in AAA represents distinctive tracts of the two atherosclerotic diseases.

Our study brings to light several novel genes that need a strong effort to better comprehend their involvement in the pathophysiology of CAS and AAA and their possible role as markers of progression or targets for therapeutic intervention.

Ethical Approval

Yes, please see Materials and Methods section.

Conflict of Interest

No.

Acknowledgements

This work was supported by grants from 'Genopolis' government FIRB project RBLA038RMA_008 and from Ente Cassa di Risparmio di Firenze to Fiorgen Foundation, Florence, Italy.

Appendix A
Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejvs.2010.07.006

References

1 Barnett HJ, Meldrum HE. The outlook for patients with carotid stenosis. *Cerebrovasc Dis* 2000;**10**:30—5.
2 Faries PL, Chaer RA, Patel S, Lin SC, DeRubertis B, Kent KC. Current management of extracranial carotid artery disease. *Vasc Endovascular Surg* 2006;**40**:165—75.
3 Hansson GK, Libby P, Schönbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res* 2002;**91**:281—91.
4 Bagnato C, Thumar J, Mayya V, Hwang SI, Zebroski H, Claffey KP, et al. Proteomics analysis of human coronary atherosclerotic plaque: a feasibility study of direct tissue proteomics by liquid chromatography and tandem mass spectrometry. *Mol Cell Proteomics* 2007;**6**:1088—102.
5 Bijnens AP, Lutgens E, Ayoubi T, Kuiper J, Horrevoets AJ, Daemen MJ. Genome-wide expression studies of atherosclerosis: critical issues in methodology, analysis, interpretation of transcriptomics data. *Arterioscler Thromb Vasc Biol* 2006;**26**:1226—35.
6 Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005;**352**:1685—95.
7 Giusti B, Rossi L, Lapini I, Magi A, Pratesi G, Lavitrano M, et al. Gene expression profiling of peripheral blood in patients with abdominal aortic aneurysm. *Eur J Vasc Endovasc Surg* 2009;**38**:104—12.
8 Barnett HJ, Meldrum HE, Eliasziw M. North American Symptomatic Carotid Endarterectomy Trial (NASCET) collaborators. The appropriate use of carotid endarterectomy. *CMAJ* 2002;**166**:1169—79.
9 Mancia G, De Backer G, Dominiczak A, Cifkova R, Fagard R, Germano G, et al. Guidelines for the management of arterial hypertension: the task force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *J Hypertens* 2007;**25**:1105—87.
10 National Cholesterol Education Program (NCEP) Expert Panel on Detection. Evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III). Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation* 2002;**106**:3143—421.

11 Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 2003;**26**:5—20.
12 Giusti B, Fibbi G, Margheri F, Serratì S, Rossi L, Poggi F, et al. A model of anti-angiogenesis: differential transcriptosome profiling of microvascular endothelial cells from diffuse systemic sclerosis patients. *Arthritis Res Ther* 2006;**8**:R115.
13 Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, et al. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res* 2002;**30**:e15.
14 Camon E, Magrane M, Barrell D, Lee V, Dimmer E, Maslen J, et al. The gene ontology annotation (GOA) database: sharing knowledge in Uniprot with gene ontology. *Nucleic Acids Res* 2004;**32**:262—6.
15 Camon E, Barrell D, Lee V, Dimmer E, Apweiler R. The gene ontology annotation (GOA) database an integrated resource of GO annotations to the UniProt knowledgebase. *In Silico Biol* 2004;**4**:5—6.
16 Han J, Shui JW, Zhang X, Zheng B, Han S, Tan TH. HIP-55 is important for T-cell proliferation, cytokine production, and immune responses. *Mol Cell Biol* 2005;**25**:6869—78.
17 Fu S, Zhao H, Shi J, Abzhanov A, Crawford K, Ohno-Machado L, et al. Peripheral arterial occlusive disease: global gene expression analyses suggest a major role for immune and inflammatory responses. *BMC Genomics* 2008;**9**:369.
18 Holm J, Hansson GK. Cellular and immunologic features of carotid artery disease in man and experimental animal models. *Eur J Vasc Surg* 1990;**4**:49—55.
19 Bui QT, Prempeh M, Wilensky RL. Atherosclerotic plaque development. *Int J Biochem Cell Biol* 2009;**41**:2109—13.
20 Blasi C. The autoimmune origin of atherosclerosis. *Atherosclerosis* 2008;**201**:17—32.
21 Heximer SP, Knutsen RH, Sun X, Kaltenbronn KM, Rhee MH, Peng N, et al. Hypertension and prolonged vasoconstrictor signaling in RGS2-deficient mice. *J Clin Invest* 2003;**111**:445—52.
22 Semplicini A, Lenzini L, Sartori M, Papparella I, Calò LA, Pagnin E, et al. Reduced expression of regulator of G-protein signaling 2 (RGS2) in hypertensive patients increases calcium mobilization and ERK1/2 phosphorylation induced by angiotensin II. *J Hypertens* 2006;**24**:1115—24.
23 Schmidt R, Bültmann A, Ungerer M, Joghetaei N, Bülbül O, Thieme S, et al. Extracellular matrix metalloproteinase inducer regulates matrix metalloproteinase activity in cardiovascular cells: implications in acute myocardial infarction. *Circulation* 2006;**113**:834—41.
24 Sluijter JP, Pulskens WP, Schoneveld AH, Velema E, Strijder CF, Moll F, et al. Matrix metalloproteinase 2 is associated with stable and matrix metalloproteinases 8 and 9 with vulnerable carotid atherosclerotic lesions: a study in human endarterectomy specimen pointing to a role for different extracellular matrix metalloproteinase inducer glycosylation forms. *Stroke* 2006;**37**:235—9.
25 Velcheva I, Antonova N, Titianova E, Damianov P, Dimitrov N, Ivanov I. Hemorheological parameters in correlation with the risk factors for carotid atherosclerosis. *Clin Hemorheol Microcirc* 2006;**35**:195—8.
26 Tamariz LJ, Young JH, Pankow JS, Yeh HC, Schmidt MI, Astor B, et al. Blood viscosity and haematocrit as risk factors for type 2 diabetes mellitus: the atherosclerosis risk in communities (ARIC) study. *Am J Epidemiol* 2008;**168**:1153—60.