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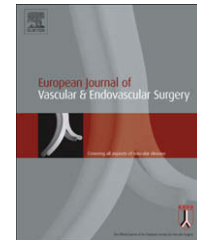
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Gene Expression Profiling of Peripheral Blood in Patients with Abdominal Aortic Aneurysm

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KEYWORDS

Abdominal aortic aneurysm;
Gene expression profile;
Erythrocyte genes;
LRP5

Abstract *Object:* Abdominal aortic aneurysm (AAA) pathogenesis remains poorly understood. This study investigated the gene expression profile of peripheral blood from patients with AAA using microarray technology.

Methods and results: We determined gene expression profiles in pooled RNA from 10 AAA patients and 10 matched controls with arrays representing 14,000 transcripts. Microarray data for selected genes were confirmed by real-time PCR in two different AAA ($n = 36$) and control ($n = 36$) populations and integrated with biochemical data. We identified 91 genes which were differentially expressed in AAA patients. Gene Ontology analysis indicated a significant alteration of oxygen transport (increased hemoglobin gene expression) and lipid metabolism [including monoglyceride lipase and low density lipoprotein receptor-related protein 5 (LRP5) gene]. LRP5 expression was associated inversely with serum lipoprotein(a) [Lp(a)] concentration.

Conclusions: Increased expression of hemoglobin chain genes as well as of genes involved in erythrocyte mechanical stability were observed in the AAA RNA pools. The association between low levels of LRP5 gene expression and increased levels of Lp(a) in AAA patients suggests a potential role of LRP5 in Lp(a) catabolism. Our data underline the power of microarrays in identifying further molecular perturbations associated with AAA.

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Introduction

The prevalence of abdominal aortic aneurysm (AAA) varies ranging from 4.1% to 11.5% in European men.¹ This disorder is characterized by localized structural deterioration of the

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aortic wall, leading to progressive dilatation and eventual aortic rupture.² Rupture of AAA is responsible for 1.5% of the total mortality in males over 55 years of age.³

There are several factors such as smoking, hypertension, hypercholesterolemia and male sex,⁴ which are well known risk factors for the development of AAA.^{5–7} However, better understanding of molecular mechanisms is an important step toward clarification of the pathophysiology, identification of genetic and molecular biomarkers and development of new therapeutic strategies for AAA.

Gene expression profiling studies by microarray technologies are particularly appropriate to investigate and create working hypotheses to understand the pathophysiology of complex genetic tracts such as AAA. Previous microarray studies of AAA utilized RNA derived from aortic tissue samples.^{8–11} The use of tissue samples has several disadvantages, including the difficulty of obtaining control samples and bias introduced by use of normal specimens from non-age-matched cadavers, organ-transplant donors or patients with different diseases. Peripheral blood is a complex fluid with a high cellular turnover rate that provides physiological connectivity between tissues. Environmental or disease perturbations in the body may leave molecular signatures detectable by analyzing blood-derived RNA.^{12,13} Most importantly, since blood samples can be obtained readily and with little discomfort to patients, biomarkers derived from blood RNA provide an easier integration to clinical and imaging data for the diagnosis and prognosis of AAA.

This study aimed to investigate the gene expression profile of venous whole peripheral blood obtained from AAA patients by using microarray technology to provide insight into systemic pathophysiological processes involved in this disease.

Materials and Methods

Subjects

We enrolled 46 patients with AAA referred to the Unit of Vascular Surgery of the University of Florence. Familial and inflammatory AAAs were excluded from the study. Familial AAA was defined when one or more first-degree family members were affected by AAA, whereas inflammatory AAA was diagnosed on the basis of preoperative features (lumbar or abdominal pain, elevated erythrocyte sedimentation rate, and weight loss) and operative appearance (presence of extensive perianeurysmal and retroperitoneal fibrosis and dense adhesions to adjacent abdominal organs). A group of 46 healthy subjects matched for age and gender were used as controls. All controls had a negative history of vascular diseases. All subjects underwent duplex scanning examination using an Acuson Sequoia Color Duplex System (Mountain View, CA). Ultrasound scanning examination was then confirmed in all patients with high resolution computed tomography scan examination (Siemens CT, Somatom HIQ Type 600). The aneurysm diameter recorded was the largest measured in either the anterior–posterior or the transverse plane. All AAA patients had a maximum aortic diameter larger than 5 cm. Digital subtraction angiography was performed only in patients

with concomitant peripheral arterial disease. Patients were considered to have hypertension if were taking antihypertensive drugs or in the presence of systolic pressure ≥ 140 mmHg and/or diastolic pressure ≥ 90 mmHg. Dyslipidemia was defined in the presence of total cholesterol levels >4.9 mmol/l and/or LDL-cholesterol >3.0 mmol/l and/or HDL <1 mmol/l and/or triglyceride levels >1.7 mmol/l according to the Third Report of the National Cholesterol Education Program. Patients were considered to have diabetes if were taking hypoglycaemic drugs or in the presence of fasting glucose levels ≥ 7.0 mmol/l on at least two separate occasions.

To assess the presence of other atherosclerotic localizations, all subjects underwent clinical and diagnostic examination (electrocardiography, echocardiography, duplex scanning of carotid arteries and ankle/brachial pressure index evaluation). Severe carotid stenosis was defined according to the North American Symptomatic Carotid Evaluation Trial criteria.¹⁴

Peripheral arterial disease was documented as present if ankle/brachial pressure index was less than 0.9. The study was approved by the local Ethical Committee and written consent was obtained from each enrolled subject.

Haematological parameters

Blood samples were taken from all subjects for blood counts [red blood cells (RBC), white blood cells (WBC), hemoglobin (Hb), hematocrit (Hct), reticulocytes] and for serum erythropoietin (EPO) level measurement. The RBC, WBC and reticulocytes count, and Hb and Hct were evaluated using an automated counter, while EPO was determined by DSL1100 Erythropoietin RIA (Diagnostic Systems Laboratories, Texas, USA). Lipoprotein(a) [Lp(a)] concentrations were determined on serum by an ELISA method [Mercodia Apo(a), Mercodia AB, Uppsala, Sweden]. We considered levels of Lp(a) determining an increased risk for atherothrombosis as plasma levels >300 mg/L.¹⁵

Total RNA preparation

Total RNA was extracted from venous peripheral blood of 46 AAA patients and 46 controls using PAXgene Blood RNA Kit (Qiagen, Germany). We integrated the RNA extraction protocol with a DNase treatment to remove contaminating genomic DNA. Moreover, the absence of contaminating genomic DNA was confirmed by PCR analysis with specific primers. The quality of the RNA was analyzed with the 2100 Bioanalyzer (Agilent, CA, USA). RNA with a RNA Integrity Number (RIN) ≥ 6.5 was used for microarray and real-time-PCR experiments. Equal quantities of total RNA from 10 AAA patients and 10 age- and gender-matched controls were pooled in 4 different pools according to the experimental design reported below.

Experimental design and microarray gene expression analysis

We established two different pools for 10 AAA 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). The demographic and clinical characteristics of each pool are provided as supplemental data (Table A). Two replicates with Cy3 and Cy5 fluorochrome inversion (dye swap) of the two microarray experiments were performed.

Experiment 1:

AAA pool A Cy3 labelled vs control pool C Cy5 labelled
AAA pool A Cy5 labelled vs control pool C Cy3 labelled
(dye swap);

Experiment 2:

AAA pool B Cy3 labelled vs control pool D Cy5 labelled
AAA pool B Cy5 labelled vs control pool D Cy3 labelled
(dye swap).

Microarray experiments were performed as described previously.^{16,17} We used arrays representing 14,000 genes (70mer oligonucleotides; Human AROS v1.1, Operon Technologies, CA, USA).

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 normalization.¹⁸

Normalization procedure was done by Statistical Microarray Analysis package (<http://stat-www.berkeley.edu/users/terry/zarray/Software/smacode.html>).

Then data were analyzed by "Significant Analysis of Microarray" (SAM) algorithm.¹⁹ In SAM analysis we choose a delta value of 0.8 that allowed us to identify 91 differentially expressed genes with a false discovery rate (FDR) of 2.2%. The full data set is available at ArrayExpress (E-mexp-1346 in <http://www.ebi.ac.uk/microarray-as/ae/browse.html>).

Gene Ontology analysis

To analyze the involvement of differentially expressed genes in different biological functional groups, all the genes present on the microarray were annotated for their role in biological processes. In our study we used one of the three ontologies produced by the Gene Ontology (GO) consortium, the biological process ontology.^{20,21} The term "biological process" should be interpreted as a biological function to which the gene product contributes. The actual mapping of genes to GO terms is provided by the Gene Ontology Annotation Database. The mappings were downloaded from <ftp://ftp.ncbi.nlm.nih.gov/gene/DATA/gene2go.gz>. Briefly, given a set of genes and one ontology, we first found the set of all unique GO terms within the ontology that were associated with one or more of the genes of interest. Next, we determined how many of the differentially expressed genes were annotated at each term and how many of the genes that were assessed (all the genes represented on the microarray) were annotated at the term. The test evaluated if there were more genes of interest at the term than would be expected by chance alone. Due to the small number of genes in some categories, two-sided Fisher's exact test was applied. The statistical analyses were implemented in the R environment using Bioconductor packages.

Real-time PCR based gene expression analysis

In order to confirm results obtained by microarray analysis, the expression of ten selected genes was also measured by real-time (RT)-PCR, both in subjects included in pools and in 36 additional patients and controls. To quantify the transcribed low density lipoprotein receptor-related protein 5 (*LRP5*), basigin (*BSG*), hemoglobin alpha 2 (*HBA2*), hemoglobin delta (*HBD*), hemoglobin epsilon 1 (*HBE1*), hemoglobin theta 1 (*HBQ1*), hemoglobin beta (*HBB*), hemoglobin gamma (*HBG*), aminolevulinic acid synthase 2 (*ALAS2*), dematin (*EPB49*), glycophorin c (*GYPC*) and stomatin (*STOM*) genes, we performed TaqMan RT-PCR on an ABI Prism 7700 instrument (Applied Biosystems, CA, USA). We used TaqMan pre-developed assays (Applied Biosystems, CA, USA) (Supplementary Data). Reactions were performed in duplicate with 50 ng cDNA. The "delta-delta Ct method" was used for comparing relative gene expression results (PE Applied Biosystems, Perkin-Elmer, CA). ΔCt values of the samples were determined by subtracting the average of the duplicate Ct values of the target genes from the average of the duplicate Ct values of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (reference). The relative gene expression levels were determined by subtracting the average ΔCt value of the target from the average ΔCt value of the calibrator. The amount of target (expressed as fold-change), standardised to an endogenous reference and relative to a calibrator, was given by $2^{-\Delta\Delta Ct}$.

Statistical analysis

Statistical analyses were carried out using statistical tests as implemented in SPSS software (v.11.5). All values were expressed as median and range. When comparing groups, statistical significance was determined by using non-parametric Mann-Whitney test. In order to test correlation between different parameters the Spearman's correlation was used. Multivariate logistic regression analysis was used to evaluate the independent association of different gene expressions with the AAA disease [odds ratio (OR) and 95% confidence interval (CI)]. A p value less than 0.05 was considered statistically significant.

Results

Demographic and clinical characteristics

In Table 1 demographic and clinical characteristics of AAA patients and healthy subjects were reported. AAA patients are characterized by high prevalence of both cardiovascular risk factors and chronic obstructive pulmonary disease (COPD, 58.7%).

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 analyzed transcripts numbered 3981.

The SAM analysis showed 91 genes differentially expressed in venous peripheral whole blood of AAA patients

Table 1 Demographic and clinical characteristics of AAA patients and controls enrolled in pools used for gene expression profiling and for validation analyses on a second larger population.

	AAA in pool (N = 10)	CTR in pool (N = 10)	<i>p</i>	AAA patients (N = 36)	CTR subjects (N = 36)	<i>p</i>
Age	68 (60–82)	66 (52–85)	–	69 (55–84)	70 (52–86)	–
Sex (male) <i>N</i> (%)	8 (80)	8 (80)	–	32 (88.9)	32 (88.9)	–
Smoking <i>N</i> (%)	8 (80)	2 (20)	0.012	24 (66.6)	12 (33.3)	0.005
Diabetes <i>N</i> (%)	1 (10)	0	0.499	3 (8.3)	0	0.119
Hypertension <i>N</i> (%)	6 (60)	2 (20)	0.085	27 (75.0)	8 (22.2)	7.46×10^{-6}
Dyslipidemia <i>N</i> (%)	7 (70)	1 (10)	0.009	25 (69.4)	2 (5.5)	2.14×10^{-8}
CAD <i>N</i> (%)	1 (10)	0	0.499	13 (36.1)	0	6.80×10^{-5}
CAS <i>N</i> (%)	1 (10)	0	0.499	7 (19.4)	0	0.005
PAD <i>N</i> (%)	1 (10)	0	0.499	8 (22.2)	0	0.003
COPD <i>N</i> (%)	6 (60)	0	0.005	21 (58.3)	0	5.18×10^{-8}

AAA = abdominal aortic aneurysm; CTR = control; CAD = coronary artery disease; CAS = carotid artery stenosis; PAD = peripheral obstructive artery disease; COPD = chronic obstructive pulmonary disease.

with respect to controls: 76/91 genes with increased expression and 15/91 genes with decreased expression (Table 2).

Gene Ontology analysis of differentially expressed genes in AAA patients

According to GO analysis adjusted by using the FDR multiple testing correction, we observed 3 significant biological processes (terms) associated with genes differentially expressed in AAA patients (Table 3). Statistically significant enriched GO biological processes were “oxygen transport”, “positive regulation of protein kinase activity”, and “lipid metabolic process”.

We observed that, apart from the 4 genes coding for erythrocyte hemoglobin chains associated to the GO term “oxygen transport”, other erythrocyte genes or genes involved in erythropoiesis showed increased expression in AAA patients including *EPB49*, *GYPC*, *STOM*, flotillin 1 (*FLOT1*), solute carrier family 25-member 37 (*SLC25A37*) and *ALAS2*.

Six genes differentially expressed in AAA patients were associated with the GO term “lipid metabolic process”: 5 out of 6 with increased expression [acyl-Coenzyme A dehydrogenase C-2 to C-3 short chain, *ACADS*; cell death-inducing DFFA-like effector a, *CIDEA*; monoglyceride lipase, *MGLL*; adiponectin receptor 1, *ADIPOR1*; hydroxysteroid (17-beta) dehydrogenase 14, *HSD17B14*], and 1 out of 6 with decreased expression (*LRP5*).

Microarray data validation by RT-PCR

To validate microarray data, mRNA expression of 12 genes was independently examined by real-time RT-PCR. We selected 5 genes involved in oxygen transport (*HBA2*, *HBD*, *HBE1*, *HBQ1*, *ALAS2*); 3 genes coding for cellular components of erythrocytes which play a role in the regulation of the mechanical stability of red cells (*EPB49*, a membrane skeleton protein; *GYPC*, a transmembrane glycoprotein; *STOM*, a cytoskeleton component); 1 gene associated with the “lipid metabolic process” (*LRP5*); 1 gene known to be an inducer of metalloproteinases (*BSG*), and 2 genes

coding for the other two hemoglobin chains (*HBB*, *HBG*), which we could not analyze by microarray analysis (due to a failure in oligonucleotide deposition during array construction). We evaluated these genes in the total RNAs of patients and controls used for comparative microarray experiments and in two different larger populations of AAA patients and controls (Table 4). The correlation between gene expression evaluated by microarray experiments and RT-PCR in validated genes narrowly failed to achieve statistical significance (Spearman’s correlation 0.62, $p = 0.057$).

RT-PCR analysis on subjects included in the pools confirmed data obtained by microarray for all the investigated genes (Table 4).

Analysis of further, separate AAA patients and controls showed increased expression of *HBB*, *HBD*, *HBQ1*, *ALAS2*, *BSG* and *GYPC* genes and decreased expression of *LRP5* gene in AAA patients with respect to controls (Table 4).

Multivariate logistic regression analysis with AAA as dependent variable and expression of validated genes, and traditional cardiovascular risk factors (age, gender, hypertension, dyslipidemia, smoking habit) and COPD as independent variables, was performed. *HBB*, *HBQ1*, *HBD* and *LRP5* gene expression levels were significantly and independently associated with AAA [2.18 (95%CI 1.09–4.37), $p = 0.028$; 3.12 (95%CI 1.14–8.55), $p = 0.026$; 2.19 (95%CI 1.22–3.95), $p = 0.009$; 0.15 (95%CI 0.02–0.90), $p = 0.039$, respectively]. Association of *ALAS2* and *BSG* gene expression with AAA disease did not reach statistical significance [1.90 (95%CI 0.96–3.76), $p = 0.065$ and 2.33 (95%CI 0.97–5.59), $p = 0.059$, respectively].

Relationship between *LRP5* gene expression and Lp(a) serum levels in AAA patients

Due to the possible role of LRP5 protein in the catabolism of Lp(a), we investigated the relationship between *LRP5* expression and serum Lp(a) concentrations. Lp(a) concentrations in AAA patients (median 248 mg/L, interquartile range 78–416 mg/L) were significantly higher than in controls (median 105 mg/L, interquartile range 58–203 mg/L), $p = 0.049$. Serum Lp(a) concentration was

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

Gene name	Symbol	Accession No	GeneID	d
<i>Genes with increased expression in AAA patients</i>				
Hemoglobin, alpha 2 (*)	HBA2	V00488	3040	5.30
Monoglyceride lipase	MGLL	U67963	11343	4.79
Guanylate kinase 1	GUK1	L76200	2987	3.52
Hemoglobin, epsilon 1 (*)	HBE1	NM_005330	3046	3.49
Cell death-inducing DFFA-like effector a	CIDEA	AF041378	1149	3.34
ATPase, Na ⁺ /K ⁺ transporting, alpha polypeptide-like 1	ATP12A	L42563	479	3.21
PDZ domain containing RING finger 4	PDZRN4	AL133067	29951	2.92
CGI-69 protein	CGI-69	NM_016016	51629	2.88
Hemoglobin, delta (*)	HBD	V00505	3045	2.78
Defensin, alpha 1, myeloid-related sequence	DEFA1	M26602	1667	2.48
Hemoglobin, theta 1 (*)	HBQ1	NM_005331	3049	2.44
Zinc finger and BTB domain containing 39	ZBTB39	AB002350	9880	2.44
Immunoglobulin lambda joining 3	IGLJ3	X57812	28831	2.42
Makorin, ring finger protein, 1	MKRN1	NM_013446	23608	2.40
Cyclin B3	CCNB3	AL137550	85417	2.36
Zinc finger protein 193	ZNF193	U62392	148022	2.29
Toll-like receptor adaptor molecule 1	TICAM1	AF070530	7746	2.29
SEC24 (<i>Saccharomyces cerevisiae</i>) related gene family, member D	SEC24D	AB018298	9871	2.27
Serologically defined colon cancer antigen 1	SDCCAG1	NM_004713	9147	2.24
Ubiquitin-conjugating enzyme E2M (homologous to yeast UBC12)	UBE2M	AF075599	9040	2.19
Free fatty acid receptor 2	FFAR2	AF024690	2867	2.18
Glycophorin C (Gerbich blood group) (*)	GYPC	NM_002101	2995	2.16
Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting1	PIN1	U49070	5300	2.14
Three prime repair exonuclease 2	TREX2	AF151107	11219	2.12
MTERF domain containing 2	MTERFD2	BX648184	130916	2.07
Flotillin 1	FLOT1	AF089750	10211	2.01
Interleukin 1 receptor accessory protein-like 1	IL1RAPL1	AJ243874	11141	2.00
Chorionic somatomammotropin hormone-like 1	CSHL1	BC029365	1428	1.99
Solute carrier family 25, member 37	SLC25A37	AF223466	1444	1.99
Glioma-associated oncogene homolog (zinc finger protein)	GLI1	X07384	2735	1.99
Crystallin, mu	CRYM	L02950	51312	1.99
Nucleoporin 88 kD	NUP88	Y08612	2039	1.93
Erythrocyte membrane protein band 4.9 (dematin) (*)	EPB49	U28389	4927	1.93
Splicing factor, arginine/serine-rich (transformer 2 <i>Drosophila</i> homolog) 10	SFRS10	U68063	6434	1.91
ATG5 autophagy related 5 homolog (<i>S. cerevisiae</i>)	ATG5	Y11588	9474	1.90
Chromosome 20 open reading frame 29	C20orf29	AK002030	55317	1.90
S100 calcium-binding protein A6 (calcyclin)	S100A6	J02763	6277	1.90
Lipocalin 2 (oncogene 24p3)	LCN2	X99133	3934	1.89
Potassium channel tetramerisation domain containing 12	KCTD12	AF359381	115207	1.87
Small EDRK-rich factor 2	SERF2	NM_005770	10169	1.86
Platelet factor 4 variant 1	PF4V1	M26167	5197	1.85
Ribosomal protein L18	RPL18	L11566	6141	1.84
Erythrocyte membrane protein band 7.2 (stomatin) (*)	STOM	U33931	2040	1.84
Paralemmin	PALM	Y16270	5064	1.80
Myelin transcription factor 2	MYT2	AF006822	8827	1.79
Adiponectin receptor 1	ADIPOR1	AK001484	51094	1.78
Interferon, alpha 13	IFNA13	BC069427	3447	1.78
SMC (mouse) homolog, X chromosome	SMCX	L25270	8242	1.77
Nuclear factor I/B	NFIB	U85193	4781	1.76
Similar to HSPC323	LOC284422	AF161441	284422	1.75
Phospholipase A2, group IB (pancreas)	PLA2G1B	M21054	5319	1.75
Zinc finger protein 145 (Kruppel-like, expressed in promyelocytic leukemia)	ZBTB16	Z19002	28904	1.73
Immunoglobulin kappa variable 1D-8	IGKV1D-8	M63438	7704	1.73
Potassium voltage-gated channel, shaker-related subfamily, member 3	KCNA3	M85217	51171	1.72
Hydroxysteroid (17-beta) dehydrogenase 14	HSD17B14	NM_016246	3738	1.72
Mitogen-activated protein kinase kinase 3	MAP2K3	D87116	509	1.71

Table 2 (continued)

Gene name	Symbol	Accession No	GeneID	d
ATP synthase, H ⁺ transporting, mitochondrial F1 complex, gamma polypeptide 1	ATP5C1	D16562	5606	1.71
Translocase of inner mitochondrial membrane 8 (yeast) homolog A	TIMM8A	U66035	1678	1.71
HUS1 (<i>Saccharomyces pombe</i>) checkpoint homolog	HUS1	AF076844	3364	1.69
Transcription elongation factor A (SII), 2	TCEA2	D50495	6919	1.69
Tumor necrosis factor type 1 receptor-associated protein	TRAP1	NM_016292	10131	1.69
Karyopherin alpha 4 (importin alpha 3)	KPNA4	AB002533	3840	1.68
Chaperonin containing TCP1, subunit 6A (zeta 1)	CCT6A	L27706	908	1.67
Immunoglobulin lambda variable 1-47	IGLV1-47	Z73663	28822	1.67
MutL homolog 3 (<i>Escherichia coli</i>)	MLH3	AB039667	50801	1.67
Potassium inwardly-rectifying channel, subfamily K, member 4	KCNK4	AF247042	27030	1.67
RNA-binding protein S1, serine-rich domain	RNPS1	L37368	10921	1.66
Zinc finger protein 384	ZNF384	U80738	7177	1.66
Tryptase, alpha	TPSAB1	M30038	171017	1.66
Ring finger protein 44	RNF44	AB029023	51374	1.65
Apoptosis related protein APR-3	APR3	NM_016085	22838	1.65
Basigin (*)	BSG	X64364	35	1.64
Aminolevulinate, delta-, synthase 2 (sideroblastic/hypochromic anemia) (*)	ALAS2	X60364	212	1.64
Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	ACADS	Z80345	682	1.64
Sarcolipin	SLN	U96094	6588	1.63
Myogenic factor 6 (herculin)	MYF6	X52011	4618	1.62
<i>Genes with decreased expression in AAA patients</i>				
Eukaryotic translation elongation factor 1 beta 2	EEF1B2	X60489	1933	-1.97
Regulator of G-protein signaling 2, 24 kD	RGS2	L13463	5997	-1.97
Protein tyrosine phosphatase, receptor type, c polypeptide	PTPRC	Y00062	5788	-2.04
ARP2 (actin-related protein 2, yeast) homolog	ACTR2	AF006082	10097	-2.17
Similar to chloride intracellular channel protein 1 (Nuclear chloride ion channel 27) (NCC27) (p64 CLCP) (Chloride channel ABP)	LOC390363	XM_495936	390363	-2.28
High-mobility group (nonhistone chromosomal) protein 1 like 10	HMG1L5	L08048	10354	-2.37
Receptor-associated protein of the synapse, 43 kD	RAPSN	Z33905	5913	-2.46
Chromosome 12 open reading frame 35	C12orf35	AK000703	55196	-2.59
Homo sapiens Bruton's tyrosine kinase	BTK	U78027	695	-2.76
Ribosomal protein L17	RPL17	X53777	6139	-2.85
Complement factor H-related 4	CFHR4	X98337	10877	-2.94
Immunoglobulin superfamily containing leucine-rich repeat	ISLR	AB024536	3671	-3.44
Low density lipoprotein receptor-related protein 5 (*)	LRP5	AF077820	4041	-3.46
Apoptosis antagonizing transcription factor	AATF	AJ249940	26574	-4.30
Anti-Mullerian hormone receptor, type II	AMHR2	U29700	269	-5.62

d-value = significance analysis of microarrays (SAM) t-statistic; (*) = genes validated by real-time PCR.

correlated with *LRP5* gene expression [Spearman's coefficient -0.411 , $p = 0.00034$; Spearman's coefficient after removal of the outliers (>1000 mg/L) -0.339 , $p = 0.004$] (Fig. 1). AAA patients with Lp(a) levels higher than 300 mg/L had reduced *LRP5* expression levels versus patients with Lp(a) levels lower than 300 mg/L ($p = 0.050$).

Haematological parameters in AAA patients and controls

In order to investigate the possible mechanisms and/or effects of expression changes in erythrocyte genes, we evaluated some haematological parameters in AAA patients

Table 3 List of all the Gene Ontology (GO) significant terms (biological processes) in AAA patients.

GO ID	p value	N	n	GO Term	Genes
GO:0015671	<0.0001	9	4	oxygen transport	<i>HBA2</i> (↑), <i>HBD</i> (↑), <i>HBE1</i> (↑), <i>HBQ1</i> (↑)
GO:0045860	0.029	3	2	positive regulation of protein kinase activity	<i>MAP2K3</i> (↑), <i>PTPRC</i> (↓)
GO:0006629	0.049	190	6	lipid metabolic process	<i>ACADS</i> (↑), <i>CIDEA</i> (↑), <i>LRP5</i> (↓), <i>MGLL</i> (↑), <i>ADIPOR1</i> (↑), <i>HSD17B14</i> (↑)

n, number of differentially expressed genes annotated to the GO term; N, number of genes represented on the array annotated to the GO term; p values were adjusted by using the false discovery rate (FDR) multiple testing correction.

Table 4 Fold-change of increase or decrease expression of genes analyzed by RT-PCR in AAA patients versus controls.

Gene	AAA patients and controls in pools <i>n</i> = 10 AAA patients vs <i>n</i> = 10 controls			AAA patients and controls <i>n</i> = 36 AAA patients vs <i>n</i> = 36 controls	
	Fold-change (range)	<i>p</i>	<i>M</i>	Fold-change (range)	<i>p</i>
<i>LRP5</i>	0.29 (0.21–0.42)	0.0008	−1.58	0.63 (0.29–1.37)	0.025
<i>HBA2</i>	4.82 (4.50–5.17)	0.001	2.93	1.15 (0.72–1.84)	0.537
<i>HBB</i>	6.82 (5.94–7.83)	0.00001	–	4.72 (0.47–47.50)	7.5 × 10^{−5}
<i>HBD</i>	6.54 (5.50–7.78)	0.00005	2.41	12.21 (2.19–68.12)	3.0 × 10^{−5}
<i>HBG</i>	1.36 (1.18–1.56)	0.0008	–	2.23 (0.55–9.06)	0.188
<i>HBQ1</i>	9.99 (9.00–11.08)	0.0001	2.19	2.38 (0.29–19.16)	0.003
<i>HBE1</i>	3.55 (2.89–4.38)	0.0009	2.65	1.44 (0.54–3.86)	0.178
<i>GYPC</i>	5.73 (4.50–7.31)	0.0006	1.87	2.20 (0.89–5.46)	0.025
<i>STOM</i>	2.25 (1.89–2.67)	0.001	1.29	1.17 (0.65–2.11)	0.390
<i>EPB49</i>	5.39 (5.20–5.58)	0.0009	2.11	0.89 (0.36–1.90)	0.539
<i>ALAS2</i>	3.60 (2.52–4.37)	0.0004	0.89	2.69 (0.63–11.52)	2.9 × 10^{−4}
<i>BSG</i>	1.85 (1.29–2.06)	0.0008	1.85	2.06 (0.61–6.90)	8.2 × 10^{−4}

RT-PCR = Real-Time Polymerase Chain Reaction, mean of the *M* values = mean of log₂ Cy5/Cy3 fluorescence intensities for each gene; non-parametric Mann–Whitney test was used for determining statistical significance.

and controls (Table 5). AAA patients had significantly higher Hct values and WBC counts. No differences were observed for reticulocyte counts.

Discussion

In this study, we analyzed gene expression profiles of venous peripheral whole blood of AAA patients. Based on Gene Ontology and other classification criteria, we found that AAA patients differentially express a variety of transcripts involved in oxygen transport, regulation of the mechanical stability of the red cells and lipid metabolism. The decreased expression of *LRP5* gene was associated with increased levels of Lp(a), a known atherothrombotic risk factor, previously associated with AAA.^{15,22} Moreover, a large panel of erythrocyte genes was differentially expressed in AAA patients.

Even though previous microarray studies of AAA focused on gene expression profile from aortic tissue samples,^{8–11}

some genes appear to be differentially expressed both in tissue¹¹ and peripheral blood, including *LRP5*, mitogen-activated protein kinase kinase 3 (*MAP2K3*), *EPB49*, *STOM*, regulator of G-protein signaling 2 (*RGS2*) (see also supplementary data Table C). Further data are required to investigate the potential role of these genes in the AAA pathophysiology.

Our data, showing decreased *LRP5* gene expression in the peripheral blood cells of AAA patients, are echoed in the microarray profiling of the aortic wall of AAA patients reported in a previous study.¹¹ The *LRP5* gene is involved in bone metabolism^{23,24} as well as lipoprotein and glucose metabolism.²⁵ Experimental studies have shown that apoE/Lrp5 double knockout mice developed multiple atheromatous aortic lesions manifesting a hump structure, which were associated with cholesterol deposits, fibrosis,

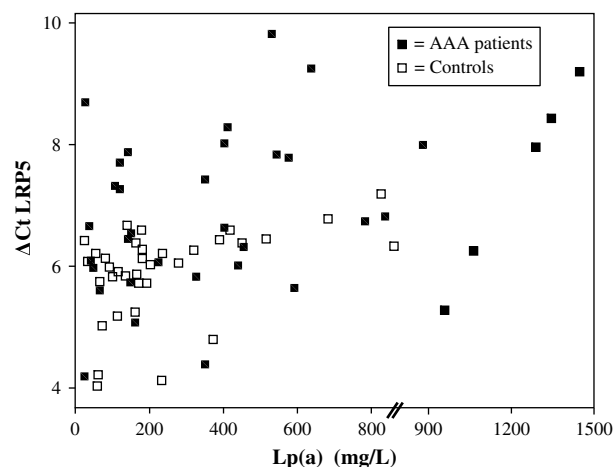


Figure 1 Correlation between Lp(a) serum concentrations and *LRP5* gene expression in AAA patients and controls.

Table 5 Haematological parameters in AAA patients and controls.

Haematological parameters	Controls (<i>N</i> = 36)	Patients (<i>N</i> = 36)	<i>p</i>
White Blood Cells (10 ³ /μL)	5.6 (3.0–8.3)	7.0 (3.5–11.6)	0.034
Red Blood Cells (10 ⁶ /μL)	4.5 (3.9–5.3)	4.7 (3.5–5.7)	0.171
Hemoglobin (g/dL)	13.9 (11.8–15.9)	14.4 (11.0–16.8)	0.331
Hematocrit (%)	39.8 (34.7–44.5)	42.7 (35.2–51.7)	0.033
Reticulocytes (%)	0.89 (0.46–1.5)	0.92 (0.54–2.14)	0.986
Erythropoietin (mU/mL)	11.5 (7.6–24.1)	13.2 (5.6–30.5)	0.224

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.

and elastolysis, some even showing the destruction of internal elastic lamina and the degenerative changes in aortic smooth muscle.²⁶

We demonstrated, for the first time, an association between decreased expression levels of *LRP5* gene and increased levels of Lp(a) in AAA patients, which suggests a potential role of *LRP5* in Lp(a) catabolism. Several studies have demonstrated that high serum Lp(a) concentrations play a role in the genesis of systemic atherosclerosis, thrombosis, and related disorders.²⁷ The mechanisms implicated in the atherogenicity of Lp(a) include the tendency to self-aggregate and precipitate, binding to glycosaminoglycans and other structures in the vascular wall, impaired fibrinolysis due to its structural homology with plasminogen, regulation of synthesis of plasminogen activator inhibitor-1 and the induction of smooth muscle cell proliferation.²⁸

Our data highlight the role of the alteration of a large number of erythroblast genes involved in oxygen transport and red cell structure stability. Of particular interest was the finding of an increased expression in AAA patients of *HBQ1* gene, which encodes the embryonic theta hemoglobin chain. This might suggest a switch toward the reactivation, in AAA patients, of the expression of genes not normally expressed in adult humans. We also observed increased expression of *ALAS2*. This mitochondrial enzyme catalyses the first step in the haem biosynthetic pathway. Murine erythroleukemia cells, exposed to hypoxia, showed an up to 3-fold increased expression of *ALAS2* mRNA levels and an increase in cellular haem content.²⁹ The increased expression of hemoglobin 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, in particular the higher hematocrit in AAA patients lends support to the hypothesis that increased expression of red cell genes is, at least in part, a response to chronic hypoxia.

Other genes identified in the microarray analysis also may be relevant to the pathophysiology of AAA. Of particular interest is the *BSG* gene coding for basigin or EMMPRIN, a 58 kDa membrane glycoprotein member of the immunoglobulin superfamily, which had increased gene expression in our AAA patients. This may be the first observation that this important inducer of several metalloproteinases (MMPs) and in particular of MMP9 is expressed in peripheral blood cells.

Our study has several limitations, in particular the variation in number and types of the different cell populations in the samples³⁰ as well as confounding factors (recognised and unrecognised) resulting from the different demographic profiles of AAA patients and controls. For example, due to the higher prevalence of traditional cardiovascular risk factors and other clinical manifestations of atherosclerosis in our AAA patients (CAD, CAS and PAD), we cannot confirm that the differentially expressed genes identify biomarkers of AAA versus atherosclerosis. In this respect, further gene expression profiling studies, enrolling patients suffering from atherosclerotic lesions in different localization such as carotid artery disease or peripheral arterial disease, might provide further information.

In conclusion, gene profiling of peripheral whole venous blood cells may provide an interesting way to explore the complex pathophysiology of AAA. Our findings suggest that the decreased expression of *LRP5* gene and the increased expression of erythrocyte genes might be potential molecular alterations characterising aneurysmal disease.

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Conflict of Interest

None.

Appendix Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejvs.2009.01.020.

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