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Clinica Chimica Acta 421 (2013) 184–190

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

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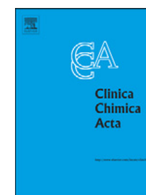

Novel genes detected by transcriptional profiling from whole-blood cells in patients with early onset of acute coronary syndrome

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A R T I C L E I N F O

Article history:
Received 26 November 2012
Received in revised form 17 February 2013
Accepted 6 March 2013
Available online 25 March 2013

Keywords:
Myocardial infarction
Acute coronary syndrome
Biomarker
Transcriptome

A B S T R A C T

Background: Genome-wide expression analysis using microarrays has been used as a research strategy to discovery new biomarkers and candidate genes for a number of diseases. We aim to find new biomarkers for the prediction of acute coronary syndrome (ACS) with a differentially expressed mRNA profiling approach using whole genomic expression analysis in a peripheral blood cell model from patients with early ACS.
Methods and results: This study was carried out in two phases. On phase 1 a restricted clinical criteria (ACS-Ph1, n = 9 and CG-Ph1, n = 6) was used in order to select potential mRNA biomarkers candidates. A subsequent phase 2 study was performed using selected phase 1 markers analyzed by RT-qPCR using a larger and independent casuistic (ACS-Ph2, n = 74 and CG-Ph2, n = 41). A total of 549 genes were found to be differentially expressed in the first 48 h after the ACS-Ph1. Technical and biological validation further confirmed that *ALOX15, AREG, BCL2A1, BCL2L1, CA1, COX7B, ECHDC3, IL18R1, IRS2, KCNE1, MMP9, MYL4* and *TREML4*, are differentially expressed in both phases of this study.
Conclusions: Transcriptomic analysis by microarray technology demonstrated differential expression during a 48 h time course suggesting a potential use of some of these genes as biomarkers for very early stages of ACS, as well as for monitoring early cardiac ischemic recovery.
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1. Introduction

Cardiovascular diseases, including acute coronary syndrome (ACS), are the leading cause of death in developed countries. Inappropriate diagnosis of ACS, particularly acute myocardial infarction (AMI), may lead to high mortality rates, while unnecessary admissions to cardiac care units may waste financial and medical resources [1]. The identification of new circulating biomarkers that provide early and specific diagnosis of acute cardiac injury has been proposed to improve diagnosis [2,3].

Genomic wide expression analysis using microarray has become a useful tool for the detection of new biomarkers, with multiple applications, such as the classification of different subtypes of ovarian cancer [4], the definition of transcriptional changes associated with smoking [5] and the evaluation of drug response [6].

Microarray studies of human disease are often limited by challenges in obtaining human tissues and by the lack of models that effectively capture clinically relevant disease features [7]. In this context, transcriptional profiling from whole-blood cells by microarray analysis has become an alternative in a search for genetic biomarkers of heart failure [8]. The dynamic and interactive properties of blood give rise to the possibility that subtle changes occurring within the body, such as changes in association with a disease process or in response to an injury, may leave 'footprints' in blood [7]. This approach has emerged in recent years as surrogate markers of several complex diseases including inflammatory process and malignant diseases [9].

In this study, our main goal was to identify new biomarkers with a differentially expressed mRNA profiling approach using whole genomic expression analysis in a peripheral blood cell model from patients with early ACS followed-up over the first 48 hour-period after admission in a hospital emergency room.

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http://dx.doi.org/10.1016/j.cca.2013.03.011

2. Material and methods

An expanded Methods section is available online as a Supplementary material.

2.1. Study population

Eighty-three individuals with ACS, including acute ST-segment elevation myocardial infarction (STEMI, n = 16), non-ST segment elevation myocardial infarction (non-STEMI, n = 41) and unstable angina (UA, n = 25), were selected at the emergency room of the Institute Dante Pazzanese of Cardiology. Diagnosis of ACS at admission was defined in Supplementary material.

This study included subjects suffering their first ACS (1ACS) and those with a second one within one year after the first event (2ACS). 2ACS individuals were taking multiple cardiac related medications (aspirin, clopidogrel, beta-blockers, diuretics, statins, nitrates, anti-arrhythmics, angiotensin converting enzyme inhibitors, calcium channel blockers and angiotensin II receptor blockers) [10].

A control group (n = 47) was selected among blood donor volunteers at the Fundação Pró-Sangue Hemocentro de São Paulo. All controls without ACS had normal ergometric test and serum cardiac biomarkers.

This study was approved by the Institutional Ethics Committee of Faculty of Pharmaceutical Sciences of the University of Sao Paulo and Institute Dante Pazzanese of Cardiology of Sao Paulo, and written informed consents were obtained from all the participants, which conforms to the Declaration of Helsinki.

2.2. Experiment design

This study was made in two phases. On phase 1 a restricted clinical criteria (ACS-Ph1, n = 9 and CG-Ph1, n = 6) was used for entrance and a genomic wide expression analysis performed in order to select potential mRNA biomarker candidates. A subsequent study phase 2 was applied to the first-round markers using a larger and independent casuistic (ACS-Ph2, n = 74 and CG-Ph2, n = 41) (Fig. 1).

Blood samples from ACS-Ph1 were obtained from an antecubital vein at six time points following STEMI diagnosis. The first time (T_0) was on patient admission to the emergency room prior to receiving any medication. The second time (T_2) blood collection was taken was after successful mechanical reperfusion and artery stenting within 2 h after T_0. After 12 h a subsequent sample collection was made at 12 h (T_{12}), 24 h (T_{24}), 36 h (T_{36}) and 48 h (T_{48}) of first blood collection. For CG-Ph1, peripheral blood was collected at one time point [11].

On phase 2 seventy-four ACS patients with acute ischemic-type chest pain under 48 h, diagnosed as previously described were evaluated by a single peripheral blood sample collection, just after their admission at the emergency room prior to receiving any medication.

2.3. Total RNA extraction from peripheral whole-blood cells

Total RNA was isolated from whole blood samples collected in PAXgene tube using the PAXGene Blood RNA extraction system (PreAnalytiX, Hilden, Germany) following the manufacturer's protocols including a RNase-free DNase (Qiagen, Valencia, CA, USA) step

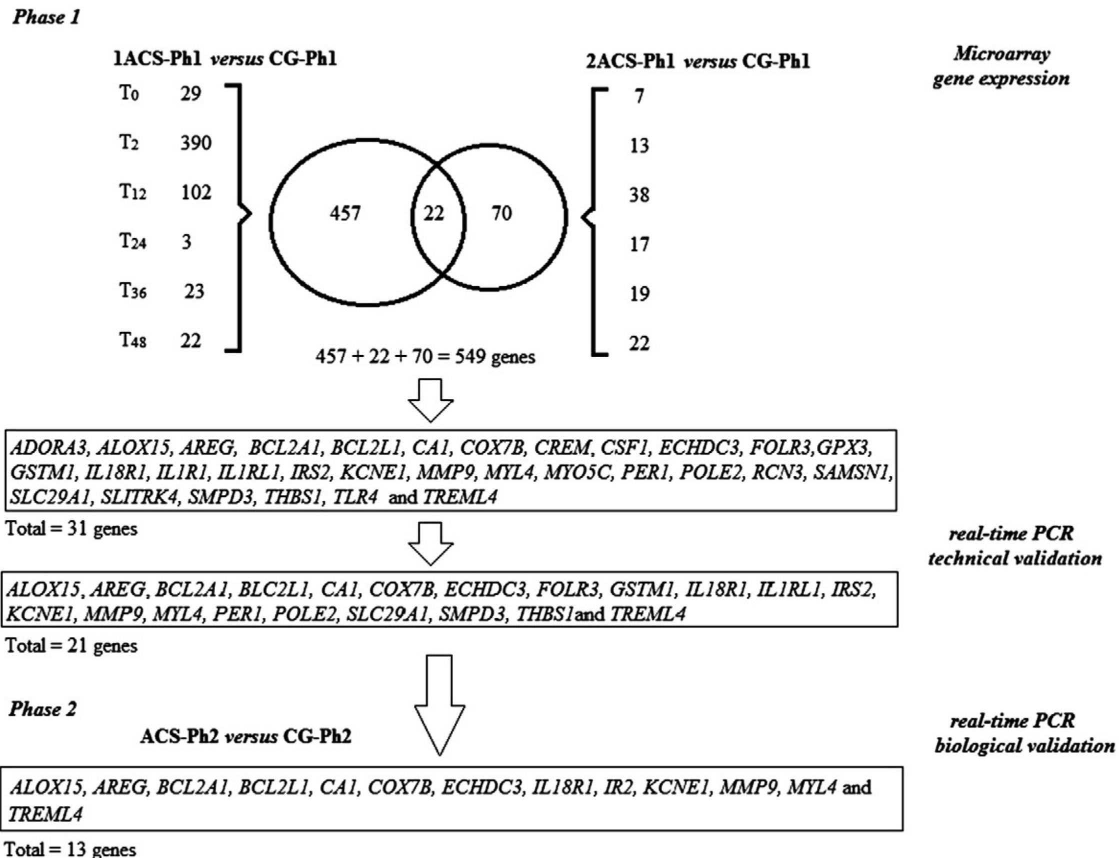

Fig. 1. Candidate gene expression biomarkers history of heart ischemia CG-Ph1: control group from phase 1; 1ACS-Ph1: patient with acute coronary syndrome without previously historic of heart ischemia from phase 1; 2ACS-Ph1: patient with acute coronary syndrome with previously history of heart ischemia from phase 1; CG-Ph2: control group from phase 2;ACS-Ph2: patient with acute coronary syndrome from phase 2; T_0: first stage; T_2: second stage; T_{12}: third stage; T_{24}: fourth stage; T_{36}: fifth stage; T_{48}: sixth stage.

to remove genomic DNA. PAXgene tubes were stored at −80 °C until RNA isolated procedure, not exceeding 6 months of storage. The quantity of RNA was measured using a Nanodrop ND-1000 Spectrophotometer to give the yield and a 260/280 ratio (Nanodrop Technologies, Delaware, USA). Agilent Bioanalyser Lab-on-a-chip RNA chips (Agilent Technologies, Waldbronn, Germany) were used for each sample to evaluate the quality of RNA using the RNA Integrity Number (RIN) scores and for check of evidence for lack of contamination of DNA. The RNA samples were stored at −80 °C until later cDNA conversion.

2.4. Transcriptomic analysis study

Transcriptomic analysis was performed in GeneChip® Human Exon 1.0 ST array (Affymetrix, CA, USA), following manufacturer's protocols. After obtaining microarray data, all filtering procedures as well as statistical validation analysis were performed in oneChannelGUI Bioconductor package [12]. Ingenuity Pathway Analysis Application (IPA) (Ingenuity Systems, California, USA) was used to identify modules of functionally related genes involved in specific pathways. Significant gene expression was set for $P < 0.001$ and fold-change ≥1. See the Supplementary material for more information about bioinformatics data analysis.

Microarray data reported in the manuscript was described in accordance with MIAME guidelines and were deposited in the GEO database (http://www.ncbi.nlm.nih.gov/projects/geo/) as GSE29532 series.

2.5. Validation of gene differently expressed by RT-qPCR

Total RNA was converted to cDNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) using Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems). cDNA was prepared from 1 μg of total RNA, with random hexamer primers into 20 μL of reaction volume in triplicate, RNA was converted to cDNA using the following conditions: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 s, and 4 °C until further processing or storage. cDNA samples were kept at −80 °C for further RT-qPCR analysis. For technical validation, the cDNA samples were preamplified using TaqMan PreAmp Master Mix (Applied Biosystems) following the manufacturer's instructions. The preamplified cDNA samples were kept overnight at 4 °C.

Putative biomarkers selected for technical and biological validation were measured by Inventoried TaqMan® gene expression assays which were carried out using primer and probe sets from Applied Biosystems. Each assay was designed using ABI's primer/probe selection algorithm and bionformatics pipeline which includes access to both public and Celera DNA sequence databases. Glyceraldehyde-3-phosphate dehydrogenase gene (*GAPD*) as reference gene, by presenting the average logarithmic intensity of expression close to target genes and a low coefficient of variation among all samples studied based on geNorm software [13], through the analysis of data from microarray experiments.

Hundred nanograms cDNA templates were used in duplicated, in a total reaction volume of 25 μL. The cDNA was amplified using 90 nM of specific primers, 250 nM of probes and 1× Gene Expression Mater Mix (Applied Biosystems), with the following cycling parameters: 40 cycles at 95 °C for 15 s and at 60 °C for 1 min using 7500 Real-Time PCR System (Applied Biosystems). Sample quantification cycle (Cq) values were determined from plots of normalized fluorescence versus number of PCR cycles during exponential amplification by Sequence Detection Software v. 2.0.1 (Applied Biosystems). The relative quantification value of each target gene was analyzed using a comparative Ct method [14].

3. Results

3.1. Biodemographic and clinical laboratory measure

The prevalence of risk factors for myocardial infarction in the ACS-Ph1, ACS-Ph2, CG-Ph1 and CG-Ph2 is shown in Table 1. Several parameters, including mean age, body mass index (BMI), hypertension, dyslipidemia, cigarette smoking, sedentary lifestyle, serum concentrations of total cholesterol, HDL cholesterol, LDL cholesterol, VLDL cholesterol, triglycerides and glucose were similar between phase 1 groups ($P > 0.05$).

Serum concentrations of CK and CK-MB (Table S2) showed that values greater than the upper reference limit were found at T_{12}. Significant increase of these biomarkers was detected only after approximately 12 h post ACS-Ph1 diagnosis ($P < 0.05$). No statistical differences of cardiac markers were observed among 1ACS-Ph1 and 2ACS-Ph1 ($P > 0.05$).

The mean age and BMI were higher in patients with 1ACS-Ph2 and 2ACS-Ph2 than CG-Ph2 ($P < 0.05$). Moreover, differences were found in the number of individuals with diabetes, dyslipidemia, cigarette smoking, sedentary lifestyle and UA among phase 2 groups ($P < 0.05$). Serum concentration of HDL was higher in CG-Ph2 than 1ACS-Ph2 and 2ACS-Ph2 ($P < 0.05$) while VLDL cholesterol, triglycerides and glucose concentrations were lower ($P < 0.05$). No statistical differences

Table 1
Clinical and laboratory data of the study phases.

Variable	Phase 1				Phase 2			
	CG-Ph1 (6)	1ACS-Ph1(5)	2ACS-Ph1(4)	*P*	CG-Ph2 (41)	1ACS-Ph2 (45)	2ACS-Ph2 (29)	*P*
Age, years	48 ± 8	53 ± 10	53 ± 11	0.42	40.4 ± 6.9	59.2 ± 10.7	65.0 ± 11.2	<0.01
BMI, kg/m²	26 ± 2	27 ± 5	26 ± 3	0.97	24.3 ± 3.4	27.9 ± 4.9	29.3 ± 4.4	<0.01
Gender male, %	100 (6)	100 (5)	100 (4)	ND	80 (33)	78 (35)	76 (22)	0.03
Diabetes, %	0 (0)	0 (0)	0 (0)	ND	0 (0)	20 (9)	48 (14)	0.01
Hypertension, %	33 (2)	40 (2)	100 (5)	0.58	0 (0)	60 (27)	83 (24)	0.04
Smoking, %	67 (4)	60 (3)	80 (4)	0.78	7 (3)	38 (17)	29 (8)	<0.01
Sedentary lifestyle, %	83 (5)	40 (2)	60 (3)	0.33	2 (1)	70 (30)	89 (25)	<0.01
UA, %	0 (0)	0 (0)	0 (0)	ND	0 (0)	24 (11)	52 (15)	0.02
STEMI, %	0 (0)	100 (5)	100 (5)	ND	0 (0)	13 (6)	3 (1)	0.16
non-STEMI,%	0 (0)	0 (0)	0 (0)	ND	0 (0)	62 (28)	45 (13)	0.14
Total cholesterol, mmol/l	5.7 ± 1.2	5.3 ± 1.2	4.8 ± 0.7	0.41	4.8 ± 0.9	5.2 ± 1.5	4.3 ± 1.3	0.10
HDL cholesterol, mmol/l	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.16	0.6 ± 0.2	0.5 ± 0.3	0.4 ± 0.1	<0.01
LDL cholesterol, mmol/l	3.7 ± 1.1	3.7 ± 1.1	3.1 ± 0.7	0.53	2.8 ± 0.8	3.2 ± 1.3	2.8 ± 1.3	0.15
VLDL cholesterol, mmol/l	0.5 ± 0.2	0.3 ± 0.3	0.4 ± 0.2	0.44	0.2 ± 0.2	0.4 ± 0.2	0.3 ± 0.1	<0.01
Triglycerides, mmol/l	2.3 ± 0.8	1.7 ± 1.5	2.0 ± 0.8	0.48	1.1 ± 0.6	2.4 ± 2.1	2.2 ± 2.5	<0.01
Glucose, mmol/l	5.4 ± 0.4	5.5 ± 0.6	5.4 ± 0.6	0.88	5.0 ± 0.6	5.9 ± 2.7	6.3 ± 1.8	0.02

Number of individuals in parenthesis. Continuous variables are presented as mean ± SD and were compared by Kruskal–Wallis test. Categorical variables were compared by chi-square test, null values were not considered for statistic test. ND: not determinate.

were found in the number of patient with STEMI and non-STEMI among 1ACS-Ph2 and 2ACS-Ph2 ($P > 0.05$).

3.2. Microarray differentially expressed gene

The complexity of the dataset was reduced by removing non-significant probe sets (i.e. those without expression or change). This filtering procedure reduced the initial set from 21,806 to 16,417 probes that were used for further analysis.

Four hundred and seventy-nine genes were differentially expressed between 1ACS-Ph1 and CG-Ph, the time course analyzed revealed different expressions of 29, 390, 102, 3, 23 and 22 genes at T_0, T_2, T_{12}, T_{24}, T_{36} and T_{48}, respectively ($P < 0.001$; Tables S3–S8; Fig. S1 gene at T_0).

Ninety-two genes were differentially expressed between 2ACS-Ph1 and CG-Ph1, the time course analysis revealed different expressions of 7, 13, 38, 17, 19 and 22 genes at T_0, T_2, T_{12}, T_{24}, T_{36} and T_{48}, respectively ($P < 0.001$; Tables S9 to S14; Fig. S2 gene at T_0).

Therefore the total sum of genes differentially expressed at phase 1 was 549 genes ($P < 0.001$). Among them twenty-two genes were found at both situations at 1ACS-Ph1 and 2ACS-Ph1, their signal and intensity of expression are shown in Fig. S3.

Gene expression of 31 representative genes in all samples of phase 1 was assessed using RT-qPCR (Table S16). No further genes were selected because of the limited amount of RNA obtained from whole-blood cell extraction. Among these genes, six genes (*COX7B, ECHDC3, FOLR3, RCN3, SLITRK4* and *SLC29A1*) were selected because they were differently expressed at T_0; the other 5 genes (*CREM, GPX3, IRS2, PER1* and *THBS1*) because they were significantly expressed by ACS-Ph1 at T_0 compared with CG-Ph1 previously associated with cardiovascular disease according the IPA analysis; 4 genes (*AREG, CSF1, SAMSN1* and *TREML4)* because they were significantly expressed at T_0 according to a conservative false discovery rate test (FDR <0.05); eleven genes (*ADORA3, ALOX15, BCL2A1, BCL2L1, CA1, GSTM1, IL1R1, IL1RL1, KCNE1, MMP9* and *TLR4)* because they were significantly regulated at more than one time point of ACS-Ph1, compared with controls previously associated with cardiovascular disease by other studies; and lastly, 5 genes (*IL18R1, MYL4, MYO5C, POLE2* and *SMPD3*) were included because they were significantly expressed at T_0 not previously associated with cardiovascular disease.

3.3. Technical validation by RT-qPCR (phase 1)

Twenty-one out of the 31 genes differentially expressed by microarray analysis were positively correlated with RT-qPCR data and were considered technically validated at the phase 1 of the study (Table S16, $P < 0.05$, $r > 0.50$). The genes were *ALOX15, AREG, BCL2A1, BLC2L1, CA1, COX7B, ECHDC3, FOLR3, GSTM1, IL18R1, IL1RL1, IRS2, KCNE1, MMP9, MYL4, PER1, POLE2, SLC29A1, SMPD3, THBS1* and *TREML4.*

3.4. Biological validation by RT-qPCR (phase 2)

Biological validation was carried out using the ACS samples from phase 2. Eight genes, *ALOX15, CA1, COX7B, ECHDC3, IL18R1, KCNE1, MMP9* and *MYL4*, were significantly regulated at 1ACS-Ph2 and 2ACS-Ph2, when each group was compared with CG-Ph2 ($P < 0.05$, Table 2) irrespectively of previous history of cardiac events and medications.

The *AREG* and *TREML4* genes were differently expressed between 1ACS-Ph2 versus CG-Ph2 ($P < 0.02$) and 2ACS-Ph2 versus CG-Ph2 ($P < 0.01$), respectively. Moreover the *BCL2A1* and *BCL2L1* genes were differentially expressed when comparing 2ACS-Ph2 versus CG-Ph2 and *IRS2* in 1ACS-Ph2 versus CG-Ph2, and when at least some of the divergent variables between the casuistic proposed in phase 1 and phase 2 were excluded, such as patients with UA, women and older patients (ages > 65 years; Table 2). Therefore 13 genes were considered biologically validated at study phase 2.

3.5. Time course analysis of validated genes and association with ACS

IPA analysis was used to investigate the biological relevance of the observed gene expression pattern by categorizing the data set into distinct groups depending on the biological function and/or disease (Fig. 2A). Therefore categories corresponding to cell death (*ALOX15, AREG, BCL2A1, BCL2L1, IRS2* and *MMP9*), cardiovascular disease (*ALOX15, BCL2L1, CA1, IRS2, KCNE1* and *MMP9*), cardiovascular system

Table 2
Relative mRNA expression of the twenty genes selected for biologic validation by qPCR in study phase 2.

Gene	*CG-Ph2*	*1ACS-Ph2*	*P*	*2ACS-Ph2*	*P*
ALOX15	0.055 (0.026–0.074)	**0.021 (0.008–0.062)**	**0.03**	**0.025 (0.003–0.063)**	**0.05**
AREG	0.003 (0.001–0.016)	**0.008 (0.004–0.015)**	**0.02**	0.004 (0.002–0.010)	0.49
BCL2A1[a]	1.481 (0.814–3.534)	1.032 (0.621–2.682)	0.28	**1.006 (0.570–1.183)**	**0.03**
BLC1L1[b]	0.037 (0.028–0.065)	0.033 (0.018–0.061)	0.44	**0.022 (0.016–0.031)**	**<0.01**
CA1	0.006 (0.002–0.012)	**0.002 (0.001–0.007)**	**0.02**	**0.001 (0.001–0.003)**	**<0.01**
COX7B	0.202 (0.150–0.268)	**0.128 (0.113–0.161)**	**<0.01**	**0.139 (0.116–0.221)**	**<0.01**
ECHDC3	0.005 (0.003–0.008)	**0.010 (0.006–0.028)**	**<0.01**	**0.012 (0.007–0.020)**	**0.00**
FOLR3	0.050 (0.031–0.210)	0.061 (0.032–0.259)	0.61	0.061 (0.032–0.117)	0.90
GSTM1	0.008 (0.001–0.017)	0.007 (0.001–0.015)	0.40	0.009 (0.002–0.016)	0.93
IL18R1	0.008 (0.007–0.014)	**0.018 (0.008–0.036)**	**<0.01**	**0.017 (0.008–0.028)**	**0.01**
IL1R1	0.002 (0.001–0.004)	0.001 (0.001–0.003)	0.31	0.002 (0.000–0.003)	0.45
IRS2[c]	0.186 (0.111–0.259)	**0.325 (0.131–0.911)**	**0.07**	0.369 (0.213–0.747)	0.186
KCNE1	0.135 (0.105–0.395)	**0.048 (0.017–0.253)**	**<0.01**	**0.061 (0.018–0.140)**	**<0.01**
MMP9	0.276 (0.223–0.532)	**0.505 (0.288–1.066)**	**0.01**	**0.569 (0.345–0.956)**	**0.02**
MYL4	0.006 (0.002–0.013)	**0.003 (0.002–0.005)**	**0.03**	**0.002 (0.001–0.006)**	**<0.01**
PER1	0.031 (0.017–0.063)	0.043 (0.029–0.088)	0.11	0.034 (0.022–0.077)	0.40
POLE2	0.002 (0.002–0.004)	0.003 (0.002–0.004)	0.60	0.003 (0.002–0.004)	0.70
SMPD3	0.013 (0.007–0.020)	0.009 (0.004–0.026)	0.23	0.011 (0.005–0.018)	0.45
THBS1	0.075 (0.049–0.272)	0.062 (0.047–0.134)	0.21	0.080 (0.059–0.182)	0.71
TREML4	0.008 (0.002–0.017)	0.008 (0.001–0.019)	0.99	**0.028 (0.016–0.039)**	**0.01**

Expression values are represented with means and quartiles range (25–75). The genes are named in accordance with HUGO (*Human Genome Organization*). In bold, gene with $P < 0.05$ for *Mann–Whitney* nonparametric test between CG-Ph2 vs. 1ACS-Ph2 and CG-Ph2 vs. 2ACS-Ph2.
[a] Women and subjects > 65 years old were excluded from the statistical analysis;
[b] Patients > 65 years old were excluded from the statistical analysis;
[c] Women, subjects > 65 years old and those with unstable angina were excluded from the statistical analysis.

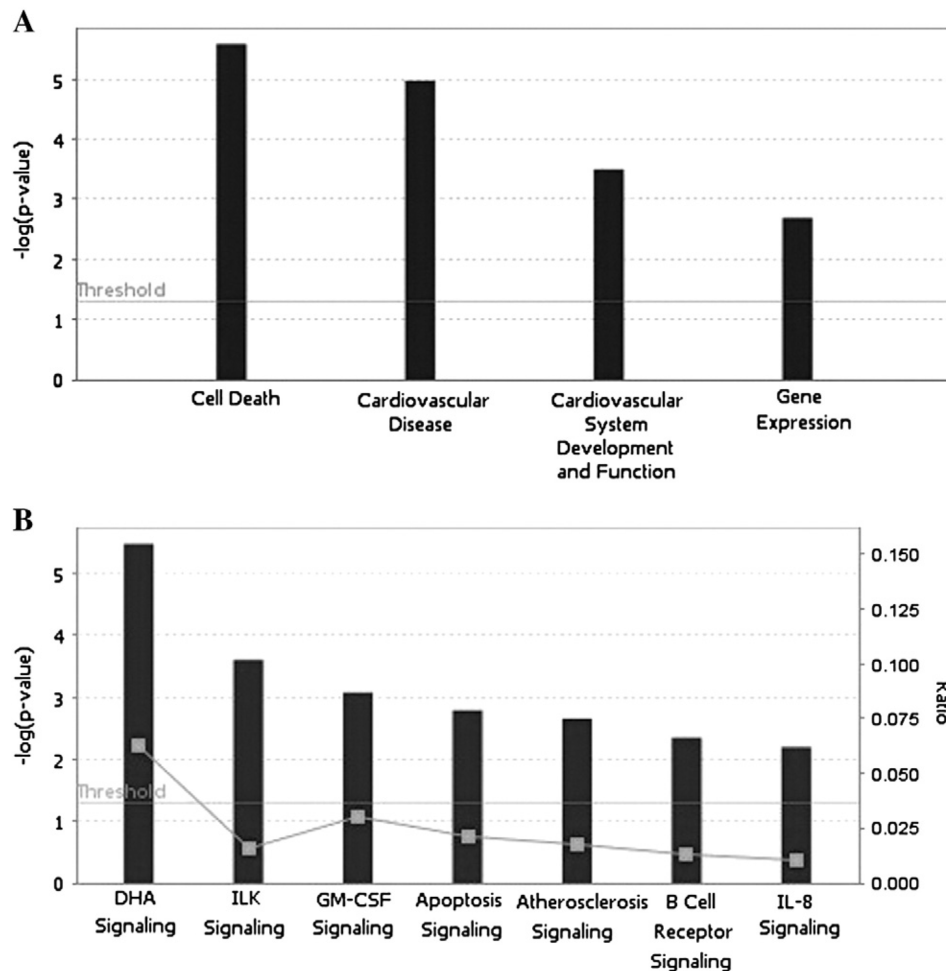

Fig. 2. Ingenuity Pathway Analyses. A: Top functional categories and B: canonical pathways from the present study data set based on significance.

development and function (*BCL2A1, BCL2L1, KCNE1* and *MMP9*) and gene expression (*BCL2L1* and *IL18R1*) were formed. Gene lists were also searched and a number of significant canonical pathways form IPA library identified, including pathways involved in docosahexaenoic acid (DHA) signaling (*ALOX15, BCL2A1* and *BCL2L1*), ILK signaling (*IRS2, MMP9* and *MYL4*), GM-CSF signaling (*BCL2A1* and *BCL2L1*), apoptosis signaling (*BCL2A1* and *BCL2L1*), atherosclerosis signaling (*ALOX15* and *MMP9*), B cell receptor signaling (*BCL2A1* and *BCL2L1*) and IL-8 signaling (*BCL2L1* and *MMP9*) (Fig. 2B). Network analysis was also performed to provide a graphical representation of genes having a known biological relationship. The first top five networks were related to the *ALOX15, AREG, BCL2A1, BCL2L1, IL18R1, IRS2* and *MMP9* (Fig. 3A), while the next four top networks were related to the *COX7B, MYL4, CA1* and *KCNE1.* A merged network can be seen in Fig. 3B.

The time-course of the 13 validated genes were evaluated using the microarray data set (phase 1, Fig. S1). The results revealed that these genes were expressed with significant differences within 12 h after 1ACS-Ph1 and 2ACS-Ph1 and the level of expression tends to return to normal values after the peak of expression, except for the *TREML4* gene, which was up regulated during the 48 h.

A comparison of the expression of all 13 genes between UA-Ph2, non-STEMI-Ph2 and STEMI-Ph2 versus CG-Ph2 is shown in Fig. S2. *ECHDC3* mRNA expression was significantly increased in the following order: UA-Ph2 < non-STEMI-Ph2 < STEMI-Ph2 ($P < 0.05$). In addition, *CA1*, *COX7B*, *KCNE1* and *MYL4* were significantly down regulated in UA-Ph2 and non-STEMI-Ph2 ($P < 0.05$). Moreover *ALOX15* and *MMP9* were differently expressed down and up regulated in non-STEMI-Ph2, respectively ($P < 0.05$).

4. Discussion

Transcriptome technologies have provided new opportunities to identify gene expression profiles related to cardiovascular disease. We have analyzed previously white blood cell transcriptome since gene expression patterns in peripheral blood have been validated in humans [15] as a basis for the detection and diagnostic biomarkers for chronic [16,17] and acute heart failure [18], and asymptomatic left ventricular dysfunction [8]. Indeed, the blood is a dynamic and interactive tissue that communicates with all cells of the body and can therefore display perturbations indicative of disease [8].

In the present study, peripheral blood cell transcriptome profiles of ACS patient were analyzed by microarray over a pre-determined time course with the aim to identify potential cardiac ischemia-related biomarkers during the first 48 hour-period after admission to an emergency room.

The first step to achieve the objective of this study was to select patients with similar clinical variables between ACS-Ph1 and CG-Ph1 for large scale transcriptomic studies. Controlling the biologic variables in microarray studies is important, in order to obtain more reliable results. Data processing of microarray and analysis was performed separating the phase 1 casuistic in two subgroups according to previous cardiovascular history as previously proposed in our proteomic study [11]. We observed ~85% fewer differentially expressed protein peaks by SELDI-TOF-MS in the 2ACS (76) compared with the 1ACS (510), with maximum differentially expressed protein values at T12 and T2 at the 1ACS and 2ACS, respectively. The pharmacological intervention may be responsible for the fold-change profile modification between

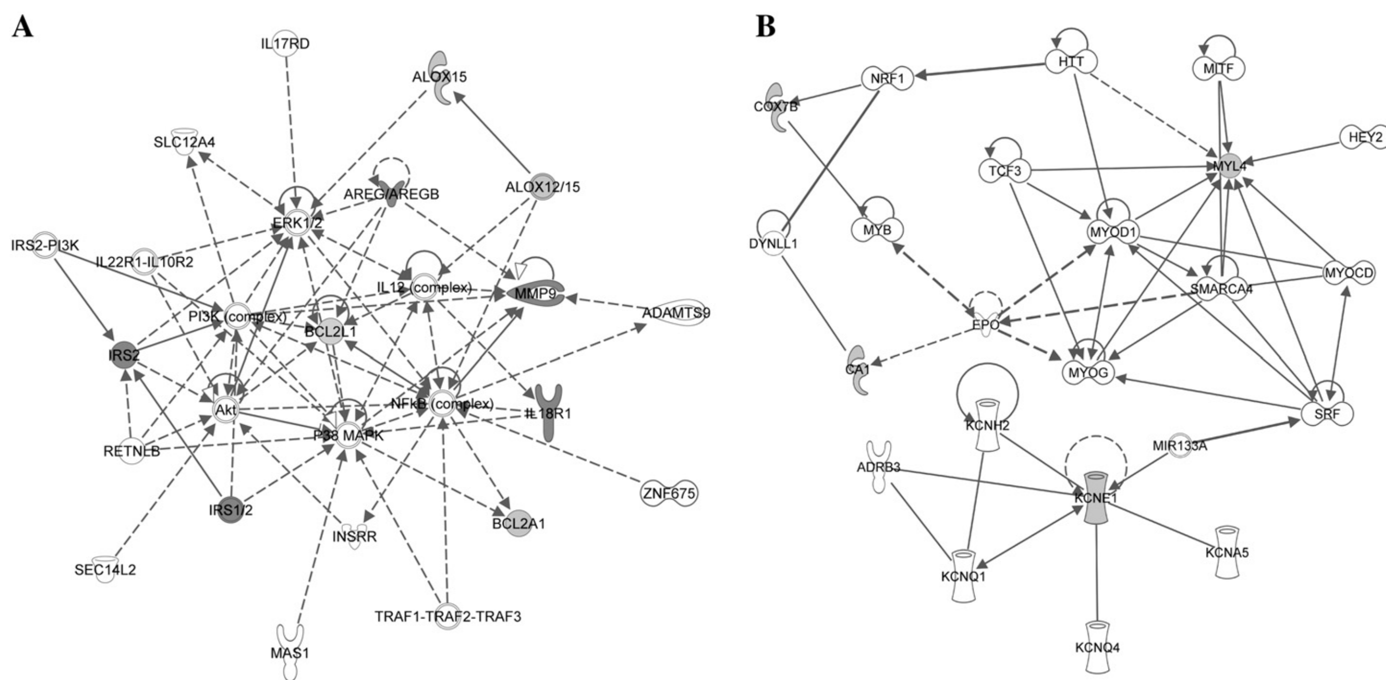

Fig. 3. Network analysis was performed to provide a graphical representation of genes having known biological relationships. A: first network (score 17, focus on 7 genes) and B: merged network (score > 2, focus on 4 genes). Genes in dark gray showed increased expression in ACS patients while genes in gray decreased their expression in ACS. Relationships are marked by arrows: dashed line arrows mark indirect interaction; filled line arrows mark direct interaction.

the ACS groups. The same was observed in this transcriptomic study, we observed 81% fewer differentially gene expressed in the 2ACS-Ph1 (92) compared with the 1ACS-Ph1 (479). Therefore the revascularization using stent and pharmacological treatment might change the dynamic of response in the inflammatory response and an injury changing the kinetic of mRNA and protein profile delivery in the circulation. However with the objective to eliminate the pharmacological intervention the peripheral blood samples of all patients on phase 2 were collected just after their admission at the emergency room prior to receive any medication.

Thirteen genes were considered technical and biologically validated by real-time PCR in our study: *ALOX15, AREG, BCL2A1, BCL2L1, CA1, COX7B, ECHDC3, IL18R1, IR2, KCNE1, MMP9, MYL4* and *TREML4.* All of them were significantly differently expressed between ACS and CG in both phases of this study.

Although the role of the *ALOX15* gene in atherosclerosis is still not clear, we observed a significant down regulation of this gene within 12 h ACS patients. There are in vitro and in vivo studies that suggest pro-atherosclerotic effect and also anti-atherosclerotic effect of ALOX15 [19]. *ALOX15* is a member of the *LOX* family, responsible for the metabolism of arachidonic acid into *15-HETE* together with *GPX3* (up regulated at phase 1). Another function is to increase the uptake of oxidized LDL by macrophages through the increase in the expression of the scavenger receptor *CD36*, favoring the formation of foam cells, characterizing its atherogenic role.

On the other hand, it has also been observed that *15-HETE* and the linoleic acid when metabolized by the enzyme *LOX12* (*13-HODE*) generate a ligand of the proliferative peroxissoma isoform activator receptor alpha (*PPAR*α) of the macrophages, activating the transcription of the ATP-binding cassette, sub-family A, member 1 (*ABCA1*) gene, that promotes efflux of cholesterol and phospholipids from intracellular compartments to the extracellular environment. Cholesterol acts as opposed to the pro-atherosclerotic effect.

In the present study, a significant reduction in *ALOX15* gene expression was observed together with a significant increase in *MMP9*, when comparing control individuals with ACS patients. Some authors have also observed that the activation of *PPARs* by metabolites of *ALOX15* can down-regulate *IL-1*β, *IL-6* and *TNF*α expressions, reducing the expression of *MMP9* [20]. Thus, it leads to the question of whether the low expression of the *ALOX15* gene in the peripheral blood cells promotes the instability [21] and rupture of atherosclerotic plaque [22] by *MMP9*, or the increased expression of *MMP9* is a response to lesion, promoting the migration of endothelial progenitor cells to the site of the lesion [23] and cardiac remodeling [24].

Furthermore, the low mRNA expression of *ALOX15* may be also contribute to the low expression of *BCL2A1* and *BCL2L1,* which were associated with apoptosis signaling and the signalization pathway of IL-8 by IPA analysis.

In the present study, *IL18R1* was significantly up regulated within 2 h ACS when compared with CG. Chandraseker and collaborators (2006) demonstrated that expression of *MMP9* and *NFKB*- is induced by the cytokine *IL18*, with the gene *IL18R1* being essential for the transduction of the signal. This cytokine is responsible for the proliferation and migration of cells from the smooth muscle to the arterial vessel wall, contributing to the development and progression of atherosclerosis, and it can further promote the migration and hyperplasia of the smooth muscle cells in the arterial neointima [25].

AREG gene was also up-regulated at hospital admission. In a cellular model of head and neck cancer increased expression of the *AREG* gene is associated to a higher expression level of *MMP9* and *VEGF*, stimulating angiogenesis and metastatic processes [26]. In addition, *AREG* and *MMP9* were also involved with cell death by IPA analysis in the present study. And the genes *IRS2* (up regulated within 2 h) and *MYL4* (down regulated within 12 h) were involved in ILK signalization together with *MMP9*.

In this study, *CA1* is shown to have significantly decreased expression levels compared to controls with ACS. Alvarez and collaborators (2007) described the relationship between carbonic anhydrase (*CA*) and cardiomyocyte hypertrophy and found that cardiomyocyte cultures of rodents treated with phenylephrine and CA don't develop hypertrophy [27].

A significant increased in the expression of *TREML4* gene was also observed in patients with ACS in comparison with controls. The *TREML4* gene is part of the *TREM* (*Triggering Receptor Expressed on*

Myeloid cells) family discovered in 2000 [28]. However, their physiopathological role is still not completely understood. It is believed that they play an important role in the regulation of the innate immune response to intracellular microorganisms, together with the toll-like receptors (*TLR*) via LPS [28].

We found that expression of *ECHDC3* was correlated with the severity of ACS. A greater difference in expression was noted in patients with STEMI-Ph2 than in non-STEMI-Ph2 patients, who in turns, differed from those diagnosed with UA-Ph2. In addition, expression of *ECHDC3* was significantly increased within 2 h after ACS. This gene was described for the first time in the literature in 1996, as a new inhibitor of mitochondrial fatty acid oxidation [29]. However, up to now, little is known that could shed some light on its putative role in ACS.

One possibility is that *ALOX15*, *CA1* and *KCNE1* are involved in a protective systemic response to the development of cardiac insufficiency in the face of partial or total occlusion of the coronary artery, as a result of the reduced blood flow in the coronary arteries. *BCL2A1* and *COX7B* could be involved in the regulation of the apoptotic process of the injured cardiac and endothelial cells. *AREG*, *IL18R1*, *IRS2*, *MYL4*, *BCL2L1* and *MMP9* could be involved in the post-lesion endothelial and cardiac remodeling. Another possibility is that *ALOX15*, *IL18R1* and *MMP9* have pro-atherogenic actions involved in the progression and rupture of atherosclerotic plaque. In addition, the recently discovered *TREML4* gene may trigger the body's inflammatory cascade in the destabilization of atherosclerotic plaque caused by the possible presence of infectious agents.

Further functional experiments must be conducted with the purpose of evaluating their relation with myocardium damage or vessel occlusion status. Moreover future studies with a large casuistic of ACS patients after their condition became stabilized, example one month, six month and one year past-ACS, it should be very interesting to evaluate these potential mRNA biomarker candidates.

In conclusion, the transcriptional profiling presented here suggests a potential use of *ALOX15, AREG, BCL2A1, BCL2L1, CA1, COX7B, ECHDC3, IL18R1, IR2, KCNE1, MMP9, MYL4* and *TREML4* as gene expression biomarkers for very early stages of ACS. Further studies must be conducted with the purpose to determine possible interferences in the evaluation of the diagnostic sensitivity and specificity and their prognostic applicability.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2013.03.011.

Acknowledgments

This work is partly supported by grants from projects 2006-03487-4 from the FAPESP, Brazil, BIO2008-04212 from the Spanish Ministry of Science and Innovation and PROMETEO/2010/001 from the GVA-FEDER. The CIBER de Enfermedades Raras is an initiative of the ISCIII. V.N. Silbiger and L.G.L. Neto are recipients of fellowships from FAPESP, Brazil. V.N. Silbiger was recipients of fellowships from CAPES, Brazil and D. Decavi are recipients of fellowships from CAPES, Brazil. A.D. Luchessi, M.H. Hirata and R.D.C. Hirata are recipients of fellowships from CNPq, Brazil.

We gratefully acknowledge the dedicated efforts of investigators and staff at the Centro Nacional de Genotipado (CeGen), Centro de Investigación Príncipe Felipe and Institute Dante Pazzanese of Cardiology, especially Inés Quintela, David Montaner and Dr. Marcos Paulo Pereira. We also thank Sandro J. Souza for the comments and the language revision.

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