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Relazione attività scientifica di metà periodo
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"*Prognostic and predictive markers of acute atherothrombotic events*"

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Introduction

Atherosclerosis is a lipid-driven, chronic inflammatory disease of the vessel wall in which both innate and adaptive immune responses play a role (Hansson GK, 2002). Immune cells and their mediators directly cause the chronic arterial inflammation that is a hallmark of atherosclerosis. Macrophages, T lymphocytes and, to a lesser extent, mast cells contribute to the smoldering inflammatory response in the vessel wall (Kovanen PT, 1995).

It is now generally accepted that a specific T-cell response is crucial in determining not only the development and growth of the atherosclerotic lesion, but also its fate toward a stable or an unstable pattern of evolution (i.e., rupture or ulceration), eventually leading to the clinical occurrence of ACS (Andersson J, 2010). T-cells are able to produce a large array of pro-inflammatory cytokines. Cytokines are pleiotropic proteins that regulate the activity of many cells. The progression of plaques from a chronic state toward rupture is accompanied by activation of a complex inflammatory network, characterized by an intricate expression and secretion of many cytokines (Golino P, 2010). The effects of this complex network of inflammation on gene expression profiling within the coronary circulation is still under investigation. Thus, using gene induction and microarray technology, the aim of the present study was to evaluate the changes in gene expression

profile in endothelial cells stimulated with plasma obtained from the coronary sinus (CS) and the aorta (Ao) of patients with ACS or stable angina (SA).

Materials and methods

Patient Population

Twelve patients, divided in 2 groups, entered the study. Group I included patients with chronic stable angina (SA, n=4) undergoing elective coronary angiography; group II included patients with ACS (n=8), undergoing urgent coronary angiography and eventually PTCA. All patients in group I had a history of effort chest pain with a stress test positive for inducible myocardial ischemia. Patients with previous myocardial infarction were excluded. ACS was defined as typical chest pain at rest occurring <48 hours from hospital admission with ECG changes suggesting myocardial ischemia, with or without increase in serum markers of myocardial damage. Within the diagnosis of ACS, normal or increased serum markers of myocardial necrosis defined unstable angina (UA) or non- ST segment elevation myocardial infarction (NSTEMI), respectively. Patients with ST-segment elevation myocardial infarction were excluded from the study. As per protocol design, in both groups, only patients with TIMI flow grade ≥2 were included, as well as patients with a single vessel disease and a culprit lesion located either in the left anterior descending coronary artery or in the circumflex coronary artery, both of which drain into the coronary sinus.

Experimental Protocol

After coronary angiography was performed, and before performing PTCA, a 6F multipurpose catheter was positioned into the coronary sinus (CS). Blood samples (4.5 ml) were simultaneously obtained from the CS and the ascending aorta (Ao) and immediately placed into empty pre-chilled Vacutainer® tubes for serum separation. Serum samples were immediately centrifuged at 1,000 x g at 4 °C for 20 minutes, and the sera were stored in aliquots at -80 °C.

Cell culture

Experiments were performed on human coronary artery endothelial cells (HCAECs, Cambrex Bio Science, USA). Cells were grown in EGM 2 medium (Cambrex Bio Science, USA) with endothelial cell growth supplement and 10% fetal serum. Cells were used at passages 2–5.

Microarray gene expression analysis

Agilent microarray analyses were done to evaluate the gene expression profile in ECs exposed to plasma obtained from CS and Ao in patients with ACS and SA. These experiments were performed using a one colour labeling microarray system. The quantity of each of the total RNA samples and determination of the A260/280 nm ratio was determined by spectrophotometry and the size distribution was assessed using an Agilent Bioanalyzer. Eight hundred ng of total RNA were converted into labelled cRNA with nucleotides coupled to a fluorescent dye (either Cy3) using the Quick Amp Kit (Agilent Technologies, Palo Alto, CA) following the manufacturer's protocol. The A260/280 nm ratio and yield of each of the cRNAs were determined using an Thermofisher Nanodrop. Eight hundred twenty-five ng of cRNA-labeled from SC and Ao were hybridized to Agilent Human Whole Genome 4 × 44k Microarrays. The hybridized array was washed and scanned and data were extracted from the scanned image using Feature Extraction version 10.2 (Agilent Technologies). The raw data and associated sample information were loaded and processed by Gene Spring® 11.5X (Agilent Technologies). For identification of genes significantly altered in patients with unstable angina, total detected entities were filtered by signal intensity value (upper cut-off 100th and lower cut-off 20th percentile) and flag to remove very low signal entities. Data were analyzed using Student's t test ($p < 0.05$) with a Bonferroni multiple test correction to minimize selection of false positives. Of the significantly differentially expressed RNA, only those with greater than 2-fold increase or 2-fold decrease as compared to controls were used for further analysis. Subsequently, hierarchical clustering (condition tree) was applied to the data files. In this way, the relationships between the different groups are shown. The condition tree was displayed as

a heat map, based on expression levels of the probe sets. Functional and network analyses of statistically significant gene expression changes were performed using Ingenuity Pathways Analysis (IPA) 8.0 (Ingenuity® Systems, http://www.ingenuity.com). Analysis considered all genes from the data set that met the 2-fold (p-value < 0.05) change cut-off and that were associated with biological functions in the Ingenuity Pathways Knowledge Base. The significance of the association between the data set and the canonical pathway was measured in 2 ways: (1) Ratio of the number of genes from the dataset that map to the pathway divided by the total number of genes that map to the canonical pathway is displayed; (2) Fisher's exact test was used to calculate a P value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

Results

The first six months of my project investigated the expression of ACS-related genes, that was determined by comparing gene expression in ECs exposed to plasma obtained from Ao and SC of patients with instable and stable angina.

The results obtained show that the plasma collected by the Ao does not determine significant changes in ECs gene expression of patients with ACS and SA. However, the plasma obtained from SC causes significant changes in the two groups of patients. Only genes with 2-fold increase or decrease were considered significant. Between these genes, 684 were up-regulated and 283 down-regulated in patients with ACS (Figure 1).

Data analysis pointed out that many of the up-regulated and down-regulated genes were involved in the inflammatory process. In particular, selectin E (SELE) was the most up-regulated gene, with a 19.66 fold change. Selectin E is a glycoprotein involved in the adhesion process of leukocytes to endothelial walls and in their migration from the intravascular lumen to the injured tissue. Interestingly, chemokines (CXCL1, CXCL2, CXCL3, CCL2) genes were up-regulated as well.

Chemokines expression is triggered by pro-inflammatory cytokines stimulation and it is involved in the recruitment of monocytes, granulocytes and T cells.

It is noteworthy the increase in expression of pro-inflammatory interleukins (IL3RA, IL7R, IL8, IL11), proteins secreted by the immune system cells and the up-regulation of NFKB related genes (NFKBIA, NFKBIZ, NFKBIE). NF-kB ("nuclear factor kappa-light-chain-enhancer of activated B cells") is a transcription factor that is activated by pro-inflammatory stimuli such as stress, cytokines, free radicals, ultraviolet irradiation and bacterial or viral antigens. Accordingly, several genes involved in the inflammatory process were up-regulated, i.e. prostaglandin-endoperoxide synthase 2 (PTGS2), fibroblast growth factor 18 (FGF18), vascular endothelial growth factor A (VEGFA), intercellular adhesion molecule 1 (ICAM1) and platelet factor 4 (PF4) (Figure 2).

Moreover, many of the down-regulated genes obtained through our microarray experiment were involved in the inflammatory process. In particular, STAT1 is a cytoplasmic transcription factor that is activated by several signals, such as cytokines, transcription factors (INF-α, INF-γ), hormones and T-bet, that represents the main transcription factor in TH1 cells. STAT1 mediates also IL-23 expression, related to TH17 cells inhibition (Kimura A, 2007; Stumhofer JS, 2006). Conversely, T-bet promotes IFN-γ and IL-2 synthesis (Kimura A, 2011; Cheng X, 2008). The reduced expression of these genes confirms the unsuccessful inhibition of TH17 cells, further confirming how plaques may be affected both by the inflammation and immune system responses.

T-bet and STAT1 act as suppressors on Th17 cells production. Having a down-regulation at this level may imply higher Th17 cells production and IL-17 and IL-6 expression. IL-17 and IL-6, in turn, arrest both Treg cells and IL-10 production (Figure 3).

Conclusions and future perspectives

Our data support the evidence that in patients with ACS a significant intracoronary production of selected cytokines/chemokines occurs; this leads to the up-regulation and the down-regulation of several genes involved in different inflammatory responses. Our next goal will be functional and

network analyses of statistically significant genes obtained by the microarray screening by employing the Ingenuity Pathways Analysis 8.0 software. Finally, data will be validated by Real Time PCR experiments.

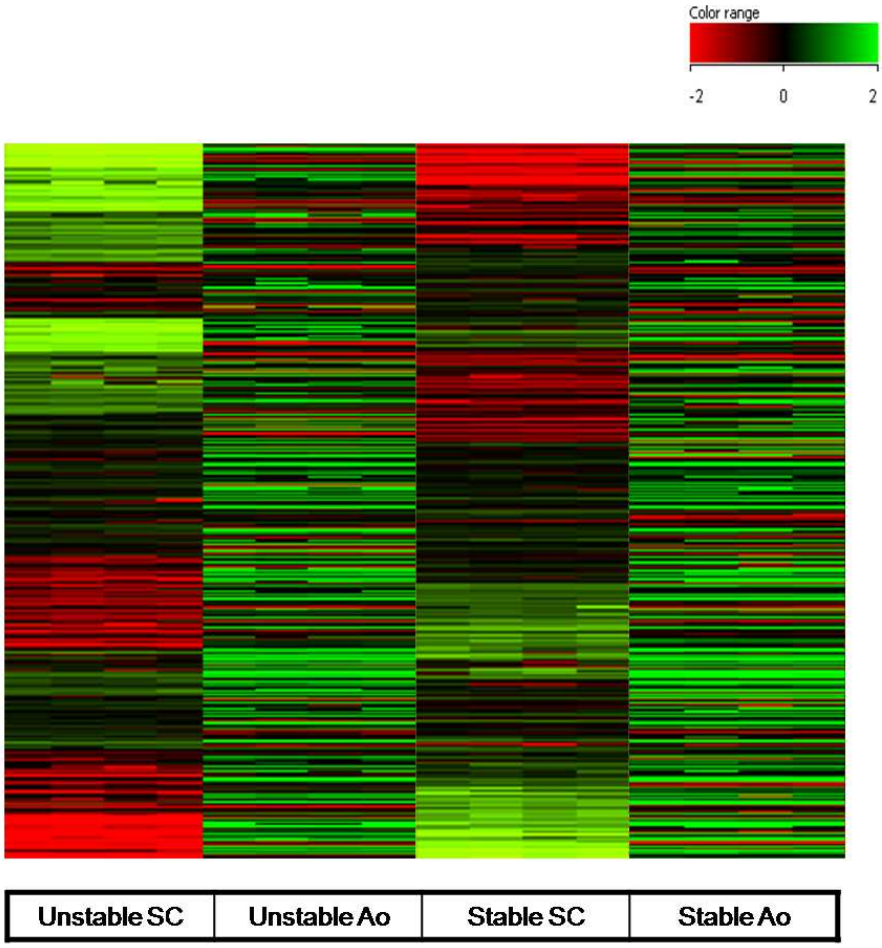

Figure 1. Different gene expression in Ao and SC of patients with ACS and SA. 684 up-regulated (green) and 283 down-regulated (red). ($P \leq 0.01$, and $\geq$2-fold, or $\leq$-2-fold difference).

Gene Symbol	Gene name	Fold Change
SELE	Selectin E	19.66
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	8.49
CCL2	Chemokine (C-C motif) ligand 2	6.32
PTGS2	Prostaglandin-endoperoxide synthase 2	5.98
CD86	CD86 molecule	4.93
CXCL2	Chemokine (C-X-C motif) ligand 2	4.92
RELB	v-rel reticuloendotheliosis viral oncogene homolog B	4.76
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	4.33
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	4.01
ID1	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	3.68
CXCL3	Chemokine (C-X-C motif) ligand 3	3.65
NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	3.36
FOS	FBJ murine osteosarcoma viral oncogene homolog	3.25
FGF18	Fibroblast growth factor 18	3.23
IL7R	IL7 receptor	3.11
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	3.02
CD83	CD83 molecule	2.86
IL8	Interleukin 8	2.84
IL11	Interleukin 11	2.82
HBEGF	Heparin-binding EGF-like growth factor	2.53
TRAF1	Protein phosphatase 4, regulatory subunit 2	2.45
NFKBIE	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	2.39
VEGFA	Vascular endothelial growth factor A	2.36
FOSL2	FOS-like antigen 2	2.34
MAP3K8	Mitogen-activated protein kinase kinase kinase 8	2.27
IL3RA	Interleukin 3 receptor, alpha	2.25
IL6	B-cell CLL/lymphoma 3	2.20
VASP	Vasodilator-stimulated phosphoprotein	2.20
GPR68	G protein-coupled receptor 68	2.13
BMPR1B	Bone morphogenetic protein receptor, type IB	2.12
FBLN1	Fibulin 1	2.11
ICAM1	Intercellular adhesion molecule 1	2.10
PF4	Platelet factor 4	2.06

Figure 2. Up-regulated genes in unstable plaque.

GeneSymbol	GeneName	Fold change
CXCR4	chemokine (C-X-C motif) receptor 4	-4.4
IL10	Interleukin 10	-4.15
CCNG2	Cyclin G2	-3.5
CDKN1B	cyclin -dependent kinase inhibitor 1B (p27, Kip1)	-2.95
CFH	complement factor H	-2.59
IL33	Interleukin 33	-2.43
NAMPT	Nicotinamide phosphoribosyltransferase	-2.15
T-BET	T box 21	-2.14
STAT1	Signal transducer and activator of transcription 1, 91kDa	-2.12
RAB5A	RAB5A, member RAS oncogene family	-2.09
CCNG1	Cyclin G1	-2.04
SENP7	SUMO1/ sentrin specific peptidase 7	-2.04
IL5	Interleukin 5	-2.03
VLDLR	Very low density lipoprotein receptor	-2.01
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	-2

Figure 3. Down-regulated genes in unstable plaque.

References

1. Hansson GK, Libby P, Schönbeck U, et al. Innate and adaptive immunity in the pathogenesis of atherosclerosis. Circ Res. 2002 Aug 23;91(4):281-91.

2. Kovanen PT. Role of mast cells in atherosclerosis. Chem Immunol. 1995;62:132-70.

3. Andersson J, Libby P, Hansson GK. Adaptive immunity and atherosclerosis. Clin Immunol. 2010 Jan;134(1):33-46.

4. Golino P, De Palma R, D'Aiuto E, Cirillo, et al. Cytokine and chemokine expression profile within the coronary circulation of patients with acute coronary syndromes. Journal of the American College of Cardiology. 2010;55:A106.E992-A106.E992.

5. Kimura A, Naka T, Kishimoto T. Il-6-dependent and -independent pathways in the development of interleukin 17-producing t helper cells. *Proc Natl Acad Sci U S A.* 2007;104:12099-12104

6. Stumhofer JS, Laurence A, Wilson EH, Huang E, Tato CM, Johnson LM, Villarino AV, Huang Q, Yoshimura A, Sehy D, Saris CJ, O'Shea JJ, Hennighausen L, Ernst M, Hunter CA. Interleukin 27 negatively regulates the development of interleukin 17-producing t helper cells during chronic inflammation of the central nervous system. *Nat Immunol*. 2006;7:937-945.

7. Kimura A, Kishimoto T. Th17 cells in inflammation. *Int Immunopharmacol*. 2011;11:319-322

8. Cheng X, Yu X, Ding YJ, Fu QQ, Xie JJ, Tang TT, Yao R, Chen Y, Liao YH. The th17/treg imbalance in patients with acute coronary syndrome. *Clin Immunol*. 2008;127:89-97.