-Università del Piemonte Orientale-

"Amedeo Avogadro"

Identification of real-time biomarkers in diseases with vascular complications

XXV Ciclo di Dottorato in Medicina Molecolare

Tutor: Prof. Sandra Brunelleschi

Dottorando: Dott. Pasquale Lista

Introduction

Metabolic syndrome (MetS) is a cluster of risk factors for atherosclerosis, including abdominal obesity, hypertension, insulin resistance, dyslipidemia with high triglycerides and low high-density lipoprotein cholesterol [1]. Affected patients have a significantly increased risk of developing cardiovascular disorders. This is probably due to a blood hypercoagulability as well as to endothelial cell activation [2]. Furthermore, several epidemiological studies, the Framingham in particular [3], have investigated into the evolution of cardiovascular disease (CVD) hypothesizing the presence of a gender difference in the pathogenetic and progression determinants detectable in men and women. For example, women were found to outlive men and to experience fewer atherosclerotic cardiovascular events, with an incidence lagging behind that in men by 10 to 20 years [4]. In the present pilot study we analyzed in detail several blood cell and blood plasma parameters in samples from patients with metabolic syndrome and subclinical atherosclerosis but without any sign of coronary artery disease.

Purpose

The main objective of this study is to determine new peripheral bioindicators gender associated of possible diagnostic value. Last year, some red blood cell parameters in samples from patients with metabolic syndrome and subclinical atherosclerosis, but without any sign of coronary artery disease, have been analyzed. In particular, three different “indicators” of red blood cell injury and aging have been evaluated: glycophorin A, CD47 and phosphatidylserine. Two of these determinants (CD47 and Phosphatidylserine externalization) appeared significantly modified and displayed gender differences. These results are in accord with several literature data [5] that suggest RBC as real time biomarkers of disease progression and pathogenetic determinants in cardiovascular diseases. RBCs can in fact contribute to atherosclerotic plaque formation [6] and can behave as pro-oxidants, thus contributing to the pathogenetic mechanisms of vascular diseases [7**].**

On the basis of these results, in the second year several plasmatic biomarkers have been taken into consideration: i) fibrinogen and C-reactive protein (CRP), that are considered classical biomarkers; and ii) antioxidant power (PAP) of blood plasma, oxidized form of LDL (ox-LDL), Annexin V, P-Selectin, and chemokine superfamily member MCP-1, that are considered new biomarkers.

Methods

Study population

The study population consisted of 60 ambulatory subjects with metabolic syndrome (30 men, aging 35-75 years, and 30 women, aging 47-74 years) and age-matched healthy donors (HD) (22 men and 18 women). All patients and HD were caucasian. Only post-menopausal women have been included in this study. All study subjects underwent a complete cardiovascular evaluation which has included: history and physical examination, heart rate, blood pressure, fasting serum glucose; fasting plasma lipids; Fibrinogen; CRP; comprehensive two-dimensional echocardiogram, carotid echo-color-Doppler and exercise ECG testing. Healthy donors were identified on the basis of the absence of CVD risk factors and a completely normal CVD screening.

MetS was diagnosed according to the amended National Cholesterol Education Program's Adult Treatment Panel III (ATP-III) guidelines in individuals meeting three or more of the criteria reported elsewhere [8].

Plasma samples

For plasma isolation, blood was centrifuged at 3000x g for 10 min at room temperature. Plasma was removed, aliquoted and frozen until analyses.

Biochemical analyses of the blood plasma

All biomarkers were measured in the plasma using commercially available immunoassay kits: antioxidant power (Total antioxidant Power, Oxford Biomedical Research, USA); antibodies to oxidized LDL (MED.DIA S.R.L., San Germano Vercellese, Italy); monocyte chemotactic protein 1 (Human MCP-1, Quantikine, R&D Systems, Germany); annexin V detection (ZYMUTEST Annexin V, HYPHEN BioMed, France). For all biomarkers the following Units have been used: PAP (mmol/L); ab-ox-LDL (U/L); MCP-1 (pg/ml); annexin V and P-Selectin (ng/ml).

Statistical analyses

Clinical data were analyzed with SPSS software v. 15.0 (SPSS Inc., Chicago Illinois). The continuous variables were calculated as the average value considering the standard deviation, while those categorical as percentages. The differences between HD and MetS patients were analyzed with the Student t test for independent samples or with Mann Whitney Test if variables did not show a normal distribution. The differences between categorical variables were analyzed with 2 test of Pearson. To study the gender difference between the two groups the analysis of variance analysis and post hoc with Bonferroni correction has been used. Differences were considered statistically significant at a p-value ≤ 0.05. Cytofluorimetric results were statistically analyzed by using the non-parametric Kolmogorov-Smirnov test using Cell Quest Software. Morphometric data (reported as

mean ± standard deviation, SD, from at least four separate experiments) were analyzed by using the Student t test. Only $p \leq 0.05$ was considered as significant.

Results

Evaluation of plasmatic proteins

Markers of MetS that do not display gender differences. As reported in **Figure 1** we found that the level of fibrinogen, a well known determinant in vascular diseases, significantly ($p<0.05$) increased in the plasma of MetS patients. Moreover, the level of CRP, a marker of inflammation recognized as a useful predictor of future cardiovascular events [9], was found significantly ($p<0.01$) increased in MetS patients (**Figure 2**). However, no significant gender difference was appreciable for both these parameters (**Figures 1, 2**). As concerns antibodies against ox-LDL, these are considered as a heterogeneous group of auto-antibodies produced following oxidative modification of LDL [10]. High levels of these proteins were found in patients having acute coronary syndrome where they positively correlated with CRP increase [11]. In our study we found that the levels of ab against ox-LDL increased significantly ($p<0.01$) in plasma from MetS patients with respect to healthy donors (**Figure 3**). However, no significant gender difference was detected in blood samples from male and female patients. Similarly, Annexin V calcium dependent protein significantly increased in plasma from MetS patients (both women and men) with respect to HD (**Figure 4).** Finally, results obtained with antioxidant power (PAP) of blood plasma clearly indicated that no significant difference was detectable in the blood plasma from MetS patients with respect to that from healthy donors and that no significant sex-based differences were observable (see **Table 1**).

Markers of MetS that display gender differences. Further analyses were performed on MCP-1, a monocyte chemotactic and activating factor. As shown in **Figure 5**, the levels of this protein increased significantly in plasma from patients with MetS. More interestingly, when the results obtained were analyzed taking into account patient sex, we found that only women with MetS displayed a significant increase of plasmatic MCP-1. Further studies have been conducted on circulating cell adhesion molecule P-Selectin. We found significantly increased values in patients with MetS. Furthermore, importantly, when results obtained from male and female patients were analyzed separately, significantly increased levels of P-Selectin were especially detected in plasma from men with MetS (**Figure 6**).

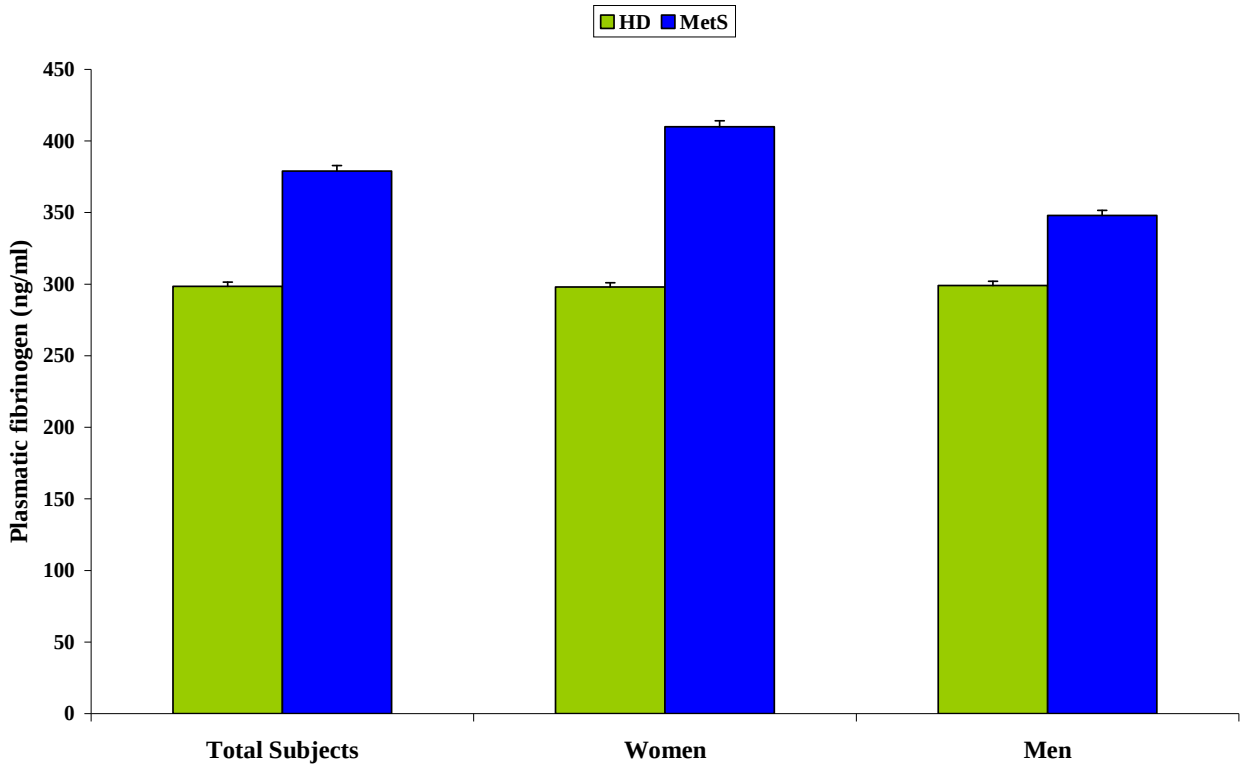

Figure 1. Histogram ± SD representing plasmatic levels of fibrinogen. With respect to HD, in MetS patients a significant ($p<0.05$) increase of this protein was detected. Note that no gender differences were instead observable. The study population consisted of 60 ambulatory subjects with metabolic syndrome (30 men and 30 women) and age-matched healthy donors (HD) (22 men and 18 women).

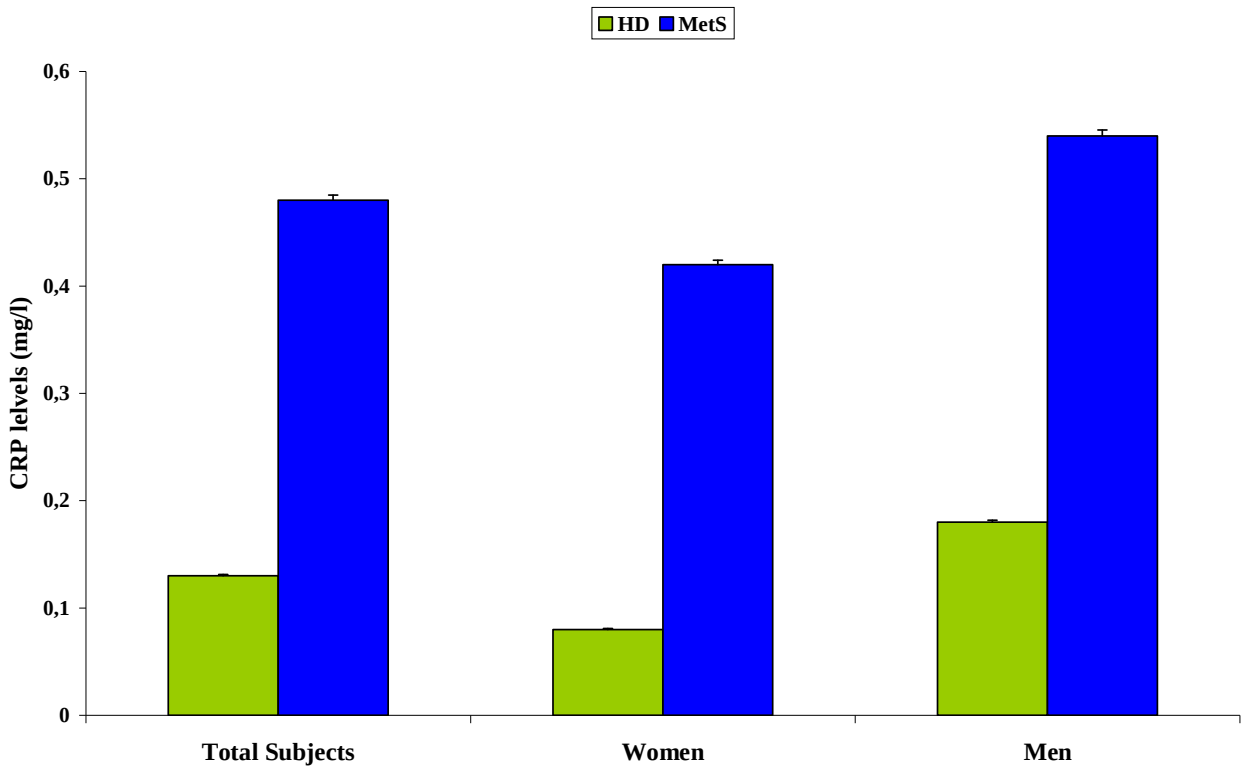

Figure 2. Histogram ± SD representing plasmatic levels of CRP. With respect to HD, in MetS patients a significant

(p<0.01) increase of this protein was detected. Note that no gender differences were instead observable. The study population consisted of 60 ambulatory subjects with metabolic syndrome (30 men and 30 women) and age-matched healthy donors (HD) (22 men and 18 women).

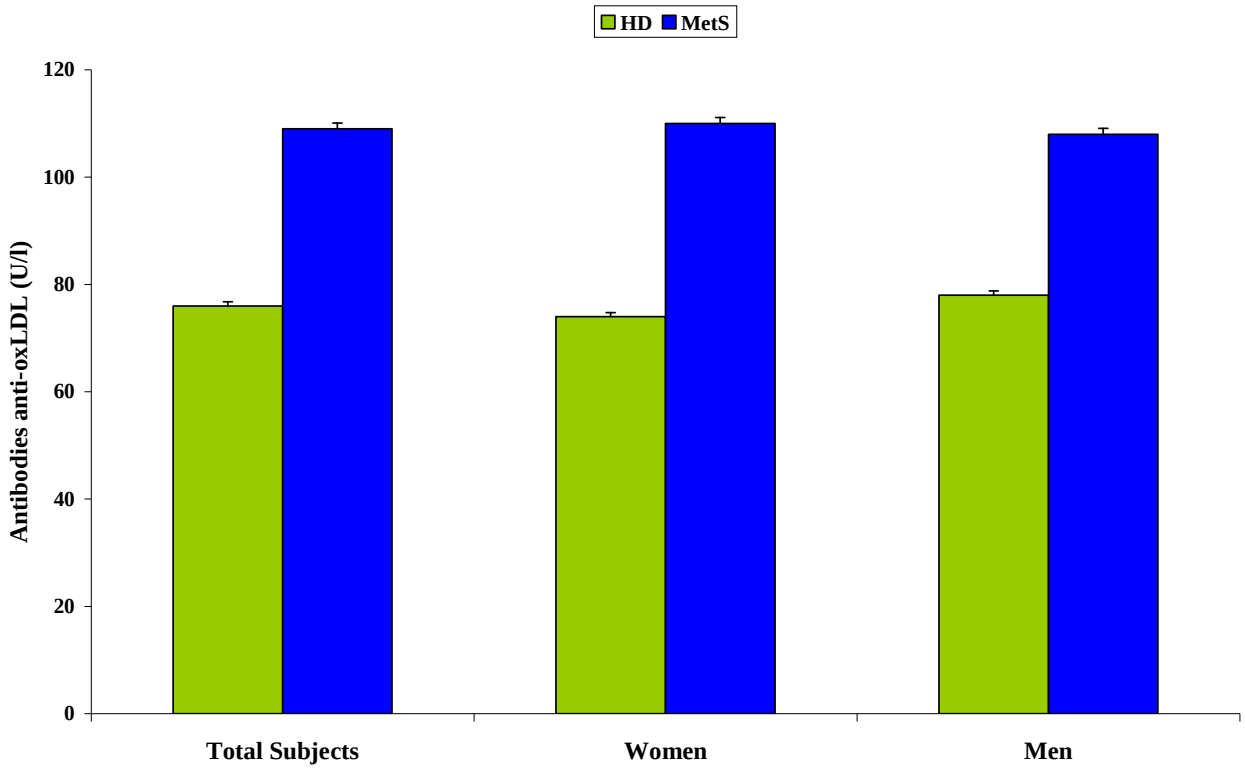

Figure 3. Histogram ± SD representing plasmatic levels of anti-oxLDL. With respect to HD, in MetS patients a significant (p<0.01) increase of these antibodies was detected. Note that no gender differences were instead observable. The study population consisted of 60 ambulatory subjects with metabolic syndrome (30 men and 30 women) and age-matched healthy donors (HD) (22 men and 18 women).

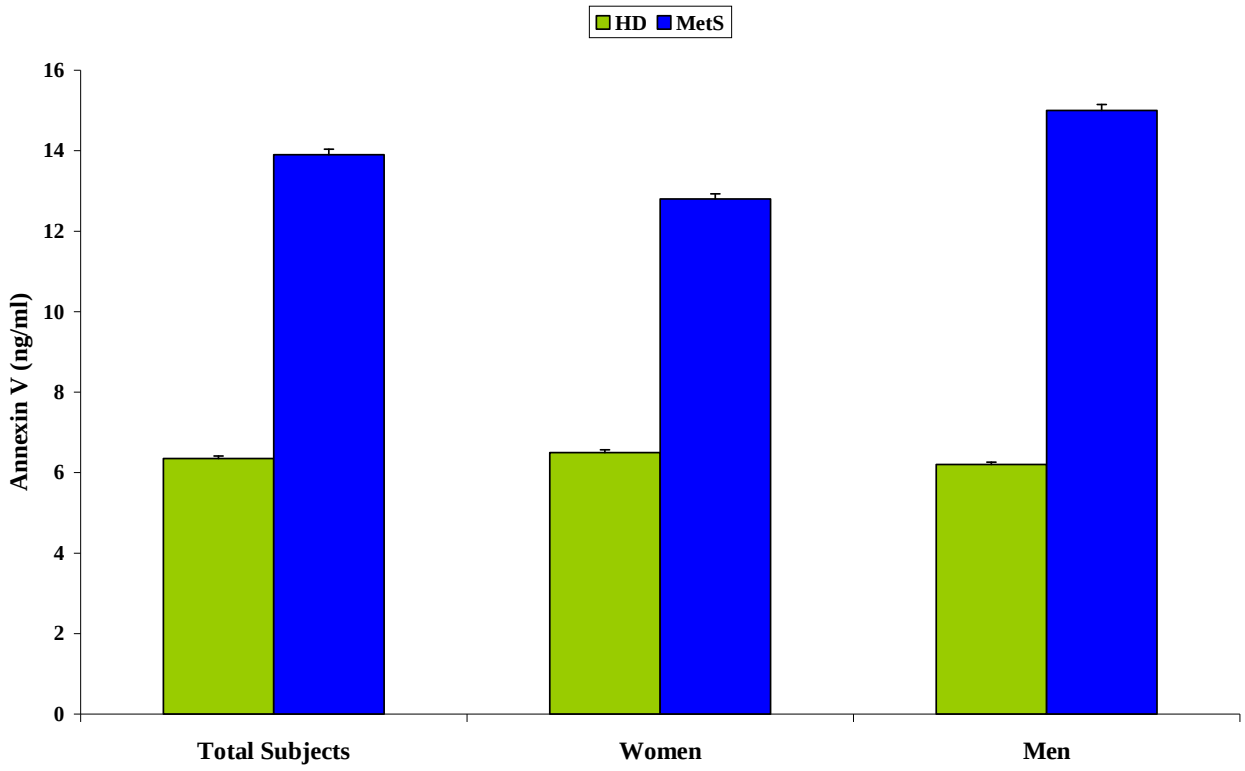

Figure 4. Histogram ± SD representing plasmatic levels of Annexin V. With respect to HD, in MetS patients a significant ($p<0.01$) increase of this protein was detected. Note that no gender differences were instead observable. The study population consisted of 60 ambulatory subjects with metabolic syndrome (30 men and 30 women) and age-matched healthy donors (HD) (22 men and 18 women).

	Males	**Females**
Healthy donors	919±175	770±198
MetS	1165±212	1156±233

Table 1. Antioxidant power in the blood plasma**.** The results are expressed in μmoles/L±SD. Standard values: 1069±145 μmoles/L. No significant differences were detected between men and women. The study population consisted of 60 ambulatory subjects with metabolic syndrome (30 men and 30 women) and age-matched healthy donors (HD) (22 men and 18 women).

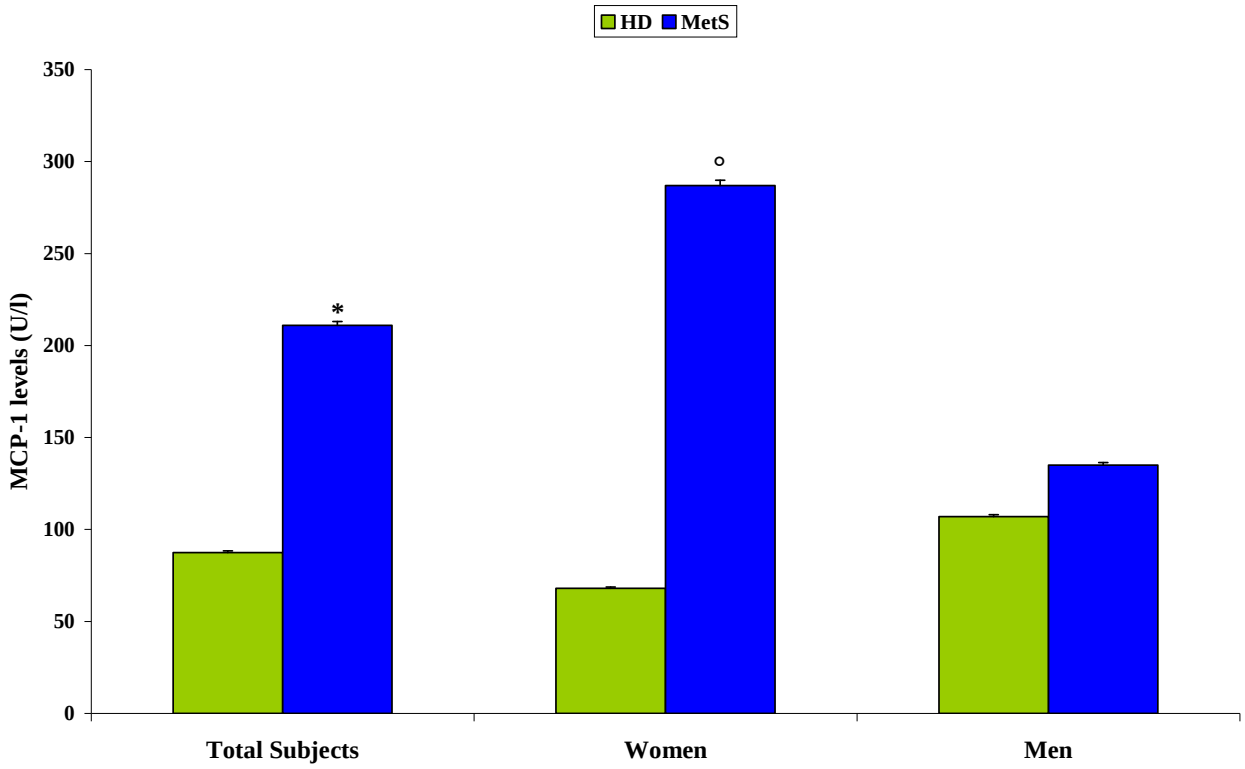

Figure 5. Histogram ±SD representing plasmatic levels of MCP-1 chemokine. The levels of MCP-1 increase significantly in plasma from MetS patients, with respect to HD. (*) $p<0.01$, patients with MetS *vs* HD. Moreover, a significant increase of MCP-1 was detected in women with MetS. (°) $p<0.01$, women with MetS *vs* men with MetS. The study population consisted of 60 ambulatory subjects with metabolic syndrome (30 men and 30 women) and age-matched healthy donors (HD) (22 men and 18 women).

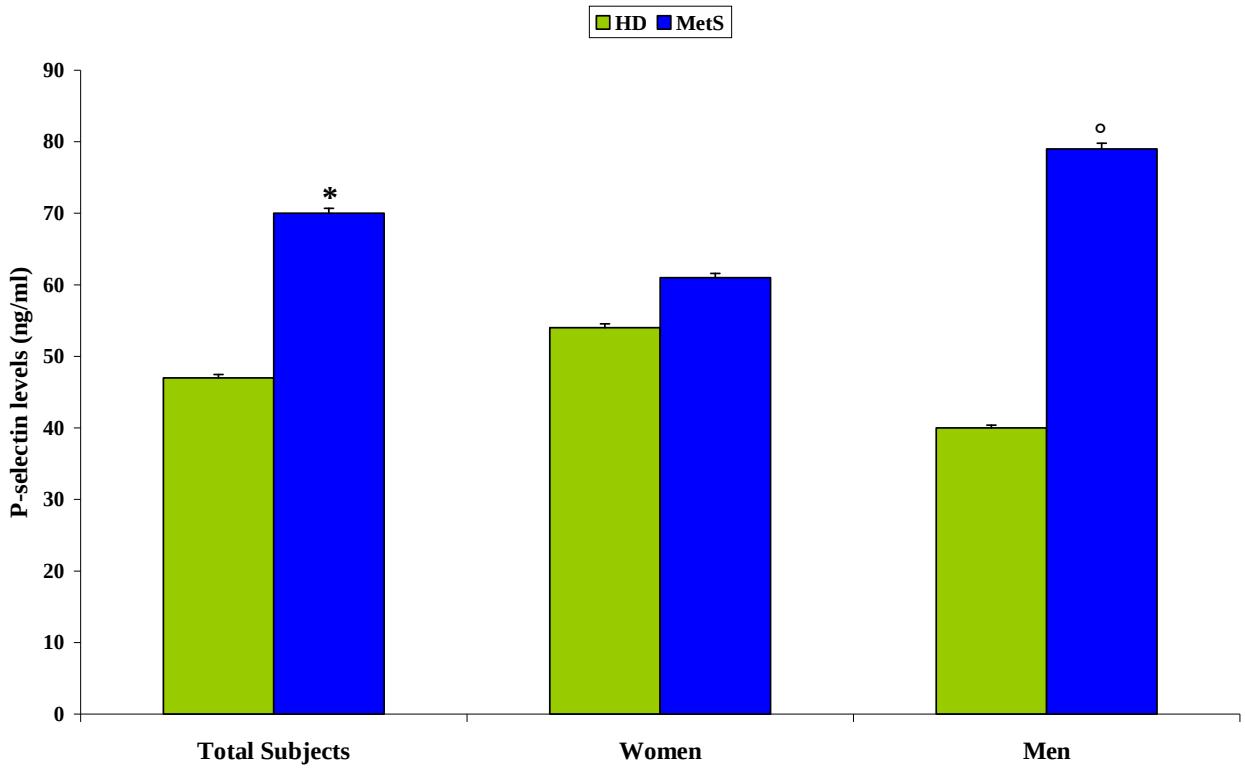

Figure 6. Histogram ±SD representing plasmatic levels of soluble P-Selectin. The levels of P-Selectin increase significantly in plasma from MetS patients, with respect to HD. (*) $p<0.01$, patients with MetS *vs* HD. Moreover, a significant increase of P-Selectin only in men with MetS. (°) $p<0.01$, men with MetS *vs* women with MetS. The study population consisted of 60 ambulatory subjects with metabolic syndrome (30 men and 30 women) and age-matched healthy donors (HD) (22 men and 18 women).

Discussion

In the present pilot study we analyzed in detail several blood plasma parameters in samples from patients with metabolic syndrome manifesting extra-cardiac atherosclerotic disease but without any clinical sign of symptomatic coronary artery disease. In particular, we investigated about possible gender differences detectable in this pre-clinical state. To this aim diverse blood determinants were evaluated in male and female patients. To be included in the study these patients should have at least 3 major criteria for MetS and a pathologically abnormal carotid intima-media thickness (IMT) in the absence of patent with coronary artery disease (CAD). Carotid IMT has been linked to many cardiovascular outcomes, including cerebral and coronary events and it has been proposed as an index of sublicnical atherosclerosis [12]. This selection allowed us to investigate some of the key determinants that could be associated with cardiovascular complications associated with metabolic syndrome. From a clinical point of view, despite a similar incidence of risk factors and intima-media thickness, males with MetS showed a significantly higher LV function and

structure involvement in the absence of patent CAD symptoms. Analytical cytology analyses allowed: i) the identification of some markers from blood plasma; ii) a gender specific alteration of some of these parameters.

Blood plasma determinants. There are a plethora of possible blood determinants or indicators that have been described or hypothesized so far as concerns the MetS. Among these are several clinical or biological parameters. However, no gender differences were detected as concerns these parameters. More interesting are the results obtained by the evaluation of MCP-1 chemokine and P-Selectin adhesion molecule. They appear as novel and of particular interest. The alteration of these molecules in the blood plasma appears in fact as associated with patient gender, being the first increased in female patients and the second in male patients. MCP-1 is a macrophage chemoattractant that has been associated with inflammation. Recently, a gender-association in atherothrombotic disease in the coronary arteries in post myocardial infarction patients has been hypothesized for MCP-1 [13]. As concerns soluble P-Selectin, this was found increased in cortical stroke as compared with lacunar infarction patients or controls since many years [14] but no data are so far available as concerns gender differences. It is however well established that soluble P-Selectin could represent a cardiovascular risk factor, e.g. in atherosclerotic disease [15]. We cannot rule out the possibility that these two soluble proteins, MCP-1 and P-Selectin, besides their putative use as gender-associated peripheral blood biomarkers, could also exert a pathogenetic role in vascular complications of MetS.

Conclusions

On the basis of the results obtained we hypothesize that some determinants could differ between males and females and that these may represent gender-associated risk factors. These findings also provide novel and useful hints in the research for gender-based real-time biondicators in the progression of metabolic syndrome towards coronary artery disease.

BIBLIOGRAFIA

[1] Grundy S.M., Cleeman J.I., Daniels S.R., et al. American Heart Association; National Heart, Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Circulation (2005) 112:e298.

[2] Ay C., Tengler T., Vormittag R., et al. Venous thromboembolism – a manifestation of the metabolic syndrome. Haematologica (2007) 92:374–80.

[3] Ingelsson E., Pencina M.J., Tofler G.H., et al. Multimarker approach to evaluate the incidence of the metabolic syndrome and longitudinal changes in metabolic risk factors: the Framingham Offspring Study. Circulation (2007) 28:984–92.

[4] Mølstad P. Coronary heart disease in women: less extensive disease and improved long-term survival compared to men. Scand. Cardiovasc. J. (2008) 10:1–7.

[5] Berliner S, Rogowski O, Aharonov S, et al. Erythrocyte adhesiveness/aggregation: a novel biomarker for the detection of low-grade internal inflammation in individuals with atherothrombotic risk factors and proven vascular disease. *Am Heart J.* 2005;149:260-267.

[6] Modun D, Music I, Vukovic J, et al. The increase in human plasma antioxidant capacity after red wine consumption is due to both plasma urate and wine polyphenols. *Atherosclerosis.* 2008;197:250-256.

[7] Minetti M, Agati L, Malorni W. The microenvironment can shift erythrocytes from a friendly to a harmful behavior**:** pathogenetic implications for vascular diseases. *Cardiovasc Res.* 2007;75:21-28.

[8] Grundy S.M., Cleeman J.I., Daniels S.R., et al. Diagnosis and management of the metabolic syndrome: An American Heart Association/National Heart, Lung, and Blood Institute scientific statement: Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive summary. Circulation. (2005) 112:e285–290.

[9] Ridker P.M., Jupiter Study Group. Rosuvastatin in the primary prevention of cardiovascular disease among patients with low levels of low-density lipoprotein cholesterol and elevated high-sensitivity C-reactive protein: rationale and design of the JUPITER trial. Circulation. (2003) 108:2292-2297.

[10] Piarulli F., Lapolla A., Sartore G., et al. Autoantibodies against oxidized LDLs and atherosclerosis in type 2 diabetes. Diabetes Care. (2005) 28:653-657.

[11] Soltesz P., Veres K., Laczik R., et al. Evaluation of antibodies to oxidized low-density lipoprotein and assessment of C-reactive protein in acute coronary syndrome and stable coronary artery disease. Thromb Haemost. (2007) 98:413-419.

[12] Lorenz MW, Markus HS, Bots ML, Rosvall M, Sitzer M. Prediction of Clinical Cardiovascular Events With Carotid Intima-MediaThickness: A Systematic Review and Meta-Analysis. *Circulation* 2007;115:459-67.

[13] Samnegård A, Hulthe J, Silveira A, Ericsson CG, Hamsten A, Eriksson P. Gender specific associations between matrix metalloproteinases and inflammatory markers in post myocardial infarction patients. *Atherosclerosis* 2009;202:550-6.

[14] Bath PM, Blann A, Smith N, Butterworth RJ. Von Willebrand factor, P-selectin and fibrinogen levels in patients with acute ischaemic and haemorrhagic stroke, and their relationship with stroke sub-type and functional outcome. *Platelets* 1998;9:155-9.

[15] Langer HF, Gawaz M. Platelet-vessel wall interactions in atherosclerotic disease. *Thromb Haemost* 2008;99:480-6.

PARTECIPAZIONI A CONVEGNI E SEMINARI

1. Convegno PREVENIRE LE COMPLICANZE DEL DIABETE: DALLA RICERCA DI BASE ALL'ASSISTENZA organizzato da ISTITUTO SUPERIORE DI SANITA'; Roma, 18 e 19 Febbraio 2010.
2. International Congress RARE DISEASES AND ORPHAN DRUGS organizzato da ISTITUTO SUPERIORE DI SANITA' (Centro Malattie Rare); Roma, 22- 25 Febbraio 2010.
3. Seminario: "Manteniamoci (i mitocondri) in forma! Una questione di vita o di morte" tenuto dal *Prof. Luca Scorrano* presso l'Università degli Studi di Roma "La Sapienza"; Roma, 4 Maggio 2010.
4. Seminario: "The Role of Aldosterone in Vascular Function and Disease: Lessons from Clinical Trials" and "The Power and Promise of Proteomics and Biomarker Discovery". Tenutosi presso l'IRCCS San Raffaele Pisana di Roma, il 12 Maggio 2010.
5. Convegno "Contributi delle Microscopie allo Sviluppo delle Nanotecnologie in Campo Biomedico: Nanodrug Delivery". Organizzato dall'Istituto Superiore di Sanità (ISS) in collaborazione con la Società Italiana di Scienze Microscopiche (SISM); tenutosi presso l'Istituto Superiore di Sanità di Roma, il 12 Maggio 2010.
6. Convegno "Resveratrolo e dintorni: prospettive terapeutiche future". Organizzato dal Dip. di Tecnologie e Salute – Istituto Superiore di Sanità di Roma, il 22 Giugno 2010.
7. Riunione operativa del Progetto Strategico "La medicina di genere come obiettivo strategico per la sanità pubblica: l'appropriatezza della cura per la tutela della salute della donna" – Istituto Superiore di Sanità di Roma, 28 Settembre 2010.

8. 52nd Annual Meeting of the Italian Cancer Society – *Lost in translation: brinding the gap between cancer research and effective therapies* – Roma, 4-7 Ottobre 2010.
9. Meeting Nazionale di Virologia – Istituto Superiore di Sanità di Roma, 22 Novembre 2010.
10. Convegno "Le Immunodeficienze: implicazioni diagnostico-cliniche, comunicativo-relazionali e gestione assistenziale"; Istituto Superiore di Sanità, Roma, 24 Novembre 2010.
11. Convegno *Sostanze Naturali, Farmaci e Alimenti: Azioni e interazioni*; Istituto Superiore di Sanità – Roma, 14 Dicembre 2010.
12. IV Seminario Nazionale Farmaci e Donne – *Salute e Medicina in una prospettiva di genere* – Istituto Superiore di Sanità, Roma, 20 Gennaio 2011.
13. Workshop "Postgenomics of Psychiatric Diseases: Imaging, Genes and Endogenus Retroviruses; Istituto Superiore di Sanità di Roma; 7 febbraio 2011.

PUBBLICAZIONI

1. Straface E, Gambardella L, Canali E, Metere A, Gabrielli N, Arcieri R, Mattatelli A, **Lista P**, Agati L, Malorni W. *P-Selectin as a new gender associated biomarker in patients with metabolic syndrome*. **Int J Cardiol.** 2010, 145(3):570-1.

2. Straface E, **Lista P**, Gambardella L, Franconi F, Malorni W. *Gender-specific features of plasmatic and circulating cell alterations as risk factors in cardiovascular disease.* **Fundam Clin Pharmacol.** 2010, 24(6):665-74.

3. **Lista P**, Straface E, Brunelleschi S, Franconi F, Malorni W. *On the role of autophagy in human diseases: a gender perspective.* **J Cell Mol Med**. 2011, 15(7):1443-57.