-Università del Piemonte Orientale-

"Amedeo Avogadro"

Identification of real-time biomarkers in Metabolic Syndrome with vascular complications

XXV Ciclo di Dottorato in Medicina Molecolare

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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 United States Food and Drug Admnistration (FDA) defines a biomarker as a characteristic that is “objectively measures and evaluated as an indicaror of normal biologic processes, pathogenic processes, or pharmacologic responses to therapeutic intervention”. Although biomarkers have been used in drug development and treatment of disease for long time, the identification of new predictive safety and efficacy biomarkers is expected to reduce the time and cost of druf development. Biomarkers are currently being developed to identify patients at risk for disease and to predict potential treatment responders, adverse event occurrences, and favorable clinical outcomes for many disease states. In fact, biomarkers have already established important applications in the selection of therapies in which the drug targets are also the biomarkers. As a result, tissue-based expression of the biomarker directs the molecularly targeted therapeutic course of treatment.

The main objective of this study is to determine new peripheral bioindicators gender associated of possible diagnostic value. During the first 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**]**.

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.

This last year, we focused our attention on P-Selectin and CD47 protein expression on platelets. P-Selectin is a transmembrane protein present in the alpha granules of platelets that, following activation, is rapidly translocated to the cell surface and then released in the blood flow [8]. It is also involved in platelet-platelet interactions, i.e. platelet aggregation, which is a major factor in arterial thrombosis [8].

CD47, or integrin-associated protein (IAP), is a 50-kDa glycoprotein expressed on all mammalian cells that has been initially described as a molecule physically associated with integrins and able to regulate their functions. CD47 has been described as physically associated with β1 (3) and β3 integrins, thrombospondin-1 (TSP-1)/CD47 interaction was demonstrated to activate the integrin $\alpha IIb\beta_3$, which resulted in platelet spreading on immobilized fibrinogen **[**9**]** and to activate the integrin α2β1, involved in the early activation of platelets on adhesion to collagen **[**10**]**. CD47 on human platelets in whole blood significantly contributes to platelet adhesion **[**11**]**.

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.

Isolation of platelets

Platelet-rich plasma was prepared by centrifugation of whole blood at 100x g for 15 min at room temperature. The platelet-rich plasma supernatant was carefully removed and was used to isolate platelets by stepwise centrifugation as previously described **[**12**]**.

Quantitative and qualitative analyses of platelet proteins

For P-Selectin detection, samples were fixed with 4% paraformaldehyde and stained with a specific monoclonal antibody IgG FITC-conjugated (Chemicon International, Inc. Temecula, CA, USA). For CD47 detection, samples were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, Milano, Italy) and stained with a spcific monoclonal antibody (Santa Cruz Biotechnology, San Diego, CA) followed by an anti-mouse FITC-conjugated (Sigma). Finally, all samples were analyzed with a Nikon Microphot fluorescence microscope or with intensified video microscopy (IVM) by a CCD camera (Carl Zeiss, Germany). Alternatively, platelets were also analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) for quantitative analyses.

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 circulating cells

Platelets. Several risk factors for vascular diseases, including diabetes and hypercholesterolemia, have been associated with platelet changes and activation **[**8**,**13**]**. Hence, in order to evaluate the possible implication of platelets as determinants of MetS, their activation and aggregation features have been considered. In particular, we focused our attention on P-Selectin and CD47 proteins expression. As shown in Figure 1A, quantitative analyses conducted by flow cytometry clearly indicated a decreased surface expression of P-Selectin in patients with MetS. More interestingly, a gender difference was also detected. In fact, the data obtained showed that: i) in healthy donors the P-Selectin molecule was significantly more expressed at the surface of platelets obtained from men with respect to those from women and ii) this expression significantly decreased in platelets from men with MetS. To note, no significant differences were detected in P-Selectin expression in platelets from women with MetS with respect to HD women. In Figure 1B two representative micrographs obtained by immunofluorescence microscopy were reported: a "representative" surface labeling of P-Selectin as detectable in control platelets (right panel) and in platelets taken from a male patient with MetS (left panel). As concerns CD47 expression, as shown in Figure 2A , its expression is increased significantly in platelets from patients with MetS. However, gender analysis indicated that this increase was apparently due to women. Considering the role of CD47 in cell adhesion, immunofluorescence analyses were also carried out. Representative micrographs are reported in Figure 2B where CD47 staining by immunofluorescence is shown (platelets from a female healthy donor, right panel, and from a female with MetS, left panel; note the increased fluorescence).

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 cell determinants. It is a matter of fact that circulating blood cells, including platelets and erythrocytes, could contribute to the pathogenesis of cardiovascular diseases **[**2,5,7,14**]**. Both cell types have also been considered as bioindicators in cardiovascular disease. In particular, changes in platelet aggregability in terms of homotypic (platelet-platelet) as well as heterotypic (platelet-RBC or platelet-endothelial cells) interaction have been considered **[**15**]**. Hence, the most promising finding derived from our experimental analyses concerns changes of P-Selectin that, fittingly with the results obtained from the blood plasma samples (see above), was found significantly increased (conceivably released by platelets and endothelial cells) but, also, displayed a gender difference. However, the most novel insights in this scenario seem to come from the analysis of RBCs. The shape maintenance as well as mechanical deformability and elasticity of RBCs (7μm) are essential pre-requisites for their circulation, specifically in small blood vessels (about 5μm). If the RBC is altered, its aggregability and adhesive properties change, thus contributing to vascular damage **[**5**]**. In our study we detected differences, in terms of cell adhesion and/or aggregation, in RBCs from MetS patients with respect to those of healthy donors. In particular, we observed that in both women and men with MetS, erythrocytes lost the capacity to pile and appeared to have an increased adhesiveness to a substrate. This is probably associated with the increased PS externalization, a well-known marker of RBC aging and death, that has been associated with increased cell adhesion properties. More interestingly, as concerns gender, we also found significant gender differences (morphological alterations, adhesion-associated molecules CD47 and PS). These results are in accord with several literature data that suggest RBC as real time biomarkers of disease progression and pathogenetic determinants of cardiovascular diseases. It should also be considered that the RBCs can contribute to atherosclerotic plaque formation and can behave as pro-oxidants thus contributing to the pathogenetic mechanisms of vascular diseases **[**7**]**.

Conclusions

Altogether these results are in line with other data indicating that cardiovascular risk factors could differ between sexes. We can hypothesize that: i) some determinants could differ between males and post menopausal females and that these may represent gender-associated risk factors, and ii) a

sort of “plasmatic memory” could be maintained in women. This “memory” is associated with peculiar and sex-associated bioindicators. Hence, at least from a “plasmatic point of view” women can become similar to men only in latest years of their lifespan (manuscript in preparation). Our data seem to add new insights in this scenario by demonstrating that: i) further plasmatic and cellular determinants/risk factors can significantly differ between the two sexes and ii) some risk factors, e.g. platelet CD47, can be evidenced only when a gender analysis was carried out. The results presented here thus represent the first "proof of concept" that gender impacts on MetS progression biomarkers. However, we cannot rule out the possibility that these determinants could also play a pathogenetic role. Hence, the so-called gender medicine could include, in a near future, the MetS, its diagnosis and monitoring.

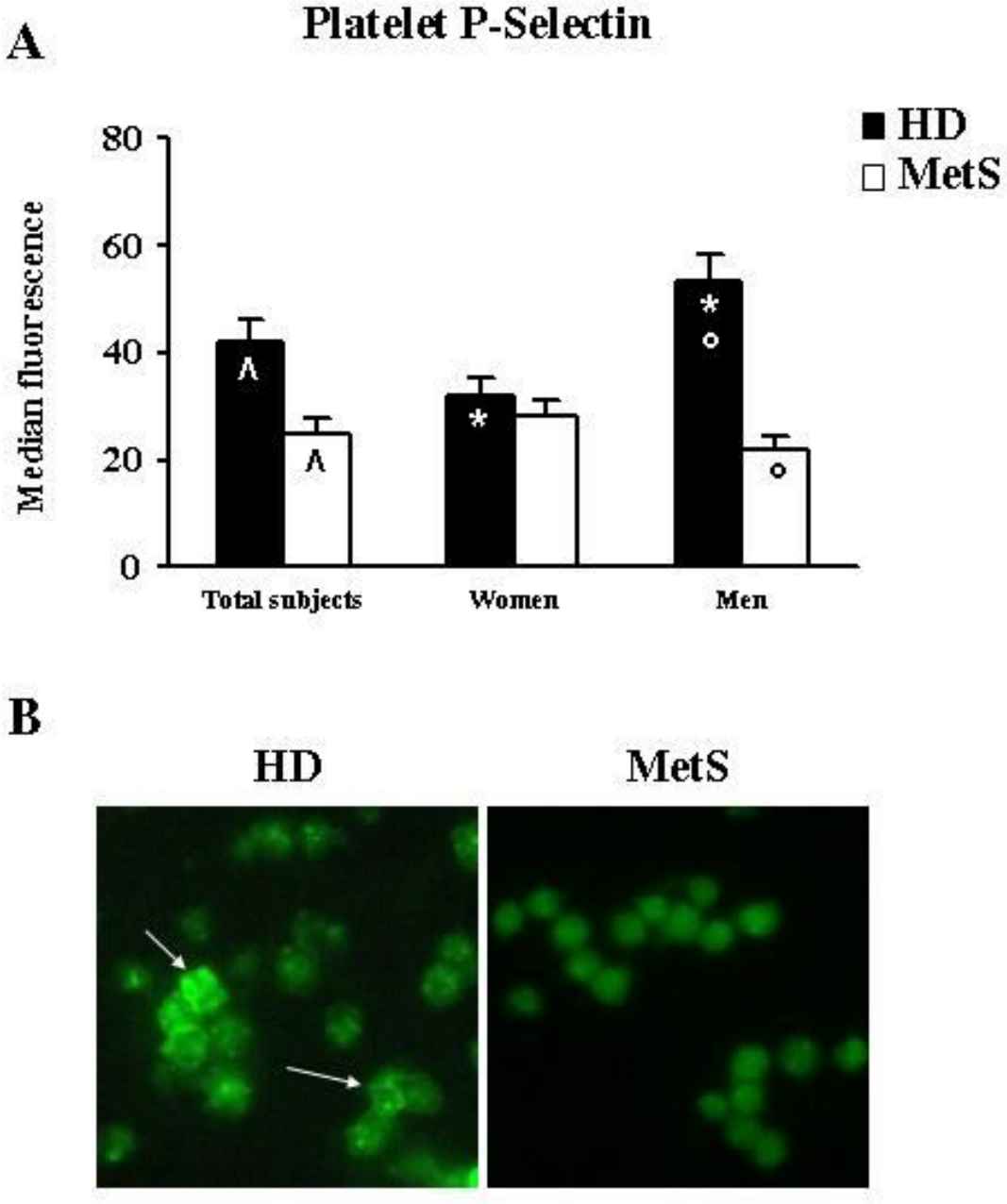
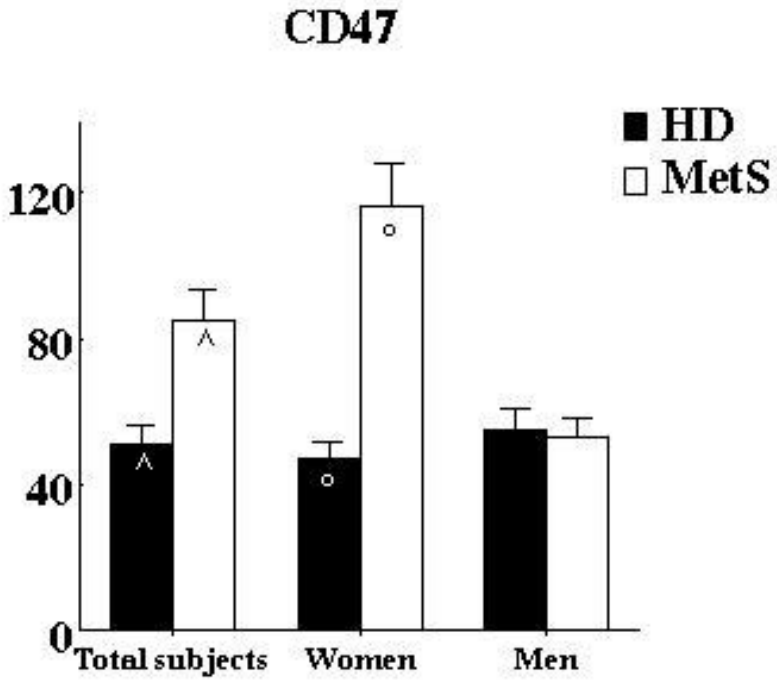

A
Platelet P-Selectin
HD
MetS
Median fluorescence
80
60
40
20
0
Total subjects
Women
Men
B
HD
MetS

Figure 1

A



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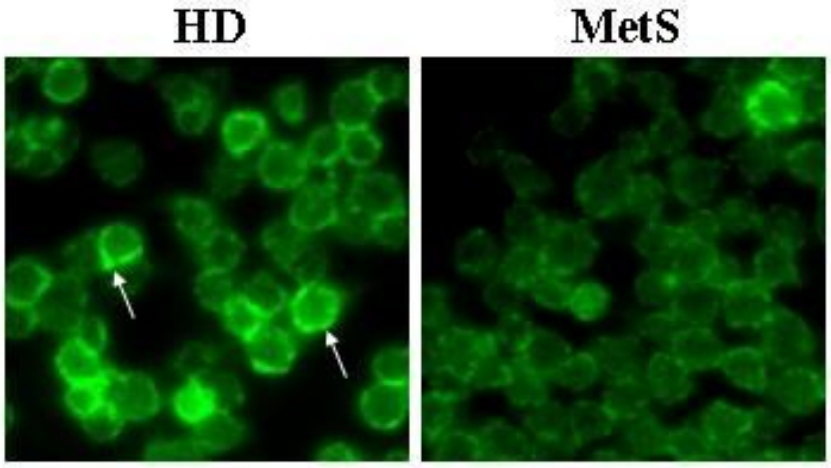

Figure 2

Figure legends

Figure 1. **A**) Cytofluorimetric analysis of P-Selectin expression in platelets. P-Selectin was more expressed at the surface of platelets in HD men with respect to those from HD women. This expression significantly decreased in platelets from men with MetS. No significant differences were detected in P-Selectin expression in platelets from women with MetS with respect to HD women. Numbers represent median values of fluorescence intensity histograms ± SD. **B**) Two representative micrographs obtained by immunofluorescence microscopy of P-Selectin distribution detected in platelets from a male HD (left panel) and with MetS (right panel). Note the presence of P-Selectin-positive clumps (arrows) in platelets from HD and the absence of positive clumps in platelets from one representative patient with MetS.
(^) $P < 0,01$, HD *vs* MetS; (*) $P < 0,01$, HD women *vs* HD men; (°) $P < 0,01$, HD men *vs* MetS men.

Figure 2. **A**) Cytofluorimetric analyses of CD47 expression in platelets. CD47 expression was increased in platelets from MetS patients with respect to those from HD. This increase was more evident in platelets from women with MetS with respect to platelets from women HD. Numbers represent median values of fluorescence intensity histograms ± SD. **B**) Representative micrographs showing CD47 staining by immunofluorescence. In particular, platelets from a female HD (right panel) and from a female with MetS (left panel) were shown.
(^) $P < 0,01$, HD *vs* MetS; (°) $P < 0,01$, HD women *vs* MetS women.

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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.

14. Conference NANODRUG DELIVERY FROM THE BENCH TO THE PATIENT; Istituto Superiore di Sanità, Roma, 10-13 Ottobre 2011.

15. III Convegno "Il trattamento con l'ormone somatotropo in Italia"; Istituto Superiore di Sanità, Roma, 30 Novembre 2011.

16. Conferenza "Mantenere i propri mitocondri in forma: una questione di vita o di morte"; Accademia Medica di Roma – Policlinico Umberto I, Roma, 1 Dicembre 2011.

17. XX Seminario Nazionale LA VALUTAZIONE DELL'USO E DELLA SICUREZZA DEI FARMACI: ESPERIENZE IN ITALIA. Istituto Superiore di Sanità, Roma, 12-13 Dicembre 2011.

18. Convegno "Complessità emergente in medicina: la centralità della persona nelle nuove frontiere etiche e scientifiche – un concorso di idee". Istituto Superiore di Sanità, Roma, 14 Dicembre 2011.

19. Convegno "Le nuove frontiere nell'immunoterapia dei tumori: realtà e prospettive". Istituto Superiore di Sanità, Roma, 19 Aprile 2012.

20. Convegno NANOMATERIALI E SALUTE. Istituto Superiore di Sanità, Roma, 10 e 11 Maggio 2012.

21. Corso di Formazione per i Lavoratori "*Il rischio in laboratorio: identificazione e prevenzione*". Istituto Superiore di Sanità, Roma, 26 Settembre 2012.