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Proteomic profiles in hyperandrogenic syndromes

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ABSTRACT. ***Background:*** **Polycystic ovary syndrome (PCOS) and congenital adrenal hyperplasia (CAH) represent the most common causes of hyperandrogenism. Although the etiopathogeneses of these syndromes are different, they share many clinical and biochemical signs, such as hirsutism, acne, and chronic anovulation. Experimental data have shown that peripheral T-lymphocytes function as molecular sensors, being able to record molecular signals either at staminal and mature cell levels, or hormones at systemic levels.** ***Methods:*** **Twenty PCOS women and 10 CAH with 21-hydroxylase deficiency, aged between 18-35 yr, were studied. T-cells purified from all patients and 20 healthy donors have been analyzed by 2-dimensional gel electrophoresis. Silver-stained proteomic map of each patient was compared with a control map obtained by pooling protein samples of the 20 healthy subjects.** ***Results:*** **Spots of interest were identified by peptide mass fingerprint. Computer analysis evidenced several peptidic spots significantly modulated in all patients examined. Some proteins were modulated in both syndromes, others only in PCOS or in CAH. These proteins are involved in many physiological processes as the functional state of immune system, the regulation of the cytoskeleton structure, the oxidative stress, the coagulation process, and the insulin resistance.** ***Conclusion:*** **Identification of the physiological function of these proteins could help to understand ethiopathogenetic mechanisms of hyperandrogenic syndromes and its complications.**

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INTRODUCTION

Hyperandrogenism is any clinical or laboratory evidence of androgen excess in women. The most common clinical presentation of hyperandrogenism in reproductive-aged women is hirsutism or acne with or without evidence of anovulation such as oligo- or amenorrhea or dysfunctional uterine bleeding. The main causes of hyperandrogenism in reproductive aged women are represented by the polycystic ovary syndrome (PCOS) (1, 2) and adrenal steroidogenic enzyme deficiencies (3). PCOS is an endocrine disorder in pre-menopausal women, with a 6-7% prevalence worldwide, and a heterogeneous presentation whose etiology is still indefinite. In the past, the diagnosis of PCOS was based only on hyperandrogenism and chronic anovulations [National Institutes of Health (NIH) criteria]. In 2003, in a European Society for Human Reproduction's (ESHRE) and American Society of Reproductive Medicine's (ASRM) joint meeting, new criteria for the diagnosis of PCOS have been developed, establishing that the diagnosis of PCOS can be made in patients who have at least 2 of the following features: hyperandrogenism, chronic anovulation, and polycystic ovaries evidenced by ultrasound method.

The 21-hydroxylase deficiency, transmitted as recessive autosomic character, represents the most frequent enzymatic defect among the congenital adrenal hyperplasia (CAH), characterized by androgens accumulation (4). The clinical symptoms of the non-classical form present a large phenotypic variability, with signs and symptoms typical for the PCOS too. Most of non-treated patients with CAH develop hirsutism after puberty; in both groups, menstrual disorders and chronic anovulation with typical signs of the PCOS are observed (5).

Various *in vitro* and *in vivo* studies have demonstrated a close relationship between hyperandrogenism and insulin resistance, suggesting that, independently on the obesity state, the hyperandrogenic women can be at risk of developing hyperinsulinemia (2). Different genetic factors could be involved in the hyperandrogenism etiopathogenesis, even if the results are so far disappointing (6). Although previous studies have showed a close link between some steroidogenic genes and androgen protein levels in PCOS and CAH (7), nevertheless the molecular mechanisms underlying these phenomenons are little known. The analysis of proteomic profiles in PCOS and CAH deficit-affected patients allows to identify common genes, potentially modulated by the androgens excess and putative candidate genes for the pathogenesis of the complications of hyperandrogenic disease.

In this study, we have analyzed the patterns of expression of patients affected by hyperandrogenic syndromes (PCOS and CAH). To this purpose, we have utilized lymphocytes from peripheral blood of 30 hyperandrogenic patients. Experimental data have shown that T-lymphocytes could represent molecular sensors both at staminal and at mature level cell, able to record different molecular signals, as well as hormones, at systemic level (8). The peripheral lymphocyte utilization permits the comparative analysis of dif-

*SM and AS gave equal contribution to this work.

Key-words: Congenital adrenal hyperplasia, polycystic ovary syndrome, proteomics, T-lymphocytes.

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ferent pathologies through an innovative and less invasive methodological approach.

MATERIALS AND METHODS

Patients

Twenty PCOS women and 10 CAH with 21-hydroxylase deficiency, aged 18-35 yr, were studied. Informed consent was obtained by all patients, the Ethics Committee approved the study protocol and all the procedures were in accordance with the Helsinki Declaration of 1975, as revised in 2000. The patients were enrolled at the our Endocrinology Unit of the Sant'Andrea Hospital. Diagnosis of PCOS was based on presence of at least 2 of the following features: clinical and/or biochemical signs of hyperandrogenism, oligo- or anovulation, and polycystic morphology of the ovary, after exclusion of other etiologies (Cushing's syndrome, late-onset 21-hydroxylase deficiency, thyroid dysfunction, hyperprolactinemia or androgen secreting tumors), according to recommendations, both Rotterdam PCOS Consensus Workshop Group (The Rotterdam ESHRE 7ASRM-Sponsored PCOS Consensus Workshop Group 2004) and NIH criteria with a clinical and/or biochemical signs of hyperandrogenism in order to stress the androgens role in the lymphocyte pattern modifications.

Twenty healthy (mean age 27±7), non-hirsute women, with regular ovulatory menses every 26-32 days, normal weight [body mass index (BMI) 22±3], no smoking, no antiandrogenic therapy, no pregnancy, no assumption of oral contraceptive, no acne, no alopecia, and serum androgens in the normal range, were studied as controls (Table 1).

All recruited subjects underwent a complete medical assessment, including family and personal medical history, physical examination for body weight, height, waist/hip ratio, blood pressure, hirsutism score, and standard blood and urine analyses. Blood pressure was measured by a mercury sphygmomanometer with the subject in the sitting position, after at least 5-min rest. Hirsutism score was graded by the Ferriman and Gallwey method, as modified by Hatch et al. (9).

Hormone assay

Basal endocrine profile, carried out in the early follicular phase or after at least 3 months of amenorrhea, including assays of serum gonadotropins (FSH, LH), 17β-estradiol (E2), testosterone (T), PRL, SHBG, DHEAS, and 17α-hydroxyprogesterone (17OHPG), androstenedione (Δ^4A), cortisol, oral glucose tolerance test (OGTT) for glucose and insulin and free androgen index (FAI) was calculated (10). In non-amenorrheic women, ovulatory function was assessed, in at least 2 cycles, by progesterone assay in the luteal phase. In subjects with baseline serum 17OHPG>6 nmol/l, ACTH stimulation was also performed, to rule out a late-onset 21-hydroxylase congenital adrenal deficiency (11).

The diagnosis of CAH 21-hydroxylase was performed by adrenal steroid assay, in particular 17OHPG (higher 2 ng/ml) and ACTH dynamic test (250 μg iv synthetic ACTH 1-24 "tetracosactid") and 17OHPG dosage after 30 and 60 min to injection. The CAH suspected subjects were submitted to DNA extraction by peripheral blood for genotype characterization (data not shown). The new "Single Nucleotide Primer Extension or Minisequencing" (12) method for identification of point mutation in the human genoma was utilized.

Table 1 - *Clinical features of the normal control subjects and hyperandrogenic patients. The data were presented as means±SD.*

	Normal control subjects	PCOS patients	CAH patients	*p*
No.	20	20	10	
Age (yr)	27±7	23±8	29±8	
Body mass index (kg/m^2)	22±3	29±5	27±3	<0.05
Regular menstrual rate	Yes	No	No	<0.05
Oligomhenorrea (%)	-	60%	100%	<0.05
Amhenorrea (%)	-	40%	0%	<0.05
Acne	-	60%	40%	<0.05
Hirsutism score (mean)	-	11	12	<0.05

PCOS: polycystic ovary syndrome; CAH: congenital adrenal hyperplasia.

T-cell isolation

About 25 ml of EDTA-anti coagulated peripheral blood were collected and immediately processed for purification of peripheral blood mononuclear cells (PBMC) by density gradient centrifugation over Ficoll-Hypaque (Nycomed Pharma, Oslo, Norway), according to the manufacturer's protocol. T-cells were then isolated by negative selection (mean purity 95%) using a magnetic beads system (Pan T Cell Isolation Kit II, human, Miltenyi Biotec, Auburn, CA), according to manufacturer's instructions. Briefly, non-T-cells are indirectly labeled with a cocktail of biotin-conjugated monoclonal antibodies, as primary labeling reagent, and anti-biotin monoclonal antibodies conjugated to magnetic micro beads, as secondary labeling reagent. The magnetically labeled non-T-cells are depleted by retaining them on a magnetizable separation column which is placed in a strong permanent magnet, while the unlabeled T-cells pass through the column. The PBMC and isolated T-cells samples were counted on an Advia Hematology Analyzer (Bayer Diagnostics).

Protein preparation for mass spectrometry

Purified T-cells were lysed in 0.1% sodium dodecyl sulphate (SDS)/2.3% dithioerythrol (DTE). Proteins were then precipitated with 80% (v/v) cold acetone followed by centrifugation (20,000 g for 10 min at 4 C). Pellets were dissolved in the rehydration solution (8 M urea/4% CHAPS) and protein content was determined by the Bradford assay. To minimize the experimental bias introduced by inter-individual variability of protein expression due to biological and environmental factors, equal amounts of protein derived from the healthy subjects were mixed to make a reference control pool.

Two-dimensional gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a subsequent combination of two electrophoresis. In the first dimension (isoelectrofocusing), separation is based on the isoelectric point (IP) of proteins. Further separation of proteins with similar IP is achieved by a second electrophoresis (orthogonal to the first) based on molecular weight (SDS-PAGE).

Isoelectric focusing (IEF) was carried out on an Ettan IPG-Phor system (Amersham Biosciences, Uppsala, Sweden), at 16 C and under a current limit of 50 μA/strip. Sixty micrograms of proteins in a final volume of 350 μl of lysis buffer, containing 65 mM DTE, 0.5% (v/v) ampholine pH 3-10 NL and a trace of bromophenol blue, were loaded onto 18 cm pH 3-10 NL Immobiline DryStrip (IPG strip, Amersham Biosciences, Uppsala, Sweden). The strip rehydration step was performed at 16 C at a constant voltage of 30 V for 4 h and additional 50 V for 5 h. Damp electrode pads

Table 2 - *Hormonal profile of the normal control subjects and hyperandrogenic patients. The data were presented as means±SD.*

	Normal control subjects	PCOS patients	CAH patients	*p*
No.	20	20	10	
FSH (mIU/ml)	6.3±2.3	5.5±3.2	5.0±3.9	
LH (mIU/ml)	4.3±3.1	11.6±4.8	8.2±6.9	<0.05
E2 (pg/ml)	35±10.3	16.1±8.1	29±22.7	<0.05
T (ng/ml)	0.4±0.1	1.0±0.4	0.9±0.2	<0.05
PRL (ng/dl)	7.2±1.5	10.2±7.3	10.3±9.4	
DHEAS (ng/ml)	3.1±1.1	4.0±2.1	7.4±6.3	
17OHPG (ng/ml) <0.05*	1.0±0.8	1.6±1.0	6.3±4.5	
Δ^4A (ng/ml)	1.5±0.7	3.3±2.2	4.1±3.9	<0.05
Cortisol (nmol/l)	222±89	298±90	250.5±76	
2-h OGTT glucose (mg/dl)	98±37.3	111±32	107±28	
2-h OGTT insulin (μU/ml)	8.3±2.5	22.7±13.7	15.8±11	<0.05
Free androgen index	3.5	7	7.1	<0.05

*p-value attributable only to congenital adrenal hyperplasia (CAH) patients. PCOS: polycystic ovary syndrome; E2: 17β-estradiol; T: testosterone; 17OHPG: 17α-hydroxyprogesterone; Δ^4A: androstenedione; OGTT: oral glucose tolerance test.

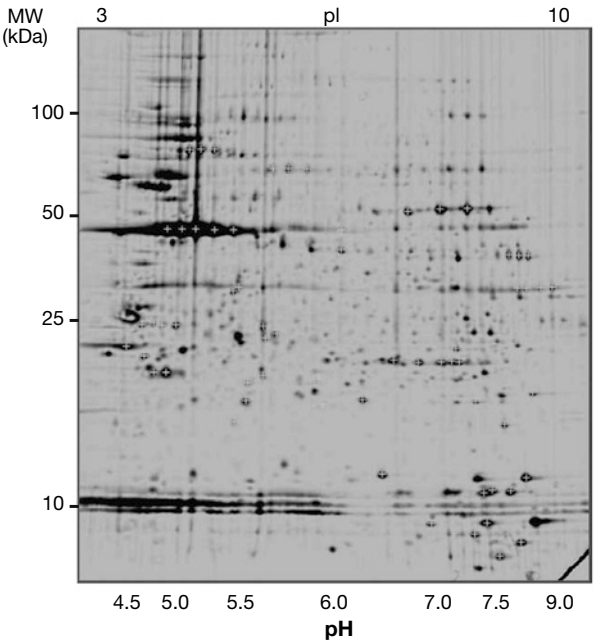

Fig. 1 - *Two-dimensional map of peripheral T-lymphocyte obtained by healthy subjects. Spots marked by crosses correspond to proteins identified.*

were positioned under the re-hydrated strip over the electrodes. The IEF step was performed using the following parameters: 400 V, 2 h; 800 V, 1 h; 1200 V, 2 h; 3000 V, 3 h; 8000 V, 6-8 h, until the total voltage reached 70 kVh. Immediately after the IEF run, IPG strips were equilibrated for 12 min in 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris-HCl, pH 6.8, 2% (w/v) DTE, and for 5 min in a similar solution in which 2% DTE was replaced with 2.5% (w/v) iodoacetamide. The second dimension electrophoresis was run on 9-16% linear gradient polyacrylamide gels (18 cm × 20 cm × 1.5 mm) at 40 mA/gel constant current at

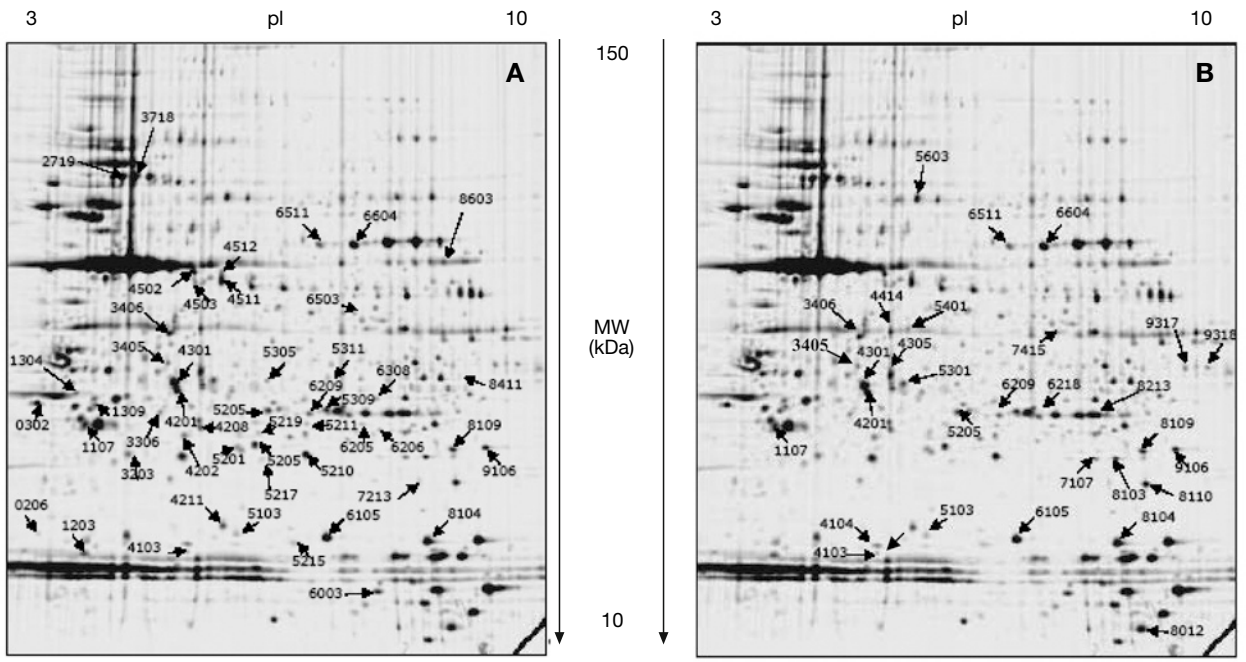

Fig. 2 - *Two-dimensional maps of peripheral T-lymphocyte. Spots marked by arrows and numbers correspond to differentially expressed proteins in congenital adrenal hyperplasia (A) and in polycystic ovary syndrome (B) patients. Proteins identified by peptide mass fingerprinting are listed in Table 3.*

Table 3 - *Protein spots isolated from peripheral blood lymphocytes obtained from normal subjects and polycystic ovary sundrome (PCOS) and congenital adrenal hyperplasia (CAH) patients and identified by mass spectrometry.*

Spot no.	Proteins PCOS and CAH	Accession no.	Theoretic al pI/MW	No. of matched peptides	Sequenze coverage	Mascot Score*	t-test *p* PCOS	t-test *p* CAH
1107	Rho GDP-Dissociation Inhibitor 1	P52565	5.02/23.3	9	29%	81	1.45×10^{-6}	1.56×10^{-4}
3405	F-actin capping protein alpha-1 subunit	P52907	5.45/33.1	7	25%	78	2.19×10^{-4}	1.65×10^{-2}
6105	Cofilin 1	P23528	8.22/18.7	7	39%	90	8.3×10^{-4}	3×10^{-4}
6604	Alpha-enolase	P06733	7.01/47.5	11	37%	111	1.2×10^{-4}	3.19×10^{-3}
9106	Peroxiredoxin-1	Q06830	9.27/22.1	6	43%	98	1.3×10^{-2}	3.49×10^{-2}
	Protein PCOS							
4414	Cathepsin D	P07339	6.10/43	8	20%	77	5×10^{-3}	
5301	3-hydroxyisobutyrate dehydrogenase mithocondrial	P31937	6.2/30.0	7	34%	98	7.79×10^{-8}	
5603	Protein disulfideisomerase A3	P30101	5.98/57.2	9	24%	84	6.9×10^{-3}	
8110	Raf kinase inhibitor protein (RKIP)	P30086	8.8/21.2	7	49%	103	8×10^{-5}	
8012	Platelet basic protein (precursor)	P02775	8.6/10.3	8	35%	107	6.38×10^{-3}	
8103	Superoxide dismutase [Mn] mitochondrial (precursor)	P04179	8.35/21.8	8	38%	104	1.07×10^{-3}	
	Protein CAH							
4202	Glutatione Stransferase P	P09211	5.43/23.6	6	53%	77		1.02×10^{-4}
4208	Proteasome subunit β type 4	P28070	5.72/29.3	8	48%	103		3.2×10^{-2}
5210	Peroxiredoxin-3	P30048	7.67/21.0	8	44%	114		1.6×10^{-2}
5219	Growth factor receptor-bound protein 2	P29354	6.2/29.5	7	29%	83		4.2×10^{-3}
5217	CCG1-interacting factor B	Q96IU4	5.94/20.6	8	56%	105		1.6×10^{-2}
5215	Prefoldin subunit 2	Q9UHV9	6.78/17.9	8	37%	97		3.7×10^{-3}

*Mascot score represents the probability that the observed match is a random event. Protein scores greater than 76 are significant ($p<0.05$).

10 C for approximately 5 h until the dye front reached the bottom of the gel. All samples were run in technical triplicate. Gels were silver stained as described by Schevchenko (13).

Gel imaging and analysis

Gels were acquired on a BioRad GS-800 Calibrated Imaging Densitometer (Bio-Rad, Veenendaal, the Netherlands) and image analysis was performed using the Bio-Rad PDQuest software, version 7.1.0. Spot volume was normalized to the total density in valid spots. The average spot quantities derived from each replicate group (≤5%) was used to perform differential analysis. Protein spots which were at least 2-fold up- or down-regulated and with a t-test *p*-value <0.05 were selected for identification by Peptide Mass Fingerprinting (PMF).

Matrix-assisted laser ionization time-of-flight mass spectrometry analysis

Protein spots were manually excised from the gel and de-stained with a 7.5 mM potassium ferricyanide/25 mM sodium thiosulfate solution. After extensive washing in H_2O, spots were washed 20 min in 200 mM NH_4HCO_3 and dehydrated with 100% acetonitrile. The gel pieces were incubated at room temperature in 20 µl of 40 mM NH_4HCO_3/10% acetonitrile containing 25 ng/µl trypsin (Trypsin Gold, mass spectrometry grade, Promega). After 1 h, 50 µl of 40 mM NH_4HCO_3/10% acetonitrile were added and digestion proceeded overnight at 37 C. The generated peptides were extracted with 50% acetonitrile/5% trifluoroacetic acid (TFA) (2 steps, 20 min at room temperature each), dried by vacuum centrifugation, suspended in 0.1% TFA, passed through micro ZipTip C18 pipette tips (Milllipore, Bedford, MA, U.S.A.) and directly eluted with the MS matrix solution (10 mg/ml cyano-4-hydroxycinnamic acid in 50% acetonitrile/5% TFA). Mass spectra of the tryptic peptides were obtained using a Voyager-DE matrix assisted laser ionization time-of-flight (MALDI-ToF) mass spectrometer (Applied Biosystems). Peptide mass fingerprinting database searching was performed using MASCOT searching

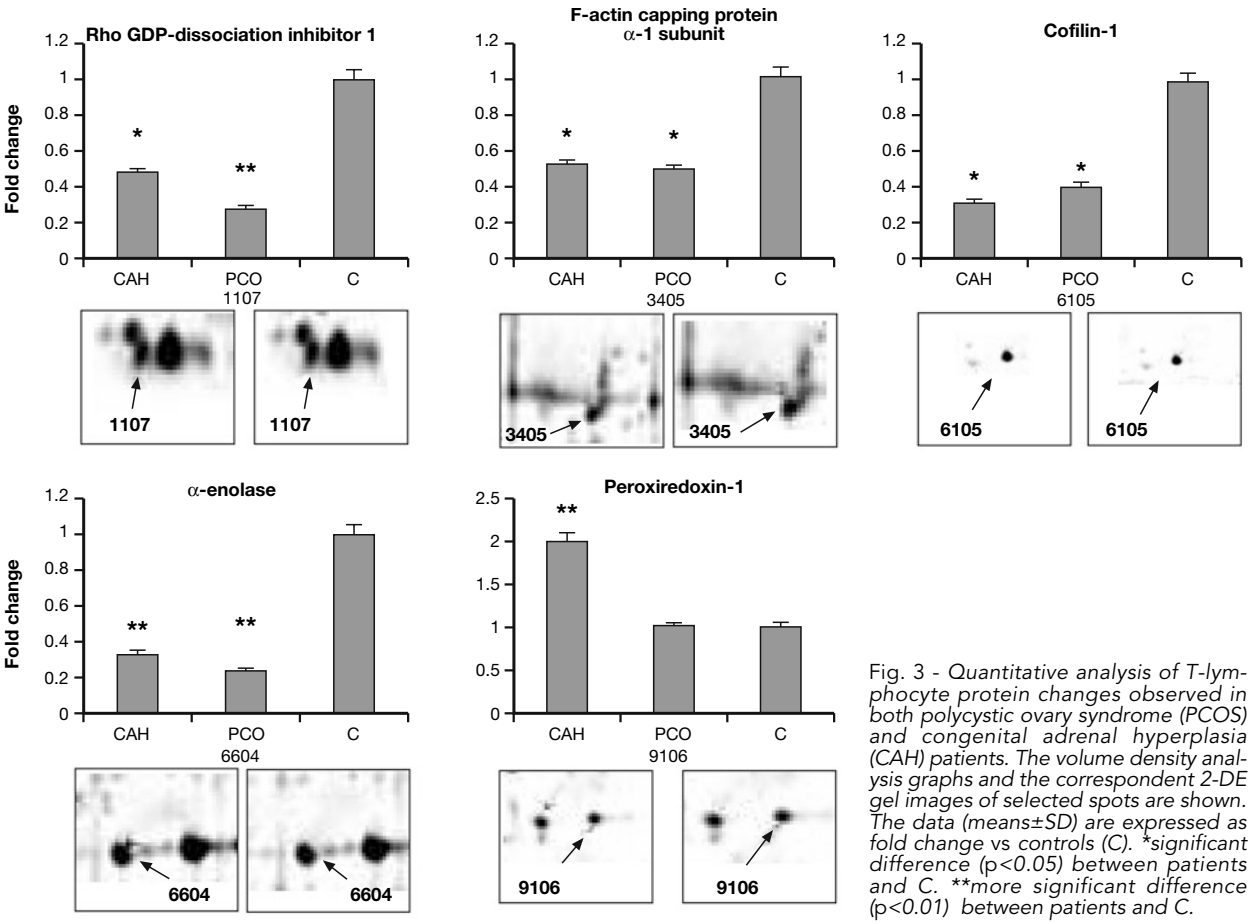

Fig. 3 - *Quantitative analysis of T-lymphocyte protein changes observed in both polycystic ovary syndrome (PCOS) and congenital adrenal hyperplasia (CAH) patients. The volume density analysis graphs and the correspondent 2-DE gel images of selected spots are shown. The data (means±SD) are expressed as fold change vs controls (C). *significant difference (p<0.05) between patients and C. **more significant difference (p<0.01) between patients and C.*

engine (http://www.matrixscience.com) in the NCBInr/Swiss-Prot databases. Parameters were set to allow one missed cleavage per peptide, a mass tolerance of 0.5 Da and considering carbamido-methylation of cysteines as a fixed modification and oxidation of methionines as a variable modification. The criteria used to accept identification included the extent of sequence coverage, number of matched peptides and probabilistic score as detailed in Table 2.

Statistical analysis

The data concerning clinical features and hormonal profiles were presented as means±SD. A comparison of the individual treatment was conducted by using one-way analysis of variance, followed by Tukey *post hoc* analyses. A *p*-value <0.05 was considered significant.

RESULTS

Protein spots differentially expressed in both PCO and CAH T-cells

Analysis of proteome profiles has been performed by comparison of maps obtained by two-dimensional electrophoresis. In this method, a semi-quantitative measure of protein level is obtained by acquisition of gel image by a densitometer followed by computer-assisted analysis of spot volume. Since 3 replicates gels are run for each samples, an average quantity for each spot is calculated. This value is compared among different classes of samples (control, PCOS, CAH) and a cut-off of 2-fold-change is used to detect differentially expressed proteins. SD of average quantities is also considered for selection of variant spots.

Proteomic analysis was performed on T-lymphocytes coming from 20 female healthy subjects, either singularly or on a pool of them with the aim of lowering the variability level. This analysis permitted to obtain 50 protein spots detectable. We have produced a reference map of female healthy subjects (Fig. 1), also available on http://w3.uniroma1.it/biocmed2/dima/.

The obtained control T-lymphocytes map was matched with the maps derived by T cell extracts of PCOS and CAH patients (Fig. 2A, B). This comparison led to the evidence of several spots differentially expressed in both diseases. In Table 3 are listed 17 spots unambiguously identified by mass spectrometry analysis, whereas the other spots are still unknown. Five proteins were modulated in both syndromes, 6 only in PCOS and 6 only in CAH (Table 3). Among the common proteins, 4 showed a down-expression in both syndromes: the cofilin 1, F-actin capping protein α-1 subunit, and Rho GDP dissociation inhibitor 1. These are involved in the functional state of the immune

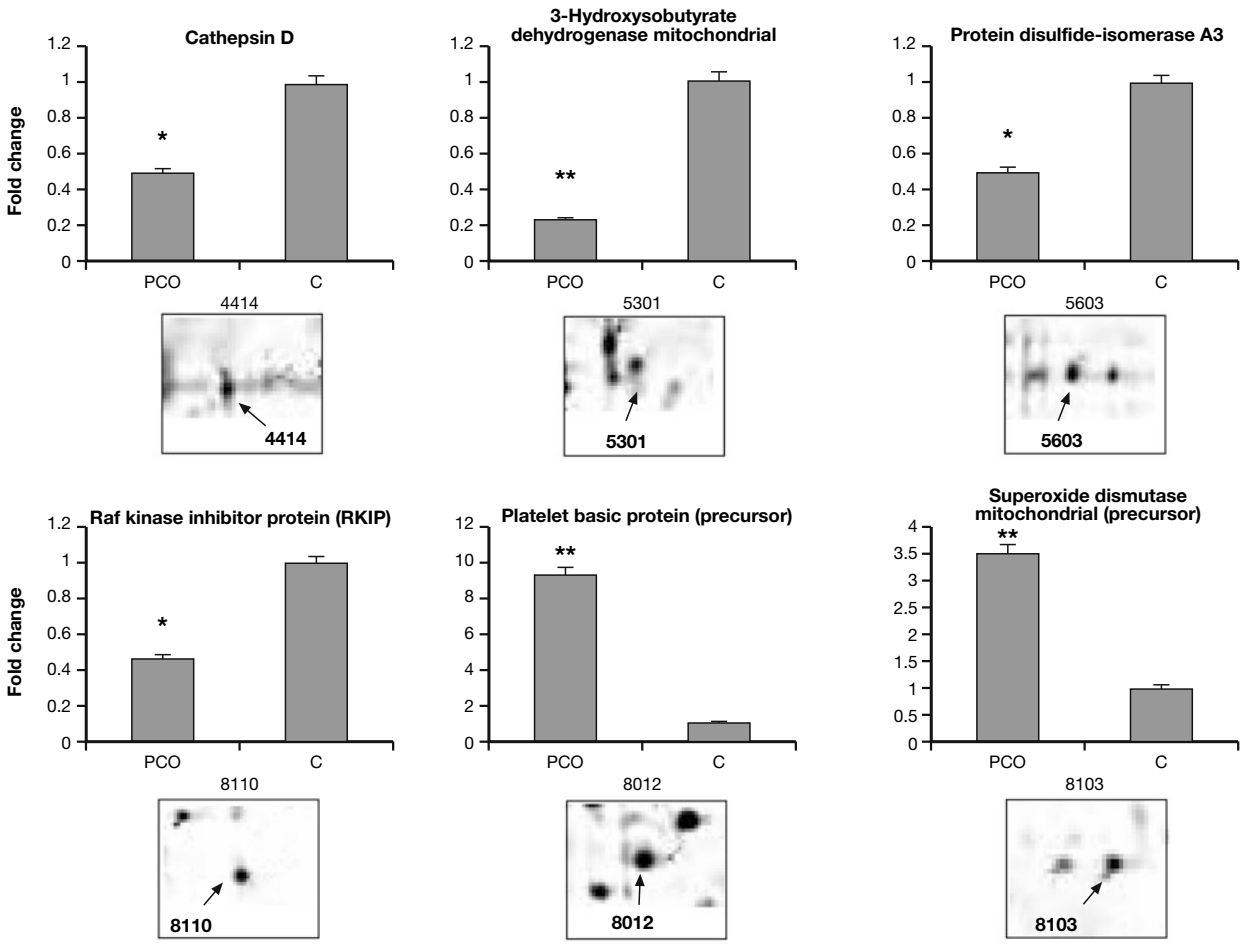

Fig. 4 - *Quantitative analysis of T-lymphocyte protein changes observed only in polycystic ovary syndrome (PCOS) patients. The volume density analysis graphs and the correspondent 2-dimensional gel electrophoresis gel images of selected spots are shown. The data (means±SD) are expressed as fold change* vs *controls (C). *significant difference (*p*<0.05) between patients and control subjects. **more significant difference (*p*<0.01) between patients and control subjects.*

system and in the regulation of the cytoskeleton structure (14-17). The α enolase, also down-expressed in both pathologies, is a glycolytic enzyme and plays important roles in many processes, including the initiation of disease conditions, acting as a plasminogen surface receptor (18). Peroxiredoxin-1 instead, was up-regulated in the CAH patients. It is an antioxidant enzyme involved in controlling the redox cellular state (19) (Fig. 3).

Proteomic analysis in PCOS-derived T-cells

Among the spots differentially expressed only in PCOS compared to healthy control, 6 proteins were identified (Fig. 4). Four of these were down-expressed: cathepsin D, an aspartyl lysosomal protease involved in apoptosis, angiogenesis and proliferation processes (20). Raf kinase inhibitor protein, a factor involved in several signaling cascades, which can inhibit activation of nuclear factor κB (NF-κB) (21). Three-hydroxyisobutyrate dehydrogenase mitochondrial and protein disulfide-isomerase A3 both involved in insulin resistance and in adipocyte differentiation, were differentially expressed (22, 23). *Vice versa* the superoxide dismutase, another protein involved in insulin metabolism (24) and platelet basic protein, the precursor of coagulation proteins as connective tissue-activating peptide III, thromboglobulin and neutrophil-activating peptide 2 (25), were up-regulated. The comparative analysis of the androgen levels and biochemical and metabolic characteristics (IGT, obesity) observed in PCOS group, indicated that 70% of these patients showed a significative modulation of 3-hydroxyisobutyrate dehydrogenase mitochondrial, protein disulfide-isomerase A3 and superoxide dismutase.

Proteomic analysis in CAH derived T-cells

Most proteins modulated in these patients were down-regulated, apart from CCG1-interacting factor B (CIB), that resulted up-regulated (Fig. 5). CIB has been isolated as a molecular partner of CCG1 (cell cycle arrest in G1), the largest subunit of TFIIB, a multi-protein complex with a central role in transcription process (26). The proteins down-regulated were: peroxiredoxin 3, glutathione S-transferase P, both involved in oxidative stress (19, 27). In-

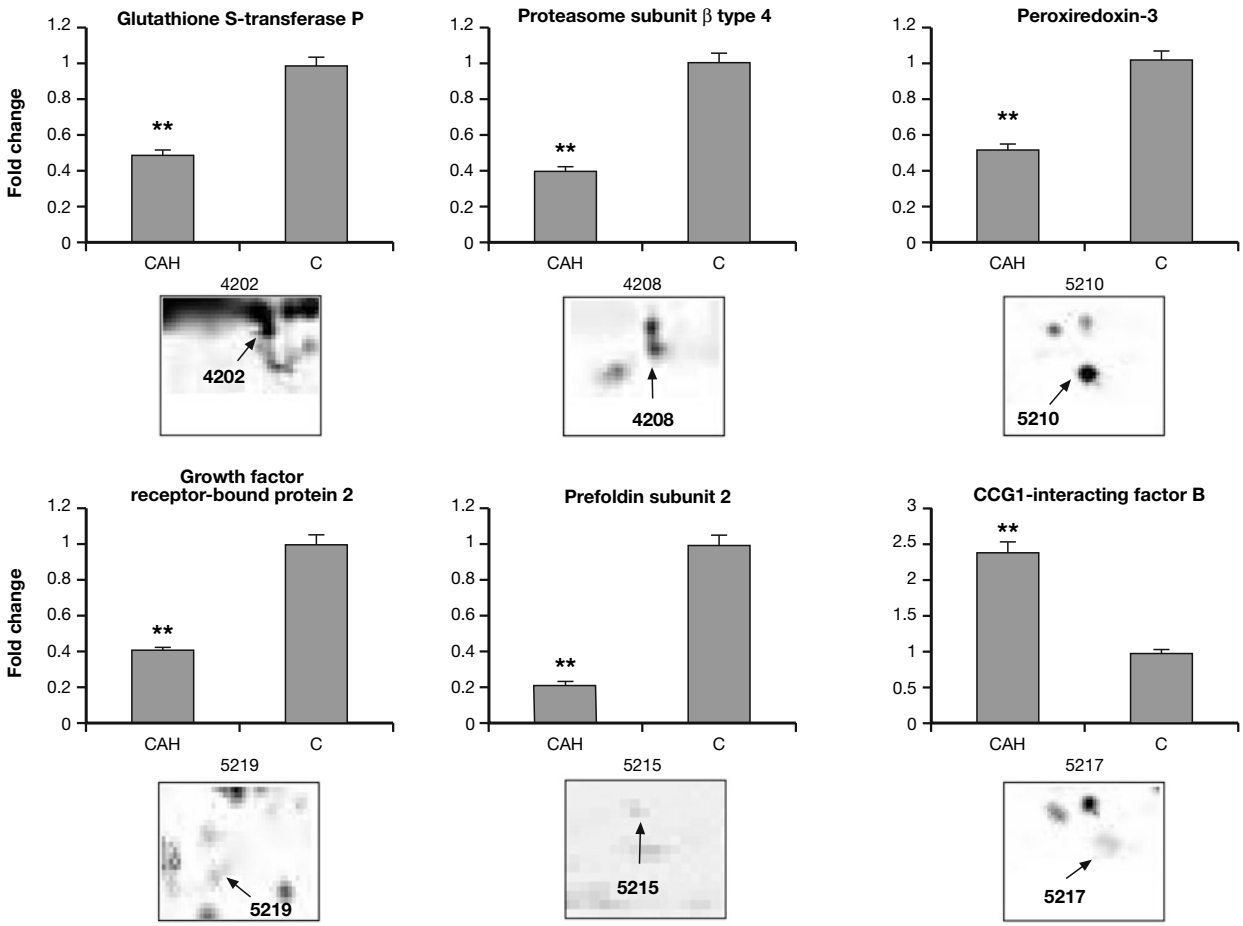

Fig. 5 - *Quantitative analysis of T-lymphocyte protein changes observed only in congenital adrenal hyperplasia (CAH) patients. The volume density analysis graphs and the correspondent 2-dimensional gel elctrophoresis gel images of selected spots are shown. The data (means±SD) are expressed as fold change* vs *controls (C). **more significant differences (p<0.01) between patients and control subjects.*

terestingly, proteasome subunit β type 4, growth factor receptor-bound protein 2, prefoldin subunit 2, show a transactivation function and are involved in activation of T-lymphocytes and in turnover of the androgen receptor (28, 29).
The α-enolase, down-regulated in both PCOS and CAH series and, the platelet basic protein up-regulated only in PCOS patients did not correlate with any coagulation defect.

DISCUSSION

PCOS is a common complex endocrine genetic disorder, which involves overproduction of androgens, leading to heterogeneous range of symptoms and associated with increased metabolic and cardiovascular morbidity (1). Recently, a strong interest has arisen in applying proteomics to foster a better understanding of disease processes, mechanisms of action, and new pharmacological targets of drugs (30). Proteomic study performed on protein extracts of T cells derived from patients affected by PCOS and CAH, showed protein spots differentially expressed in the examined samples. To this purpose, we utilized lymphocytes by hyperandrogenic patients, showing that these cells can represent sensors able to record different molecular signals, at systemic level (8, 31).
Since T-lymphocytes play a critical role in many disease pathogeneses, a rising interest in studying molecular mechanisms underlying specific actions of these immune cells has emerged, along with the availability of high-throughput technologies for broad-range analysis of gene and protein expression, such as DNA microarrays and proteomics. This approach has been applied to investigation of pathologies such as autoimmune disorders and neural disorders (8, 32, 33), and represents a promise to PCOS studies. Previous genomic studies of PCOS to date have focused only on ovarian tissues and gene expression changes at theca cells from size-matched follicles in PCOS women (34). Plasma and serum have an extensive and rich proteome that is representative not only of events in the circulatory system, but also of many organs within the body. Pathogenic disease response mechanisms can often be detected in patient plasma or serum samples owing to tissue leakage following cellular death or damage or alterations in the endocrine system during disease progression (34, 35). PCOS and CAH syn-

dromes share a number of clinical signs and biochemical parameters, so that they are generally thought to elicit similar effects on the organism homeostasis. From a clinical point a view, the peripheral lymphocyte utilization permits the comparative analysis of different pathologies through an innovative and easily methodological approach.

The results presented here evidenced that many T-cells proteins are differentially regulated, compared to the reference map, exclusively in PCO or in CAH. Previous microarray and proteomic analyses published on PCOS so far have been performed on whole ovaries, isolated theca cells, and on intra-abdominal adipose tissue (36, 37). On the contrary, the purpose of the present study was the identification of proteins modulated by the high circulating androgen levels at systemic level. Aim of this study was to evaluate the proteins potentially involved in metabolic and vascular damages described in hyperandrogenic diseases. At this purpose, the capacity of T-lymphocyte to record the hormonal variation represented a good approach to investigate the hypothetic proteins involved in the pathogenesis of these diseases.

This study allowed to elucidate putative proteins involved in studied diseases leading to the identification of 17 protein spots modulated in both classes of hyperandrogenic women enrolled. Study of functions of some of these proteins could be correlated with androgen effects on metabolic and cardiovascular mechanisms. The modulation of 3-hydroxyisobutyrate dehydrogenase, protein disulfide-isomerase A3, superoxide dismutase involved in the insulin resistance molecular mechanism and in adipocyte differentiation (21, 23, 24), could suggest the pivotal role of the androgens in the induction or in the worsening of the metabolic disease associated with hyperandrogenic disorders (38, 39). Alpha- enolase and, the platelet basic protein are involved in a significant manner in the coagulation cascade (18, 25), well known to be crucial in the early phases of atherogenesis, platelet aggregation and increase of intima media thickness. The Peroxiredoxin 1 and 3 and the Glutathione S-transferase P are involved in the stress oxidative processes (19, 27), and were modulated in the patients enrolled in the study. The involvement of these proteins could be due to the direct increase of androgen levels or *vice versa* to be a reaction caused by different stimuli (40). Growth factor receptor-bound protein 2, Prefoldin subunit 2, and proteasome subunit β type 4, all involved in the turnover of the androgen receptor (28, 29) resulted modulated in our samples.

The modulation observed suggests that the androgens are probably able to regulate the androgen receptor expression relatively to their circulating levels. These data supply an additional evidence that lymphocytes are responsive to hormonal stimulation. The involvement of the proteins cofilin-1, Factin and Rho GDP suggests the role of androgen in immune system induction through lymphocyte activation. Finally, the modulation of catepsin D, Raf kinase inhibitor, and CCG1-interacting factor B (CIB) (20, 22, 26), transactivating proteins involved in cellular differentiation, in apoptosis process and in cell cycle, could suggest the direct involvement of androgens in neoplastic complications described for these syndromes. The modulation of the proteins described in hyperandrogenic patients are involved in the mechanisms of endothelial damage, in the oxidative stress, in the turn-over of the androgen receptor, in cell cycle control, in signal transduction and in cellular architecture. It is important to consider that the proteomic profiles obtained reflect only partially the clinical assessment of the patients. It is difficult to establish a direct correlation between the modulation of some proteins and the future metabolic alterations, not present at the moment, because of the patient's young age. However, the modulation of protein involved in oxidative stress, in the cell cycle, in cytoskeleton, and in the turnover of androgen receptor could be able to induce and/or complicate the hypherandrogenic syndromes (27, 28, 38, 40). These results represent an encouraging basis for the development of the future studies in order to define specific molecular phenotype of hypherandrogenic patients, to elucidate new mechanisms involved in androgens production, and to identify proteins candidate potentially involved in the pathogenesis of hyperandrogenism.

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