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Proteomic analysis of human plasma and peripheral blood mononuclear cells in Systemic Lupus Erythematosus patients

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List of abbreviations

ACN	Acetonitrile
ACR	American College of Rheumatology
ACTH	Adreno Cortico Tropic Hormone
ADs	Autoimmune diseases
ANA	Anti-nuclear antibodies
A.O.U.	Azienda Ospedaliera Universitaria
APC	Antigen Presenting Cell
APO	Apolipoprotein
AUC	Area Under the Curve
BANK1	B-cell scaffold protein with ankyrin repeats 1
BILAG	British Isles Lupus Assessment Group Scale
BLK	B lymphoid tyrosine kinase
C2	Complement component 2
C4	Complement component 4
CD40LG	CD40 Ligand
sCD40L	soluble CD40 ligand
CD70	CD70 molecule
aCL	anticardiolipin antibodies
CICs	Circulating immune complex
CLU	clusterin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHCA	α-cyano-4-hydroxycinnamic acid
anti-dsDNA	anti-double stranded DNA
DIL	drug-induced lupus
DTT	Dithiothreitol
2DE	Two dimensional electrophoresis
EBV	Epstein-Barr virus
ECLAM	European Consensus Lupus Activity Measure
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EGF	Epidermal growth factor
GST	glutatione S-transferase
GWAS	Genome-wide association studies
FCGR2A	Fc fragment of IgG, low affinity IIa, receptor
HIV	human immunodeficiency virus
HLA	Human leukocyte antigen
HP	Haptoglobin
HPV	human papilloma virus
HSV-1	Herpes simplex virus 1
IEF	Isoelectric focusing
IFN	Interferon
IL	Interleukin
IRAK1	Interleukin-1 receptor-associated kinase 1
IRF5	Interferon regulatory factor 5

IPG strip	Immobilized PH Gradient strip
ITGAL	integrinalpha L (antigen CD11A (p180)lymphocyte function-associated antigen 1 alpha polypeptide)
ITGAM	Integrin alpha M
IP-10	Interferon gamma-induced protein 10
LAC	lupus anticoagulant
LC MS/MS	Liquid chromatography tandem mass spectrometry
MCP	monocyte chemoattractant protein
MHC	major histocompatibility complex
MIP	Macrophage inflammatory protein
MS	Multiple sclerosis
OD	Optical density
aPL	Antiphospholipid antibody
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate-buffered saline
PDIA	Protein disulfide isomerase family A
PMF	Peptide Mass Fingerprinting
PK	Piruvate Kinase
PRDX2	Peroxiredoxin 2
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
PPP2CA	Protein Phosphatase 2, Catalytic Subunit, Alpha Isozyme
PXK	PX domain containing serine/threonine kinase
RA	Reumatoid Arthritis
ROC	receiver operating characteristic
snRNPs	small nuclear ribonucleoproteins
SDS	Sodium Dodecyl Sulphate
SLE	Systemic Lupus Erythematosus
SA-PE	Streptavidin-Phycoerythrin
SSc	Systemic Sclerosis
SSP	Standard spot number
SPP1	secreted phosphoprotein 1
SIS	SLE Index Score
SLAM	Systemic Lupus Activity Measure
SLEDAI	SLE Disease Activity Index
STAT4	Signal transducer and activator of transcription 4
TALDO	Transaldolase
TCA	Trichloroacetic acid
TGF	Transforming growth factor
TFA	Trifluoroacetic acid
Th	T helper cell
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
Treg	T Regulator cell
TREX1	Three prime repair exonuclease 1
TRIS	2-Amino-2-(hydroxymethyl)-1,3-propanediol
VEGF	Vascular endothelial growth factor

Chapter 1

INTRODUCTION

Autoimmune Disease

The purpose of the immune system is to protect the body against infectious agents. When the immune system does not work correctly, it may cause various diseases.

Autoimmune diseases (ADs) are a wide range of related diseases in which the immune system produces an impaired response against its own cells, tissues and/or organs, causing inflammation and tissues damage. In most cases, the beginning of attacks against the body's self-molecules is unknown. There are more than 80 different autoimmune diseases, and they vary from common to very rare [1].

ADs can be divided into tissue-specific (such as Hashimoto's thyroiditis, Type I or Immune Mediated Diabetes Mellitus), where unique tissue-specific antigens are targeted, or systemic (for example Systemic Lupus Erythematosus, Rheumatoid Arthritis, Systemic Sclerosis), in which multiple tissues are affected, and a variety of apparently ubiquitously expressed autoantigens are targeted [2].

The development of AD depends on a combination of both genetic and environmental factors. The concordance rate of the disease is generally higher in monozygotic twins compared with dizygotic twins or at least compared to other family members, suggesting a genetic contribution [3]. However, the concordance in identical twins is often incomplete, and this indicates the presence of additional factors, presumably from the environment [4].

From the point of view of genetic predisposition, autoimmunity is associated with certain genes; some also are multigenic ADs, in which different genes contribute to the production of the abnormal phenotype [5]. Among the major candidates there are:

human leukocyte antigen (HLA) allele (s) genes, T-cell receptor genes, cytokine and cytokine receptor genes, immunoglobulin genes and immunoglobulin Fc receptor genes for various autoimmune diseases.

Some of these genes confer a high level of risk than others, for instance, the strongest allelic association was shown by the HLA genes, in many cases, they are considered as primary susceptibility factors for many autoimmune diseases [6].
From the standpoint of environmental exposure to chemicals, pharmacological products and pathogens there is a significant link with ADs in both human and animal models. [7]
Some ADs may arise in response to pharmacological treatment. For example, drugs such as procaineamide and hydralazine can induce autoantibodies and lupus-like disorders in patients [8]. Penicillamine has been associated with myasthenia gravis and a-methyldopa is known to cause a form of hemolytic anemia. However, in some cases of AD's drug-induced, ADs symptoms disappeared once the prescribed medication is discontinued [9].

Infections from certain viruses, bacteria, and mycoplasma can cause the onset of systemic AD in genetically predisposed individuals. In addition, a bacterial or viral infection can trigger a severe increase in antibodies and autoreactive T cells, which may cause a worsening of existing symptoms. The viruses most involved in the onset of various ADs are: HSV-1, Coxsackie virus, EBV, HIV, HPV, or influenza virus [2]. Exposure to UV radiation, particularly UV-B rays, has been associated with the presence in flare-ups in lupus patients. In fact, in vitro studies suggest that exposure of DNA and snRNPs to UV-B cause autoreactive lymphocytes and their activation. The mechanism by which these environmental factors induce autoimmunity includes epigenetic changes (such as DNA methylation and histone modification), which can cause reaction with the self component to generate novel antigens, aberrant cell death releasing cellular material that can lead to inflammasome activation and production of pro-inflammatory cytokines and molecular mimicry [10].

Many ADs occur more frequently in women, but the reason has not yet been fully clarified. Several studies suggest a role for female sex hormones [11], which can directly interact with cells of the immune system through receptors, and influence by altering the production of antibodies and the proliferation of immune cells [9]. Reserchers have

suggested that women have a genetic predisposition to autoimmunity due to their second X chromosome [12,13]. Since one X chromosome is silenced by epigenetic mechanisms in women, it is possible that these epigenetic mechanisms contribute to the predisposition to autoimmunity through effects on the inactive X [14].

The ADs incidence appear to vary from country to country: this variation may be due to the uneven prevalence of an HLA allele linked to a particular AD (due to ethnic differences) or of a triggering pathogen or chemical agent (due to geographic or environmental differences) [2].

Systemic Lupus Erythematosus

Introduction

SLE is a systemic autoimmune disease with a broad spectrum of clinical presentations and incompletely understood pathogenesis. This AD is characterized by alterations in both the innate and adaptive immune system that lead to the loss of immunologic tolerance and occurrence of auto-antibodies against nuclear material [15]. The term "*lupus*" from the Latin "wolf" has been widely used in the eighteenth century to describe a wide variety of skin lesions that was considered similar to that caused by the "wolf's bite". Biette et al produced one of the first descriptions of lupus in 1833. In 1846 Ferdinand von Hebra introduced the butterfly term to describe the malar rash. Later in 1872 Kaposi et al founded the systemic nature of the disease. The discovery of the LE phenomenon by Hargraves et al. in 1948 and Friou et al in 1958 provided the first bases on its pathogenesis [16].

Epidemiology

The incidence rates in North America, South America, and Europe range from 2 to 8 cases per 100,000 per person-year. The SLE sex ratio is 6-10:1 (female:male) pri-

marily affecting women in the fertile age. The prevalences of SLE vary considerably depending on ethnicity [17]. In Caucasians is estimated a prevalence of around 20 cases/100,000 per person-year and an incidence of 2-8 new cases per 100,000 person-year. For North America's black population these data should be tripled. The disease appears to be more common in urban than rural areas [18].

Natural course and aetiology

SLE is a chronic disease with a variety of disease manifestations, and with eterogenity of presentations, which often delay diagnosis. Common manifestations include rashes, photosensitivity, arthritis, pleuritis, pericarditis, nephritis, neuropsychiatric disorders, and hematological disorders. The disease course is characterized by periods of remission and exacerbation.

SLE is characterized by autoantibody non-organ specific production (anti-nuclear antibodies ANA, anti-dsDNA and anti-phospholipid antibody). The aetiology of SLE includes both genetic and environmental components with female sex strongly influencing pathogenesis. [18]

Genetic factors

Genetic factors are implicated in pathogenesis with a concordance rate for lupus of 24%–60% among monozygotic twins and 2%–5% among dizygotic twins or siblings [19]. The sibling recurrence risk ratio in SLE patients is 29-fold higher than in the general population, and the higher concordance among monozygotic than dizygotic twins indicates the importance inheritance. [20]

Genome-wide association studies (GWAS) have progressively improved the understanding of which genes are most critical to the potential for SLE: there are approximately 30 susceptibility loci for SLE. The most important genes associated are implicated in immune response and inflammation (HLA-DR, PTPN22, STAT4, IRF5, BLK,

OX40L, FCGR2A, BANK1, SPP1, IRAK1, TNFAIP3, C2, C4, CIq, PXK), DNA repairs (TREX1), adherence of inflammatory cells to the endothelium (ITGAM), and tissue response to injury (KLK1, KLK3). [21]

Epigenetic effects

The risk for SLE may be influenced by epigenetic effects such as DNA methylation and post-translational modifications of histones (acetylation, deacetylation and methylation), which can be either inherited or environmentally modified. The regulatory regions of some genes known to be involved in the disease's pathogenesis (for example ITGAL, CD40LG, CD70, and PPP2CA) have been reported to be hypomethylated in SLE [22].

Environmental factors

Epidemiological studies have identified a number of environmental factors suspected of modulating susceptibility to SLE; some of these factors include viruses (such as EBV that has been identified as a possible factor in the development of lupus), hormones, smoking, alcohol intake and exposure to ultraviolet light (the most obvious environmental factor that may exacerbate SLE sintoms), silica, organic solvents, including various drugs like hydralazine and INF-α. It is well established that certain drugs induce autoantibodies in a significant number of patients. Newer biologics and antiviral agents have been reported to cause drug-induced lupus (DIL) [23]. Socioeconomic factors have been associated with poorer outcomes and higher disease activity, [24] although it remains unclear whether they play a role in disease susceptibility or subsequent progression.

Hormonal factors

The role of sex hormones (estrogens and androgens) in the development of SLE is suggested by the marked prevalence of females and from studies in animal models, where the addition of hormones can lead to autoimmune disease with an increase in auto-reactive B cells. In clinical practice, patients receiving oral contraceptives had flares; but a randomized controlled trial found out that combined oral contraceptives do not confer a higher risk of symptoms exacerbations of the disease in women with SLE clinically stable. [25]

In postmenopausal women with SLE who have used hormone replacement therapy it was observed that there is a higher risk of getting mild to moderate lupus flares [26]. Pregnancy may aggravate SLE, in fact, the levels of estradiol and progesterone are lower during the second and third trimesters in comparison with healthy pregnant women. [27].

Pathogenesis

Disease pathogenesis in SLE manifests itself as systemic acute and cronic inflammation that affects many organ systems. Furthermore, its onset can vary from patient to patient.

In SLE there is an aberrant innate immune response that causes tissue injury by released infiammatory cytokines and by impaired activation of T and B cells. IFNα and tumour necrosis factor (TNF), contribute to affected tissue injury and inflammation. These mediators, together with the cells producing them (macrophages, leucocytes, dendritic cells and lymphocytes), are the subject of investigation as potential therapeutic targets in lupus [28].

Most lupus patients have high titers of auto-reactive antibodies (such as dsDNA) associated with their autoantigens and to complememt factor, which form circulating immune complex (CICs). CIC deposition leads to infiammation [29]. Immune complexes and complement activation pathways are central players in tissue injury. In healthy in-

dividuals, immune complexes are cleared by Fc and complement receptors, in patients with autoimmune disease they are not cleared because the Fc and complement receptors are functionally deficient. Tissue damage is mediated by recruitment of inflammatory cells, reactive oxygen intermediates, production of inflamatory cytokines, and modulation of the coagulation cascade [30]. The pathogenesis of SLE involves a multitude of cells and molecules that participate in apoptosis, innate and adaptive immune responses.

Briefly, we can summarize the abnormalities in the immune system in:

- ✓ impaired clearance of immune complexes;
- ✓ altered activity of networks idiotype-antidiotype that are unable to inactivate T and B lymphocytes;
- ✓ Increased activity of T-helper to produce the antibodies by B-lymphocytes;
- ✓ The suppressor T cells do not function properly;
- ✓ alterations of the mechanisms of immunological tolerance.

Classification criteria

Consensus guidelines provided by the American College of Rheumatology (ACR) offer the basis for accurate and standardized diagnosis of SLE. The original recommendations published in 1982 were updated in 1997 and contain 11 diagnostic categories (Table 1). The presence of any four of these criteria, either concurrently or consecutively, confirms the diagnosis of SLE. The major change in the 1997 revision was the inclusion of newer immunological tests, namely, antiphospholipid (aPL) antibodies, anticardiolipin (aCL) antibodies, and lupus anticoagulant (LAC), and the removal of redundant histological preparations [31,32].

Table 1: ACR criteria

SLE Diagnostic Criteria

Clinical criteria

Malar rash	A rash on the cheeks and nose, often in the shape of a butterfly
Discoid rash	A rash that appears as red, raised, disk-shaped patches
Photosensitivity	A reaction to sunlight that causes a rash to appear or get worse
Oral ulcers	Sores in the mouth
Arthritis	Joint pain and swelling of two or more joints
Serositis	Inflammation of the lining around the lungs (pleuritis) or inflammation of the lining around the heart (pericarditis)
Kidney disorder	Persistent protein or cellular casts in the urine
Neurologic disorder	Seizures or psychosis

Laboratory criteria

Blood disorder	Anemia (low red-cell count), leukopenia (low whitecell count), lymphopenia (low level of specific white cells), or thrombocytopenia (low platelet count)
Immunologic disorder	Positive test for anti–double-stranded DNA, anti ENA, or antiphospholipid antibodies
Abnormal antinuclear antibodies	Positive antinuclear-antibody test(ANA) targeting nuclear antigens

Activity indices

The disease course is characterized by periods of remission, followed by progressive exacerbations, therefore it is important to categorize disease activity. Discerning in an appropriate manner the disease activity is important for improving both prognosis and treatment. The indices validated and used are the following: the European Consensus Lupus Activity Measure (ECLAM), the British Isles Lupus Assessment Group Scale (BILAG), the National Institutes of Health SLE Index Score (SIS), the Systemic Lupus Activity Measure (SLAM), and the SLE Disease Activity Index (SLEDAI). These indices have been developed in the context of long-term observational studies and have been shown to be strong predictors of damage and mortality, and reflect change in disease activity [18].

Bertsias et al [28] recommend the use of the ECLAM and the SLEDAI whom are more convenient for daily practice.

Proteomic and SLE

In SLE, the presence of specific serological markers is limited, and the pathogenesis has not yet been fully elucidated. The diagnosis of SLE during the initial stages may be mistaken, this happens because the symptoms may be similar to those of other rheumatic diseases (such as RA and SSc); moreover, it requires a complex interpretation of the ACR criteria.

The disease is also characterized by periods of remission and flare, and, being systemic, it could also manifests itself in different sites, and so far there are no specific tests to indicate accurately the picture.

From the present literature [33-39], we note the importance of proteomics, which can help to find new therapeutic targets and it also could help to better understand the cellular mechanisms.

Proteins are indeed important executors of physiological and cellular functions; changes in their expression reflect the different physiological conditions (e.g. healthy and sick). Observing the variations may help to better understand the pathogenesis in order to improve diagnosis and treatment.

Plasma and Peripheral Blood Mononuclear Cells (PBMCs) can be obtained easily and non-invasively. Both the matrices are subjected to rapid changes in response to different signals, such as during inflammatory states. Plasma is one of the most widely used blood component, while in recent years we have seen a greater interest in PBMCs.

PBMCs are mainly composed of monocytes and lymphocytes, these cells are the most involved in the immune response. Observing the changes in their proteome may help us to have a deeper knowledge of their function in various diseases [40-42].

Cytokines and SLE

Cytokines are molecules produced by various types of cells and secreted into the surrounding medium, usually in response to a stimulus. The development of an effective immune response involves lymphoid, inflammatory and hematopoietic cells [43].

The term cytokine includes a large and diverse family of polypeptide regulators widely produced throughout the body by cells of different embryonic origin.

Cytokines are essential for the development and operation of both innate and adaptive immune response, even though not limited to the immune system. A large variety of cells is able to secrete cytokines, but the two main sources of these mediators are T helper and macrophages.

The general characteristics of the cytokines may be briefly summarized as follows:

- proteins with molecular weight comprised between 10 and 50 kD;
- cytokines act on their target cells via specific receptors, usually equipped with high or very high binding affinity;

- the actions of cytokines can be divided into "autocrine" (when the target cell of a specific cytokine is represented by the same cell that produces it), "paracrine" (when the target cell is located in the proximity of the cell responsible for the production) or more rarely "endocrine" (if the target cell is located remote from the cell responsible for the cytokine secretion and reaches the target via the bloodstream);
- cytokines can direct their action towards many different cell types. This feature is known with the term *pleiotropism*. Pleiotropism means also that a cytokine can perform different actions on the same cell line;
- One of the features of cytokines is the redundancy of the actions of these molecules, in fact many of these soluble molecules share the same actions, sometimes even against the same cell type [43].

These mediators are involved in the pathogenesis of autoimmune diseases, including SLE and RA. In SLE, several of these cytokines are overexpressed and contribute to the pathogenesis, in the activity or organ involvement of the disease.

The most involved cytokines are those produced by the interaction of different T cell subsets. For example Th1 (IL-1, IL-6, IFN-γ, TNF-α) and Th17 (IL-17, IL-21,IL-22) cytokines subset generally drive pathogenic effector responses while. Th2 (IL-4, IL-5, IL-13) and Treg (TGF-β and IL-10) cytokines mediate regulatory response (44).
Even chemokines (chemoattractan cytokines) that exert their effects by attracting inflammatory cells, including monocytes, neutrophils and T lymphocytes, to sites of inflammation, are implicated in the pathogenesis of SLE, and in some studies, the expression was found to be increased and correlated with renal involvement and disese activity [45,46].

The measurement of cytokines in SLE can be useful in order to monitor the disease more accurately. In fact, it may be useful for identifying flare, assistance in monitoring response to therapy, provide information regarding the organs involvement and identification of possible therapeutic targets. Indeed, some therapies that inhibit their

action have been successfully used for the treatment of other rheumatic autoimmune diseases (RA and Crohn's disease) [47, 48].

Chapter 2

OBJECTIVES

In ADs, particularly in SLE, early diagnosis, flare or remission phases can be difficult to identify, because of complex etiopathogenesis, heterogeneous presentation of symptoms, and unpredictable course. In addition, the initial symptoms may include signs or symptoms common among different ADs.

The definition of specific serological markers is essential for early differential diagnosis. There are no set of biomarkers that can be used reliably to confirm the presence of SLE or to monitor its progression with specificity and sensitivity.

Starting from these assumptions, the aim of this study was to observe the changes in the proteome and in cytokines expression of SLE patients, in order to increase our knowledge about pathogenesis and to find possible diagnostic markers and/or therapeutic targets for improving diagnosis and treatment.

Chapter 3

METHODS

Patients

Twenty one SLE patients, (20F:1M, 48,2±6,14 years), twelve RA patients (12F, 64,15±12,17 years, used as pathological control) and twenty one healthy volunteers (20F:1M, 42,14±9.58 years) were enrolled in the study.

All subjects were recruited from the Cit.Il.AKeA Project (This project was supported *by Regione Autonoma della Sardegna, Legge regionale 7/2007, "Le interleuchine nella longevità, nelle patologie sistemiche e neoplastiche: semplici marcatori o fattori predittivi per la risposta terapeutica?" Cit. Il.AKeA [49], P.I. Luca Deiana*, approved by the local bioethics) in collaboration with the unit of Medical Pathology A.O.U. University of Sassari and all signed a written consent prior to blood sampling.

SLE diagnosis is based on the 1987 American College of Rheumatology criteria [50]. Each SLE patient had the same disease activity, calculated with SLEDAI (Score ≤6, inactive disease). All partecipants were Caucasians people. Blood samples were collected into vacutainers containing potassium EDTA as anticoagulant (Vacutest Kima). For 2D analyses we have used pooled samples for each group; while for ELISA and Western Blot validations we used single samples.

All SLE patients were on treatment using standard protocol incorporating glucocorticoids and immunosuppressive agents while healthy subjects didn't suffered from any autoimmune disease.

Plasma samples

The red cell fraction was separated from plasma by centrifugation at 2500 g at 4°C for 15 min and the clear plasma supernatant was stored in aliquots frozen at -80°C

until use. For cytokines analysis plasma samples were centrifuged again at 10000g for 10min at 4°C to remove completely platelets and precipitates.

Removal of the high-abundance proteins from plasma samples

For each group (SLE, RA and Healthy), were created plasma pools.

For protein extraction we used the ProteoMiner™ ProteinEnrichment Kits (Biorad). The The Proteominer is a new tool used to reduce the concentration of proteins in complex biological samples. The presence of high-abundance proteins in biological samples (such as albumin and immunoglobulins) makes the detection of medium and low abundance proteins very difficult. The technology is based on the interaction of the samples with hexapeptides related to chromatographic supports (Figure 1). The high-abundance proteins are washed during procedures, while the low-abundance proteins are focused on their specific ligands.

In the first step was removed the storage buffer from columns by washing them with 600 μl of wash buffer (30-60 sec) twice after this process the column conteined 100 μl of settled beads, ready for sample binding.

The plasma samples were centrifuged at 10000g for 10 min to clarify.

One ml of sample was added to each column. The columns were positioned on a rotational shaker for 2 hr at room temperature. Then, the columns were washed for 3 times with 600 μl of wash buffer. After, all the buffer was removed, 600 μl of deionized water was added and the columns have been rotatedend-to-end for 1 min. The material was discarded. Then 100 μl of rehydrated elution reagent was added and the columns were incubated at room temperature and lightly vortex several times over a period of 15 min. In the next step the eluate was placed in a clean collection tube and centrifuged at 1000g for 30–60 sec. The eluate contains the proteins at medium and low abundance ; this step was repeated two more times.

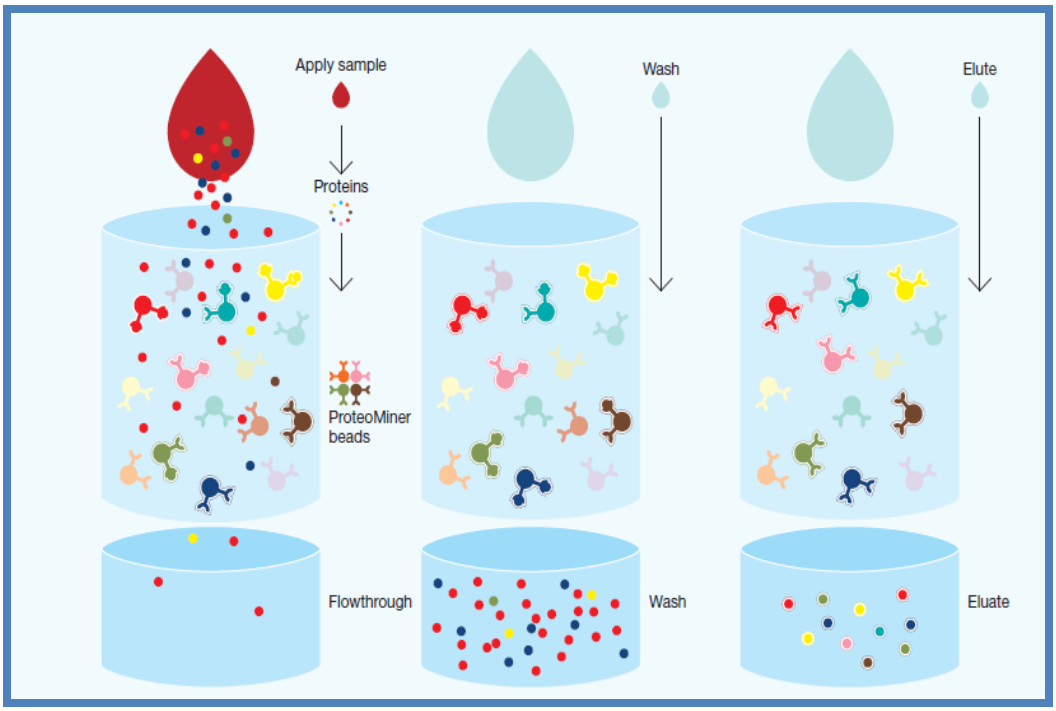


Figure 1: High-abundance proteins saturate their ligands (yellow and red beads) and excess protein is washed out during the procedure. Low-abundance proteins are concentrated on their specific ligands (pink and teal beads)

PBMCs samples

PBMCs were isolated using a Ficoll-Paque Plus density gradient (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

The cells were resuspended in phosphate-buffered saline (PBS). Soluble proteins were extracted from PBMCs by incubation on ice in 200 µl of lysis buffer (7M urea, 2M thiourea, 4% CHAPS, DNasi (3µl/ml), RNasi (3µl/ml)and Complete Protease Inhibitor Cocktail(5µl/ml) for 10 min. Were carried 2 freeze-thaw cycles at -80°C for 5 min. Samples were centrifuged at 14000g for 30 min at 4°C and supernatant containing proteins recovered. For proteomics analyses the protein extract of PBMCs was pooled for each group (SLE,RA and Healthy). The pool is prepared by mixing defined units of protein extract (50 µg of protein) belonging to the same group.

Protein quantification

The concentration of plasma and PBMCs proteins were determined using the RC/DC Protein Assay (BioRad) according to the manufacturer's instructions.

The RC/DC Protein Assay uses a Lowry method modified to reduce agent compatible (*RC*) as well as detergent compatible (*DC*). The assay based on the reaction of proteins with an alkaline copper tartrate solution and Folin reagent where the color development is primarily due to the aminoacids tyrosine and tryptophan, and to a lesser extent, cystine and histidine. Proteins reduce the Folin reagent by loss of 1, 2 or 3 oxygen atoms, thus producing one or more of several possible reduced species which have a characteristic blue colour with a maximum absorbance at 750 nm. A standard curve was constructed using albumin as standard sample in a range between 0.2 and 2mg/ml.

Two dimensional SDS PAGE

Total protein extracts of plasma and PBMCs samples were separated by 2D-PAGE gels. Analytical gels contained 150 µg of total protein extracts; for preparative gels 1000 µg of protein were applied. Three experimental replicates were performed for each sample pool belonging to the three different groups.

Isoelectrofocusing (IEF)

Plasma: The first dimension (Isoelectric Focusing, IEF) was performed on IPG (Immobilized pH gradient) strips linear pH 3-10, 17 cm from BioRad. At first, the strips were rehydrated for 20 hours at 20°C without voltage in rehydration buffer (Urea 8M, Tiourea 2M, 4% CHAPS, Destreak reagent, 10mM DTT, 1% carrier ampholyte pH 3-10 and 0.05% bromophenol blue) and covered with mineral oil. The proteins were separated using the Protean IEF Cell system (Biorad) according to the following protocol: 2

hr at 250 V, 2 hr at 500 V, 2 hr at 750 V, 2 hr at 1000 V, 2 hr at 5000 V, 2 hr at 8000 V, at 8000 V for 50.000 Volthours, for a total of 80.000 V). Focused IPG strips were equilibrated in two steps (15 min each) in 1 ml freshly prepared sample buffer (50 mM Tris–HCl pH 8.8, 6 M urea, 20% (v/v) glycerol and 2% (w/v) SDS, 1% (w/v) bromophenol blue) supplemented with 2% dithiothreitol and 2.5% iodoacetamide respectively [51].

PBMCs: IEF was performed on IPG strips linear pH 4-7, 17 cm from BioRad. At first, the strips were rehydrated for 21 hours in rehydration buffer (Urea 8M, Tiourea 2M, 4% CHAPS, Destreak reagent, 10mM DTT, 1% carrier ampholyte 4-6 and 0.05% bromophenol blue) and covered with mineral oil. The proteins were separated in the first dimension according to the following protocol: 2 hr at 250 V, 2 hr at 500 V, 2 hr at 750 V, 2 hr at 1000 V, 2hr at 5000 V, 2 hr at 8000 V and at 8000 V for 50.000 Volthours for a total of 80.000 Vh. After isoelectric focusing the IPG strips were equilibrated in the same protocol of plasma.

SDS-PAGE

To separate the protein in the second dimension we performed a size denaturing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE). Equilibrated IPG strips were embedded into 0.5% agarose on top of SDS polyacrylamide gels. The anionic detergent sodium dodecyl sulfate (SDS) denatures the proteins and provides the protein with a negative net charge.

For the second dimension 13,5% (for plasma proteins) and 12,5% (for PBMCs proteins) SDS-polyacrylamide gels were used. The gels were made using the Multicasting Chamber from BioRad; using this apparatus we were able to produce ten gels simultaneously with a high reproducibility. The gels were run on a Protean Multicell (BioRad) were six gels at a time can be run. The instrument is connected to a cooling bath to avoid excessive heating of the buffer. The lower chamber was filled with buffer containing Tris 0.125M, Glycine 0.96M, SDS 1%, while the upper chamber had the same composition but with the SDS concentration doubled (2%). This modification was

suggested by the work of Weirner et al [52] who proved that in order to minimize gel smearing the concentration of SDS in the cathodic buffer needs to be increased.

After completion of the run, analytical gels were stained in SYPRO® Ruby Protein Gel Stain whereas preparative gels in Coomassie staining solution. SYPRO Ruby stain is ready to-use and is a ultrasensitive, luminescent stain for the detection of proteins separated by polyacrylamide gel electrophoresis.

After electrophoresis, the gel was placed in a clean container with 100 ml of fix solution (50% methanol, 7% acetic acid) and put in an orbital shaker for 30 minutes. After that, we added the SYPRO® Ruby stain and incubated in an orbital shaker overnight. The day after, the gels were transferred to a clean container and washed in 100 ml of wash solution (10% methanol, 7% acetic acid) for 30 minutes. The transfer phase helps to minimize background-staining irregularities and stain speckles spot on the gel. Before imaging the gels were rinse in ultrapure water. The gels were scanned using Chemidoc™ XRS (BioRad).

Coomassie staining is cheap, quantitative and reproducible. The staining solution used was composed of 0.05% coomassie Blue R-250 dissolved in 50% methanol, 10% acetic acid and H_2OmilliQ.

Gels were then washed with H_2OmillQ and destained in destaining solution containing the same components of the staining solution except for the dye. Gels were scanned using a GS-800 densitometer (BioRad).

Image analysis

Images were analysed using the PDQuest Advance 2D Analysis Software (Bio-Rad). We performed the image analysis following the steps summarized below:

- *Scanning*: gels are turned into pixel data and then each map is turned into a series of pixels describe by their optical density value (OD).
- *Filtering*: this step eliminates noise, background effect.

- *Automated spot detection:* identification of spots present in each gel independently, then spots are located on the gel image fitted by ideal gaussian distributions and quantified by the sum of the OD values within each gaussian distribution.
- *Matching of protein profiles*: 2D gels are then edited and matched to one another in a "match set", to achieve this, landmarks are used, consisting of reference spots used by PDQuest to align and position the match set members for matching.
- *Normalization*: is used to compensate gel to gel variations due to sample preparation and loading, staining and destaining procedures.
- Spots were first detected and matched automatically to a master gel selected by the software and then we corrected manually the errors in the editing end matching steps.

- Spot volumes were normalised with the option "Local Regression Model" method (LOESS); this method uses the raw quantity of each spot in a member gel, multiplied by a factor based on the local regression method of the matched spots. In order to exclude artifacts and false positive we manually confirmed both matching and data quality of all the spots: only those spots that were detectable in all gels of a sample set were considered for evaluation. The quantity table was exported to a spreadsheet file and submitted to statistical analyses.

Western blot

For western blot analysis were analysed the same protein extracts used for 2D PAGE.

The samples were boiled in laemli buffer (TrisHCl 1M pH6.8, glicerol, 10% SDS, 0.5% bromophenol blue) for 4 minutes at 95°C and then loaded on a 12.5% acrylamide gel. After SDS PAGE the gel was transferred to a nitrocellulose membrane using the Trans Blot Plus Cell system from BioRad. The gel size was measured and two whatman filter papers and one nitrocellulose membrane of the same size were prepared. The filter papers and nitrocellulose were soaked in transfer buffer (25mM Trizma base, 200mM glycine in H_2O milliQ). Then the soaked nitrocellulose and the polyacrylamide gel were placed on to one of the filter papers and covered with the second filter paper. This stack was placed between two sponge cloths before they were placed into the holding device of the blot chamber.

Then, blotting was performed at 60V for 1 hour. During the blotting procedure the instrument was connected to a cooling bath to avoid excessive heating of the buffer.

After disassembling, the membrane was soaked in blocking buffer containing 3% albumin in PBS-T (PBS 10X, 20% (v/v) methanol and 0.05% (v/v) Tween-20 in ddH2O) for 1 hour at room temperature. It was then shortly rinsed with washing buffer (PBS-T) and the primary antibody (mouse monoclonal antibody peroxiredoxin2, dilution 1:3000, Pierce) was applied in the same buffer overnight at 4°C. The blots were washed 3 times for 10 min in washing buffer before the secondary horseadish peroxidase labelled goat anti-mouse antibody was applied in the same buffer at a dilution of 1:2000 for 80 minutes at RT. The membranes were washed five times in PBS-T before the blots were developed using the ECL Immunoblotting Detection Kit from Amersham. The images were acquired with the Chemidoc ™ XRS from BioRad. The exposure time was set at 60 sec and then the image analyzed for densitometric studies.

Mass spectrometry and protein identification

Mass spectrometry is the most common, high throughput method for identifying proteins from 2D gels. It accurately weights peptides after digestion with a suitable enzyme and the masses of these peptides can then be matched against the theoretical peptide masses of known proteins for identification.

In the present work, protein spots with a statistically significant variation ($p \leq 0.05$, ANOVA), showing an over two-folds difference in volume, were selected as differentially expressed and analyzed by MS and MS/MS analyses. MS analyses were performed on a MALDI-TOF mass spectrometer micro MX™(Waters).

The main parameters used were: reflectron mode, positive polarity, mass range 800-4000 Da. Mass spectra were calculated using an external calibration, with the following mixture of peptides: Bradykinin fragment 1-7 (m/z 757.3997),Angiotensin II (m/z 1046.5423), $P_{14}R$ synthetic peptide (m/z 1533.8582), ACTH fragment 18-39 (m/ z 2465.1989), Insulin oxidized B chain (bovine) (m/z 3494.6513) (ProteoMass™ Peptide & Protein MALDI-MS Calibration KIT, SIGMA). One µl of tryptic peptide solution of each digested spot was mixed with an equal amount of the matrix α-cyano-4-hydroxycinnamic acid (CHCA), prepared in 0,2% Trifluoroacetic acid (TFA) and in 70% Acetonitrile (ACN), applied on a MALDI plate and dried at room temperature. All spectra were analyzed using the Mass Linx v 4.1 software (Waters). Protein identification was performed by peptide mass fingerprinting (PMF) using MASCOT software searching at www.matrixscience.com. Search parameters were restricted to Homo sapiens taxonomy using the NCBInr database. Enzyme selection was trypsin, with up to one missed cleavage permitted. Carbamidomethylation of cysteines was selected as a fixed modification; Gln -> pyro-Glu (N-term Q), Oxidation (M) as variable modifications. Protein mass was unrestricted and peptide mass tolerance typically set at ±150 ppm. Mass values were entered as monoisotopic MH+.

To get a further confirmation of the proteins identified, some of them, considered most relevant, were subjected again to identification through LC-MS/MS analyses. LC-MS/MS analyses was performed on an HPLC-Chip/Ion Trap (Agilent). All searches were performed using the Proteome Discoverer version 1.4 with the following parameters: Mascot search engine, database SwissProt , the taxonomy Homo sapiens, a maximum of 2 sites of hydrolysis missed, enzyme trypsin, oxidation of methionine and cyclization of the N-terminal glutamine to pyroglutamic as the variable changes.

ELISA

A non competitive ELISA kit (Cloud-Clone Corp. Assembled by Usnc Life Science Inc. Huston, USA) for human clusterin was used following manufacturer instructions using plasma samples.

Cytokine quantification

Plasma concentration of the cytokines was determined by the Bio-Plex MAGPIX Multireader (Biorad) instrument.

The Bio-Plex system is based on xMAP technology developed and owned by Luminex Corp, which can theoretically support up to 50 different ELISA assays for a single sample. Each test is performed on the surface of a polystyrene bead, diameter of 5.6 μm. Each bead contains inside two fluofores in different ratio, which allow the beads' classification in 100 different regions (Figure 2). Each set of beads can be conjugated with a different antibody.

The principle of the Bio-Plex Pro™ Assay (Bio-Rad) is similar to the ELISA-sandwich. The beads (50 µl) associated with the specific antibody are incubated with the sample (diluted 1:4 with Bio-Plex sample diluent) in a microplate. After one hour of incubation in the dark and agitation, 3 washes with wash buffer are performed, and the biotinylated secondary antibody (25 µl) is added. The biotinylated secondary antibody binds to another epitope of the molecule.

After 30' of incubation in the dark and stirring, the streptavidin-phycoerythrin (SA-PE) (50µl) is added for 10'. The phycoerythrin acts as a fluorescence detector (Figure 3).

After incubation, the plate is inserted into the player's Bio-Plex MAGPIX; the reader employs light-emitting diodes (LEDs) and a charge-coupled device (CCD) imager to illulinate and image a monolayer of immobilized magnetic beads. Precision fluidics align the beads in a single line from a stream that leads into a cell where two LED excite the beads individually. The red led (635 nm) excites the dye in each ball, identifying the specific beads. The green led (532 nm) excites phycoerythrin reporter that generates a signal that is read by a photomultiplier tube which allows to quantify the analyte captured (figure 4). The processor manages high-speed digital data and the Bio-Plex Manager software presents the data as mean fluorescence intensity (MFI) and as a concentration (pg/ml). Concentration of the analyte bound to each bead is proportional to the MFI of the reporter signal.

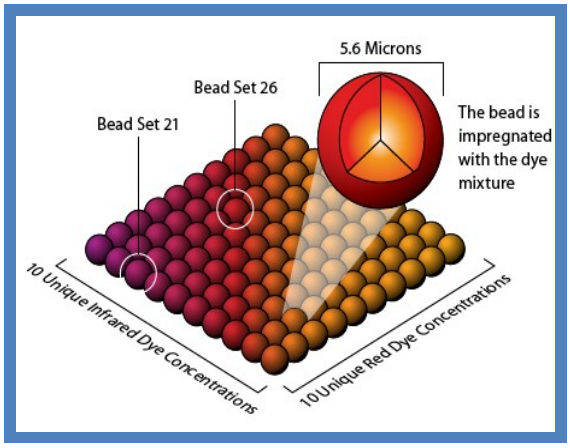


Figure 2: different bead set

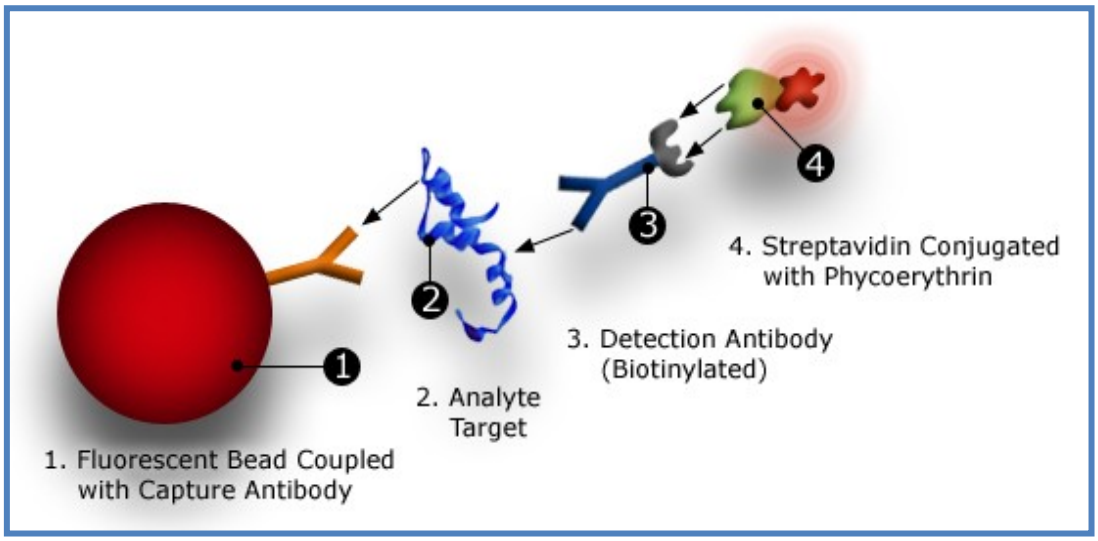


Figure 3: Bio-Plex sandwich immunoassay

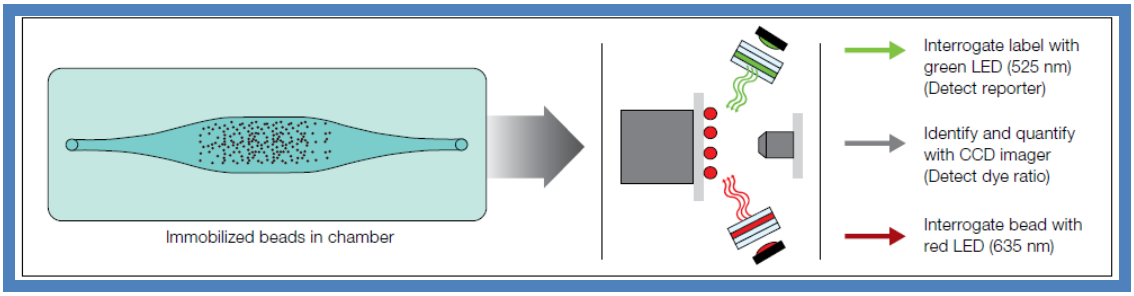


Figure 4: LED/Image-based system

Statistical analysis

All the statistical analyses were performed using STATA Release 9 and SPSS v 17 softwares. To compare changes of the mean intensity values of protein spots between healthy, SLE and RA groups a parametric test, the One Way ANOVA, was performed followed by "Fisher's least significant difference method" (LSD); p-values ≤ 0.05 were considered significant.

Among all statistically significant spots, only those that showed changes of the mean intensity values at least by a factor 2 compared to the healthy group were selected, in order to exclude the impact of experimental variability and consider more reliable data.

To compare the different cytokine expression patterns among the three groups, the Kruskall Wallis test and the Dunn' methods were permormed for pair comparisons, because the data did not follow a normal distribution.

To compare the changes in plasma clusterin concentration and in optical density of Peroxiredoxin 2 among the three groups the ANOVA test was performed. Receiver operating characteristic (ROC) curve was used to assess the diagnostic value of statistically significant cytokines.

Materials

IPG strips pH 4-7 17cm and pH3-7 17cm, Bio-Plex Pro™ Assay were purchased from BioRad Laboratories (CA, USA), Ampholyte 4-6, Ampholyte 3-10, Dithiothreitol (DTT), urea and thiourea, DNasi, RNasi ProteoMass™ Peptide & Protein MALDI-MS Calibration KIT from Sigma-Aldrich (MO, USA), COmplete Protease Inhibitor Cocktail Tablets from Roche (), Mouse Monoclonal antibody Anti-Peroxiredoxin 2 from PIERCE Thermo Fisher Scientific Inc. (IL, USA) and Goat anti-mouse Secondary antibody SC-2005 from Santa Cruz Biotechnology (CA, USA), Magic Mark XP western protein standard from Invitrogen (CA, USA), ELISA Kit for Clusterin (CLU) Human from Cloud-Clone Corp. Assembled by Usnc Life Science Inc. (TX, USA). All other reagents were of the highest quality available.

Chapter 4

RESULTS

2D-PAGE comparisons

In order to know the pattern of protein expression on matrices of plasma and PBMCs in SLE patients a proteomic approach has been used including 2D-PAGE and mass spectrometry analyses.

2D electrophoresis maps

The proteomic comparisons were based on the differences of mean intensity values of protein spots between SLE patients, RA patients and healthy subjects. For each "matchset" (3 replicates for each group) we had the same SSP (standard spot number) assigned by the software. The software used for image analysis (PD QUEST advanced) has detected about 500 and 600 valid spots in PBMCs and plasma samples respectively. Among these 13 spots and 8 spots were found to show significant changes (higher than two folds, $p \leq 0.05$, ANOVA test) in intensities compared with the healthy group in PBMCs and plasma samples respectively.

The figures 5 and 6 show the 2D PBMCs and plasma maps obtained in our laboratory through the method described in materials and methods. The images were found to be similar either between gels or between groups (Figures 7 and 8).

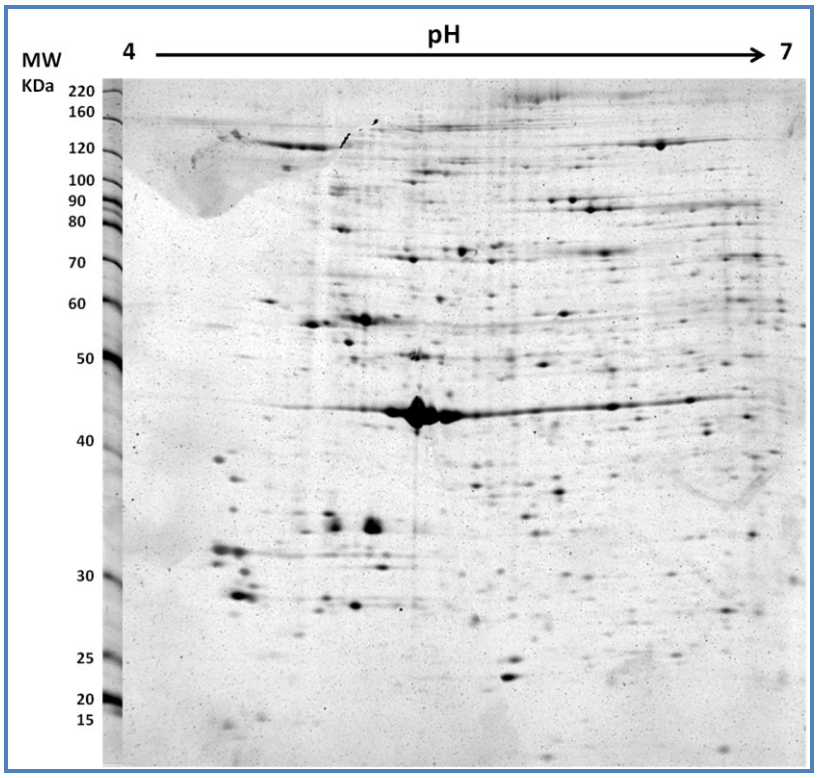


Figure 5: Typical gel image obtained in our lab for PBMCs proteins in 12.5 % gel

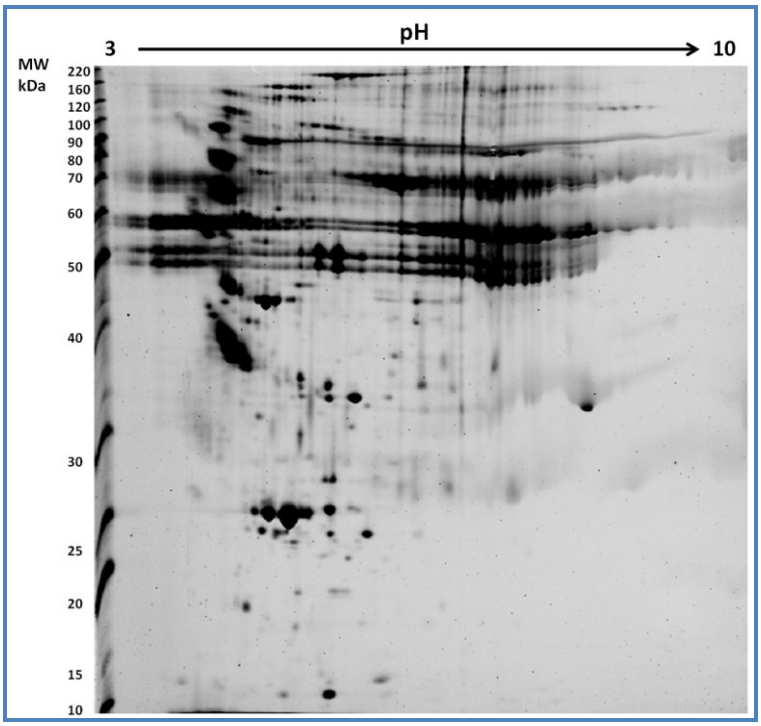


Figure 6: Typical gel image obtained in our lab for Plasma proteins after removal of high-abundance proteins in 13.5 % gel

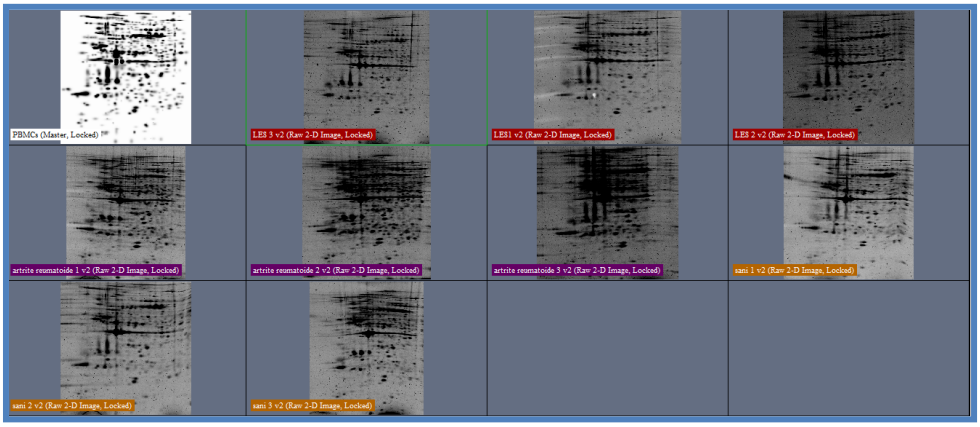


Figure 7: Matchset example created by PDQuest software for PBMCs pool sample

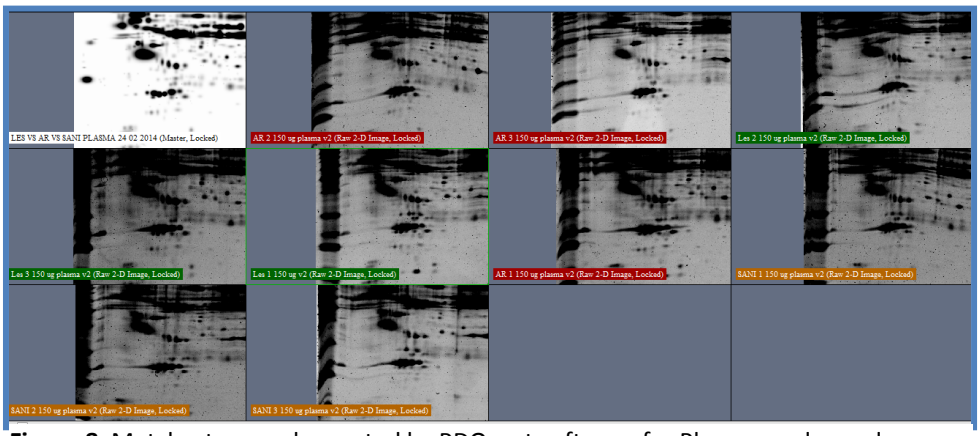


Figure 8: Matchset example created by PDQuest software for Plasma pool sample

Proteins identification by Mass Spectrometry

Differentially expressed protein spots were excised from the gels, digested with trypsin and the proteolytic fragments analyzed by MALDI-TOF mass spectrometry as described in materials and methods section.

PBMCs proteins identification

We found 13 protein spots that undergoing statistically significant changes in mean intensity values between the three groups considered (Figure 9) : six spots showed OD values higher in SLE patients than in healthy subjects; five different spots increased in intensity in RA patients compared to healthy subjects.

One spot underwent similar changes in intensity both in SLE patients and in RA patients compared with healthy controls: Finally, one spot was missing only in SLE patients. (Table 2).

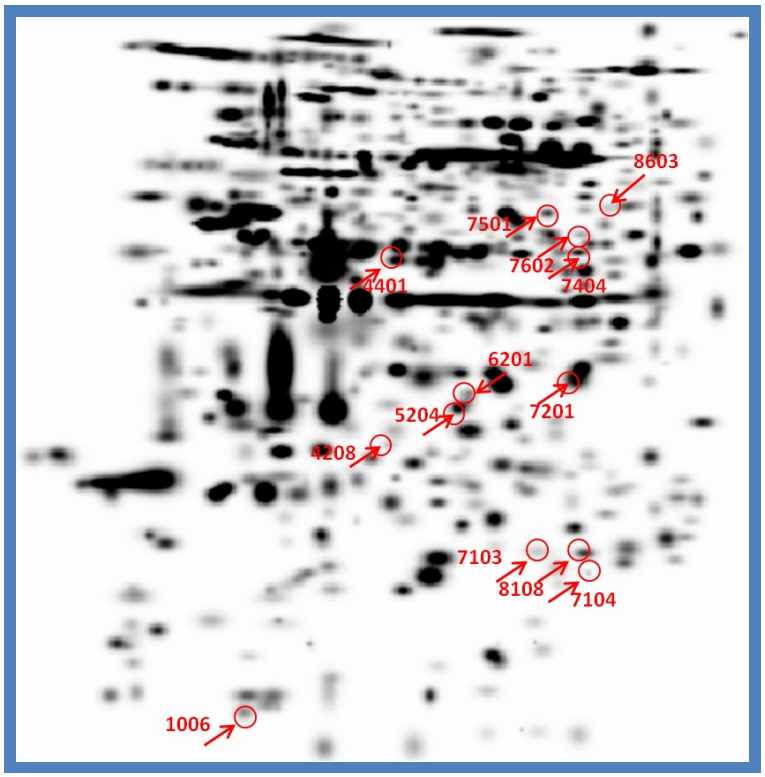


Figure 9: Differentially expressed protein spots on PBMCs gel

We observed that in SLE patients and in pathological controls the protein patterns that undergo changes compared to healthy subjects are different .

Table 2

standard spot number (SSP)	NCBInr Accession number	Protein name	Ratio	MW	pI	Mascot score	QM	%C
SLE vs HEALTHY								
Up-regulated proteins								
7104	gi|32189392	Peroxiredoxin-2	17.7	22049	5.66	86	7	39
7201	gi|48257056	TALDO1 protein, partial	4.1	37556	6.35	123	12	33
7404	gi|408535871	Chain D, Human Pkm2 With L-serine And Fbp Bound	3.37	60277	8.22	111	12	27
7501	gi|21361657	Protein disulfide-isomerase A3 precursor	2.37	57146	5.98	150	20	35
7602	gi|73535278	Chain A, Human Pyruvate Kinase M2	2.34	62570	7.01	170	20	44
8108	gi|4504183	Glutathione S-transferase P	3.38	23569	5.43	82	8	47
RA vs HEALTHY								
Up-regulated proteins								
1006	gi|119607748	hCG15971, isoform CRA_b	2.3	13907	5.24	83	6	48
4208	gi|46360168	Prohibitin	3.10	29859	5.57	224	16	53
5204	gi|4826643	Annexin A3	1.96	36524	5.63	120	15	39
6201	gi|29550921	Sulfotransferase 1A3/1A4	2.86	34288	5.68	103	13	39
7103	gi|193506758	Chain A, Crystal Structure Of E18q Dj-1	1.88	21113	6.75	98	8	57
SLE, RA vs HEALTHY								
Down-regulated proteins								
4401	gi|46593007	Cytochrome b-c1 complex subunit 1, mitochondrial	0.50	53297	5.94	135	14	30
Missing in SLE patients								
8603	gi|197692147	T-complex protein 1 subunit beta	-	57766	6.01	246	22	51

Mass spectrometric identification of spots using the Mascot search engine. In the table are reported SSP number, NCBInr accession number, protein name, their fold regulation values (ratio), molecular weight (MW) in daltons (Da), isoelectric point (pI), sequence coverage (C%) and matching peptides (QM).

The data that we consider most interesting were the absence of the spot protein T-complex protein 1 subunit beta only in SLE patients and the overexpression of peroxiredoxin 2 (fold regulation of approximately 17 times) in SLE patients than in healthy subjects (Figure 10).

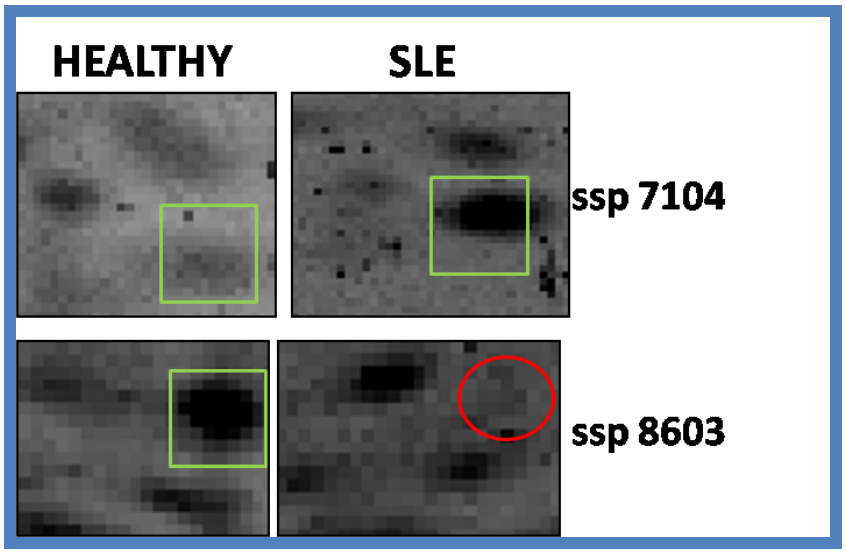


Figure 10: Zoom on the position of T-complex 1 protein spot (below in the figure) and Peroxiredoxin 2 (above in the figure).

Plasma proteins identification

Of the eight differentially expressed spots, four increased in intensity values both in SLE patients and in RA patients compared with healthy subjects (Figure 11).

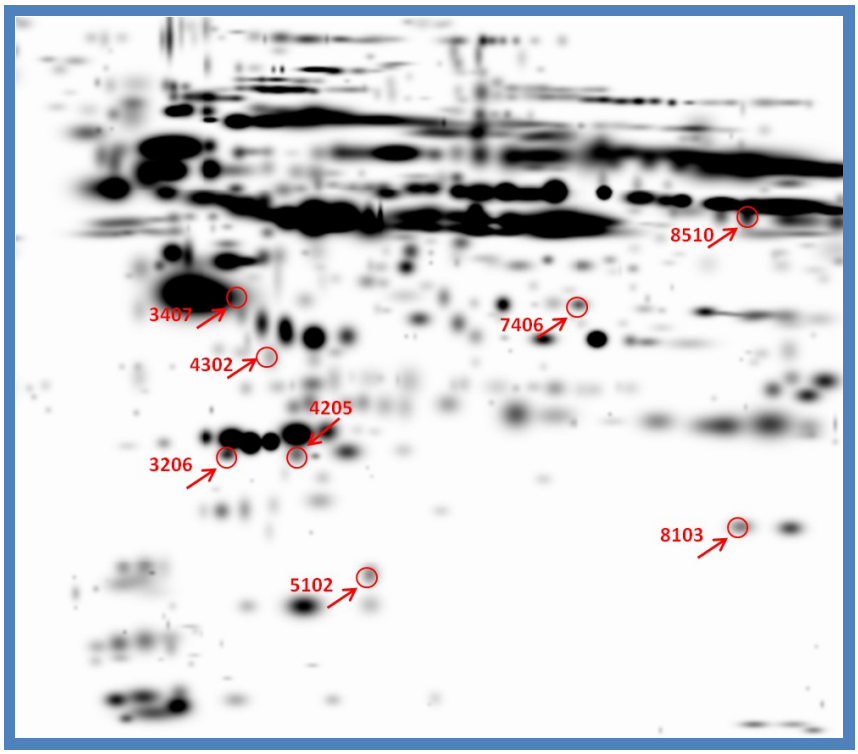


Figure 11: Differentially expressed protein spots on plasma gel

These proteins have been identified as Crystal Structure Of Lipid-Free Human Apolipoprotein A-I (two spots), apolipoprotein J precursor or clusterin, aptoglobin or HP protein, ficolin-2 isoform, Crystal Structure Of Fibrinogen Fragment D. One spot, identified as apolipoprotein E is overexpressed in RA patients compared with healthy controls; another spot identified as Chain B, Crystal Structure Of Fibrinogen Fragment D is up-regulated in SLE patients with respect to healthy subjects (Table 3).

Table 3

standard spot number (SSP)	NCBInr Accession number	Protein name	Ratio	MW	pI	Mascot score	QM	%C
SLE vs HEALTHY Up-regulated proteins								
3407	gi|178855	Apoliprotein J precursor	6.06	36184	5.04	131	15	38
4205	gi|90108664	Chain A, Crystal Structure Of Lipid-Free Human Apolipoprotein A-I	2.46	23453	5.48	187	16	53
5102	gi|78174390	HP protein	3.65	17069	5.68	95	11	34
7406	gi|61744445	ficolin-2 isoform a precursor	5.07	34436	6.31	165	11	43
8103	gi|2781208	Chain B, Crystal Structure Of Fibrinogen Fragment D	8.49	38081	5.84	122	19	41
8510	gi|2781208	Chain B, Crystal Structure Of Fibrinogen Fragment D	4.09	38081	5.84	251	26	63
RA vs HEALTHY Up-regulated proteins								
3206	gi|90108664	Chain A, Crystal Structure Of Lipid-Free Human Apolipoprotein A-I	2.03	22937	5.05	263	22	62
4205	gi|90108664	Chain A, Crystal Structure Of Lipid-Free Human Apolipoprotein A-I	2.05	23453	5.48	187	16	53
4302	gi|178853	apolipoprotein E	2.21	35320	5.35.	169	21	44
5102	gi|78174390	HP protein	3.65	17069	5.68	95	11	34
7406	gi|61744445	ficolin-2 isoform a precursor	4.25	34436	6.31	165	11	43
8103	gi|2781208	Chain B, Crystal Structure Of Fibrinogen Fragment D	5.61	38081	5.84	122	19	41

Mass spectrometric identification of spots using the Mascot search engine. In the table are reported SSP number, NCBInr accession number, protein name, their fold regulation values (ratio), molecular weight (MW) in daltons (Da), isoelectric point (pI), sequence coverage (C%) and matching peptides (QM).

Among the identified proteins, we decided to carry out further analyses only on clusterin, because it is the only protein that shows a significant increase in the mean intensity values in SLE patients compared both in healthy that pathological controls (Figure 12).

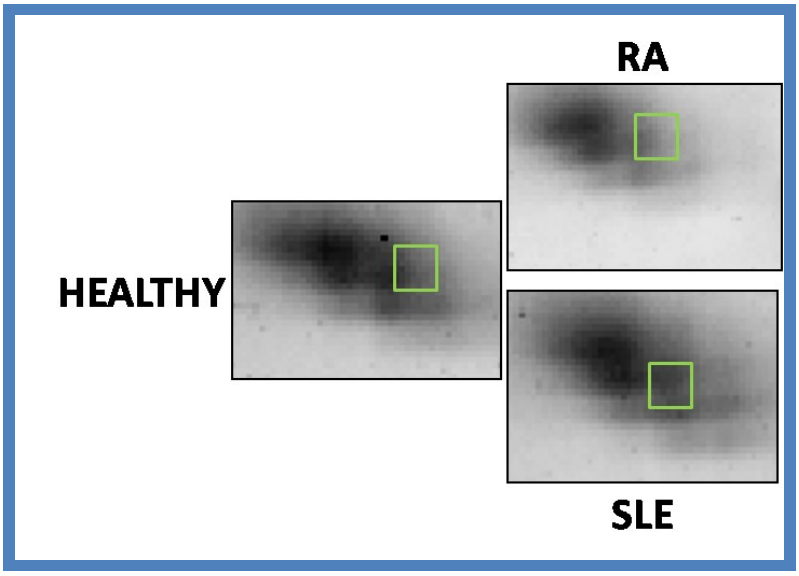


Figure 12: Zoom on the position of clusterin in Plasma gel (ssp 3407)

In Table 4 and 5 are reported the protein spots whose identifications were confirmed also with LC-MS/MS analyses

Table 4

standard spot number (SSP)	SWISS Prot Accession number	Protein name	MW in KDa	Calc pI	Mascot score	%C	Unique Peptide	Peptides	Ion Score
7104	P32119	Peroxiredoxin-2 OS=Homo sapiens GN=PRDX2 PE=1 SV=5 - [PRDX2_HUMAN]	57.8	7.20	51.98	2.06	2	2	52
7201	P37837	Transaldolase OS=Homo sapiens GN=TALDO1 PE=1 SV=2 - [TALDO_HUMAN]	37.5	6.81	209.93	21.66	7	7	73
8108	P09211	Glutathione S-transferase P OS=Homo sapiens GN=GSTP1 PE=1 SV=2 - [GSTP1_HUMAN]	23.3	5.64	230.35	21.90	5	5	92
8603	P78371	T-complex protein 1 subunit beta OS=Homo sapiens GN=CCT2 PE=1 SV=4 - [TCPB_HUMAN]	57.5	6.46	930.87	52.90	22	24	119

Mass spectrometric identification of spots using the Mascot search engine. In the table are reported SSP number, SWISS Prot accession number, protein name, molecular weight (MW) in Kilo daltons (KDa), calculated isoelectric point (Calc pI), sequence coverage (C%), and ion score.

Table 5

Standard Spot number (SSP)	SWISS Prot Accession number	Protein name	MW in KDa	Calc pI	Mascot score	%C	Unique Peptide	Peptides	Ion Score
3407	P10909	Clusterin OS=Homo sapiens GN=CLU PE=1 SV=1 - [CLUS_HUMAN]	52.5	6.27	131	24.28	12	12	61
7406	P02675	Ficolin-2 OS=Homo sapiens GN=FCN2 PE=1 SV=2 - [FCN2_HUMAN]	34	6.77	460.13	28.43	12	12	97
8103	P78371	Fibrinogen beta chain OS=Homo sapiens GN=FGB PE=1 SV=2 - [FIBB_HUMAN]	55.9	8.27	308.74	15.27	10	10	62

Mass spectrometric identification of spots using the Mascot search engine. In the table are reported SSP number, SWISS Prot accession number, protein name, molecular weight (MW) in Kilo daltons (KDa), calculated isoelectric point (Calc pI), sequence coverage (C%), and ion score.

Western blot analysis

To validate the proteomic analysis data, western blot analysis for Peroxiredoxin 2 were performed. The Figure 13 shows the bands detected by this antibody on a 1-D gel when 50 µg of total protein extracts were loaded per well.

Our data showed that expression of the Peroxiredoxin 2 (PRDX2) protein was significantly increased in the SLE PBMCs (2.4 folds) compared to healthy subjects (Figure 14, p-value= 0.025). This finding was consistent with the proteomic analysis data.

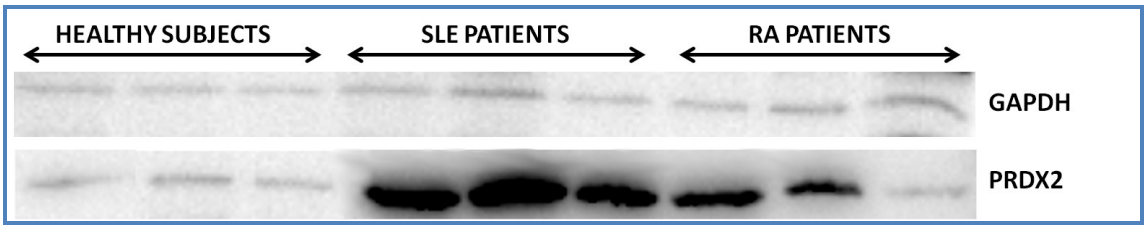


Figure 13: One-dimensional western blot analysis. PRDX2 and GAPDH immunodetection to assess PRDX2 up-expression in SLE patients.

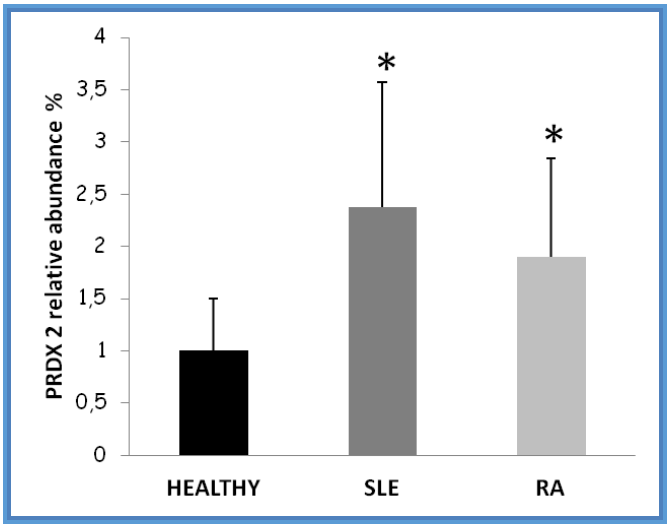


Figura 14: Bar charts reporting PRDX 2 relative abundance in Healthy, SLE and RA subjects. Following data normalization, PRDX 2 was confirmed as differentially expressed in SLE patients compared to healthy controls (p= 0.025) and also for RA patients vs healthy controls (p = 0.05).

ELISA

We wanted to quantify with a reliable technique the clusterin concentration in our plasma samples, using an available commercial kit for human clusterin.

We found significative differences between the SLE groups versus RA patients. The table below shows the ANOVA result.

Table 6: Comparison of clusterin levels among enrolled individuals.

Group	Mean (SD)	Anova	Post-hoc estimation (Sidak)
SLE	99,5 (25.3)	0,006	0.02*
RA	70.9 (32.8)		0.31**
Healthy	85.6 (28.3)		0.40***

***** SLE vs RA ** SLE vs Healthy *** RA vs Healthy

Cytokines Analysis

Table 7 shows the cytokines analysed, and figures 15-17 show the box plots for each analyte.

Cytokine levels that were not detectable were considered to be zero. To investigated the reproducibility of cytokine levels in patients with SLE, RA and healthy controls we used a two-tailed Kruskal–Wallis test. The p-values less than 0.05 were considered to be statistically significant. Table 8 reports the cytokines statistically significant with the correspondig p-values.

Table 7

Bio Plex Pro Assays		
Th17 Cytokines	**Others Cytokines**	**Chemokines**
Hu IL-1β	Hu IL-1ra	Hu IL-8
Hu IL-4	Hu IL-5	Hu MCP-1(MCAF)
Hu IL-6	Hu IL-7	Hu MIP-1α
Hu IL-10	Hu IL-9	Hu MIP-1β
Hu IL-17A	Hu IL-12(p70)	Hu IP-10
Hu IL-17F	Hu VEGF	
Hu IL-22		
Hu IL-25		
Hu IL-31		
Hu IL-33		
Hu IFN-γ		
Hu sCD40L		
Hu TNF-α		

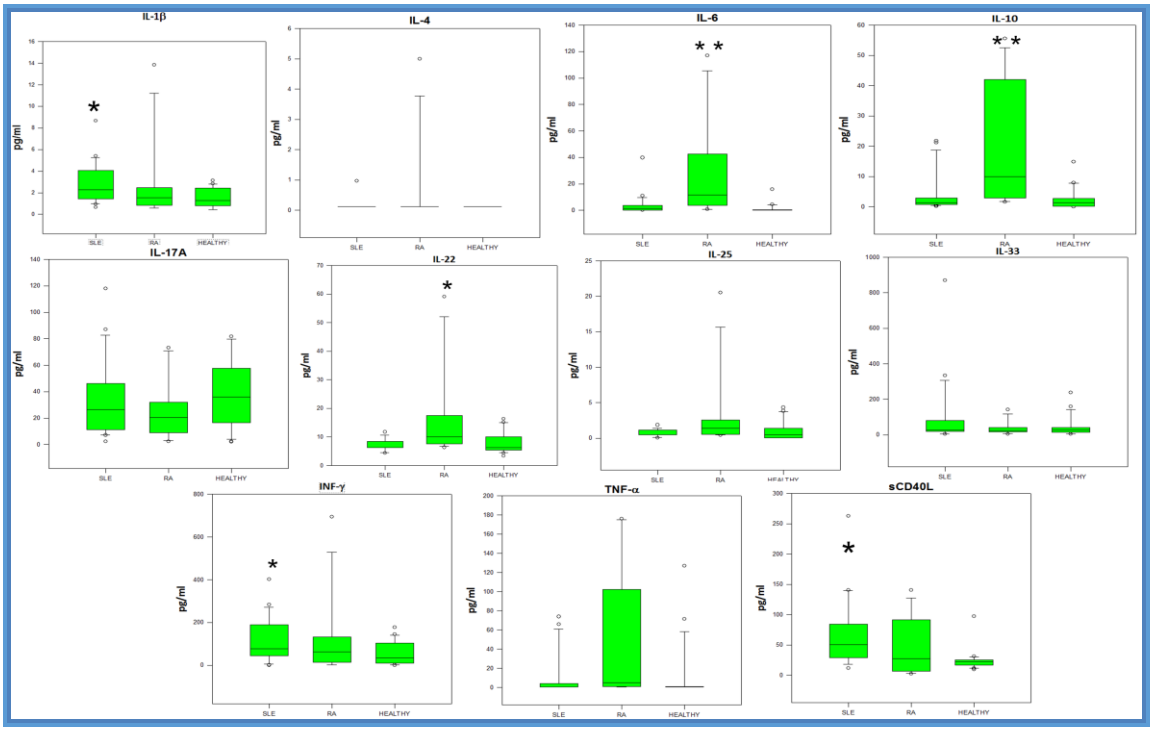


Figure 15: Levels of plasma Th17 cytokines in the different groups. Cytokines are expressed as pg/ml. The median levels in the three groups (SLE, RA, Healthy) were respectively: *IL-1β* 2.26, 1.53, 1.29; *IL-4* 0.12, 0.12, 0.12; *IL-6* 1.4, 11.46, 0.2; *IL-10* 1.38, 9.94, 1.38; *IL-17A* 26.64, 20.40, 35.87; *IL-22* 6.34, 10, 6.34; *IL-25* 0.45, 1.38, 0.45; *IL-33* 26.56, 35.17, 26.56; *IFN-γ* 76.40, 62.87 34.93; *TNF-α* 0.72, 4.97, 0.72; *sCD40L* 50.37, 27.93, 22.88; *p-value<0,05, **p-value<0,001.

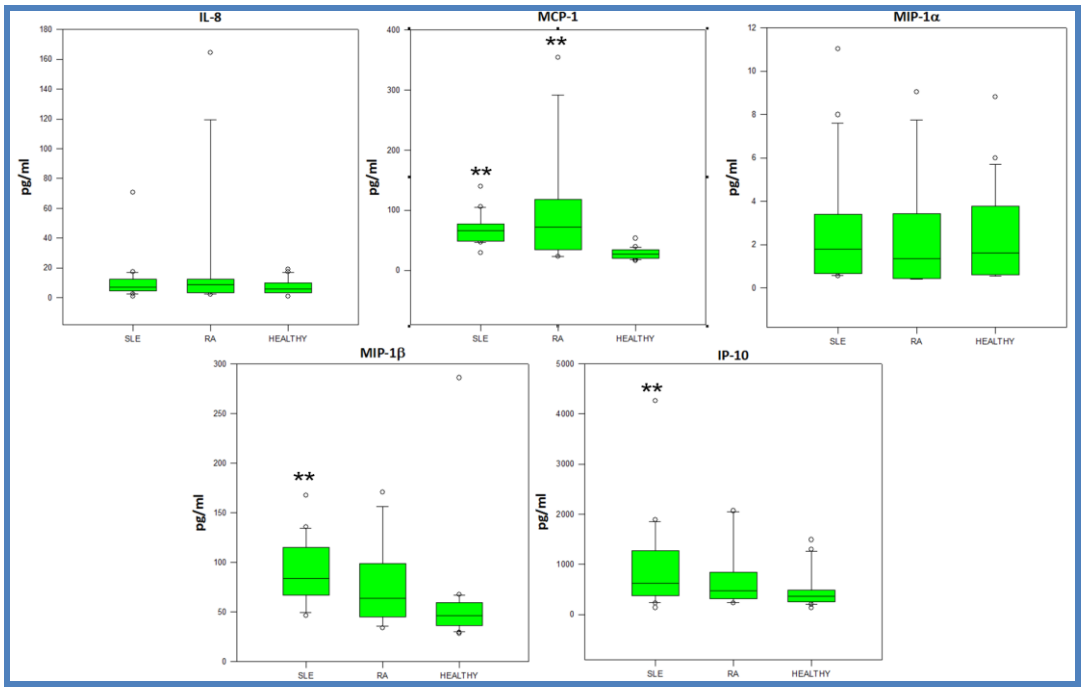


Figure 16: Levels of plasma chemokines in the different groups. Cytokines are expressed as pg/ml. The median levels in the three groups (SLE, RA, Healthy) were respectively: *IL-8* 7.32, 8.73, 5.95; *MCP-1* 66.67, 72.46, 27.39; *MIP-1α* 1.78, 1.37, 1.62; *MIP-1β* 83.78, 63.72, 46.38; *IP-10* 621.38, 476.36, 363.34. *p-value<0,05, **p-value<0,001.

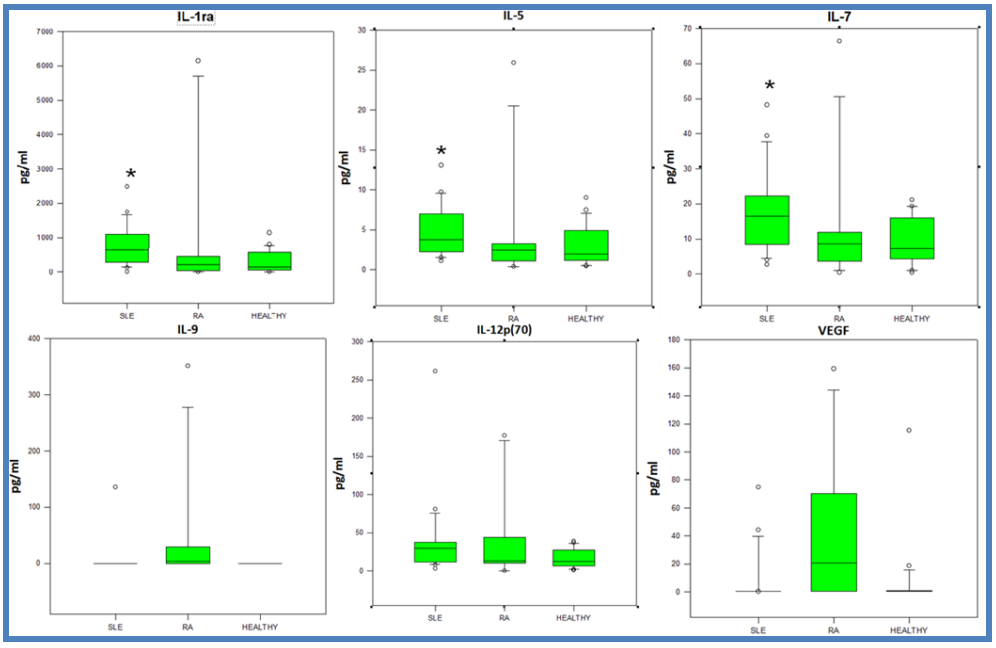


Figure 17: Levels of others cytokines in the different groups. Cytokines are expressed as pg/ml. The median levels in the three groups (SLE, RA, Healthy) were respectively: *IL-1ra* 645.91, 223.150, 150.95; *IL-5* 3.75, 2.49, 1.98, *IL-7* 16.47,8.55, 7.35; *IL-12(p70)* 29.44, 13.51, 12.19; *VEGF* 0.62, 20.785, 0.62.*p-value<0,05.

Table 8: cytokines statistically significant with the correspondig p-values

SLE vs HEALTHY	Kruskall Wallis	Post-hoc estimation (Dunn)
Hu MIP-1β	<0,001	<0,001
Hu IL-5	0,029	0,035
Hu IL-7	0,02	0,034
Hu IL-1ra	0,016	0,016
Hu IP-10	0,021	0,02
Hu IFN-γ	0,031	0,026
Hu IL-1β	0,019	0,016
Hu MCP-1(MCAF)	<0,001	<0,001
Hu-sCD40L	0,002	<0,001
RA vs HEALTHY	**Kruskall Wallis**	**Post-hoc estimation (Dunn)**
Hu IL-6	<0,001	<0,001
Hu IL-10	<0,001	<0,001
Hu IL-13	0,006	0,005
Hu MCP-1(MCAF)	<0,001	<0,001
Hu IL-22	0,002	0,005
Hu IL-25	0,014	0.027

Receiver Operating Characteristic (ROC) curve

In order to validate the diagnostic discriminating power of cytokines we performed further statistical analyses using the Receiver Operating Characteristic (ROC) curve.

This technique include sensitivity, specificity, positive predictive power, negative predictive power and accuracy. ROC analysis quantifies the accuracy of diagnostic tests or other evaluation modalities used to discriminate between two states or conditions, which are here referred to as healthy and SLE disease. The discriminatory accuracy of a diagnostic test is measured by its ability to correctly classify known normal and abnormal subjects.

ROC curve shows the characteristics of a diagnostic test by graphing the false-positive rate (1-specificity) on the x-axis and the true-positive rate (sensitivity) on the y-axis for various cutoff values. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. A test with perfect discrimination (no overlap in the two distributions) has a ROC curve that passes through the upper left corner (100% sensitivity, 100% specificity).

Accuracy is measured by the area under the ROC curve (AUC). An area of 1 (perfect diagnostic ability) represents a perfect test; an area of 0.5 (no diagnostic ability) represents a worthless test. A rough guide for classifying the accuracy of a diagnostic test is the traditional academic point system:

- AUC=0.5 test is not informative
- 0.5<AUC≤0.7 inaccurate test
- 0.7<AUC≤0.9 moderately accurate test
- 0.9 <AUC≤1 highly accurate test
- AUC=1.0 perfect test

Usually it is considered appropriate to use a diagnostic test with an area under the curve ≥ 0.8 (Figure 18).

ROC curves were constructed for all the cytokines that have undergone statistically significant changes of the concentration values among the 3 groups (Figure 19).

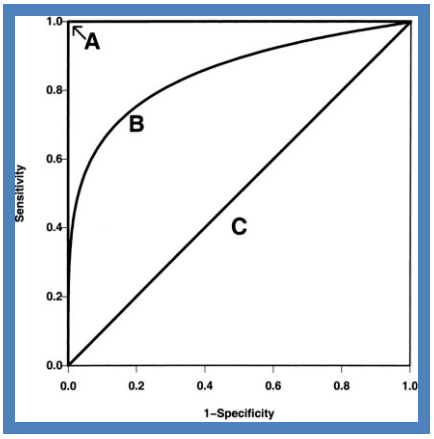


Figure 18: Example of ROC Curve; for A the AUC is 1, for B the AUC is 0,8 and for C the AUC is 0,5

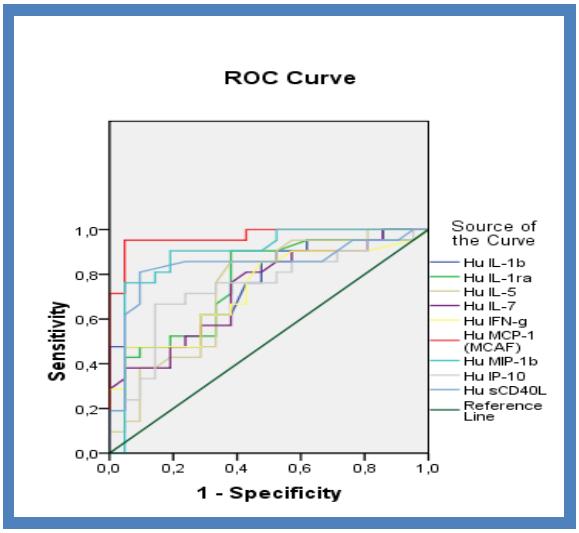
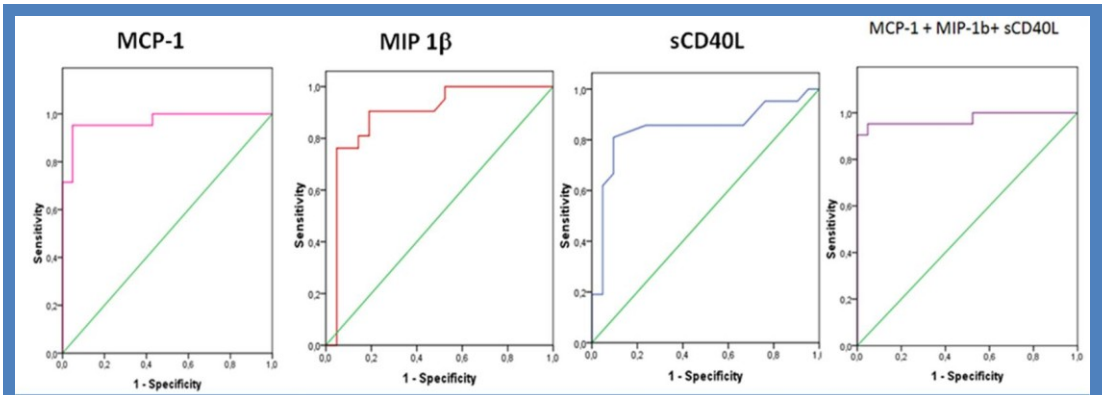


Figure 19: The ROC curves graph for nine cytokines. The AUC values are: *IL-1β*=0.761, *IL-1ra*=0.764, *IL-5*=0.735, *IL-7*=0.731, *IFN-γ*=0.734, *MCP-1*=0.968, *MIP-1β*=0.890, *IP-10*=0.744, *sCD40L*=0.842

The analyses revealed that the area under curves (AUC) of MCP-1, MIP-1b and sCD40L are greater than those of the other cytokines investigated (Figure 20).



Test	Sensitivity	1-Specificity	AUC (95% CI)	PPV	NPV	TP	FP	TN	FN
MCP-1	0,952	0,190	0,968 (0,000-1,000)	0,833	0,944	20	4	17	1
MIP-1β	0,904	0,238	0,890 (0,780-1,000)	0,792	0,889	19	5	16	2
sCD40L	0,900	0,238	0,842 (0,709-0,976)	0,782	0,895	18	5	16	2
MCP-1 + MIP-1b+ sCD40L	0,952	0,190	0,973 (0,000-1,000)	0,833	0,944	20	4	17	1

Figure 20: Test set ROC curves for SLE patients vs Healthy subjects for the panel of MCP-1, MIP-1b and sCD40L. Sensitivity=(TP/P), Specificity=(1-TN/(FP+TN)) PPR= Positive Predictive Rate (TP/TP+FN); FPR= Negative Predictive Rate (FP/FP+TN); TP=true positive; FP=false positive; TN=true negative; FN=false negative

Correlation between drugs dosage and clusterin level.

The effects of different drug treatments on the expression of the clusterin and the cytokines (with p-values ≤ 0.05) were analyzed by Spearman's rank correlation where drug doses were available, by dividing the patients into subgroups based on the presence or absence of a certain drug treatment and comparing the means of analysed parameters between the two groups (Tables 9-12).

Using these approaches, we found no evidence for any effect of different drug treatments on the analysed parameters.

Table 9: Correlation between cytokines levels and glucocorticoid drug dosage

Variables	rho	p-value
Hu IL-1β	0.800	0.333
Hu IL-1ra	0.800	0.333
Hu IL-5	0.800	0.333
Hu IL-7	1	0.083
Hu IFN-γ	0.800	0.333
Hu MCP-1(MCAF)	0.400	0.750
Hu MIP-1β	-0.800	0.333
Hu IP-10	-0.400	0.750
Hu sCD40L	-0.200	0.917

Table 10: Correlation between cytokines levels and immusuppressant drug dosage

Variables	rho	p-value
Hu IL-1β	0.800	0.333
Hu IL-1ra	0.800	0.333
Hu IL-5	0.800	0.333
Hu IL-7	0.400	0.750
Hu IFN-γ	-0.200	0.917
Hu MCP-1(MCAF)	0.400	0.750
Hu MIP-1β	0.400	0.750
Hu IP-10	-0.200	0.917
Hu sCD40L	0.200	0.917

Table 11: Correlation between glucocorticoid dosage and clusterin level

rho	p-value
0.240	0.170

Table 12: Correlation between immusuppressant dosage and clusterine level

rho	p-value
0.160	0.590

Chapter 5

DISCUSSION

In this study, first of all, a proteomic approach has been used to identify the pattern of protein expression on matrices of plasma and PBMCs in SLE patients. The proteomic comparisons were based on the differences of mean intensity values of protein spots among SLE patients, RA patients and healthy subjects. We decided to use patients with rheumatoid arthritis (another systemic autoimmune disorder) as pathological control, because the first SLE symptoms can be misdiagnosed; therefore, having a similar control can be useful to exclude alterations in common between the two syndromes.

In PBMCs, 6 proteins were upregulated (Protein disulfide-isomerase A3, Glutathione S-transferase , Peroxiredoxin-2, Transaldolase and pyruvate kinase), one was down regulated (Cytochrome b-c1 complex subunit 1) and another protein was missing (T-complex protein 1 beta subunit) in SLE patients compared to healthy subjects (Table 2).

Proteins that have undergone major changes in their expressions, especially in SLE patients, can be grouped into three functional categories: cell stress (T-complex protein 1 beta subunit, Protein disulfide-isomerase A3), redox-regulation (Cytochrome b-c1 complex subunit 1, Glutathione S-transferase p and Peroxiredoxin-2) and energy metabolism (Transaldolase and pyruvate kinase m2).

In the group of proteins associated with oxidative stress a remarkable change was found. Among them, we confirmed increased expression of PRDX2 with western blot analysis.

Glutathione S-transferases (GSTs) are a family of Phase II detoxification enzymes that work to protect cellular macromolecules from attack by reactive electrophiles. Specifically, GSTs catalyse the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds. Glutathione conjugation is the first step in the mercapturic acid pathway that leads to the elimination of toxic compounds. [53]

While the ligand-binding function remains unclear, sequestering molecules may serve a regulatory role, preventing cytotoxic ligands from interacting with their targets. Recent studies have demonstrated a regulatory role for the p and m classes of GSTs in the mitogen-activated protein (MAP) kinase pathway that participate in cellular survival and death signalling. Specifically, Glutathione S-transferases isoform p (GST) was shown to be an endogenous inhibitor of c-Jun N-terminal kinase 1 (JNK1), a kinase involved in stress response, apoptosis, and cellular proliferation [54,55].

In chronic lymphocytic leukemia patients, GST levels were elevated in individuals responding to chlorambucil and corticosteroids, and this expression was further enhanced in patients who had received multiple rounds of therapy and whose disease was classified as resistant to chlorambucil/steroid treatment [56]. Y Dai et al.[36] showed that GSTp expression in SLE patients was downregulated.

The protein disulfide isomerase A3 (PDIA3), also known as Erp57, acts as a chaperone into the endoplasmatic reticulum (ER). The expression level of PDIA3 increases in response to cellular stress due to its function. [57,58]. Lately a study, showed that PDIA3 had an antiapoptotic effect in the melanoma cell line A375 after ER stress was induced [59]. PDIA3 probably plays a role in the malignant transformation of prostate and cervical cancer [60,61]. The expression of this gene is induced during neoplastic transformation, possibly leading to redox-dependent modulation of cancer-relevant regulatory factors [62,63]. In pterygium the increase in PDIA3 protein expression is most likely due to elevated cellular stress [64]. In addition, PDIA3 has recently gained attention due to its function as a component of the peptide-loading complex of the major histocompatibility complex (MHC) class I pathway [65]. In PDIA3-deficient mice, this complex is impaired and negatively influences presentation of antigenic peptides [66].

Cytochrome b-c1 complex subunit 1 is a component of the ubiquinol-cytochrome c reductase complex (complex III or cytochrome b-c1 complex), which is

part of the mitochondrial respiratory chain. This protein may mediate formation of the complex between cytochromes c and c1.

Transaldolase 1 (TALDO 1) is a key enzyme of the nonoxidative pentose phosphate pathway providing ribose-5-phosphate for nucleic acid synthesis and NADPH for lipid biosynthesis. This pathway can also maintain glutathione at a reduced state and thus protect sulfhydryl groups and cellular integrity from oxygen radicals.

Recently, it was proved that TALDO 1 is involved in different autoimmune diseases (MS and RA) and other malignancies. In MS patients, recombinant TALDO1 stimulates the aggregate formation and proliferation of T lymphocytes [67,68]. Peptides of TALDO1 bind to MHC of T cells and induce cytotoxic T cell response in MS patients [69].

In colorectal cancer, by proteomic approaches, Ma et al showed that TALDO 1 was overexpressed. [70]. Previous studies showed that TALDO-deficient mice exhibited oxidative stress and mitochondrial dysfunction in hepatocytes [71].

The pyruvate kinase M2 isoform (PKM2) is an enzyme that catalyses the transphosphorylation from phosphoenolpyruvate to ADP as the last step of glycolysis to generate ATP.

PKM2 is a ubiquitous prototype enzyme present in all tissues during the embryonic stage and it is gradually replaced by other isozymic forms in specific tissues during development. PKM2 is also involved in many other nonglycolytic functions; in fact, PKM2 interacts with a variety of biological molecules, such as molecules involved in intracellular membrane trafficking, signaling and transcription factors and it is also known to interact with pathogenic proteins [72]. In a recent report, PKM2 is found to be highly immunomodulatory by interacting with SOCS3 (suppressor of cytokines signaling 3) and during interaction with APC (depending upon CD28 receptor on surface) [73,74]. PKM2 is shown to interact with IgE receptor on the cells, resulting in the inhibition of its activity [75].

T-complex protein 1 is a molecular chaperone; assists the folding of proteins upon ATP hydrolysis. As part of the BBS/CCT complex, it may plays a role in the assem-

bly of BBSome, a complex involved in ciliogenesis regulating transports vesicles to the cilia. T-complex protein 1 plays a role, in vitro, in the folding of actin and tubulin [76]. In our study, we found that the expression of the beta subunit of T-complex protein 1, was abolished in SLE patients. Interestingly, mutations of this protein have been shown to be associated with cytoskeletal disorganization and increased sensitivity to microtubule destabilizing agents.

The peroxiredoxin 2 (PRDX2) is a cytoplasmic enzyme that reduces intracellular reactive oxygen species (ROS) levels. In cardiomyocytes, PRDX2 overexpression protects from peroxide-induced apoptosis and necrosis, while downregulation of this enzyme promotes injurious effects of oxidative stress [77]. The antiapoptotic role of this enzyme has been established in red blood cells as well as in pancreatic cells [78,79].
In our study, the intracellular levels of PRDX2 protein in PBMCs were greatly increased in SLE patients compared with healthy subjects (2.4 folds). This fact suggests the possibility that PRDX2 may be elevated as part of the inflammatory response in SLE PBMCs [80]. PRDX2 is a redox enzyme with extensive roles in immune regulation: Moon et al showed [81] that in mice, PRDX2 inhibits immune cell responses by scavenging ROS, and Yang et al [82] showed that intracellular PRDX2 is a negative regulator of the pro-inflammatory toll-like receptor 4 (TLR4) signaling pathway. PRDX2 is also involved in the pathway of T cell receptor signaling by consuming H_2O_2 [83]. Moreover, PRDX2 is an inhibitor of apoptosis [84]. It is unclear whether the observed increase in the intracellular PRDX2 content of the PBMCs from SLE patients plays a protective or pathological role. It is probable that in SLE, PRDX2 and its regulation of ROS are critical in controlling T cell responses.

In plasma samples, protein expression patterns of SLE patients and the pathological controls with RA were similar; in fact, of the eight differentially expressed spots between patients and controls, four spots increased in intensity both in SLE and in RA patients. These proteins belong to the Apolipoprotein AI/A4/E, Clusterin, Peptidase S1, Ficolin 2, Fibrinogen alpha/beta families. The only proteins differentially expressed be-

tween SLE and RA patients that change compared to healthy subjects are Clusterin (SLE vs Healthy) and Apolipoprotein E (RA vs Healthy).

Our results show that in SLE patients five protein spots are differentially expressed: Apolipoprotein A1, Haptoglobin, Fibrinogen, Ficolin 2 and Clusterin (Table 3). Among all, Clusterin expression was validated by ELISA.

Apolipoprotein A1 (APO A1) is a protein that, in humans, is encoded by the APOA1 gene. Apo A1 is a major constituent of the high-density lipoprotein (HDL) complex and has a specific role in lipid metabolism; furthermore, apo A1 has anti-inflammatory properties both in acute and chronic inflammation . This protein promotes cholesterol efflux from tissues to the liver for excretion and it is a cofactor of lecithin cholesterol-acyltransferase (LCAT) which is responsible for the formation of most plasma cholesteryl esters [85]. In some autoimmune diseases, apo A-I is considered to be an immune regulator and can suppress pro-inflammatory cytokines generated by activated T cell [86]. Malle et al [87] demonstrated an enhanced intracellular biosynthesis and secretion of apolipoproteins SAA and A-I in HUH-7 cells in response to the agonists IL-1α, IL-6, butyrate and dexamethasone, alone or in combination. García-Gómez et al [88] found that a low dose of glucocorticoids, in RA patients, was associated with an increase in HDL-c and HDL2-c levels, without significantly affecting other lipoprotein fractions; in fact, no change was detected in the major protein constituent of HDL, APO A-I.

Some researchers believe that serum apoA-I concentrations should be declined during active phase of autoimmune diseasesand this protein has played an important role in anti-inflammation, such as RA and SLE [89-91]. Our results, contrary to the hypothesis of above mentioned studies, showed an increase in plasma levels of the protein in RA and SLE patients rather than in healthy subjects. This difference could be due to the fact that all tested patients showed an inactive phase of the disease.

Haptoglobin (Hp) is a positive member of the acute phase proteins (APPs) and it is mainly synthesized in the liver; the protein is responsible for transporting hemoglo-

bin. Its synthesis is induced by IL-6, IL-1β and TNF and exerts antioxidant properties. It also plays an important role in maintaining homeostasis and in the damaged tissues repair mechanisms. Hp also has anti-inflammatory effects in autoimmune diseases [92]. The HP22 genotype was shown to be over-represented in inflammatory diseases such as RA, SLE, primary sclerosing cholangitis (PSC) and diabetes mellitus type 2 (T2D)[93,94]. In general, serum levels of Hp increase during inflammatory processes [95] or glucocorticoids admnistration [96].

Plasma fibrinogen is an important component of the coagulation cascade, as well as a major determinant of blood viscosity and blood flow. Fibrinogen has a biological half-life of about 100 h and it is synthesized predominantly in the liver. As a clotting factor, fibrinogen is an essential component of the blood coagulation system, being the precursor of fibrin. Plasma fibrinogen is an acute-phase protein, and it is therefore likely to increase with inflammation or tissue necrosis. Interpretation of raised fibrinogen may be complicated by its behavior as an acute phase reactant [97]. Impaired fibrinolysis has been reported in patients with SLE and may contribute to both the development of hypercoagulability and an increased risk of thrombosis [98]. In their study, Spengler et al [99] showed an increase of immunoglobulin and plasma fibrinogen, which induced red blood cells of SLE patients to agggregate at higher rate, with aggregates greater size rather than healthy subjects. Epidemiological studies suggest that elevated plasma fibrinogen levels are associated with an increased risk of cardiovascular disorders, such as stroke, ischemic heart disease and other thromboembolism [100-101].

Ficolin-2 or L-Ficolin is a serum molecule, structurally and functionally related to collectins. It is able to opsonize infecting microorganisms and to activate the lectin pathway of complement in cooperation with mannan-binding lectin (MBL)-associated serine proteases (MASPs) . Recently, Ficolin -2 has been the subject of several disease association studies, providing evidence that ficolin-2 complements MBL is an impor-

tant component of innate immunity in the circulation. In healthy subjects the distribution of serum ficolin 2 is perfectly Gaussian [102].

Conversely, Watanabe et al [103] observed that serum levels of L-ficolin in patients with SLE were significantly lower when compared with those in healthy subjects. Our results are in agreement with Østergaard et al [104] because they showed an increase in plasma levels of ficolin-2 in SLE patients compared to healthy subjects.

In a recent study [105], the serum levels of Ficolin-2 were found to be significantly higher in RA patients than in healthy subjects, which is also consistent with our results.

Clusterin (CLU) is a glycoprotein with a nearly ubiquitous tissue distribution that has been reported to be implicated in several physiological processes such as lipid transportation, complement inhibition, tissue remodelling, membrane recycling, cell-cell interaction and promotion or inhibition of apoptosis.

CLU may have a protective role against disease activity in SLE, since disturbances in apoptosis and complement function play an important part in the pathogenesis of SLE [106,107]. Newkirk MM et al [108] showed an inverse correlation between plasma and/or serum CLU and the activity of disease. In our study, we found an increase of Clusterin plasma levels in SLE patients compared with healthy subject, and this might be related to the fact that the patients were in a stable phase of the disease.

As a second aim of this study, we compared the expression pattern of cytokines of SLE and RA patients with healthy subject. Numerous studies suggest the importance of monitoring plasma levels of cytokines in patients with various autoimmune diseases and point out, in particular, the crucial role of cytokines in the pathogenesis of SLE.

All the cytokines described in literature are molecules potentially involved in the pathogenesis of SLE. Cytokines are soluble factors that act as mediators for the differentiation, maturation and activation of the various immune cells. Altered levels of cytokines in the plasma would be a major cause of an immune dysregulation followed by local inflammatory processes and tissue damage. Observing the plasma cytochines

variations, may help us to understand SLE mechanisms as well as to find new ideas for designing biomarkers and therapeutic agents.

We decided to observe changes in the expression of cytokines produced by Th1, Th2 and Th17, and some chemokines. This analysis showed that some cytokine plasma concentrations (Table 8) were significantly higher in SLE patients than in healthy subjects (IL-1β 1.29, 2.26 pg/ml, IFN-γ 34.93, 76.40 pg/ml, IP-10 363.34, 621.38 pg/ml, IL-1ra 150.95, 645.91 pg/ml, IL-5 1.98, 3.75 pg/ml, IL-7 7.35, 16.47 pg/ml, MCP-1 27.39, 66.67 pg/ml, MIP-1β 46.38, 83.78 pg/ml, sCD40L 22.88, 50.37 pg/ml; in Healthy and SLE respectively).

In order to validate any diagnostic power of the cytokines, we have carried out the ROC curves analyses. Our results suggests that the combination of MCP1, MIP-1β and sCD40L cytokines might be used as potential biomarkers (AUC=0.973).

The higher plasma levels of cytokines and chemokines that we found in SLE patients are in agreement with some reports [109,110]. For example, Eriksson et al. [109] found higher plasma levels of MIP-1β, MCP-1, and higher serum levels of RANTES in SLE patients when compared with healthy individuals. In contrast, Kaneko et al. [111] did not find differences in serum concentrations of MIP-1β between SLE patients and healthy subjects. Narumi et al [112] showed that serum levels of IP 10 increased in SLE patients and are strongly correlated with the disease activity. Another study showed that plasma concentrations of IP10 and MCP-1 were increased in SLE patients. They also showed a strong positive correlation between IP-10 levels and SLE disease activity and suggested a possible correlation between SLE renal involvement and IP-10 [113].
In some studies it has been observed an increase in the serum concentration of IFN–γ, and that its serum levels are correlated with the disease activity [114,115].

The major functions of IL-1β, is to act as mediators of the host inflammatory reaction to infections (116). The principle source of IL-1β is the activated macrophage. At higher concentrations, IL-1 exerts endocrine effects, inducing fever and acute-phase proteins. Of the IL-1 family members, IL-1R antagonist (IL-1Ra) have been the most

studied in autoimmune diseases. Cigni et al (117) also showed IL-1 serum levels higher in SLE group than in the control group

sCD40L is considered a proinflammatory and prothrombotic cytokine in cardiovascular diseases [118,119]. The major sources of the soluble form of sCD40L are activated CD4 lymphocytes [120] and activated platelets [121]. Increased levels of sCD40L have been found in SLE [118,122], RA [123], and systemic sclerosis [124]. Increased levels of sCD40L have been correlated with the disease activity in SLE [120] and many studies have documented the effectiveness of anti-CD40L therapy in lupus nephritis at many murine models [125,126].

IL-7 is a 25-kd glycoprotein and was first isolated based on its inductive effect to differentiate immature B cell that depends on IL-7 in the program of its development [127]. This cytokine is mainly secreted by stroma cells in primary lymphoid tissues and organs including bone marrow and thymus [128,129]. The proliferation of T cell containing autoreactive T cell is regulated by IL-7, which may probably be related with the increase of self-antigen [130]. Ben-David et al. have found that IL-7 is over-expressed in SLE afflicted mice [131]. Furthermore, a recent study has also revealed that SLE patients have a high level of sIL-7R in sera, which is associated with SLE disease activity index scores, and the serological level of sIL-7R is reduced after immunosuppressive therapy, a main and effective treatment for SLE currently [132].

IL 5 is a cytokine that acts as a growth and differentiation factor for both B cells and eosinophils. The increased production of this cytokine may be related to pathogenesis of eosinophil-dependent inflammatory diseases. In one study unusual IL-5 overproduction resulted in SLE patients with eosinophilia [133].

Several studies showed that IL-6 and TNF-α plasma levels are higher in SLE patients. In our study the plasma levels of these cytokines were as low as in healthy subjects; the lower levels of secreted TNF-α and IL-6 observed in our SLE patients, might be explained partly by the medication that our patients received. In fact, glucocorticosteroids can affect the expression of several cytokines [134,135]. This in-

formation is important for an appreciation of our results, since all patients were receiving low doses of prednisone. Brink et al. reported that even low doses of steroids can inhibit cytokine synthesis in patients with SLE [136]. In another study, Swaak et al. observed that patients with SLE taking prednisone at a dose of 15 mg/day showed a reduction in ''ex-vivo'' production of IL-6 and TNF-α [137].

Conclusions

In summary, a comparative proteomic study was performed to analyse the differential protein expression in the human PBMCs and plasma of SLE patients and healthy subjects.

We have identified the significant protein spots using MALDI-TOF and LC-MS/MS analyses, and confirmed by Western blotting and ELISA analyses.

Several proteins were differentially expressed in the PBMCs from SLE patients. Among these, PRDX2 may be used as candidate biomarker or target protein for further investigations. In plasma, we showed that plasma clusterin levels increased, but this increase is not statistically significant.

These proteomic results provide suggestions for understanding the molecular mechanisms of SLE, as well as the physiological changes correlated with SLE disease.

From cytokines analyses, we attributed to MCP-1, MIP-1β and sCD40L a hypothetical role as biomarkers with diagnostic performance optimized with two different statistical methods. These results suggest the potential usefulness of these cytokines in SLE as potential biomarkers, able to discriminate with a significant diagnostic performance.

Further studies are required to confirm the results presented. SLE patients should be expanded in order to have the possibility to analyse data both from treated patients and from patients in different phases of disease.

Chapter 6

REFERENCES

1. Smith, D A, Germolec, D R.(1999). *Introduction to Immunology and Autoimmunity*. Environmental Health Perspectives. 107(5): 661-665.
2. Ray S, Sonthalia N, Kundu S, Ganguly S. (2012). Autoimmune Disorders: An Overview of Molecular and Cellular Basis in Today's Perspective, J Clin Cell Immunol, S10
3. Goris A, Liston A. (2012). The immunogenetic architecture of autoimmune disease. Cold Spring Harb Perspect Biol. Mar 1;4(3). pii: a007260.
4. Alarcon-Riquelme ME. (2007). Recent advances in the genetics of autoimmune diseases. Ann N Y Acad Sci;1110:1–9.
5. Encinas JA, Kuchroo VK. (2000). Mapping and identification of autoimmunity genes. Curr Opin Immunol; Dec; 12:691-7
6. Reed AM, Yetterberg, SR (2002). Genetic and environmental risk factors for idiopathic inflammatory myopathies. Rheumatic disease clinics of North America. Understanding Autoimmune Disease – a review article for the layman 28: 891-916.
7. Dooley MA, Hogan SL. (2003). Environmental epidemiology and risk factors for autoimmune disease. Curr Opin Rheumatol 15: 99-103
8. Antonov D, Kazandjieva J, Etugov D, Gospodinov D, Tsankov N. (2004) Drug-induced lupus erythematosus. Clin Dermatol. Mar-Apr;22(2):157-66.
9. DeLisa Fairweather. (2007) Autoimmune Disease: Mechanisms, Johns Hopkins University, Bloomberg School of Public Health, Baltimore, Maryland, USA
10. Reeves WH, Lee PY, Weinstein JS, Satoh M, Lu L. (2009). Induction of autoimmunity by pristane and other naturally occurring hydrocarbons. Trends Immunol. Sep;30(9):455-64.
11. Ahmed SA, Hissong BD, Verthelyi D, Donner K, Becker K, Karpuzoglu-Sahin E. (1999) Gender and risk of autoimmune diseases: possible role of estrogenic compounds. Environ Health Perspect;107 (Suppl 5):681–6
12. Herrera BM, Cader MZ, Dyment DA, Bell JT, Deluca GC, Willer CJ, Lincoln M R, Ramagopalan S V, Chao M, Orton S M, Sadovnick A D, Ebers G C. (2007). Multiple sclerosis susceptibility and the X chromosome. Mult Scler;13:856–64.
13. Yin X, Latif R, Tomer Y, Davies TF. (2007). Thyroid epigenetics: X chromosome inactivation in patients with autoimmune thyroid disease. Ann N Y Acad Sci;1110:193–200.
14. Hewagama A, Richardson B. (2009). The genetics and epigenetics of autoimmune diseases. J Autoimmun;33:3e 11.
15. Rahman A, Isenberg DA. (2008). Systemic lupus erythematosus. N Engl J Med; 358(9):929-39.
16. http://www.reumatologiaonline.com/main/medici/les.php
17. Gaubitz M. (2006). Epidemiology of connective tissue disorders. Rheumatology (Oxford); 45 Suppl 3:iii3-iii4.
18. Matucci Ceneric M, Romagnani S, Consiglio Sanitario Regionale Regione Toscana Linee guida sulla diagnosi e cura di patologie di interesse immuno-reumatologico.
19. Simard JF, Costenbader KH. (2007) What can epidemiology tell us about systemic lupus erythematosus? Int J Clin Pract. Jul;61(7):1170-80.

20. Deng Y , Tsao BP. (2010). Genetic susceptibility to systemic lupus erythematosus in the genomic era. Nat Rev Rheumatol. Dec;6(12):683-92

21. Jonsen A, Bengtsson AA, Nived O, Truedsson L, Sturfelt G. (2007). Gene-environment interactions in the aetiology of systemic lupus erythematosus. Autoimmunity; 40(8):613-7.

22. Tenbrock K, Juang YT, Leukert N, Roth J, Tsokos GC. (2006). The transcriptional repressor cAMP response element modulator alpha interacts with histone deacetylase 1 to repress promoter activity. J Immunol;177:6159-64.

23. Jonsen A, Bengtsson A A, Nived O, Truedsson L, Sturfelt G. (2007) Gene-environment interactions in the aetiology of systemic lupus erythematosus. Autoimmunity ; 40(8):613-7.27

24. Sutcliffe N, Clarke A E, Gordon C, Farewell V, Isenberg DA. (1999) The association of socioeconomic status, race, psychosocial factors and outcome in patients with systemic lupus erythematosus. Rheumatology;38:1130–7 28

25. Petri M, Kim MY, Kalunian KC, Grossman J, Hahn BH, Sammaritano LR, Lockshin M, Merrill JT, Belmont H M, Askanase A D, McCune W J,Hearth-Holmes M, Dooley MA, Von Feldt J, Friedman A, Tan M, Davis J, Cronin M, Diamond B, Mackay M, Sigler L, Fillius M, Rupel A, Licciardi F, Buyon JP. OC-SELENA Trial. (2005) Combined oral contraceptives in women with systemic lupus erythematosus. N. Engl. J. Med., 353, 2550–2558

26. Buyon JP, Petri M A, Kim MY, Kalunian KC, Grossman J, Hahn B H, Merrill JT, Sammaritano L, Lockshin M, Alarcón GS, Manzi S, Belmont HM, Askanase AD, Sigler L, Dooley MA, Von Feldt J, McCune WJ, Friedman A, Wachs J, Cronin M, Hearth-Holmes M, Tan M, Licciardi F. (2005). The effect of combined estrogen and progesterone hormone replacement therapy on disease activity in systemic lupus erythematosus: A randomized trial. Ann. Intern. Med., 142, 953–962.

27. Doria A, Cutolo M, Ghirardello A, Zampieri S, Vescovi F, Sulli A, Giusti M, Piccoli A, Grella P, Gambari PF. (2002). Steroid hormones and disease activity during pregnancy in systemic lupus erythematosus. Arthritis Rheum 2002;47:202-9.

28. Bertsias G, Carvera R, Boumpas TD. (2012). Systemic lupus erythematosus: pathogenesis and clinical features. W: Bijlsma JWJ . red. EULAR Textbook of rheumatic diseases, first edition. London: BMJ Group;. s. 476–505.

29. Choi J, Kim S T, Craft J. (2012) The pathogenesis of systemic lupus erythematosus-an update. Curr Opin Immunol 24 (6):651-657

30. Kimberly R P , Salmon J E, Edberg J C, Gibofsky A. (1989) The role of Fc gamma receptors in mononuclear phagocyte system function. Clin Exp Rheumatol. Sep-Oct;7 Suppl 3:S103-8.

31. Yu C, Gershwin M E, Chang C. (2014) Diagnostic criteria for systemic lupus erythematosus: a critical review. J Autoimmun. Feb-Mar;48-49:10-3

32. Tsokos GC. (2011) Systemic lupus erythematosus. N Engl J Med. Dec 1;365(22):2110-21.

33. Pavón EJ , Muñoz P, Lario A, Longobardo V, Carrascal M, Abián J, Martin AB, Arias SA, Callejas-Rubio JL, Sola R, Navarro-Pelayo F, Raya-Alvarez E, Ortego-Centeno N, Zubiaur M, Sancho J. (2006) Proteomic analysis of plasma from patients with systemic lupus erythematosus: increased presence of haptoglobin alpha2 polypeptide chains over the alpha1 isoforms. Proteomics. Apr;6 Suppl 1:S282-92.

34. Schulz M, Dotzlaw H, Mikkat S, Eggert M, Neeck G. (2007). Proteomic analysis of peripheral blood mononuclear cells: selective protein processing observed in patients with rheumatoid arthritis. J Proteome Res. 2007 Sep;6(9):3752-9.

35. Kim WK , Cho HJ, Ryu SI, Hwang HR, Kim DH, Ryu HY, Chung JW, Kim TY, Park BC, Bae KH, Ko Y, Lee SC. (2008) Comparative proteomic analysis of peripheral blood mononuclear cells from atopic dermatitis patients and healthy donors. BMB Rep. 41(8):597-603.

36. Dai Y, Hu C, Huang Y, Huang H, Liu J, Lv T. (2008) A proteomic study of peripheral blood mononuclear cells in systemic lupus erythematosus. Lupus. Sep;17(9):799-804.

37. Zhang L, Jia X, Zhang X, Sun J, Peng X, Qi T, Ma F, Yin L, Yao Y, Qiu C, Lu H. (2010) Proteomic analysis of PBMCs: characterization of potential HIV-associated proteins. Proteome Sci. Mar 12;8:12.

38. Rana A, Minz RW, Aggarwal R, Sharma S, Pasricha N, Anand S, Singh S. (2012). A comparative proteomic study of sera in paediatric systemic lupus erythematosus patients and in healthy controls using MALDI-TOF-TOF and LC MS-A pilot study. Pediatr Rheumatol Online J. 17;10(1):24.

39. Wang L, Dai Y, Qi S, Sun B, Wen J, Zhang L, Tu Z. (2012) Comparative proteome analysis of peripheral blood mononuclear cells in systemic lupus erythematosus with iTRAQ quantitative proteomics. Rheumatol Int. Mar;32(3):585-93.

40. Maes E, Landuyt B, Mertens I, Schoofs L. (2013) Interindividual variation in the proteome of human peripheral blood mononuclear cells. PLoS One. Apr 11;8(4):e61933.

41. de Roos B. (2008) Proteomic analysis of human plasma and blood cells in nutritional studies: development of biomarkers to aid disease prevention. Expert Rev Proteomics. Dec;5(6):819-26.

42. de Roos B, Duthie SJ, Polley AC, Mulholland F, Bouwman FG, Heim C, Rucklidge GJ, Johnson IT, Mariman EC, Daniel H, Elliott RM. (2008) Proteomic methodological recommendations for studies involving human plasma, platelets, and peripheral blood mononuclear cells. J Proteome Res. 2008 Jun;7(6):2280-90.

43. Abul K. Abbas AK, Lichtman AH, Pillai S (2012) Immunologia cellulare e molecolare. Masson-Edra editore

44. Davis LS,Hutcheson J,Mohan C. (2011)The role of cytokines in the pathogenesis and treatment of systemic lupus erythematosus. J Interferon Cytokine Res. Oct;31(10):781-9.

45. Vilá LM, Molina MJ, Mayor AM, Cruz JJ, Ríos-Olivares E, Ríos Z. (2007)Association of serum MIP-1alpha, MIP-1beta, and RANTES with clinical manifestations, disease activity, and damage accrual in systemic lupus erythematosus. Clin Rheumatol. 2007 May;26(5):718-22.

46. Lit LCW, Wong CK, Tam LS, Li EKM, Lam CWK (2005) Raised plasma concentration and ex vivo production of inflammatory chemokines in patients with systemic lupus erythematosus. Ann Rheum Dis 65:209–215

47. Taylor PC. (2003) Anti-cytokines and cytokines in the treatment of rheumatoid arthritis. Curr Pharm Des.;9(14):1095-106

48. Cui D, Huang G, Yang D, Huang B, An B (2013) Efficacy and safety of interferon-gamma-targeted therapy in Crohn's disease: a systematic review and meta-analysis of randomized controlled trials. Clin Res Hepatol Gastroenterol. Nov;37(5):507-13

49. Deiana L, Ferrucci L, Pes GM, Carru C, Delitala G, Ganau A, Mariotti S, Nieddu A, Pettinato S, Putzu P, Franceschi C, Baggio G. (1999) AKEntAnnos. The Sardinia Study of Extreme Longevity. Aging (Milano). Jun;11(3):142-9.

50. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS. (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis and Rheumatism.;31:315–324.

51. Pasella S, Baralla A, Canu E, Pinna S, Vaupel J, Deiana M, Franceschi C, Baggio G, Zinellu A, Sotgia S, Castaldo G, Carru C, Deiana L. (2013). Pre-analytical stability of the plasma proteomes based on the storage temperature. Proteome Sci. Mar 21;11(1):10. doi: 10.1186/1477-5956-11-10.

52. Weinert BT, Timiras PS. (2003). Invited review: Theories of aging. J Appl Physiol. 95, 1706-1716.

53. Townsend DM, Tew KD. (2003). The role of glutathione-S-transferase in anti-cancer drug resistance Oncogene (2003) 22, 7369–7375. doi:10.1038/sj.onc.1206940

54. Adler V, Yin Z, Fuchs SY, Benerza M, Rosario L, Tew KD, Pincus MR, Sardana M, Henderson CJ, Wolf CR, Davis RJ and Ronai Z. (1999). Regulation of JNK signaling by GSTp. EMBO J., 18, 1321–1334.

55. Yin Z, Ivanov V, Habelhah H, Tew KD and Ronai Z. (2000). Glutathione S-transferase p elicits protection against H2O2-induced cell death via coordinated regulation of stress kinases. Cancer Res. (Adv. Brief), 60, 4053–4057.

56. Schisselbauer JC, Silber R, Papadoupoulos E, Abrams K, LaCreta FP and Tew KD. (1990). Characterization of glutathione S-transferase expression in lymphocytes from chronic lymphocytic leukemia patients. Cancer Res., 50, 3569–3673.

57. Ni M, Lee AS. (2007) ER chaperones in mammalian development and human diseases. FEBS Lett, 581:3641-51.

58. Corazzari M, Lovat PE, Armstrong JL, Fimia GM, Hill DS, Birch-Machin M, Redfern CP, Piacentini M. (2007) Targeting homeostatic mechanisms of endoplasmic reticulum stress to increase susceptibility of cancer cells to fenretinide-induced apoptosis: the role of stress proteins ERdj5 and ERp57. Br J Cancer; 96:1062-71.

59. Aureli C, Gaucci E, Arcangeli V, Grillo C, Eufemi M, Chichiarelli S. (2013) ERp57/PDIA3 binds specific DNA fragments in a melanoma cell line. Gene, 524:390-5.

60. Pressinotti NC, Klocker H, Schäfer G, Luu VD, Ruschhaupt M, Kuner R, Steiner E, Poustka A, Bartsch G, Sültmann H. (2009). Differential expression of apoptotic genes PDIA3 and MAP3K5 distinguishes between low- and high-risk prostate cancer. Mol Cancer; 8:130

61. De Marco F, Bucaj E, Foppoli C, Fiorini A, Blarzino C, Filipi K, Giorgi A, Schininà ME, Di Domenico F, Coccia R, Butterfield DA, Perluigi M. (2012). Oxidative stress in HPV-driven viral carcinogenesis: redox proteomics analysis of HPV-16 dysplastic and neoplastic tissues. PLoS ONE; 7(3):e34366.

62. Cicchillitti L, Di Michele M, Urbani A, Ferlini C, Donat MB, Scambia G, Rotilio D. (2009) Comparative proteomic analysis of paclitaxel sensitive A2780 epithelial ovarian cancer cell line and its resistant counterpart A2780TC1 by 2D-DIGE: the role of ERp57. J Proteome Res; Apr;8(4):1902-12.

63. Coe H, Michalak M. (2010). ERp57, a multifunctional endoplasmic reticulum resident oxidoreductase. Int J Biochem Cell Biol; 42:796-9.

64. Kim SW, Lee J, Lee B2, Rhim T. (2014) Proteomic analysis in pterygium; upregulated protein expression of ALDH3A1, PDIA3, and PRDX2. Mol Vis. Aug 27;20:1192-202.

65. Garbi N, Hammerling G, Tanaka S. (2007) Interaction of ERp57 and tapasin in the generation of MHC class I-peptide complexes. Curr Opin Immunol; 19:99-105.

66. Garbi N, Tanaka S, Momburg F, Hammerling GJ. (2006) Impaired assembly of the major histocompatibility complex class I peptide-loading complex in mice deficient in the oxidoreductase ERp57. Nat Immunol; 7:93-102.

67. Banki K, Colombo E, Sia F, Halladay D,Mattson DH, Tatum AH, Massa PT, Phillips PE, Perl A. (1994). Oligodendrocytespecific expression and autoantigenicity of transaldolase in multiple sclerosis. J Exp Med. Nov 1;180(5):1649-63.

68. Colombo E1, Banki K, Tatum AH, Daucher J, Ferrante P, Murray RS, Phillips PE, Perl A. (1997). Comparative analysis of antibody and cell-mediated autoimmunity to transaldolase and myelin basic protein in patients with multiple sclerosis. J Clin Invest. Mar 15;99(6):1238-50.

69. Niland B, Banki K, Biddison WE, Perl A. (2005) CD8+ T cell-mediated HLA-A*0201-restricted cytotoxicity to transaldolase peptide 168–176 in patients with multiple sclerosis. J Immunol. Dec 15;175(12):8365-78.

70. Ma Y, Peng J, Huang L, Liu W, Zhang P, Qin H. (2009) Searching for serum tumor markers for colorectal cancer using a 2-D DIGE approach, Electrophoresis. Aug;30(15):2591-9.

71. Hanczko R, Fernandez DR, Doherty E, Qian Y, Vas G, Niland B, Telarico T, Garba A, Banerjee S, Middleton FA, Barrett D, Barcza M, Banki K, Landas SK, Perl A. (2009) Prevention of hepatocarcinogenesis and increased susceptibility to acetaminophen-induced liver failure in transaldolase-deficient mice by N-acetylcysteine, J Clin Invest. Jun;119(6):1546-57.

72. Gupta V, Bamezai RN. (2010). Human pyruvate kinase M2: a multifunctional protein. Protein Sci. Nov;19(11):2031-44.

73. Frauwirth KA, Riley JL, Harris MH, Parry RV, Rathmell JC, Plas DR, Elstrom RL, June CH, Thompson CB. (2002). The CD28 signaling pathway regulates glucose metabolism. Immunity 16:769–777.

74. Matarese G, La Cava A (2004). The intricate interface between immune system and metabolism. Trends Immunol 25:193–200

75. Oak MH, Cheong H, Kim KM (1999) Activation of Fc epsilon RI inhibits the pyruvate kinase through direct interaction with the gamma-chain. Int Arch Allergy Immunol 119:95–100.

76. Kubota H, Hynes G, Willison K. (1995). The chaperonin containing t-complex polypeptide 1 (TCP-1): multisubunit machinery assisting in protein folding and assembly in the eukaryotic cytosol. Eur J Biochem 230: 3–16.

77. Zhao W, Fan GC, Zhang ZG, Bandyopadhyay A, Zhou X, Kranias EG. (2009). Protection of peroxiredoxin II on oxidative stress-induced cardiomyocyte death and apoptosis. Basic Res Cardiol; 104:377-89.

78. Nagababu E, Mohanty JG, Friedman JS, Rifkind JM. (2013). Role of peroxiredoxin-2 in protecting RBCs from hydrogen peroxide-induced oxidative stress. Free Radic Res; 47:164-71.

79. Zhao F, Wang Q. (2012). The protective effect of peroxiredoxin II on oxidative stress induced apoptosis in pancreatic beta-cells. Cell Biosci. Jun 18;2(1):22.

80. Jikimoto T1, Nishikubo Y, Koshiba M, Kanagawa S, Morinobu S, Morinobu A, Saura R, Mizuno K, Kondo S, Toyokuni S, Nakamura H, Yodoi J, Kumagai S. (2001) Thioredoxin as a biomarker for oxidative stress in patients with rheumatoidarthritis. Molecular Immunology; 38:765‑72.

81. Moon EY1, Noh YW, Han YH, Kim SU, Kim JM, Yu DY, Lim JS. (2006). T lymphocytes and dendritic cells are activated by the deletion of peroxiredoxin II (Prx II) gene. Immunol Lett. Feb 15;102(2):184-90.

82. Yang CS1, Lee DS, Song CH, An SJ, Li S, Kim JM, Kim CS, Yoo DG, Jeon BH, Yang HY, Lee TH, Lee ZW, El-Benna J, Yu DY, Jo EK. (2007). Roles of peroxiredoxin II in the regulation of proinflammatory responses to LPS and protection against endotoxin-induced lethal shock. J Exp Med, Mar 19;204(3):583-94. Epub 2007 Feb 26.

83. Kwon J, Devadas S, Williams MS. (2003). T cell receptor-stimulated generation of hydrogen peroxide inhibits MEK-ERK activation and lck serine phosphorylation. Free Radic Biol Med. Aug 15;35(4):406-17.

84. Zhang P, Liu B, Kang S, Seo M, Rhee S, Obeid L. (1997). Thioredoxin peroxidase is a novel inhibitor of apoptosis with a mechanism distinct from that of Bcl-2. J Biol Chem. Dec 5;272(49):30615-8.

85. http://www.ncbi.nlm.nih.gov/gene/335

86. Burger D, Dayer JM. (2002). High-density lipoprotein-associated apolipoprotein A-I: the missing link between infection and chronic inflammation? Autoimmun Rev. Feb;1(1-2):111-7.

87. Malle E, Leonhard B, Knipping G, Sattler W. (1999). Effects of cytokines, butyrate and dexamethasone on serum amyloid A and apolipoprotein A-I synthesis in human HUH-7 hepatoma cells. Scand J Immunol. Aug;50(2):183-7.

88. García-Gómez C,Nolla JM, Valverde J, Narváez J, Corbella E, Pintó X. (2008). High HDL-cholesterol in women with rheumatoid arthritis on low-dose glucocorticoid therapy. Eur J Clin Invest. Sep;38(9):686-92.

89. Barry B, Martina G, Oliver FG, Jean MD. (2004). Apolipoprotein A-I infiltration in rheumatoid arthritis synovial tissue: a control mechanism of cytokine production? Arthritis Res Ther, 6:563-66.

90. Abe H, Tsuboi N, Suzuki S, Sakuraba H. (2001). Anti-apolipoprotein A-I autoantibody: characterization of monoclonal autoantibodies from patients with systemic lupus erythematosus. Rheumatol, 28(5):990-95.

91. McMahon M, Grossman J, Chen W, Hahn BH. (2006). Inflammation and the pathogenesis of atherosclerosis in systemic lupus erythematosus. Lupus 2006, 15:59-69.

92. Levy AP, Asleh R, Blum S, Levy NS, Miller-Lotan R, Kalet-Litman S, Anbinder Y, Lache O, Nakhoul FM, Asaf R, Farbstein D, Pollak M, Soloveichik YZ, Strauss M, Alshiek J, Livshits A, Schwartz A, Awad H, Jad K, Goldenstein H. (2010). Haptoglobin: basic and clinical aspects. Antioxid Redox Signal, 12:293-304

93. Langlois MR, Delanghe JR. (1996). Biological and clinical significance of haptoglobin polymorphism in humans. Clin Chem, 42:1589-600; PMID:8855140

94. Pavón EJ, Muñoz P, Lario A, Longobardo V, Carrascal M, Abián J, Martin AB, Arias SA, Callejas-Rubio JL, Sola R, Navarro-Pelayo F, Raya-Alvarez E, Ortego-Centeno N, Zubiaur M, Sancho J. (2006), Proteomic analysis of plasma from patients with systemic lupus erythematosus: increased presence of haptoglobin alpha2 polypeptide chains over the alpha1 isoforms. Proteomics 2006; 6(Suppl 1):S282-92

95. Morimatsu M, Syuto B, Shimada N, Fujinaga T, Yamamoto S, Saito M, Naiki M. (1991). Isolation and characterization of bovine haptoglobin from acute phase sera. J Biol Chem, 266:11833-7; PMID:1904872

96. Baumann H1, Prowse KR, Won KA, Marinković S, Jahreis GP. (1988). Regulation of acute phase protein genes by hepatocyte-stimulating factors, monokines and glucocorticoids. Tokai J Exp Clin Med. Dec;13(6):277-92.

97. Kamath S, Lip GY. (2003). Fibrinogen: biochemistry, epidemiology and determinants. QJM. Oct;96(10):711-29.

98. Dhillon PK , Adams MJ. (2013). Thrombosis in systemic lupus erythematosus: role of impaired fibrinolysis. Semin Thromb Hemost. Jun;39(4):434-40.

99. Spengler MI, Svetaz MJ, Leroux MB, Bertoluzzo SM, Carrara P, Van Isseldyk F, Petrelli D, Parente FM, Bosch P. (2011). Erythrocyte aggregation in patients with systemic lupus erythematosus. Clin Hemorheol Microcirc. 47(4):279-85.

100. Meade TW, Mellows S, Brozovic M, Miller GJ, Chakrabarti RR, North WR, Haines AP, Stirling Y, Imeson JD, Thompson SG. (1986) Haemostatic function and ischaemic heart disease: principal results of the Northwick Park Heart Study. Lancet, Sep 6;2(8506):533-7.

101. Wilhelmsen L, Svardsudd K, Korsan-Bengtsen K, Larsson B, Welin L, Tibblin G. (1984). Fibrinogen as a risk factor for stroke and MI. N Engl J Med 1984; 311:501–5.)

102. Kilpatrick DC, Chalmers JD (2012). Human L-ficolin (ficolin-2) and its clinical significance. J Biomed Biotechnol 2012:138797)

103. Watanabe H, Saito R, Asano T, Sato S, Iwadate H, Kobayashi H, Ohira H. (2012) Serum L-ficolin levels in patients with systemic lupus erythematosus. Mod Rheumatol. Nov;22(6):899-902.

104. Østergaard O ,Nielsen CT, Iversen LV, Tanassi JT, Knudsen S, Jacobsen S, Heegaard NH. (2013). Unique Protein Signature of Circulating Microparticles in Systemic Lupus Erythematosus Arthritis Rheum. Oct;65(10):2680-90.

105. Yongjing Cheng, Yuling Chen, Xiaolin Sun, Yuhui Li, Cibo Huang, Haiteng Deng, Zhanguo Li.(2014). Identification of Potential Serum Biomarkers for Rheumatoid Arthritis by High-Resolution Quantitative Proteomic Analysis. Inflammation. Oct;37(5):1459-67.

106. Walport MJ, Davies KA, Morley BJ, Botto M. (1997). Complement deficiency and autoimmunity. Ann N Y Acad Sci; 815: 267–81.

107. Andrade F, Casciola-Rosen L, Rosen A.(2000). Apoptosis in systemic lupus erythematosus. Clinical implications. Rheum Dis Clin North Am 2000;26: 215–27, v.

108. Newkirk MM, Apostolakos P, Neville C, Fortin PR. (1999). Systemic lupus erythematosus, a disease associated with low levels of clusterin/apoJ, an antiinflammatory protein. J Rheumatol 1999; 26: 597–603.

109. Eriksson C, Eneslätt K, Ivanoff J, Rantapää-Dahlqvist S, Sundqvist KG (2003). Abnormal expression of chemokine receptors on T-cells from patients with systemic lupus erythematosus. Lupus 12:766–774

110. Vilá LM, Molina MJ, Mayor AM, Cruz JJ, Ríos-Olivares E, Ríos Z. (2007). Association of serum MIP-1alpha, MIP-1beta, and RANTES with clinical manifestations, disease activity, and damage accrual in systemic lupus erythematosus. Clin Rheumatol. May;26(5):718-22. Epub 2006 Aug 19.

111. Kaneko H, Ogasawara H, Naito T, Akimoto H, Lee S, Hishikawa T, Sekigawa I, Tokano Y, Takasaki Y, Hirose SI, Hashimoto H. (1999). Circulating levels of β-chemokines in systemic lupus erythematosus. J Rheumatol 26:568–573

112. Narumi S, Takeuchi T, Kobayashi Y, Konishi K. Serum levels of IFN-inducible protein-10 relating to the activity of systemic lupus erythematosus. Cytokine 2000;12:1561–5.

113. Lit LC, Wong CK, Tam LS, Li EK, Lam CW. (2006). Raised plasma concentration and ex vivo production of inflammatory chemokines in patients with systemic lupus erythematosus. Ann Rheum Dis;65:209–15.

114. Akahoshi M, Nakashima H, Tanaka Y, Kohsaka T, Nagano S, Ohgami E, Arinobu Y, Yamaoka K, Niiro H, Shinozaki M, Hirakata H, Horiuchi T, Otsuka T, Niho Y. (1999). Th1/Th2 balance of peripheral T helper cells in systemic lupus erythematosus. Arthritis Rheum. Aug;42(8):1644-8.

115. al-Janadi M, al-Balla S, al-Dalaan A, Raziuddin S. (1993). Cytokine profile in systemic lupus erythematosus, rheumatoid arthritis, and other rheumatic diseases. J Clin Immunol. Jan;13(1):58-67.

116. Dinarello CA. (2011) Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. Blood; Apr 7;117(14):3720-32.

117. Cigni A, Pileri PV, Faedda R, Gallo P, Sini A, Satta AE, Marras R, Carta E, Argiolas D, Rum I, Masala A. (2014). Interleukin 1, interleukin 6, interleukin 10, and tumor necrosis factor α in active and quiescent systemic lupus erythematosus. J Investig Med; Jun;62(5):825-9.

118. Varo N1, de Lemos JA, Libby P, Morrow DA, Murphy SA, Nuzzo R, Gibson CM, Cannon CP, Braunwald E, Schönbeck U. (2003). Soluble CD40L: risk prediction after acute coronary syndromes. Circulation. Sep 2;108(9):1049-52.

119. Aggarwal A, Schneider DJ, Terrien EF, Sobel BE, Dauerman HL. (2004) Increased coronary arterial release of interleukin-1 receptor antagonist and soluble CD40 ligand indicative of inflammation associated with culprit coronary plaques. Am J Cardiol;93:6e9.

120. Kato K, Santana-Sahagún E, Rassenti LZ, Weisman MH, Tamura N, Kobayashi S, Hashimoto H, Kipps TJ. (1999) The soluble CD40 ligand sCD154 in systemic lupus erythematosus. J Clin Invest. Oct;104(7):947-55.

121. Otterdal K, Pedersen TM, Solum NO. (2004). Release of soluble CD40 ligand after platelet activation: studies on the solubilization phase. Thromb Res. 114:167e77.

122. Vakkalanka RK, Woo C, Kirou KA, Koshy M, Berger D, Crow MK. (1999)Elevated levels and functional capacity of soluble CD40 ligand in systemic lupus erythematosus sera. Arthritis Rheum. 42:871e81.

123. Tamura N, Kobayashi S, Kato K, Bando H, Haruta K, Oyanagi M, Kuriyama M, Kipps TJ, Hashimoto H. (2001). Soluble CD154 in rheumatoid arthritis: elevated plasma levels in cases with vasculitis. J Rheumatol; Dec;28(12):2583-90.

124. Komura K, Sato S, Hasegawa M, Fujimoto M, Takehara K. (2004.)Elevated circulating CD40L concentrations in patients with systemic sclerosis. J Rheumatol; Mar;31(3):514-9.

125. Daikh DI, Finck BK, Linsley PS, Hollenbaugh D, Wofsy D. (1997). Long-term inhibition of murine lupus by brief simultaneous blockade of the B7/ CD28 and CD40/gp39 costimulation pathways. J Immunol;159: 3104e8.

126. Quezada SA, Eckert M, Adeyi OA, Schned AR, Noelle RJ, Burns CM. (2003). Distinct mechanisms of action of anti-CD154 in early versus late treatment of murine lupus nephritis. Arthritis Rheum 2003;48:2541e54. Cd40 tutti da questo Elevated levels of soluble CD40 ligand (sCD40L) in serum of patientswith systemic autoimmune diseases

127. Lundström W, Highfill S, Walsh ST, Beq S, Morse E, Kockum I, Alfredsson L, Olsson T, Hillert J, Mackall CL. (2013) Soluble IL7Rα potentiates IL-7 bioactivity and promotes autoimmunity. Proc Natl Acad Sci U S A. 2013 May 7;110(19):E1761-70.

128. PillaiM, Torok-Storb B, Iwata M. (2004). Expression and function of IL- 7 receptors in marrow stromal cells. Leuk Lymphoma, 45(12):2403– 2408

129. Bikker A, Hack CE, Lafeber FP, van Roon JA (2012). Interleukin-7: a keymediator in Tcell-driven autoimmunity, inflammation, and tissue destruction. Curr Pharm Des 18(16):2347–2356

130. McElroy CA, Dohm JA, Walsh ST (2009) Structural and biophysical studies of the human IL-7/IL-7Ralpha complex. Structure 17(1):54–65 per IL 7 presi da Perspectives of the relationship between IL-7 and autoimmune diseases

131. Ben-David H1, Sharabi A, Parameswaran R, Zinger H, Mozes E. (2009). A tolerogenic peptide down-regulates mature B cells in bone marrow of lupusafflicted mice by inhibition of interleukin-7, leading to apoptosis. Immunology. 2009 Oct;128(2):245-52.

132. Badot V1, Luijten RK, van Roon JA, Depresseux G, Aydin S, Van den Eynde BJ, Houssiau FA, Lauwerys BR. (2013). Serum soluble interleukin 7 receptor is strongly associated with lupus nephritis in patients with systemic lupus erythematosus. Ann Rheum Dis. Mar;72(3):453-6.

133. Anzai M, Maezawai R, Ohara T, Kodama K, Fukuda T, Kurasawa K. (2008) Systemic lupus erythematosus associated with facial edema, overproduction of interleukin-5, and eosinophilia. J Clin Rheumatol. Dec;14(6):361-2.

134. Waage A, Bakke O. (1988) Glucocorticoids suppress the production of tumour necrosis factor by lipopolysaccharide-stimulated human monocytes. Immunology. Feb;63(2):299-302.

135. Calandra T, Bernhagen J, Metz CN, Spiegel LA, Bacher M, Donnelly T, Cerami A, Bucala R. (1995) MIF as a glucocorticoid-induced modulator of cytokine production. Nature. Sep 7;377(6544):68-71.

136. Brink I, Thiele B, Burmester GR. (1999). Effects of anti-CD4 antibodies on the release of IL-6 and TNF-a in whole blood samples from patients with systemic lupus erithematosus. Lupus.; 8:723-30,

137. Swaak AJ, van Den Brink HG, Aarden LA. (1996). Cytokine production (IL-6 and TNF-alpha) in whole blood cell cultures of patients with systemic lupus erythematosus. Scand J Rheumatol.;25(4):233-8.

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