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"STUDIO DELLE ALTERAZIONI POST-TRADUZIONALI E FUNZIONALI DEL FIBRINOGENO IN PAZIENTI CON LUPUS ERITEMATOSO SISTEMICO (LES)"

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Titolo tesi:

"*Studio delle alterazioni post-traduzionali e funzionali del fibrinogeno in pazienti con Lupus Eritematoso Sistemico (LES)*"

"Study of fibrinogen post-translational and functional modifications in Systemic Lupus Erythematosus (SLE) patients"

Summary

Haemostatic System Disorders and Risk of Thrombosis in Autoimmunity: A Brief Introduction

Haemostatic system is characterized by a complex interaction between platelets aggregation, coagulation and fibrinolysis. These processes are activated during specific temporal phases after vessel injury and strongly regulated in order to stop bleeding and promote wound healing. Physiologically, endothelial damage is the trigger factor for thrombin-induced fibrin clot assembly at the site of insult, that will be removed by plasmin-mediated fibrinolysis after tissue repair.

Genetic alterations and environment can be involved in coagulation factors impaired levels or activity, fibrinogen post-translational modifications, fibrin clot structural architecture abnormalities, resulting in haemostatic system affection. Thrombotic or hemorrhagic events represent the clinical manifestations of several haemostatic system disorders including pulmonary hypertension, myocardial infarction, venous thromboembolism, preeclampsia, cirrhosis.

However, a growing literature has recently revealed a higher risk of thrombosis also in autoimmunity. Indeed, autoimmune disorders are frequently characterized by thrombotic events, suggesting a crucial association between systemic inflammation and thrombosis [1,2]. Accordingly, Behçet Syndrome (BS) has been proposed as a model of inflammation-induced thrombosis, where therapeutic treatment with glucocorticoids and or immunosuppressants produced a reduction of patients' mortality due to arterial/venous thrombosis unlike anticoagulant drugs, according to European League Against Rheumatism (EULAR) guidelines. Based on this evidence, inflammation could be considered a trigger event for thrombosis, together with traditional cardiovascular risk factors [2]. In several diseases as Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE), an early progression of atherosclerosis has been largely observed,

supported by inflammation-enhanced Reactive Oxygen Species (ROS) production in addition to a profile of dyslipidemia [2]. In this context, cumulative data in literature revealed higher ROS-mediated oxidized low-density lipoproteins (oxLDLs) levels in SLE patients. They induce vascular smooth cells apoptosis, monocytes recruitment and pro-inflammatory cytokines production as well as nitric oxide (NO) reduction, promoting a pro-thrombotic endothelial phenotype [3,4] and the formation of atherosclerotic plaque. Accordingly, cardiovascular manifestations represent one of the principal causes of mortality in patients affected by SLE, where both traditional and disease-related cardiovascular risk factors are actively involved [5].

However, pathogenetic mechanisms of thrombosis are not completely clarified, suggesting the need of further investigations to better understand the increased cardiovascular risk in SLE patients.

Systemic Lupus Erythematosus (SLE)

Introduction to SLE

SLE is an autoimmune disease with multisystemic clinical manifestations and unpredictable course. SLE incidence has been estimated ranging 1-10 cases per 100 000 persons for year and the prevalence has been reported to range between 20 and 150 cases every 100 000 persons [6-8]. However, SLE epidemiological data are strongly influenced by the considered geographical area. Based on this evidence, SLE incidence in Italy was assessed to be 2.6/100 000 in 2002 and prevalence has been estimated about 57.9/100 000, according to the recent studies in others Mediterranean European countries as Greece and Spain [9-11]. Female predominance of SLE (9:1 female to male) and higher SLE incidence in Africans, Americans and native Americans, Hispanic and Asian individuals than Caucasians suggest the multifactorial etiology of this disorder, where both genetic and environmental factors could be involved [6,8]. It is traditionally accepted that SLE is a chronic autoimmune disorder characterized by an altered autoimmune response to self-antigens, especially mediated by adaptive immune system (T cells and B cells), that results in organ and tissue injury [6,8]. However, recent studies revealed the centrality of innate immune system components as dendritic cells (DCs) and phagocytes in SLE pathogenesis, based on their susceptibility to self-antigens in the first phases of inflammation. Indeed, as antigen presenting cells (APCs), they are crucial in antigens presentation, stimulating the adaptive immune system [6].

Epidemiology

As several epidemiologic studies revealed, lupus disorder is a rare disease where gender, age, racial and geographical variations have a crucial role in its development, suggesting

how environmental, genetic and hormonal factors could be involved in SLE pathogenesis [12].

Milder cases consideration and the higher survival of SLE patients than in the past are associated with an increased incidence and prevalence of the disorder observed in the last years [13]. As reported in table 1, SLE incidence is triplicate in USA from 1-51 per 100 000 in the 1950-79 cohort to 5-56 per 100 000 between 1980 and 1992, as indicated by D'Cruz D. P et al. in 2007 (Table.1) [14].

	Incidence (per 100 000 per year)	Prevalence (per 100 000)
USA		
All races	5·1	52·2
White	1·4	7·4
Black	4·5	19·5
Puerto-Rican	2·2	18·0
Canada		
White	1·6	20·6
First Nations	4·7	42·3
Finland	NA	28·0
France	5·0	40·0
Iceland	3·3	35·9
Italy	NA	71·0
Northern Ireland	NA	25·4
Spain		
All races	NA	91·0
White	2·2	34·1
Sweden	4·7	42·0
UK		
All races	3·8	26·2
White	3·0	20·5
Asian	10·0	47·8
Chinese	NA	92·9
Afro-Caribbean	21·9	159·4
Australia		
White	NA	19·3
Aboriginal	11·0	63·1
Japan	2·9	28·4
Martinique	4·7	64·2

NA=data not available. Adapted from reference 6 with permission from Sage Publications.

Table.1 SLE incidence and prevalence in the world [14]

A growing literature has recently shown the higher susceptibility to SLE in non-Caucasian individuals including African-Americans, Hispanics and Asians compared to the general population. African-American women show three/four-fold higher risk to develop SLE rather than Caucasian ones, potentially due to genetic predisposition and environment exposure. These elements are also crucial in influencing SLE incidence and prevalence, that has been found similar in racial groups living around the world.

Introduction

As indicated in figure 1 and 2, Afro-Caribbean population shows a higher incidence and prevalence of SLE: however, several factors show effects on the considered epidemiological parameters including population race, countries heterogeneity, differences in applied methods of investigation, methodological approaches and health management [12].

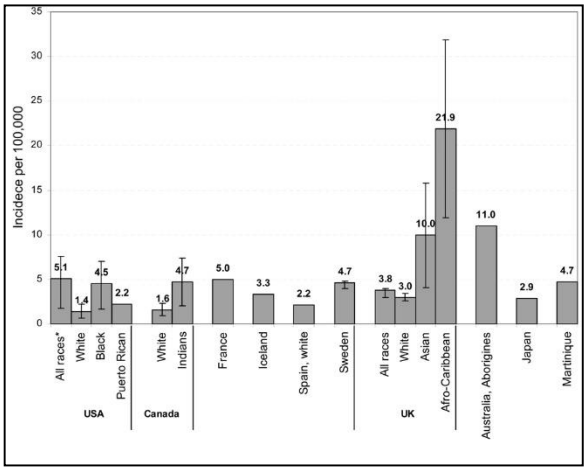

Fig.1 SLE incidence in the countries of interest [12]

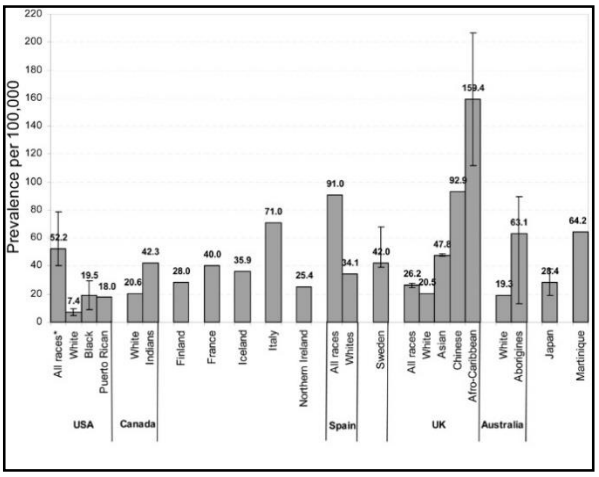

Fig.2 SLE prevalence in the countries of interest [12]

Based on worldwide data, 16-50 years is indicated as the common range of SLE onset: SLE cases before age 16 years represent a small 10-20% whereas after the age of 50 years the percentage is about 8-15%. Women show lupus development with a frequency of 6-8 times more than men, suggesting the crucial role of sex and hormonal effects [15].

In this context, the Euro Lupus Project, a multicentric prospective cohort study of 1000 patients with lupus followed up from 1991, shows that [16]

- SLE *excursus* is influenced by several factors including the age at the disease onset, the gender and the autoantibody profile;
- Therapeutic treatments and cases of SLE remission are frequently associated with the decrement of inflammatory manifestations observed during the long phase of disorder;
- Abnormalities in haemostasis and vascular events are one of the principal cause of morbidity and mortality in SLE patients.

All these elements contribute to distinguish SLE evolution in different racial groups around the world. Implementations in health care and therapeutic approaches may induce an enhancement in lupus patient survival, changing from 50% in 1950-60 to about 95% in 2000. However, mortality rate is still 2-4 fold higher in SLE compared to healthy subjects [17].

Pathogenesis

SLE pathogenesis is largely discussed. The majority of evidence describes lupus disorder as a pathological condition with a multifactorial aetiology. As shown in figure 3, genetics and environment, epigenetics, sex and hormonal factors as well as immune system deregulation seem to be actively involved.

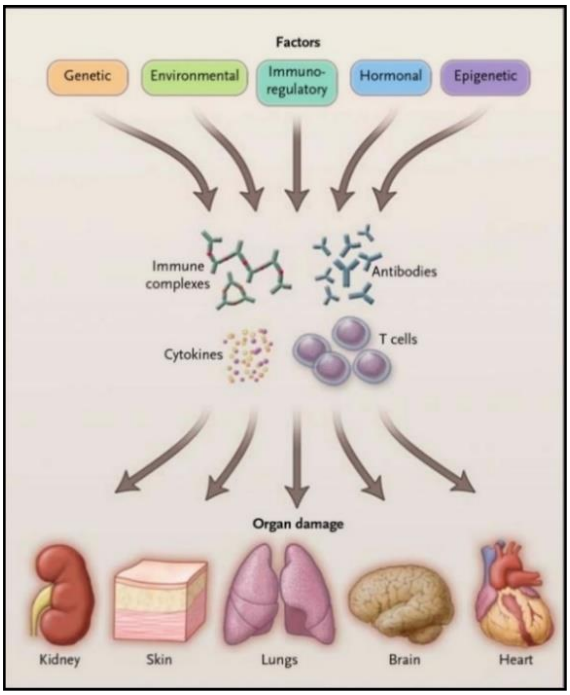

Fig.3 Overview of SLE pathogenesis [21]

Genetic Factors

The ten-fold higher risk to develop SLE in monozygotic twin than dizygotic ones and the increased frequency of SLE in families suggest the role of specific genetic mutations in lupus susceptibility [18,19]. More than 80 genetic risk factors involved in different immunological pathways were discovered by genome-wide association studies (GWAS). Particularly, immune cells processing, clearance of cellular debris, type I interferon (IFN-I) and Toll-Like Receptor (TLR) signaling as well as lymphocyte activation seem to be affected [18,19]. Moreover, several genes related to IFN system, Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB) signaling, DNA degradation, apoptosis,

phagocytosis, neutrophil, monocyte/macrophage function and signaling seem to be associated with the risk of SLE onset [19].

However, the mechanism through which they contribute to autoimmune disorders is not completely clarified yet. Accordingly, several single nucleotide polymorphism (SNPs) related to SLE occur in non-coding DNA regions of immune response-related genes [20,21].

Only a small percentage of SLE cases are due to genetic mutations following the mendelian inheritance. The major part of patients displays a combination of a larger number of genes, including some (Interferon Regulatory Factor 5-IRF5, Signal Transducer And Activator Of Transcription 4-STAT4, Interleukin 1 Receptor Associated Kinase 1-IRAK1) strongly associated with nucleic acid sensing and interferon α (IFN-α) production or others, central for the modulation of T-lymphocyte (Protein Tyrosine Phosphatase 22- PTPN22, Tumor Necrosis Factor Superfamily Member 4-TNFSF4, Programming Cell Death 1-PDCD1) or B cell (B Cell Scaffold Protein With Ankyrin Repeats 1-BANK1, B-lymphoid Tyrosine Kinase-BLK, Lck/Yes Novel Tyrosine Kinase-LYN) activity (PTPN22 regulates lymphocyte activation). Following several investigations, some genes as IRF5 and STAT4 are specifically associated with the increased risk of SLE or others autoimmune disorders (STAT4 with RA, PTPN22 with RA and diabetes Type 1) [20,21]. Moreover, among genetic risk loci for SLE pathogenesis TNIP1, PRDM1, JAZF1, UHRF1BP1 binding protein and Interleukin-10 (IL-10) can be underlined [20]. A great relevance in SLE development is also represented by mutations of genes associated with complement component 1q (C1q) as well genes located between MHC class I and II, affecting components of the classical complement system. Several polymorphisms on genes encoded for mannose-binding lectin (MBL) can occur in SLE, being associated with abnormalities in mannose-rich microorganisms opsonization as well as in macrophages and complement cascade activation [18,22].

Finally, alterations in TLR (especially TLR-7, TLR -8, TLR -9) may result in their hyperactivation, promoting immune response progress, whereas genetic variants of the Fc receptor for IgG can affect immune complexes clearance, triggering autoimmune disorder by activation of TLR and IFN system [18,22].

Moreover, polymorphisms on C- reactive protein (CRP), serum amyloid genes and in the programmed cell death 1 gene (PDCD1) seem to be associated with autoantibody production and SLE development [14].

However, only a 15% of the SLE hereditability can be attributed to the identified loci: the involvement of each single gene in the entire disease process and its contribute to the phenotype and severity of the disease is still under debate.

Environmental Factors

As an autoimmune disorder with a multifactorial etiology, environmental factors are actively involved in the pathogenesis of SLE and the principal ones can be summarized below:

- Exposure to silica, dust, dioxins, polycyclic aromatic hydrocarbons and dietary components [8];
- Alcohol and smoking [8];
- D vitamin deficiency. This condition is partly due to SLE patients' photosensitivity and their lower exposition to UV lights. The role of D vitamin lower levels is still unclear, despite polymorphisms in its receptor and the relationship among 25-hydroxy vitamin D (25-OH D vitamin) and lupus disease activity seem to be associated with harmful effects in patients. However, therapeutic supplementation with D vitamin is needed for preventing glucocorticoid-related osteoporosis: D vitamin clinical effects are so difficult to understand [19];

- Exposure to ultraviolet (UV) lights. The most negative effects of UV-light exposure are found on cutaneous tissue. Particularly, an increase in keratinocyte cells apoptosis is observed in addition to higher self-antigens production and immune complexes formation. Pro-inflammatory cytokines, including interleukin-1 (IL-1), interleukin- 6 (IL-6) and tumor necrosis factor- α (TNF-α), are produced by keratinocytes and lymphocytes promoting skin damages as well as activation of mast cells, fibroblasts and endothelial cells via chemokines and adhesion molecules release. UV lights exposure seems to be also associated with DCs recruitment in lesional cutaneous lupus skin and IFN-α production, leading to inflammatory status [18,19];
- Bacterial and viral infections (as Epstein-Barr virus-EBV, Cytomegalovirus-CMV, parvovirus B19, etc). Microorganisms are implicated in the pathogenesis of autoimmune disorders including SLE due to molecular mimicry mechanisms. Indeed, the similarity between several viral or bacterial antigens and autoantigens may cause an autoantibodies cross reaction with self-antigens, promoting autoimmune response and chronic inflammatory status [19,21]. Accordingly, the EBV Antigen 1 (EBNA-1) shows homologous regions with the autoantigen Ro: B-lymphocytes activation and antibodies production result in autoantigens attack, suggesting how sometimes infections could be involved in autoimmunity in predisposed individuals [19];
- Therapeutic treatment with several drugs including procainamide, hydralazine and quinidine. Acting as DNA demethylating agents, these drugs could be associated with autoantibodies production and autoimmune response that resolve by interrupting therapeutic supplementation [18].

Epigenetic factors

Epigenetic is characterized by several molecular mechanisms as DNA methylation, post-translational histone modifications and microRNAs (miRNAs) that act regulating genes expression without changes in nucleotide sequence. Alterations in epigenetic processes can be associated with SLE pathogenesis. Several data described an hypomethylation status in SLE patients, affecting pro-inflammatory cytokines production and B and T lymphocytes stimulation [19]. Particularly, reduced levels of DNA methylation may be due to environmental, dietary and lifestyle factors as well as smoking and drugs supplementation. These alterations can be associated with [18-20]:

- B-cells autoantibodies production and overexpression of co-stimulatory molecules including CD11a, CD70, CD40 Ligand (CD40LG) and perforin [18,20,21];
- Higher levels of pro-inflammatory cytokines (IL-6 and Interleukin-4 - IL-4). Studies on murine animal models showed the increased pro-inflammatory cytokines levels as a consequence of using DNA methylation inhibitors [18] and accordingly, DNA methylation in B-cells is inhibited in association with increased IL-6 levels [20].

Additionally, alterations in histone enzyme activity seem to be linked with abnormal expression of genes and costimulatory molecules as CD40LG in patients affected by SLE [18,20]. Further studies revealed alterations in miRNA system in SLE, affecting genes expression as well as DNA epigenetic regulation and histone modifications [18,19].

Sex and Hormonal Factors

The higher prevalence of SLE in female individuals suggests a great involvement of sex and hormonal factors in lupus pathogenesis. Indeed, increased levels of estrogens have

been assessed in women affected by SLE, potentially derived from alterations in sex hormones metabolism (an increased activity of aromatase has been observed). Moreover, sex chromosome influence in SLE expression [20] has been evaluated using on engineered mice: the expression of X chromosome seems to be strongly associated with a higher risk of developing SLE and with the severity of disorder [18,21].

Evidence in literature described several estrogen effects on T-lymphocyte cytokines production: up-regulation of Th2 cytokines such as IL-4, Interleukin-5 (IL-5), IL-6, IL-10, and Transforming Growth Factor- β (TGF-β) was observed whereas Th1 cytokines including IL-2, TNF-α, Interferon- γ (IFN-γ) were inhibited. Estrogens also promote Bcl-2 anti-apoptotic molecule, suggesting its involvement in cell survival, including autoreactive B cells. Parallel, estrogen hormone may act up-regulating the expression of CD40LG and estrogen receptor in T-cells of SLE patients as well as the activation and differentiation of DCs [23]. Additionally, recent studies revealed the estrogens' involvement in modulating the Autoimmune Regulator Gene (AIRE) expression, as a new molecular mechanism contributing to the higher risk of autoimmunity onset in female gender [24]. AIRE, expressed in the thymus by the medullary thymic epithelial cells (mTECs) and in secondary lymphoid organs, is a transcription factor with a central role in immune tolerance. It promotes the synthesis of organ-specific proteins located in peripheral tissues and the differentiation of thymic cells into Foxp3+ regulatory T(reg) cells. However, it can also induce the apoptosis of autoreactive thymocytes. Based on this evidence, hormones can be included among AIRE expression regulating factors. If AIRE expression is increased by androgens and dihydrotestosterone (DHT), as shown in *in vitro* studies and in human thymic stromal cells, it appears downregulated by estrogens in $mTEC_s$. These results support the evidence of a major risk to develop autoimmune disorders in females [24].

Immune System Alterations and SLE Development [19]

It is traditionally accepted that immune system deregulation is central in SLE pathogenesis. Autoantibodies production and pro-inflammatory cytokines release result in a chronic inflammatory status, promoting tissue injury and lupus clinical manifestations.

T-cells in SLE T-cells play a crucial role in lupus development, being implicated in pro-inflammatory cytokines production, B-cells stimulation and activation as well as autoantibodies generation [19,22].

T-cells can be divided in $CD4^+$ T cells, including Th1, Th2 and Th17 (Treg) and $CD8^+$ T cells that show cytotoxic activity. Among $CD4^+$ T cells, deregulation in cytokines generation is largely described: particularly, decreased IL-2 levels and higher content of IL-6 and Interleukin-17 (IL-17) characterize inflammatory status and promote immune cells recruitment including neutrophils [6,22]. Interleukin-2 (IL-2) and IL-17 are inversely related in SLE: low doses of IL-2 represent a biomarker of the disorder and promote T cells differentiation to Th17 subtype, leading to an imbalance with Treg lymphocytes [19].

On the contrary, $CD8^+$ T cells show a compromised cytotoxic capacity in SLE patients but further investigations are needed. Indeed, the presence of double negative T-cells ($CD4^-$ $CD8^-$) has been found in particular siti of SLE subsets as kidney, where they are involved in cytokine synthesis (IL-1β and IL-17) as well as in autoreactive B cells stimulation to produce autoantibodies [20].

B-cells in SLE. As adaptive immune system cellular components, B-cells principally act in producing antibodies against different antigens like antinuclear antigens (ANAs), but they can be also included among APCs for T-cells stimulation. De-regulation in B-cells activity can be due to abnormalities in specific signaling pathways including phosphorylation one (as calcium flux pathway).

Autoreactive lymphocytes survival and autoimmunity development is encouraged by the higher content of B lymphocyte stimulator (BAFF/ Blys) and APRIL (A proliferation-inducing ligand), as observed in several human and mice SLE models. Blys effects on B cell subsets are different. Indeed, survival of mature B cells and plasma cells is largely associated with Blys and APRIL whereas no effects on memory B lymphocytes have been reported. Additionally, leukocyte Blys mRNA expression seems to influence SLE activity, suggesting Blys and also APRIL as a new therapeutic targets in SLE treatment [6,19].

Moreover, innate immunity is actively involved in SLE development and tissue damages. Alterations in structural and functional properties of neutrophils, monocytes, macrophages and DCs, leading to abnormal phagocytosis and apoptosis, deficiencies in apoptotic debris removal, self-antigen presentation and inflammatory molecules production, seem to be largely implicated in autoimmunity and SLE pathogenesis [20,22].

***DCs*.** DCs play a physiological role as APCs, by recognizing viral or microbiological antigens via Pattern Recognition Receptors (PRRs). Their activation is associated with inflammatory cytokines production, phagocytic activity for apoptotic debris clearance and T lymphocytes stimulation. Based on this evidence, alterations in DCs functioning can be involved in breaking self-tolerance and autoimmunity [20,22]. Particularly, TLRs are able to interact with apoptotic debris, DNA, RNA or nuclear proteins in subjects with higher susceptibility to SLE, inducing DCs activation and pro-inflammatory cytokines production (as IFN-1), in absence of microorganism trigger factors.

DCs can be traditionally divided in two different subsets, based on their phenotypic and immunological properties, mechanisms of activity modulation and sites of action: myeloid DCs and plasmacytoid DCs (pDCs). pDCs produce large amounts of IFN-1, a pro-inflammatory cytokine with cytotoxic cellular side effects including cell apoptosis. The consequent release of self-antigens is one of the reasons supporting the involvement

of pDCs in SLE pathogenesis [22]. IFN-1 can stimulate *in vivo* immune response, directly acting on T cells: indeed, a modulation of T cell activation, proliferation, differentiation and survival are described. pDCs can also influence B cell functions, in antigen recognition and presentation phase, cellular migration, survival and differentiation, cytokines production and class-switch recombination as well as Blys release enhancement [19]. Based on those multiple sites of action, tissue IFN-1 is proposed as a potential target of lupus disorder therapeutic treatments [22].

Parallel, myeloid DCs are divided into tolerogenic DCs, active in apoptotic debris removal and anti-inflammatory cytokines production, and immunogenic DCs, associated with pro-inflammatory cytokines production and self-antigens processing and presentation. Alterations in content and functions of both these cellular subsets have been found in SLE patients [22].

T and B cells stimulation and autoantibodies synthesis occurs consequentially.

Neutrophils. Among innate immune system cells, also neutrophils show functional properties alterations in lupus disorder. Particularly, a reduced phagocytic and lysosomal activity, an increase in adhesion molecules (as intercellular adhesion molecule- ICAM-1, vascular cell adhesion molecule-VCAM-1) levels and in cellular aggregation are observed, together with an *in vivo* intravascular activation [22]. A reduced apoptotic debris removal as well as higher production of oxidant molecules, hydrolytic enzymes and inflammatory mediators represent further evidence of abnormalities in neutrophils biological activity, suggesting their involvement in adaptive immune system activation and tissue injury, particularly lupus nephritis [22]. In addition to the traditional neutrophilic pro-inflammatory mechanisms of action, Neutrophil Extracellular Traps (NETs) production by NETosis represents a great mediator in immune system response against microorganisms and self-antigens. NETs release can be also induced by dendritic

cells-mediated interferon stimulation, reciprocally substantiating inflammatory stimulation and representing a IFN-1 production [22].

Monocytes/Macrophages. The involvement of monocytes/macrophages in SLE pathogenesis is due to alterations in their physiological phagocytic activity and apoptotic debris elimination. These alterations are associated with immune tolerance break, promoting autoimmunity [22].

Based on these findings, a complex interaction among environmental and genetic factors is evident in the development of SLE. Defective mechanisms of apoptotic debris clearance are crucial in innate immune system stimulation, followed by an overproduction of pro-inflammatory cytokines as IFNs (type I and II) and BAFF/Blys, promoting T and B cells activation. Increased autoantibodies and IFN-1 levels are observed in SLE patients and a positive correlation between cells activation and pro-inflammatory mediator production is described, yielding to a chronic inflammatory and autoimmune status [19]. Epigenetic modulation, affecting IL-2 and IL-17 levels, have been also greatly described. In this context, anti-blood cell antibodies, anti-double stranded DNA (anti-dsDNA) and anti-phospholipid (aPLs) antibodies play a central role in tissue injury and lupus clinical manifestations via immune complex formation, leading to cytopenia, nephritis and cardiovascular affection. A great relevance in disease onset is also represented by pro-inflammatory cytokines as IL-6, Blys, IL-17, IFN-1, TNF-α, Interleukin-18 (IL-18), due to their implication in immune system de-regulation and inflammation support [19,22].

Clinical Manifestations

SLE clinical manifestations involve multiple organs and tissues and the age of onset can influence clinical and laboratory profile: constitutional symptoms (fever, weight loss, fatigue) together with malar rash, discoid and subacute cutaneous lesions, photosensitivity, oral ulcers, arthritis, serositis, myositis, nephropathy, cardiovascular/neurologic changes, abdominal pain, hepatosplenomegaly, normochromic normocytic or hemolytic anemia, leukopenia and thrombocytopenia are the principal characteristic signs [25]. Several observational studies underlined that SLE clinical phenotype is not so different among male and female gender. However, if the principal cause of mortality in SLE patients aged 20-39 years is musculoskeletal and lupus-related causes, malignancy and cardiovascular manifestations represent the main cause of death in patients aged over 40 years old [7].

Mucocutaneous Manifestations [26]

Cutaneous manifestations (cutaneous lupus erythematosus-CLE) occur in most patients with SLE in the early phase of the disease course. Particularly, a distinction among acute CLE (photosensitive malar and macular rash), subacute and chronic CLE can be established. Cutaneous vasculitis, urticarial vasculitis, livedo reticularis, Raynaud syndrome, periungueal telangiectasias, erythema multiforme and calcinosis can be also considered manifestations of lupus disorder but not strictly specific of it. Moreover, UV exposure and smoking represent trigger factors for CLE.

Topical or oral steroids, antimalarials and other immunosuppressive drugs are suggested as therapeutic approach for active CLE.

Musculoskeletal Manifestations [26]

Arthralgia and arthritis are frequently found in SLE patients, often occurring in hand and knee joints. Therapeutic treatment with nonsteroidal anti-inflammatory drugs, antimalarials, corticosteroids or immunosuppressive therapy are proposed. A deforming arthropathy called Jaccoud arthropathy can be found in a small subset of SLE patients causing ligamentous laxity and lower joint subluxation. Immunosuppressed patients or corticosteroids taking for long periods seem to be associated with avascular necrosis and joint pain and osteoporosis.

Hematological Manifestations

Hematological alterations have been described in SLE patients: leukopenia, thrombocytopenia and autoimmune hemolytic anemia are considered for the diagnosis of the disorder. Among these clinical abnormalities, lymphopenia is one of the most common hematological complication in SLE, also due to therapeutic treatment with steroids and immunosuppressive drugs. Mechanisms involved in leukopenia are not completely discovered yet, however anti-lymphocyte antibodies and impaired apoptosis seem to be largely associated with this condition. Lymphopenia can be also used as a marker of disease activity and strictly connected with opportunistic infections in SLE patients as well as neutropenia. Lupus thrombocytopenia can be derived from peripheral destruction, sequestration and decreased production of platelets and it is generally associated with neuropsychiatric lupus, lupus nephritis, Antiphospholipid Antibody Syndrome (APS) and hemolytic anemia [26]**.** The severity of thrombocytopenia may give clinicians important information about survival and response to treatment [7].

Younger patients are frequently characterized by hemolytic anemia [26].

Neuropsychiatric Manifestations

Pathogenesis, assessment and treatment of Central Nervous System (CNS) disease is still unclear [14]. It is traditionally accepted that the pathogenesis of neuropsychiatric lupus is multifactorial, varying between human subjects and involving autoantibodies, immune complexes and cytokines. Several autoantibodies can be measured in serum and cerebrospinal fluid of CNS lupus and divided in brain specific and systemic autoantibodies. Among brain specific autoantibodies those binding neuronal, brain reactive autoantibodies (BRAA), N-methyl-d-aspartate receptor (NMDA) subunits NR2a and NR2b, ganglioside, microtubule-associated protein 2 (MAP-2), neurofilament and glial fibrillary acidic proteins need to be mentioned [13]. Particularly, NR2 subunit receptor anti-DNA antibodies may induce neuronal apoptosis and its higher levels can be found in cerebrospinal fluid from patients with SLE and cognitive decline [14].

aPLs (and anti-cardiolipin in particular) are the most common in neuropsychiatric SLE patients, related to cognitive impairment, depression, psychosis, chorea, seizure and migraine [13].

Neuropsychiatric manifestations are included in the 1997 American College of Rheumatology (ACR) and Systemic Lupus International Collaborating Clinics (SLICC) criteria for diagnosis of SLE [27,28]. CNS lupus diagnosis is clinical and based on autoantibodies detection and advanced techniques as magnetic resonance spectroscopy [14]. Other neurologic criteria can be found in myelitis, cranial neuropathy, mononeuritis multiplex, peripheral neuropathy and acute confusional state. Neuropsychiatric SLE do not show a high frequency due to non-specific symptomatology [26,29].

Renal Manifestations

Lupus nephritis (LN) is considered one of the most relevant complications of SLE and the major predictor of poor prognosis. The formation of immune complexes may affect

renal physiological functioning and causes glomerulonephritis, classified as Class I-IV according to the location and the amount of immune complex deposition [7,26].

LN often develops during the first years after SLE diagnosis where persistent proteinuria greater than 0.5g per day or 3 + on dipstick and/or red blood cells, hemoglobin, granular, tubular or mixed casts on urine examination are considered as ACR criteria to define SLE [26]**.** However, the gold standard for LN diagnosis is a renal biopsy evaluating inflammatory interstitial infiltrates, interstitial fibrosis, tubulitis and tubular atrophy. Tubular atrophy appears strictly connected with interstitial fibrosis suggesting a central involvement in a worse outcome for patient [7].

Glucocorticoids and cyclophosphamide (CYC) represent the principal actors in therapeutic treatment of LN according to ACR and EULAR. Therapeutic approach with mycophenolate mofetil (MMF) has been introduced in specific human populations as African-Americans [30]. Guidelines indicate the use of the renin-angiotensin-aldosterone system (RAAS) blockers and hydroxychloroquine (HCQ) in presence of proteinuria and hypertension. Azathioprine is recommended for maintenance of remission after induction treatment with CYC or MMF or in milder cases of SLE. Rituximab can be considered in refractory patients or when other immunosuppressants are contraindicated [13].

Gastrointestinal Manifestations

SLE gastrointestinal involvement is partly due to vasculitis at the level of visceral smooth muscles and it is associated with poor prognosis for patients. Among clinical manifestations, intestinal pseudo obstruction (IPO) is considered an uncommon complication of SLE with a difficult diagnosis and delayed treatment [7].

Pleuropulmonary Manifestations [26]

Among pleuropulmonary affection, pleuritis, acute pneumonitis, interstitial lung disease (ILD), shrinking lung syndrome (SLS), pulmonary embolism, pulmonary arterial hypertension (PAH) and diffuse alveolar hemorrhage (DAH) are the principal clinical manifestations.

Pleuritis has higher prevalence, occurring in about 14% of SLE patients: chest pain, cough, shortness of breath and fever may represent the characteristic traits. ILD occurs in 3-13% of SLE patients and it is characterized by a reduced lung diffusion capacity. Acute pneumonitis is less frequent and difficult to differentiate from infection or drug-induced pneumonitis. For this reason, therapeutic treatment (based on high-dose steroids and treatment of infection simultaneously) may be delayed and mortality can reach 50%. Among less common pleuropulmonary manifestations, also DAH can be included as a consequence of capillaritis and it generally occurs in SLE patients with high disease activity.

PAH can be secondary in SLE patients and related to autoantibodies-mediated endothelial dysfunction and increased cardiovascular risk. Vasodilatory and immunosuppressive therapies can be beneficial. SLS is another uncommon complication of lupus disorder where dyspnea, small lung volumes and a reduced pulmonary functionality are the principal clinical signs. The lack of a standardized therapeutic treatment is due to the rarity of this manifestations.

Moreover, a higher risk of pulmonary embolism occurs in patients affected by SLE and it is associated with increased risk of thrombotic events in antiphospholipid antibodies positive patients.

Cardiovascular Manifestations

Patients with SLE are characterized by higher frequency of cardiovascular diseases (CVD) compared to the general population [31]. Several observational studies revealed that among immune-mediated inflammatory disorders, SLE shows the highest CVD standardized prevalence also after adjustment for demographic and traditional cardiovascular risk factors. It is generally accepted that inflammation has a central role in CVD, promoting endothelial dysfunction and atherosclerosis progression by cytokines and inflammatory mediators' production as well as white blood cells, platelets and coagulation factors recruitment. CVD represent one of the principal causes of mortality for several autoimmune disorders (RA, BS, vasculitis, psoriatic arthritis, psoriasis etc) but particularly for SLE, suggesting the involvement of traditional risk factors, chronic inflammation, SLE disease activity, SLE-related immunological factors and SLE-related medications in increasing cardiovascular risk [32,33].

As shown in figure 4, the mortality pattern is bimodal in SLE. As reported by Urowitz et al. in 1976 [34], after a long-term systematic analysis of 81 patients followed for five years at the University of Toronto Rheumatic Disease Unit and by Rubin LA et al. in 1985 [35], death seems to be firstly associated with infections, glomerulonephritis and CNS lupus in patients with early duration of disorder where SLE is active and therapeutic treatment with steroids is largely employed. On the contrary, the principal cause of mortality in SLE patients with long duration of disorder is represented by CVD (particularly myocardial infarction).

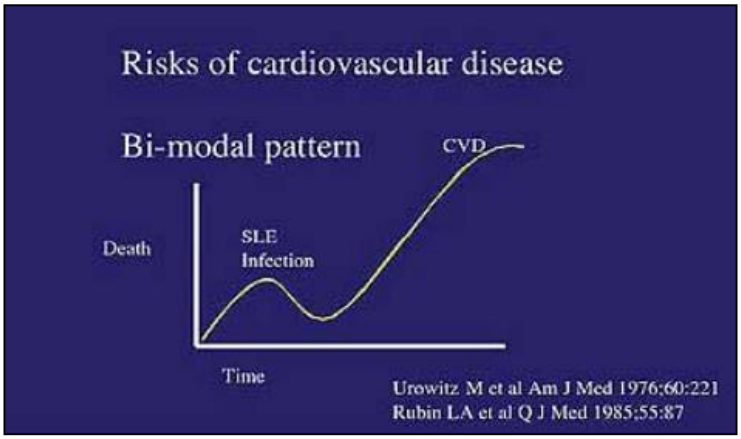

Fig.4 The bimodal pattern of mortality in SLE patients [34,35]

The major part of cardiac structures can be affected: pericardium, endocardium, myocardium, coronary arteries and conduction tissue. Commonly, immune complexes are at the base of pericarditis, one of the most characteristic cardiovascular manifestations and included in the ACR classification criteria for SLE [27]. Valvular abnormalities are observed in several cases of SLE, suggesting a role of aPL and anti-endothelial antibodies and immune complexes deposition as the main pathogenetic mechanisms.

Among CVD, myocarditis is a common manifestation: myocardial dysfunction is largely due to premature atherosclerosis-induced coronary artery disease (CAD), hypertension, renal failure, valvular disease and toxic effects of medications as CYC and chloroquine [36]. Several reports in literature show the early atherosclerosis in SLE and its association with the majority of CAD cases in affected [36,37].

Traditional and disease-related risk factors for CVD in SLE are summarized in figure 5.

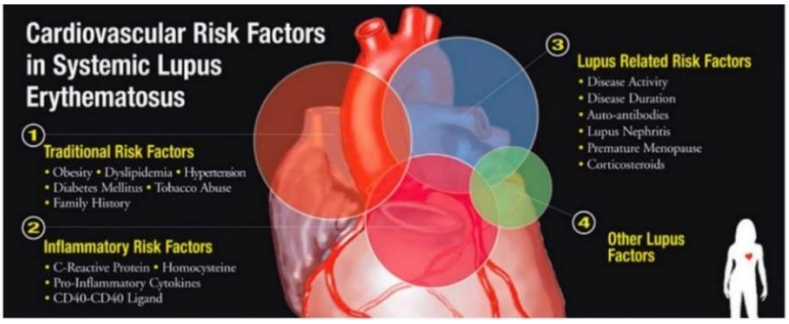

Fig.5 Cardiovascular risk factors in SLE [5]

Traditional CVD Risk Factors in SLE

Hypertension. With its prevalence of 17-52% in SLE patients and higher levels in females, hypertension may contribute to plaque formation and arterial stiffening [37,38].

Dyslipidemia. SLE patients are characterized by an atherogenic lipid profile with high levels of total cholesterol, triglycerides (TG), low-density lipoproteins (LDL), very low-density lipoproteins (VLDL) and Lipoprotein(a)-Lp(a) as well as lower levels of high-density lipoprotein (HDL) cholesterol [5], suggesting their involvement in atherosclerotic plaque formation via ROS-induced lipid oxidation. Moreover, circulating lipoprotein remnant particles seem to be a trigger factor for complement system activation and inflammatory response [37]. Additionally, alterations in HDL function were found in SLE patients. If HDL cholesterol has an anti-inflammatory and anti-atherogenic role in healthy subjects, preventing oxLDLs and foam cells formation, a pro-inflammatory HDL subtype (piHDL) has been displayed in SLE [5,37]. Indeed, piHDL generates from chronic inflammation–induced structural HDL changes that reduce HDL cholesterol ability to reverse cholesterol transport and clear oxLDL from the subendothelial space [37]. piHDL higher levels were found in SLE patients with CVD than those without [5].

Additionally, an altered activity of paraoxonase 1 (PON1), an antioxidant component of HDL able to prevent lipoprotein oxidation, has been described in lupus disorder and associated with cardiovascular and cerebrovascular events. It is probably due to auto-antibodies against PON-1: SLE patients with IgG anticardiolipin (aCL) show reduced levels of apoA-1, one of the major anti-inflammatory and atheroprotective component of HDL [5, 39]. The presence of anti-oxLDL antibodies associated with the increased risk of atherosclerosis is also displayed in [39].

Besides these factors, other contributors to the increased cardiovascular risk in SLE derive from Framingham heart studies [5,37,38]:

1. *Advanced age*
2. *Sex*
3. *Coronary artery disease and myocardial infarction*
4. *Diabetes mellitus*,
5. *Obesity*
6. *Smoking*

Pathogenesis of Atherosclerosis

Atherosclerosis is recognized as a condition strictly connected with the increased cardiovascular risk in SLE patients. Cumulative evidence in literature underlines the multiple contribute of immune system de-regulation, inflammation, traditional cardiovascular risk factors, aberrant endothelial cell functions and repair mechanisms (lower levels of endothelial progenitor cells, EPCs, has been observed in SLE patients) as well as SLE-related determinants in atherosclerosis development and progression [39]. Atherosclerosis is a chronic inflammatory disease occurring in the arterial wall. It is due to the progressive accumulation and oxidation of LDL in the subendothelial space, promoting endothelial activation and consequent inflammatory responses involved in atherosclerotic plaque formation [39-41]. Several cellular and non-cellular components play a central role in atherosclerotic process.

Briefly, shear-stress or inflammation-mediated endothelial injury promotes the upregulation of adhesion molecules as VCAM-1, intercellular adhesion molecule 1 (ICAM-1), E-selectin and P-selectin, cytokines and chemokines as monocyte chemotactic protein-1 (MCP-1), IL-6, TNF-α and also the generation of ROS. Additionally, a decrease in NO production is observed. This condition results in the activation and dysfunction of endothelium that undergoes a pro-thrombotic and pro-atherogenic phenotype. As shown in figure 6, blood flow cells (monocytes, T-cells and also platelets) are recruited to the

endothelium, migrating in the subendothelial space together with plasma lipids (LDL, VLDL) that are oxidized by ROS and converted to ox-LDL. In the subendothelial space, monocytes-macrophages engulf ox-LDL (via interaction between ox-LDL and TLR4 and CD14 on macrophages) becoming foam cells, the basis of plaque lesion development [39]. Mechanical stimulating factors may induce macrophages and platelets secretion of pro-inflammatory cytokines that amplify inflammatory response and promote proliferation and recruitment of smooth cells in the arterial intima. Despite the production of growth factors for endothelial cells and pro-inflammatory cytokines, smooth cells secrete cellular matrix components leading to the formation of a fibrous plaque, composed of a lipid core surrounded by smooth cells and connective tissue fibers [41]. Activated macrophages expose metalloproteinases (MMPs) that mediate the fibrous cap proteolytic degradation and the release of pro-thrombotic molecules promoting thrombosis [39,41].

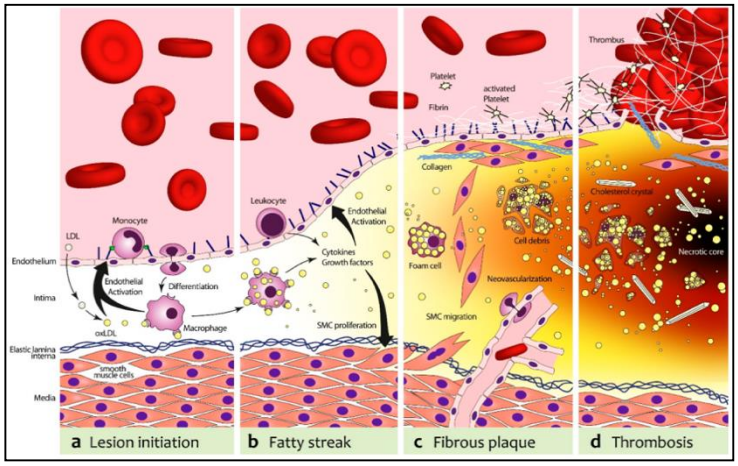

Fig.6 Process of Atherosclerosis [42]

Disease-related CVD Risk Factors in SLE

Demographics/Clinical Features. Male sex, advanced age, aPLs, renal function alterations and previous vascular events, together with chronic organ damage (according to SLICC damage index and SLEDAI (Systemic Lupus Erythematosus Disease Activity Index) are crucial in SLE cardiovascular manifestation development [37].

Particularly, aPLs antibodies are found in about 30-40% of SLE patients, suggesting how SLE could be considered the most common secondary form of Antiphospholipid Syndrome (APS) [5]. aPLs act against membrane phospholipids and proteins of several blood cells inducing platelet aggregation and improving thrombosis risk. Indeed, aPLs positivity predispose to valvular disease, arterial and venous thrombosis and pulmonary hypertension [38].

Moreover, lupus nephritis and renal disease are some of the most representative indicators of disease activity. Renal involvement is recognized in about 50% of patients with SLE. Lupus nephritis can lead to acute renal failure or chronic kidney disease and it is strongly associated with increased risk of myocardial infarction in SLE patients [38].

SLE Therapy. Therapeutic treatment with glucocorticoids reveals a double effect in affected individuals. Indeed, it shows positive results on clinical symptomatology of patients but also harmful effects, altering blood pressure, glucose and lipid profile and corporal weigh [5]. Longer duration and high cumulative dose of glucocorticoids or azathioprine are associated with increased atherosclerosis and cardiovascular risk in SLE patients. On the contrary, HCQ, cyclophosphamide, MMF, antimalarial drugs seem to have protective effects against atherosclerosis [37,38]. Many studies showed that HQC is able to inhibit aPLs-induced platelet aggregation, reducing the risk of thrombotic events [5].

Genetics. Despite data in literature are still lacking, some study displayed an involvement of genetic contributors in the pathogenetic mechanisms of atherosclerosis in SLE patients, leading to an increased CVD risk. Particularly, some specific genetic variants of STAT4 and BAFF has been associated with higher risk of arterial events and ischemic cerebrovascular disease in SLE [37].

Inflammatory/non-traditional risk factors. As a chronic inflammatory and autoimmune disorder, SLE is characterized by an overproduction of several inflammatory mediators

as cytokines, ROS and Reactive Nitrogen Species (RNS) that are strongly associated with endothelial dysfunction, atherosclerosis progression and CVD risk. Beside oxidative stress, increased levels of IFN-1 can induce EPCs depletion affecting endothelial repair mechanisms, proangiogenic IL-1 transcriptional pathways inhibition, macrophages recruitment to atherosclerotic lesions and foam cell formation as well as plaque instability promotion and platelets activation [37]. In this context, also NETs are actively involved in SLE vascular events, interacting with blood cells, coagulation factors and pro-inflammatory signaling pathways (IFN-I) and participating as molecular scaffold for thrombus formation [37].

Several pro-inflammatory cytokines and chemokines including MCP-1, interleukin-8 (IL-8), TNF- α and IL-6 are increased in SLE patients together with adhesion molecules as VCAM-1, ICAM-1 and E-selectin that promote blood cells recruitment [37]. Both TNF-α and IL-1 stimulate monocyte differentiation into macrophages/foam cells, promoting arterial inflammation and endothelial dysfunction [39]. Evidence in literature suggests the role of IL-17 in stimulating atherosclerosis in autoimmune patients as well as alterations in Treg activity seem to be strictly associated with atherosclerotic plaque formation [39].

Another relevant risk factor for cardiovascular events is represented by homocysteine (Hcy), based on the evidence that SLE patients show higher levels of Hcy compared to general population, probably due to macrophage activation [37]. Hyperhomocysteinemia is potentially involved in atherosclerosis and thrombotic risk by mediating endothelial dysfunction, proliferation of smooth muscle cells, platelet activation and atherosclerotic plaque assembly as well as inhibiting NO production [5,37].

Finally, elevated levels of circulating ox-LDL, as a consequence of reduced antioxidant HDL levels or PON1 activity, higher piHDL content or systemic oxidative stress status

in SLE [37], represent themselves a system of endothelial insult, triggering a local immune response.

Risk factors	Monitoring strategies	Management strategies
SLE Disease activity	Assess disease activity and medications at each visit	• Lowest possible dose of corticosteroids • Add steroid sparing agent if unable to lower corticosteroid dose • Consider hydroxychloroquine therapy • Consider ASA therapy
SLE Renal disease	Assess renal parameters at each visit (BP, serum albumin, creatinine, and urinalysis) • Goal BP <130/80 mmHg • Goal to normalize creatinine and albumin • Goal proteinuria <300 mg/dl	• Aggressive blood pressure control • Addition of ACE inhibitor • Consider ASA therapy
Anti-phospholipid or Lupus Anticoagulant positivity	Check anti-phospholipid, Lupus Anticoagulant, and beta 2 glycoprotein antibody status initially and as needed	• Consider hydroxychloroquine therapy • Consider ASA therapy

Table.2 SLE-specific cardiovascular risk factors [5]

SLE Diagnosis

General Diagnostic Criteria and Molecular Markers

SLE is characterized by a great variability in symptomatology as well as in clinical and serological profile. These elements suggested the elaboration of more specific and accurate diagnostic criteria especially during the early phase of the disorder when the clinical features are still quite evident [43].

The ACR revealed the most used classification criteria for SLE diagnosis in 1971, that underwent in changes and innovation in 1982 and 1997, yielding more sensitivity and specificity. According to these, SLE diagnosis was performed if at least 4 of the 11 criteria were recognized, simultaneously present or not [27]. However, based on ACR criteria, several SLE patients in early phase or limited disease were not included and the exclusion of some cutaneous (as maculopapular or polycyclic rash), neurological (as myelitis) and immunological manifestations represent an important limitation. Additionally, the lack of inclusion of low serum complement components levels in ACR criteria compromise the diagnosis for patients without any immunological criteria [15].

In 2012, the SLICC, an international group focused on SLE clinical research [28] added some variations to ACR classification criteria being more sensitive by less specific than the ACR criteria [25].

New guidelines were elaborated and used for SLE diagnosis still now (as shown in table 3). On the contrary to the ACR criteria, clinical and immunological criteria were divided and SLE diagnosis were based on lupus nephritis in the presence/absence of ANAs or anti-dsDNA antibodies or the positivity to four criteria (including at least one clinical criterion and one immunological criterion) [15,44]. In SLICC criteria, a classification of cutaneous manifestations in acute and chronic one is reported, excluding photosensitivity because of the difficult distinction with discoid rash. Articular involvement is accepted in presence of pain affecting at least two joints, whereas neurological manifestations are

evaluated according to several clinical treats reported in "ACR neuropsychiatric case definitions" of 1999 [44]. Further, SLICC criteria also updated proteinuria cut off values, including the presence of a ratio between urinary proteins and creatinine obtained in 8-24 hours comparable to 500mg of proteins in 24 hours [44].

Among immunological treats, anti-ds DNA, anti-Smith (anti-Sm) antibodies, antiphospholipid (anti-β2 glycoprotein) antibodies are considered, together with low complement levels (based on C3, C4, CH50 or total hemolytic complement levels) and the positivity to Coombs test, not in case of hemolytic anemia [15,44].

Table 1
Diagnostic criteria for systemic lupus erythematosus

SLICC Classification System[2]	1997 Update of the 1982 ACR Criteria for Classification of SLE[1]
Must meet 4 criteria, including 1 clinical and 1 immunologic	**Must meet 4 of 11 criteria**
Clinical criteria	
Acute cutaneous lupus (1 or more of the following) • Malar rash • Bullous lupus • Maculopapular rash • Photosensitive rash *Chronic cutaneous lupus* (1 or more of the following) • Classic discoid (localized vs generalized) • Hypertrophic lupus • Lupus panniculitis • Mucosal lupus • Lupus erythematous tumidus • Chilblains lupus • Discoid lupus/lichen planus overlap Oral or nasal ulcers Nonscarring alopecia *Synovitis OR tendonitis:* 2 or more joints + morning stiffness *Serositis:* pleural or pericardial Renal • Urine protein-to-creatinine ratio with more than 500 mg protein/24 h OR • Red blood cell casts *Neurologic* (1 or more of the following) • Seizures • Psychosis • Mononeuritis multiplex • Myelitis • Neuropathy • Acute confusional state *Hematologic* • Hemolytic anemia OR • Leukopenia (<4000/mm³ at least once) OR • Lymphopenia (<1000/mm³ at least once) OR • Thrombocytopenia (<100,000/mm³)	Malar rash Discoid rash Photosensitivity Oral or nasal ulcers Nonerosive arthritis: 2 or more joints *Serositis:* pleural or pericardial Renal disorder • Persistent proteinuria >500 mg/24 h OR >3 + quantification OR • Cellular cast: red cell, hemoglobin, granular, tubular or mixed Neurologic disorder • Seizure OR • Psychosis Hematologic disorder • Hemolytic anemia with reticulocytosis OR • Leukopenia (<4000/mm³ or more than 2 occasions) OR • Lymphopenia (<1500/mm³ on more than 2 occasions) OR • Thrombocytopenia (<100,00/mm³)
Immunologic criteria	
ANA positivity Anti-dsDNA positivity Anti-Sm positivity Antiphospholipid antibody positivity Low complement Direct Coombs (in absence of hemolytic anemia)	ANA positivity Anti-DNA, anti-Sm, OR antiphospholipid antibody positivity

Table.3 Diagnostic criteria for SLE [26]

SLE pathogenesis is greatly driven by immune system dysfunction and autoantibodies production, actively involved in tissue injury and systemic clinical manifestations. Based on these data, detecting the presence of antibodies against self-antigens in biological fluids is included in the clinical practice for SLE diagnosis.

Introduction

ANA antibodies, directed against nuclear components, belong to IgG class and represent the most common autoantibodies found in SLE patients but not specific for the disorder [15]. Anti-dsDNA autoantibodies, assessed in 50-60% of affected, are more specific for SLE, showing a great relevance in clinical practice. Indeed, antibodies against nucleosomes are significantly related to renal failure and disease activity. Among Extractable Nuclear Antigens (ENA) antibodies, anti-Sm, anti-nRNP (against ribonucleoproteins and also found in Sjögren Syndrome and Mixed Connective Tissue Disease-MCTD), anti-SSA/Ro and anti-SSB/La (against cytosolic or nuclear antigens, associated with photosensibility and cutaneous lesions and also found in Sjögren Syndrome and MCTD) antibodies play a central role in SLE disease. Other classes of autoantibodies can be involved in SLE pathogenesis, including those against red blood cells (associated with autoimmune hemolytic anemia), platelets (associated with autoimmune piastrinopenia) and lymphocytes (associated with lymphocytopenia) [45,46]. aPLs antibodies are an heterogenous group of autoantibodies against cellular membrane phospholipids, plasmatic proteins with high affinity for membrane phospholipids or protein-phospholipids complexes. Among this class of antibodies, aCL, anti-β2 glycoprotein and anti-phospholipid-thrombin complex represent the principal constituents. The Lupus Anticoagulant (LAC) is an immunoglobulin against cellular membrane proteins and phospholipids, found with higher percentage in SLE patients than in healthy subjects. However, patients do not necessarily develop LAC. About 30-40% of patients affected by SLE are positive for aPLs antibodies, associated with platelets aggregation and arterial/venous thrombotic events [46]. The positivity to aPLs antibodies does not necessarily lead to APS but when it happens, an increased cardiovascular risk is observed. Several data in literature reported a progression of atheroma in presence of APS as well as more damages to vascular and cardiovascular system [14].

Introduction

As a chronic inflammatory and immune-mediated disorder, an activation of complement system is reported in SLE, suggesting the assessment of C3 and C4 complement serum fractions for diagnosis and clinical monitoring. Lower levels of complement components and higher anti-dsDNA levels are used for the evaluation of disease activity.

An increase in total blood immunoglobulin amount, together with other pro-inflammatory markers (as velocity of erythrocyte sedimentation and rheumatoid factor) is also described [46].

Among novel biomarkers, an upregulation of IFN-I genes is observed [15].

Finally, imaging tests as well as biochemical analysis on urinary samples and renal biopsy are employed in order to evaluate renal, cardiac, pulmonary, neurological and articular involvement. In particular, proliferative lupus nephritis is described by higher levels of urinary B cell activating factor (uBAFF), urinary proliferation-inducing ligand (uAPRIL) and urinary osteoprotegerin (uOPG) levels than controls or SLE patients without nephritis [7]. Other urinary markers are emerging as indicators of disease activity. Urinary podocyte excretion, urine progranulin (uPGRN) and pentraxin 3 (uPTX3) levels are useful for disease progression: several data reported the significantly increase in these parameters in active lupus nephritis patients when compared to those in remission or healthy controls. Additionally, serum TNF-like weak inducer of apoptosis (TWEAK) and serum insulin-like growth factor binding protein-2 (IGFBP-2) levels seem to be associated with renal involvement and clinical activity [7].

The variability of SLE clinical manifestations led to the elaboration of disease activity index scores to better evaluate severity, prognosis and therapeutic response of SLE. The principal disease indexes are represented by The Systemic Lupus Activity Measure (SLAM) Index, the SLEDAI Index, The European Consensus Lupus Activity Measurement (ECLAM) and The British Isles Lupus Assessment Group (BILAG).

Among them, BILAG score is used to evaluate damages to organs whereas SLEDAI is associated with disease activity status [45]. These indexes are principally needed in research but they are also used for the disease clinical monitoring.

BILAG Index

BILAG Index was proposed for the first time in 1988 and the updated (BILAG 2004) in 2005 (table 4).

BILAG-2004 INDEX

- Only record manifestations/items due to SLE disease activity
- Assessment refers to manifestations occurring in the last 4 weeks (compared with the previous 4 weeks)
- TO BE USED WITH THE GLOSSARY

Record: NA Not Available
0 Not present
1 Improving
2 Same
3 Worse
4 New

Yes/No OR Value (where indicated)
*Y/N Confirm this is due to SLE activity (Yes/No)

CONSTITUTIONAL
1. Pyrexia - documented >37.5° C ( )
2. Weight loss - unintentional >5% ( )
3. Lymphadenopathy/splenomegaly ( )
4. Anorexia ( )

MUCOCUTANEOUS
5. Skin eruption - severe ( )
6. Skin eruption - mild ( )
7. Angio-oedema - severe ( )
8. Angio-oedema - mild ( )
9. Mucosal ulceration - severe ( )
10. Mucosal ulceration - mild ( )
11. Panniculitis/Bullous lupus - severe ( )
12. Panniculitis/Bullous lupus - mild ( )
13. Major cutaneous vasculitis/thrombosis ( )
14. Digital infarcts or nodular vasculitis ( )
15. Alopecia - severe ( )
16. Alopecia - mild ( )
17. Periungual erythema/chilblains ( )
18. Splinter haemorrhages ( )

NEUROPSYCHIATRIC
19. Aseptic meningitis ( )
20. Cerebral vasculitis ( )
21. Demyelinating syndrome ( )
22. Myelopathy ( )
23. Acute confusional state ( )
24. Psychosis ( )
25. Acute inflammatory demyelinating polyradiculoneuropathy ( )
26. Mononeuropathy (single/multiplex) ( )
27. Cranial neuropathy ( )
28. Plexopathy ( )
29. Polyneuropathy ( )
30. Seizure disorder ( )
31. Status epilepticus ( )
32. Cerebrovascular disease (not due to vasculitis) ( )
33. Cognitive dysfunction ( )
34. Movement disorder ( )
35. Autonomic disorder ( )
36. Cerebellar ataxia (isolated) ( )
37. Lupus headache - severe unremitting ( )
38. Headache from IC hypertension ( )

MUSCULOSKELETAL
39. Myositis - severe ( )
40. Myositis - mild ( )
41. Arthritis (severe) ( )
42. Arthritis (moderate)/tendonitis/tenosynovitis ( )
43. Arthritis (mild)/Arthralgia/Myalgia ( )
44. Myocarditis - mild ( )
45. Myocarditis/endocarditis + cardiac failure ( )
46. Arrhythmia ( )
47. New valvular dysfunction ( )
48. Pleurisy/pericarditis ( )
49. Cardiac tamponade ( )
50. Pleural effusion with dyspnoea ( )
51. Pulmonary haemorrhage/vasculitis ( )
52. Interstitial alveolitis/pneumonitis ( )
53. Shrinking lung syndrome ( )
54. Aortitis ( )
55. Coronary vasculitis ( )

GASTROINTESTINAL
56. Lupus peritonitis ( )
57. Abdominal serositis or ascites ( )
58. Lupus enteritis/colitis ( )
59. Malabsorption ( )
60. Protein-losing enteropathy ( )
61. Intestinal pseudo-obstruction ( )
62. Lupus hepatitis ( )
63. Acute lupus cholecystitis ( )
64. Acute lupus pancreatitis ( )

OPHTHALMIC
65. Orbital inflammation/myositis/proptosis ( )
66. Keratitis - severe ( )
67. Keratitis - mild ( )
68. Anterior uveitis ( )
69. Posterior uveitis/retinal vasculitis - severe ( )
70. Posterior uveitis/retinal vasculitis - mild ( )
71. Episcleritis ( )
72. Scleritis - severe ( )
73. Scleritis - mild ( )
74. Retinal/choroidal vaso-occlusive disease ( )
75. Isolated cotton-wool spots (cytoid bodies) ( )
76. Optic neuritis ( )
77. Anterior ischaemic optic neuropathy ( )

RENAL
78. Systolic blood pressure (mm Hg) value ( ) Y/N*
79. Diastolic blood pressure (mm Hg) value ( ) Y/N*
80. Accelerated hypertension Yes/No ( )
81. Urine dipstick protein (+=1, ++=2, +++=3) ( ) Y/N*
82. Urine albumin-creatinine ratio mg/mg ( ) Y/N*
83. Urine protein-creatinine ratio mg/mg ( ) Y/N*
84. 24-hour urine protein (g) value ( ) Y/N*
85. Nephrotic syndrome Yes/No ( )
86. Creatinine (plasma/serum) µmol/L ( ) Y/N*
87. GFR (calculated) mL/min/1.73 m^2 ( ) Y/N*
88. Active urinary sediment Yes/No ( )
89. Active nephritis Yes/No ( )

HAEMATOLOGICAL
90. Haemoglobin (g/dL) value ( ) Y/N*
91. Total white cell count (x 10^9/L) value ( ) Y/N*
92. Neutrophils (x 10^9/L) value ( ) Y/N*
93. Lymphocytes (x 10^9/L) value ( ) Y/N*
94. Platelets (x 10^9/L) value ( ) Y/N*
95. TTP ( )
96. Evidence of active haemolysis Yes/No ( )
97. Coomb's test positvie (isolated) Yes/No ( )

Table.4 BILAG 2004 Index

Introduction

According to the new version, it is an organ-specific 86-question assessment based on the principle of the doctor's intent to treat, which requires an assessment of improved (1), the same (2), worse (3), or new (4) over the last month. Within each organ system, multiple manifestations and laboratory tests are combined into a single score for that organ.

The resulting scores for each organ can be A through E. Grade A represents very active disease where immunosuppressive drugs and/or a prednisolone (or equivalent) dose of >20 mg daily or high-dose anticoagulation are needed. Grade B indicates moderate disease activity, suggesting lower doses of corticosteroids, topical steroids, topical immunosuppressive drugs, antimalarials, or nonsteroidal anti-inflammatory drugs. Grade C is referred to mild stable disease as well as for patients previously affected but without disease activity whereas grade E is used for cases with no disease activity.

The BILAG 2004 index susceptibility to changes in SLE disease activity legitimate its use not only for research, but also for evaluating positive or negative evolutions of the disorder. Particularly, it is useful to monitor disease outcome and treatment protocols.

This score is simple to obtain and minimally influenced by the operator.

To facilitate comparisons with global indexes, a numerical scoring system has been associated with the BILAG 2004 index. The optimal method is to convert the assessments so that an "A" = 12 points, "B" = 8 points, "C" = 1 point, and "D/E" = 0 points [47].

SLEDAI Index

SLEDAI Index, firstly proposed in 1985, is an index to evaluate disease activity in patients affected SLE; modifications performed in 2002 included cases of persistent active disease (SLEDAI-2K) and are reported in table 5.

Weight (check)	Descriptor	Definition
8 ❑	Seizure	Recent onset, exclude metabolic, infectious, or drug causes.
8 ❑	Psychosis	Altered ability to function in normal activity due to severe disturbance in the perception of reality. Include hallucinations, incoherence, marked loose associations, impoverished thought content, marked illogical thinking, bizarre, disorganized, or catatonic behavior. Exclude uremia and drug causes.
8 ❑	Organic brain syndrome	Altered mental function with impaired orientation, memory, or other intellectual function, with rapid onset and fluctuating clinical features, inability to sustain attention to environment, plus at least two of the following: perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, or increased or decreased psychomotor activity. Exclude metabolic, infectious, or drug causes.
8 ❑	Visual disturbance	Retinal changes of SLE. Include cytoid bodies, retinal hemorrhages, serous exudates or hemorrhages in the choroids, or optic neuritis. Exclude hypertension, infection, or drug causes.
8 ❑	Cranial nerve disorder	New onset of sensory or motor neuropathy involving cranial nerves.
8 ❑	Lupus headache	Severe, persistent headache; may be migrainous but must be nonresponsive to narcotic analgesia.
8 ❑	Cerebrovascular accident	New onset of cerebrovascular accident(s); exclude arteriosclerosis.
8 ❑	Vasculitis	Ulceration, gangrene, tender finger nodules, periungual infarction, splinter hemorrhages, or biopsy or angiogram proof of vasculitis.
4 ❑	Arthritis	Two or more joints with pain and signs of inflammation (i.e., tenderness, swelling, or effusion).
4 ❑	Myositis	Proximal muscle aching/weakness, associated with elevated creatine phosphokinase/aldolase or electromyogram changes or a biopsy showing myositis.
4 ❑	Urinary casts	Heme granular or red blood cell casts.
4 ❑	Hematuria	More than five red blood cells/high power field; exclude stone, infection, or other cause.
4 ❑	Proteinuria	> 0.5 g/24 hr.
4 ❑	Pyuria	More than five white blood cells/high power field; exclude infection.
2 ❑	Rash	Inflammatory type rash.
2 ❑	Alopecia	Abnormal, patchy, or diffuse loss of hair.
2 ❑	Mucosal ulcers	Oral or nasal ulcerations.
2 ❑	Pleurisy	Pleuritic chest pain with pleural rub or effusion or pleural thickening.
2 ❑	Pericarditis	Pericardial pain with at least one of the following: rub, effusion, or electrocardiogram or echocardiogram confirmation.
2 ❑	Low complement	Decrease in the complement proteins C3 and C4 or in total complement activity (CH50), below the lower limit of normal for testing laboratory.
2 ❑	Increased DNA binding	Increased DNA binding by Farr assay above normal range for testing laboratory.
1 ❑	Fever	> 38°C; exclude infectious cause.
1 ❑	Thrombocytopenia	< 100,000 platelets/$\times 10^9$/L; exclude drug causes.
1 ❑	Leukopenia	< 3,000 white blood cells/$\times 10^9$/L; exclude drug causes.
	Total score	

Table.5 SLEDAI-2K Index

It is a list of 24 items, 16 of which are clinical items such as seizure, psychosis, organic brain syndrome, visual disturbance, other neurological problems, hair loss, new rash, muscle weakness, arthritis, blood vessel inflammation, mouth sores, chest pain worse

with deep breathing and manifestations of pleurisy and/or pericarditis and fever. Eight of the 24 items are laboratory results such as urinalysis testing, blood complement levels, increased anti-DNA antibody levels, low platelets, and low white blood cell count. These items are scored based on whether these manifestations are present or absent in the previous 10 days.

Organ involvement is weighted; for example, joint pain and kidney disease are each multiplied by four, but central nervous system neurological involvement is multiplied by eight. The weighted organ manifestations are then summed into a final score, which can range from zero to 105. Scores greater than 20 are rare. A SLEDAI of 6 or more has been shown to be consistent with active disease requiring therapy. A clinically meaningful difference has been reported to be an improvement of 6 points or worsening of 8 points.

The SLEDAI was modified in the Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) trial; this modification is known as the SELENA-SLEDAI system. The SELENA-SLEDAI adds some clarity to some of the definitions of activity in the individual items, but does not change the basic scoring system. SLEDAI score defined the activity categories: A SLEDAI score >5 is associated with a probability of initiating therapy in >50% of cases [47].

Current and Future Therapies

Current guidelines suggest the use of antimalarial drugs, glucocorticoids (GCs), non-steroidal anti-inflammatory drugs (NSAIDs) for the treatment of SLE constitutional symptoms and mild cases. On the contrary, methotrexate, azathioprine, CYC and MMF are recommended for patients with severe lupus [25, 48]. Intravenous immunoglobulin (natural polyclonal antibodies-mainly IgG fraction, pooled from sera of donors) are considered in case of active disorder, but numerous are the contraindications and limitations for its use [13].

Lupus patient therapeutic treatment is also driven by D vitamin, calcium supplements and antiresorptive agents for osteoporosis prevention, antihypertensive agents and statins in order to mitigate systemic clinical alterations [48].

The principal drugs used for SLE treatment are summarized in table 6 [13]:

- *Steroids*. As immunosuppressive molecules, they induce anti-inflammatory cytokines (IL-10, IL-1Ra and annexin-1) and decrease adhesion molecules expression and pro-inflammatory cytokines (IL-2, IL-6, TNF) levels. They can also modulate lymphocyte activation by inhibiting APCs process as well as cyclo-oxygenase 2 (COX 2) and nitric oxide synthase (NOS) activity;
- *Hydroxychloroquine*. HCQ is a molecule with immune-modulative characteristics but without immunosuppressive effects. It plays a role in modulating APCs process and TLR- 9 signaling pathway. It is used in case of arthritis, skin rashes and fatigue but it has also revealed antithrombotic properties;
- *Cyclophosphamide*. CYC prevents cell division by cross-linking DNA and suppressing DNA synthesis. It is recommended for lupus nephritis and severe SLE;

- *Azathioprine*. As a purine analogue, azathioprine suppresses DNA synthesis by inhibiting xanthylic and adenylic acids assembly. Used for systemic clinical features of lupus erythematosus and for maintenance therapy of lupus nephritis, it can also help to reduce steroid requirement;
- *Methotrexate*. Methotrexate is a folate antimetabolite used in cancer therapy for its role in inhibiting DNA synthesis. Its involvement in decreased purine synthesis and cell proliferation suggests the employment of methotrexate also in SLE treatment, particularly in case of skin and joints manifestations;
- *Mycophenolate mofetil*. MMF (mycophenolic acid as active metabolite) avoids T and B cells proliferation by interfering with nucleotides synthesis and inhibiting monophosphate dehydrogenase. It is suggested in cases of lupus nephritis and in moderate to severe SLE.

Steroids
Potent immunosuppressive drugs. Induce anti-inflammatory cytokines (interleukin 10, interleukin 1Ra, and annexin-1); decrease production of the adhesion molecules and inflammatory cytokines (interleukin 2, interleukin 6, and tumour necrosis factor); inhibit processing of antigens by monocytes for presentation to lymphocytes; and inhibit cyclo-oxygenase 2 and inducible nitric oxide synthase.[57] Used for all features of systemic lupus erythematosus.

Non-steroidal anti-inflammatory drugs
Analgesic, antipyretic, and anti-inflammatory properties. Inhibit cyclo-oxygenase, types 1 and 2. Used for fever, serositis, and arthritis.

Hydroxychloroquine
Immunomodulative properties without immunosuppression. Increases lysosomal pH and interferes with antigen processing and possibly modulation of the immune response mediated by toll-like receptor 9.[58] Used for arthritis, skin rashes, and fatigue. Might have a useful role in nephritis, have antithrombotic properties, and reduce cholesterol concentrations.

Cyclophosphamide
Forms active alkylating metabolites (4-hydroxycyclophosphamide, phosphoramide mustard, and acrolein). Prevents division of the cells by cross-linking DNA and suppressing DNA synthesis. Euro-lupus protocol recommends six pulses of intravenous cyclophosphamide at a dose of 500 mg every 2 weeks. Used for lupus nephritis[48] and severe systemic lupus erythematosus.

Azathioprine
Purine analogue that suppresses DNA synthesis by inhibiting synthesis of xanthylic and adenylic acids.[58] Used for systemic features of lupus and maintenance therapy of lupus nephritis, class III and IV. An option as an induction therapy for selected patients with lupus nephritis who are very concerned about the risk of infertility associated with cyclophosphamide,[60] and helps to reduce the steroid requirement.

Methotrexate
A folate antimetabolite that inhibits DNA synthesis. Binds to dihydrofolate reductase, resulting in decreased purine synthesis and cell proliferation. Used for non-organ threatening disease manifestations such as skin and joint disease.[61]

Ciclosporin
Forms complex with cyclophylin that disrupts the activation of calcineurin (complex of phosphatases). Inhibits production of interleukin 2 and arrests T-cell cycle between G0 and G1.[63] Used for moderate to severe systemic lupus erythematosus and as a steroid-sparing drug.

Mycophenolate mofetil
Mycophenolate (mycophenolic acid as active metabolite) inhibits monophosphate dehydrogenase and blocks synthesis of guanosine nucleotides and proliferation of T and B cells. Used for induction and maintenance therapy in lupus nephritis[62] and in moderate to severe systemic lupus erythematosus.

Tacrolimus
Calcineurin inhibitor effective in treatment of lupus nephritis[64] and in cutaneous lupus.[64]

Leflunomide
Inhibits dihydro-orotate dehydrogenase necessary for pyrimidine and cellular protein kinases synthesis. Has immunosuppressive and antiviral effects.[65]

Biologics
See panel 4.

Intravenous immunoglobulin
Consists of natural polyclonal antibodies, mainly IgG fraction, pooled from the sera of thousands of donors. Off-label use in catastrophic antiphospholipid syndrome.[66] An option in patients with active disease but there are contraindications and limitations for use of immunosuppressive drugs, such as pregnancy or concomitant infections.

Table.6 Conventional drugs for SLE treatment [13]

Therapeutic supplementation with conventional and novel immunosuppressive drugs improved 5-year survival rate of SLE patients from about 50-70% in the 1950s to over

90% in the 1990s and later [25,48]. However, SLE mortality remains higher if compared to the general population, largely due to CVD and renal failure, infections and tumors. Additionally, several adverse effects have been found in association with conventional drugs use, including weight gain, osteoporosis, immunodepression, glucose intolerance and hypertension, myopathy, delayed wound healing and behavior changes [25].

Based on this evidence, in the last years new therapeutic approaches have been developed in order to improve SLE management, increase survival rate and reduce side effects and GCs administration.

As shown in table 7, these therapeutic agents can be classified in different categories according to their specific mechanism of action and molecular targets [13,25]:

1. **B-cell targeting agents**
    1.1. B-cell depleting therapy: rituximab.
    1.2. Inhibition of B-cell survival: belimumab, atacicept.
    1.3. B-cell modulating therapy: epratuzumab.
    1.4. Other potential B-cell (plasma cell) targeting strategies.
2. **T-cell/costimulatory targeting agents**
    2.1. Inhibition of T-cell function: abatacept, ruplizumab, toralizumab, lupuzor.
3. **Cytokine/innate immunity targeting agents**
    3.1. Anti-IL-6 agents: tocilizumab.
    3.2. Anti- TNF-α agents: infliximab, etanercept.
    3.3. Anti- IFN-1 agents: sifalimumab, rontalizumab.
    3.4. Complement inhibitors: eculizumab.

Targeting B cells
- B-cell depleting therapy: rituximab
- B-cell modulating therapy: epratuzumab
- Inhibition of B-cell survival: belimumab, atacicept
- Other potential B-cell (plasma cell) targeting strategies: bortezomib

Targeting T cells
- Inhibition of T-cell function: abatacept, ruplizumab, toralizumab, lupuzor

Interleukin 6
- Tocilizumab

Tumour necrosis factor α inhibitors
- Infliximab
- Etanercept

Type I interferon inhibitors
- Sifalimumab
- Rontalizumab

Complement inhibitors
- Eculizumab

Table.7 Biological treatments for SLE [13]

1.1 **Rituximab**

Rituximab is a chimeric murine/human monoclonal antibody (mAb) projected against CD20, a transmembrane protein crucial in the modulation of cell-cycle and differentiation of B cellular lineage. CD20 is found on mature B cells and their precursor but not on stem cells, pro-B or plasma cells [13,14,25]. Rituximab is traditionally used and well tolerated in the treatment of non-Hodgkin's lymphoma and RA. However, cumulative evidence in literature has recently displayed its recommendation also for SLE patients, in case of unresponsiveness to conventional and novel immunosuppressive agents as MMF [14,25]. Humans therapeutic treatment with rituximab leads to circulating B cells elimination by complement and antibody dependent cytotoxicity, resulting in decreased autoantibodies production. Beside the simple B-cells depletion, also different CD4 regulatory T cells and increased T-cells apoptosis were observed after 30 days of rituximab supplementation, suggesting a role of this therapeutic agent in modulating regulatory T cells and B cells interaction [14].

Several clinical trials are now active in order to evaluate properties and beneficial effects of therapeutic treatment with Rituximab, but results are still controversial. The EXPLORER trial assessed rituximab in 257 patients with moderate and severe non-renal SLE and undergoing conventional drugs, evaluating the presence and maintenance of clinical response (according to BILAG score) as primary endpoint. Positive effects were observed in African-American and Hispanic patients [49]. On the contrary, the LUNAR trial, a randomized double-blind, placebo controlled trial and performed on 144 patients class III or IV lupus nephritis, reported that the primary endpoint (rituximab superiority) was not achieved [13,50].

Despite the results of these two trials that failed to demonstrate the superiority of rituximab over placebo, several other clinical studies are in progress, revealing beneficial effects derived from rituximab treatment. In cases of active lupus nephritis with no response to conventional therapies, Rituximab is suggested by ACR and EULAR guidelines [13].

1.2 **Belimumab**

Belimumab is a BLys inhibitor suggested for patients with autoantibody-positive active SLE under standard therapy. Belimumab administration is generally well tolerated, it avoids flares, increases serological activity, and allows to reduce corticosteroids exposure [51]. Based on this evidence, in 2011 Belimumab was approved by the Food and Drug Administration in the USA and by the European Commission for SLE treatment. BLys (also known as BAFF), is a 285 amino acids cytokine with a central role in B-cell homeostasis and survival. It belongs to the TNF family together with its related homologue APRIL and it is expressed by several cell types including innate immune cells (neutrophils, dendritic cells, monocytes and macrophages), T and B lymphocytes [51].

The proteolytic cleavage of Blys from cellular membrane activates the molecule and promotes its interaction with three different receptors involved in NF-kB signaling pathway activation: BR3 (BAFF receptor 3), TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor) and BCMA (B-cell maturation antigen). As shown in figure 7, BLys interaction with its receptor inhibits B-cells apoptosis, promoting B-cell proliferation and differentiation into immunoglobulin (Ig) producing plasma cells [51].

Belimumab is a fully human Ig G1λ monoclonal antibody obtained using recombinant DNA technology in a mammalian cell expression system. It shows high affinity for soluble BLys, avoiding its interaction with specific receptors and the consequent signaling pathway described before. B-cell proliferation and differentiation are inhibited, apoptosis of autoreactive B-cells induced and autoantibodies circulating levels reduced [51].

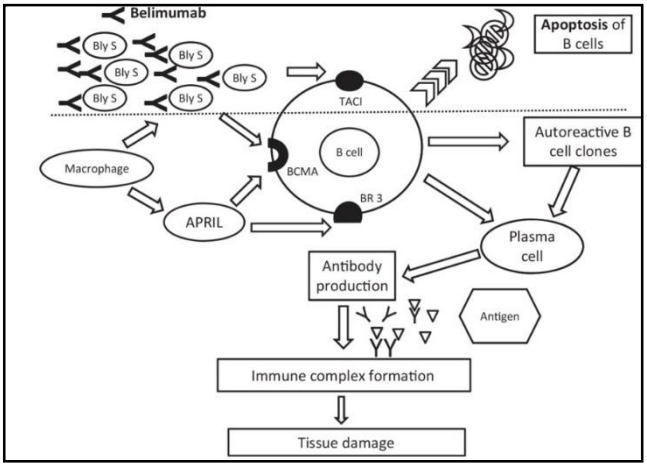

Fig. 7 Belimumab mechanism of action [51]

The pre-clinical trials and phase I trials were performed as early as 2000-2001 showing great results in terms of B lymphocytes and anti-dsDNA antibody reduction. This evidence encouraged further trials, suggesting the role of Belimumab as a new option for treatment and management of SLE [51].

Introduction

Following favorable results of Belimumab in phase II trials, two important phase III trials (BLISS-52 and BLISS-76 trials) were performed in order to assess the effect of Belimumab against placebo in autoantibody-positive active SLE patients under standard therapy, evaluating the Systemic Lupus Erythematosus Responder Index (SRI) at week 52 as primary endpoint [44].

BLISS-52 enrolled 865 patients from South America, Asia and Eastern Europe [54] while BLISS-76 recruited 819 patients from North America, Europe and Israel [53]. Both the studies reported a significantly increase in SRI rate in SLE patients after 52 weeks of supplementation with belimumab when compared to placebo group and no more adverse events were described. Consequently, these two clinical trials were essential in developing new therapeutic strategies for SLE treatment, underlying the safety profile and efficacy of belimumab in controlling SLE in a specific cohort of patients [54].

Further studies are needed to investigate the long-term safety, efficacy and tolerability and its effects on CNS, renal, lung and heart clinical complications. Data about belimumab pharmacokinetics, safety in pregnancy or in co-administration with corticosteroids as well as in combination therapies (Belimumab and Rituximab) are not completely reported [51,52]. At the same time, combined therapy with belimumab, reliable clinical biomarkers to predict positive/negative response to the pharmacological treatment and the potential existence of anti-drug antibodies associated to the loss of therapeutic efficacy represent essential future aims [55].

In addition to Belimumab, other agents with high affinity for BLys/BAFF pathway exist as Atacicept, a fusion protein between TACI and the Fc portion of IgG, is able to bind both BLys and APRIL. However, several clinical trials failed because of the excessive reduction in B cells number and serum immunoglobulin levels [6].

Redox Status in Biological Systems

Redox Homeostasis and Oxidative Stress: General Details

Oxygen has a central role in life mechanisms showing both beneficial and harmful effects on biological systems. The principal oxygen involvement is in adenosine-5-triphosphate (ATP) generation via mitochondrial oxidative phosphorylation [56]. Together with several others cellular redox processes, this reaction is also implicated in the production of ROS as well as RNS [57].

Free radicals are physiologically produced during normal cellular metabolism. A free radical can be defined as an atom or molecule containing one or more unpaired electrons in valency shell or outer orbit and capable of independent existence. These aspects determine its instability, short life and high reactivity that is described as the capacity of electron abstraction from other compounds to be more stable. Thus, the attacked molecule loses its electron and becomes a free radical itself, inducing chain reactions cascade which finally damages living cells [58].

At physiological levels, ROS/RNS play an important role in immune function (defence against pathogenic microorganisms) and in many intracellular signaling pathways, in mitogen response and in redox regulation as secondary intracellular messengers (figure 8) [8,58]. At higher concentrations, they can be responsible of molecular damages on proteins, lipids and nucleic acids (DNA, RNA) and a complex system of antioxidant defences has been evolved to maintain a redox balance and avoid biological system injury [56]. However, environmental factors, deficiencies in antioxidants, immune system dysfunctions, chronic disorders, etc can alter the balance between oxidant molecules and antioxidants, leading to a condition called *oxidative stress* or *nitrosative stress* respectively [59]. Indeed, if minor disturbances on the redox balance are likely to induce homeostatic adaptations in response to environmental changes, more major perturbations

may cause irreparable damages and cellular death [56]. Accordingly, oxidative stress is recognised to have a pathological involvement in several disorders including inflammation, atherosclerosis and cardiovascular diseases, autoimmune disorders, neurodegeneration, respiratory diseases, cancer as well as aging process [56,57,60].

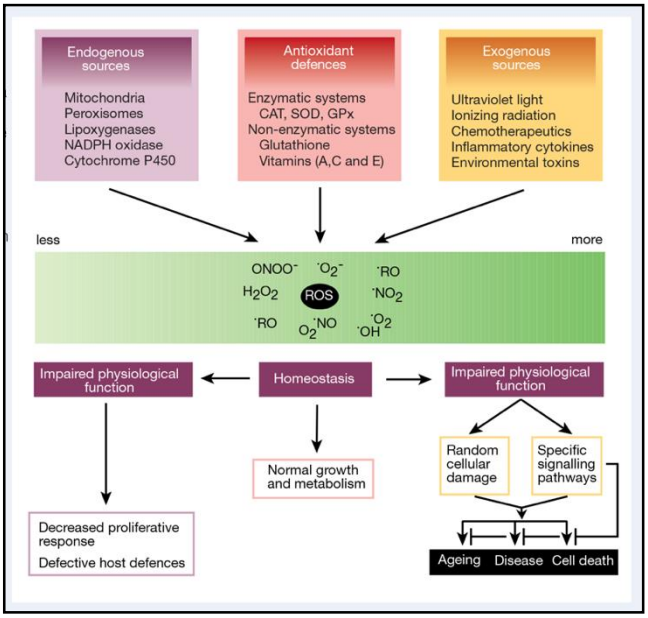

Fig.8 ROS sources and antioxidant systems [69]

ROS Classification

As shown in figure 9, the most important free radicals produced during metabolic reactions are represented by oxygen derived radicals or ROS. These elements can derive from endogenous sources as mitochondria, peroxisomes, endoplasmic reticulum but also from exogenous ones as cigarette smoke, hypoxia/hyperoxia status, ionizing radiations, heavy mental ions. Some pathophysiological processes as immune cell activation, inflammation, infection, aging, ischemia, excessive exercise and mental stress can be also included in ROS production [60].

Among ROS, Superoxide Ion Radical ($\mathbf{O_2^{\cdot -}}$), Hydroxyl Radical ($\mathbf{OH^{\cdot}}$), Peroxyl Radical ($\mathbf{ROO^{\cdot}}$) have been largely described. The non-radical species can be identified in

Hydrogen Peroxide (**H_2O_2**), Singlet Oxygen (**$^1O_2^-$**) and Hypochlorous Acid (**HOCl**): these molecules are not free radicals but they can easily lead to oxidative reactions in living organisms [58,60].

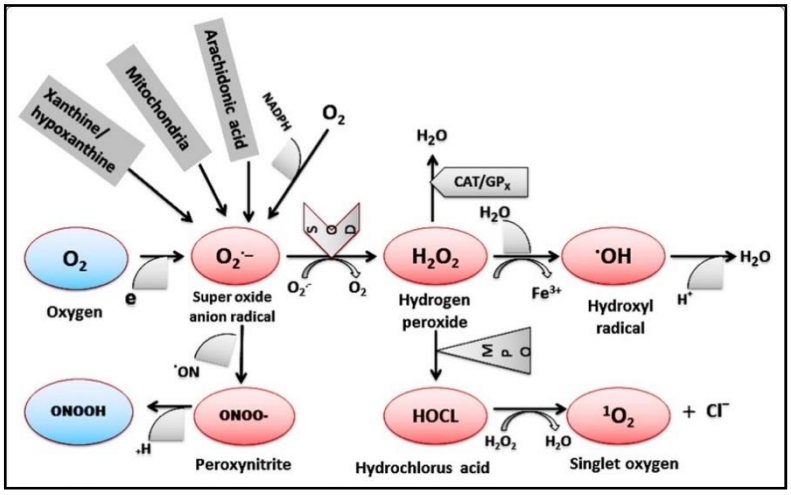

Fig.9 Free radical generation and their catabolism [120]

***Superoxide Ion Radical*.** $O_2^{\cdot-}$ is one of the most important ROS formed by enzymatic processes, auto-oxidation reactions and by a non-enzymatic electron transfer reactions in which an electron is transferred to molecular oxygen. Mitochondria are considered the principal source of $O_2^{\cdot-}$: electrons transfer along the enzymes belonged to the respiratory chain is not totally efficient and leakage of electrons on to molecular oxygen, in particular from complexes I and III, results in the generation of $O_2^{\cdot-}$ [56]. $O_2^{\cdot-}$ rate formation is determined by the number of present electrons on the chain and so is elevated under conditions of hyperoxia and raised glucose, as in diabetes. Paradoxically, it is also increased under hypoxia status, when the reduced availability of oxygen to act as the final electron acceptor for complex IV causes accumulation of electrons [56]. $O_2^{\cdot-}$ can be also generated from the shorter transport chain within endoplasmic reticulum (ER). Other sources of $O_2^{\cdot-}$ include NADPH (Nicotinamide Adenine Dinucleotide Phosphate Hydrogen) oxidase, found in polymorphonuclear leukocytes, monocytes and macrophages, cytochrome P450 and several oxide-reductase enzymes as xanthine

oxidase, lipoxygenase, cyclo-oxygenase. Various growth factors, drugs and toxins are also involved in ROS generation [56,58].

Superoxide ion is not able to come across intracellular membranes and its molecular damages are only evident in the compartment of generation. It is detoxified by the superoxide dismutase enzyme (SOD) which convert it to H_2O_2.

Hydrogen Peroxide. H_2O_2 it is not a free radical and so less reactive than $O_2^{\cdot -}$, but it is considered a reactive specie because of its involvement in free radical generation and detoxification [56]. It can easily diffuse through cellular and organelle membranes but any direct effect on DNA has been described. However, H_2O_2 can damage nucleic acid by producing **OH**$^{\cdot}$ in the presence of transition metal ions (Haber- Weiss reaction) and for this reason, the conversion of H_2O_2 to water, catalyzed by antioxidant enzymes as catalase (CAT) and glutathione peroxidase (GPx), is central in biological systems [56,58]. Xanthine oxidase, amino acid oxidase, NADPH oxidase and peroxisomes are important sources of H_2O_2 generation and particularly, granulocytic enzymes can expand the reactivity of H_2O_2 via eosinophil peroxidase and myeloperoxidase (MPO).

Additionally, H_2O_2 can biochemically react with chloride ion producing HOCl, a highly oxidative molecule and central in immune system response for pathogens killing. It can also react with DNA and proteins, inducing their oxidation [60].

Hydroxyl Radical. $OH^{\cdot}$ is the most dangerous among ROS, able to react with any biological molecule as proteins, lipids, carbohydrates and DNA causing severe damages to cells and consequently apoptosis. It derives from Fenton Reaction in which H_2O_2 react with metal ions (Fe^{2+} or Cu^{+}), given from different proteins such as ferritin (an intracellular protein of iron stocking) and ceruloplasmin (plasma copper carrying protein) or other molecules. Moreover, OH^{-} can be also generated during Haber-Weiss Reaction, where $O_2^{\cdot -}$, reacts with H_2O_2 [56,58,60] following the mechanism reported below.

Fenton Reaction: $\mathbf{H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}}$

Haber-Weiss Reaction: $\mathbf{O_2^{-} + H_2O_2 \rightarrow OH + OH^{-} + O_2}$

Peroxyl Radical. The simplest form of $ROO^{\cdot}$ is Perhydroxyl Radical ($HOO^{\cdot}$), derived from the protonation of superoxide anion. Many studies underlined the role of $ROO^{\cdot}$ in fatty acids peroxidation and cancer development [58].

Singlet Oxygen. $^1O_2$ is an electronically high excited state of molecular oxygen, *in vivo* produced by the activation of neutrophils and eosinophils. Some enzymatic reactions catalyzed by lipoxygenases, dioxygenases and lactoperoxidase are involved in $^1O_2$ generation. $^1O_2$ is a highly reactive molecule associated with DNA and tissue damage [58].

Hypochlorosus Acid. HOCl is a non-radical specie that originates from H_2O_2 oxidation of Cl^- ion catalyzed by MPO. HOCl has important microbial characteristics due to its ability of proteins, lipids and DNA chemical alterations. It can react with methionine and cysteine amino residues, leading to methione sulfoxide and disulfides involved in cross-reactions between proteins and protein-DNA. Several HOCl-mediated DNA mutations are detected as well as mono and di-chloroamines, able to come across hydrophobic microbial membranes affecting intracellular targets. Furthermore, HOCl is an important trigger factor for NETs production and release during inflammatory response [61].

NO and RNS: Classification and Sources of Production

NO is an important biological molecule with vasodilatory activity and a central role as a regulator of many cellular events as apoptosis. It acts as an intracellular second messenger, stimulating guanylate cyclase (resulting in the activation of several signaling pathways as protein kinase G (PKG) that induces phosphorylation of several intracellular proteins including calcium ion -Ca^{2+} channels) [62] and protein kinases and it is involved

in many biological activities like blood pressure regulation, smooth muscle cells relaxation, neurotransmission, leukocytes adhesion, platelets aggregation, angiogenesis and thrombosis, defence mechanisms and immune system modulation [58,63]. NO is generated in tissue by different NOS enzymes, that catalyze NO formation from molecular oxygen (O_2) and L-arginine using NADPH as an electron donor (the molecular reaction is reported below).

$$\textbf{L-Arginine} + \textbf{O}_2 + \textbf{NADPH} \rightarrow \textbf{L-Citrulline} + \textbf{NO}^{\cdot} + \textbf{NADP}^{+}$$

Three different isoforms of NOS have been found. Neuronal NOS (nNOS) and endothelial NOS (eNOS) are involved in NO production for the regulation of physiologic functions, whereas inducible NOS (iNOS) is firstly activated in macrophages and activated in response to inflammatory stimuli [63-65].

Under physiological conditions, NO is generated by eNOS, constitutively expressed in endothelium, in response to shear stress or agonist factors (as acetylcholine, bradykinin and thrombin) promoting endothelial homeostasis to environment changes and stimuli. eNOS-derived NO acts in inducing vasodilatation via NO-sensitive guanylyl cyclase activation in smooth muscle cells. Platelets adhesion to endothelium and aggregation, leukocytes adhesion as well as LDL oxidation and smooth muscle cells proliferation are consequently prevented, suggesting a role of NO in an anti-thrombotic and anti-atherogenic endothelial phenotype [66]. On the contrary, endothelial dysfunction is a non-physiological condition where inflammatory molecules and cytokines may induce iNOS upregulation and alterations of eNOS expression/activity [63]. eNOS seems to be involved in vascular dysfunction according to *eNOS uncoupling* process: indeed, eNOS switches from a NO-producing enzyme to another one able to release superoxide anion, leading to a peroxynitrite (ONOO-) overproduction [63] via the reaction

$$\textbf{NO} + \textbf{O}_2^{\cdot -} + \rightarrow \textbf{ONOO-}$$

eNOS uncoupling is recognized a central mechanism in atherogenesis development, reducing NO production and promoting oxidative stress [66].

Among RNS, ONOO- is one of the most important components together with NO and nitrogen dioxide (NO_2) and non-reactive species as nitrosamines. RNS excessive levels seem to be involved in oxidative molecular damages, particularly causing protein structure and function affection, alterations in catalytic enzyme activity and cytoskeletal organization as well as abnormalities in cell signaling transduction [64]. ONOO- is a high reactive cytotoxic molecule able to diffuse through cellular membrane and react with lipids, proteins (methionine and tyrosine) and DNA, leading to cellular membrane lipoxidation, DNA damage and cell apoptosis [65].

ROS Sources of Production

ROS and RNS can be generated by cellular enzymatic and non-enzymatic reactions based on chemical bonds breaks, radical cleavage to give another radical and via redox reactions [57]. Mitochondrial respiratory chain, phagocytosis, prostaglandin synthesis, cytochrome P450 system, NADPH oxidase, xanthine oxidase and peroxidase activity include several enzymatic processes generating free radicals [57]. On the contrary, ionizing radiations-induced oxygen reactions with organic compounds or mitochondrial oxidative phosphorylation represent non-enzymatic processes of free radicals' generation [57].

Mitochondria

Under physiological conditions, mitochondria and mitochondrial oxidative phosphorylation are one of the major sources of ROS production [67]. Superoxide anion is the most common oxygen free radical generated during the electrons transfer along complex I (NADH dehydrogenase) and complex III (ubiquinone cytochrome c reductase)

of the mitochondrial respiratory chain [56,58,69]. $O_2^{\cdot-}$ generation is particularly increased under hyperoxia or raised glucose conditions, due to the higher metabolic rate, but also during hypoxia status when the lack of oxygen as final electron acceptor for mitochondrial complex IV leads to electrons accumulation [56].

NADPH oxidase [61,66]

NADPH oxidase is a membrane-bound enzyme complex located in the extracellular space and it exists in four isoforms as NOX1, NOX2, NOX3 and NOX4. NOX2 is considered "the primary-source of immune-active ROS in neutrophils" in response to a "priming signal", as inflammatory cytokines or lipolysaccaride (LPS)[61].

Particularly, NADPH oxidase generates superoxide anion by electrons transfer across the membrane from cytosolic NADPH to extracellular molecular oxygen, according to the reaction reported below.

$$\mathbf{NADPH + 2O_2 \leftrightarrow NADP^+ + 2O_2^- + H^+}$$

Xanthine Oxidase

Xanthine oxidase is a form of xanthine oxido-reductase involved in the production of several ROS. This enzyme catalyzes the oxidation of hypoxanthine to xanthine, generating hydrogen peroxide as the molecular reaction here described [57].

$$\mathbf{hypoxanthine + H_2O + O_2 \leftrightarrow xanthine + H_2O_2}$$

Moreover, xanthine oxidase can oxidize xanthine to uric acid as indicated below.

$$\mathbf{xanthine + H_2O + O_2 \leftrightarrow uric\ acid + H_2O_2}$$

Xanthine oxidase seems to be also involved in purines, pterins and aldheydes metabolism.

Myeloperoxidase

MPO is a peroxidase expressed in neutrophil granulocytes and involved in HOCl production via H_2O_2-mediated oxidation of halides (Cl^-, Br^-) and thiocyanate (SCN^-) [61]. MPO is a lysosomal protein located in azurophilic granules of neutrophils; it is released into the extracellular space via degranulation. Many authors have recently described an important association between high levels of MPO and the severity of coronary artery diseases as well as the pathophysiological involvement of MPO deficiency in immune system defenses alterations.

An additional classification among endogenous and exogenous ROS/RNS sources can be reported. Biological systems can be exposed to environmental changes and other exogenous factors which may induce reactive species production affecting redox homeostasis. Cigarette smoke, alcohol and drugs assumption, ozone exposure, hyperoxia/hypoxia, ionizing radiation and heavy metal ions can trigger oxidative reactions and free radical production. Particularly, iron and copper metals are involved in superoxide ion and hydrogen peroxide generation by Haber-Weiss/Fenton reaction yielding $OH^{\cdot}$ [57,60]. Moreover, as several studies showed, immune cell activation, inflammation and infections, aging, ischemia, cancer and aging seem to be pathophysiologically involved in free radical generation.

Antioxidant Defence Mechanisms

Antioxidant defense mechanisms are needed for the maintenance of redox homeostasis in biological systems, preventing ROS/RNS-induced oxidative damages on several molecules. They can act interrupting oxidative reactions or decreasing the rate of their onset and promote the generation of inactive products [57].

Among antioxidants species, two categories can be identified: enzymatic and non-enzymatic molecules.

Enzymatic Antioxidants [56,60,65]

Enzymatic defenses are characterized by the presence of a transition metal in their core, central in electrons transport during the detoxification processes. Among enzymatic antioxidants, *SOD*, *CAT* and *GPx* are some of the most important molecules.

SOD exists in three isoforms. Firstly, copper-zinc containing SOD (Cu, Zn-SOD or SOD1) is a dimeric protein located both in the cytoplasm and in the mitochondrial intermembrane space. Manganese containing SOD (Mn-SOD or SOD2) is a homo-tetrameric protein restricted to the mitochondrial matrix, whereas SOD3 (EC-SOD) is found in extracellular matrix, on cellular surface and in extracellular fluids [66]. SOD plays a central role in the control of $O_2^{\cdot-}$ production by catalyzing the conversion of $O_2^{\cdot-}$ to H_2O_2 as the reaction below [56,65].

$$\mathbf{O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2}$$

Several authors revealed the anti-atherosclerotic role of SOD enzymes inhibiting oxidative damages mediated by superoxide anion $O_2^{\cdot-}$ and preventing inactivation of NO [66]. However, the functional role of SOD in atherosclerosis development is still unclear.

CAT is described as a tetramer composed of 4 identical monomers each of which contains a *heme* group in the active site. It is located in eukaryotic peroxisomes, catalyzing the detoxification of H_2O_2 to water as reported below [56,60].

$$2H_2O_2 \rightleftarrows O_2 + 2H_2O$$

H_2O_2 conversion to water is also catalyzed by GPx enzyme.

GPx is a family of enzymes divided in selenium (Se)-independent and Se-dependent groups and located in mitochondria and in cytoplasm. They are involved in the conversion of H_2O_2 to water according to the reaction here described [65].

$$2GSH + H_2O_2 \rightleftarrows GS\text{-}SG + 2H_2O$$

Gpx is present in 4 isoforms. The first isoform is a great scavenger for hydrogen peroxide whereas the fourth isoform is more active against lipid hydroperoxides [66]. The activity of GPx depends on the presence of reduced glutathione (GSH), a hydrogen donor, promoted by *glutathione reductase* and *glutathione S-transferase* enzymes. GSH is synthesized in cytosol from L-glutamate, L-cysteine and glycine and involved in sensing and buffering cellular redox conditions because of the thiol (SH) group. GSH participates in several detoxifying reactions forming glutathione disulfide (GS-SG), which is then converted back to the reduced form (GSH) by the action of NADPH dependent Glutathione Reductase [56].

Glutatione S-transferase acts against lipid peroxides and in the liver site where drugs detoxification processes can lead to free radical production.

Non-Enzymatic Antioxidants

Non-enzymatic antioxidants help enzymatic ones to neutralize free radicals improving redox status in biological systems. They are generally introduced with diet, suggesting the role of decreased antioxidants consumption in redox homeostasis alterations and several chronic and degenerative pathologies [57]. Non-enzymatic antioxidants include water-soluble molecules, able to react against oxidants in cellular cytosol or plasma and fat-soluble molecules, with a central role in membrane lipids protection from lipid peroxidation processes.

Vitamin C (or Ascorbic Acid) is a water-soluble molecule that improves intracellular and extracellular antioxidant capacity by scavenging oxygen free radicals [60]. It is converted in *Ascorbil Radical* during redox reactions, showing an anti-oxidant, anti-atherogenic, anti-carcinogenic and immunomodulator activity [57]. Particularly, C vitamin role in endothelial function is due to its ability in reducing vascular resistance and leukocytes adhesion [63]. However, at higher levels the ascorbil radical may display pro-oxidant activity due to its nature, suggesting that results about beneficial effects of C vitamin therapeutic supplementation could be controversial [68].

GSH is one of the major soluble antioxidant molecules thanks to its thiol group (SH) as a hydrogen donor and GSH/GSSG ratio is an important indicator of oxidative stress. GSH is involved in hydrogen peroxide conversion to water during a GPx-catalyzed reaction. Based on its structural features, GSH antioxidant activity can be observed in membrane lipids protection from oxidation, in converting vitamin C and E back to their active forms and in apoptosis cells prevention by modulating several pro-apoptotic and anti-apoptotic signaling pathways. Indeed, different transcription factors including Activator Protein 1 (AP-1), Nf-kB and Specificity Protein Factor 1 (Sp-1) are regulated by GSH in their active/inactive status [60].

Vitamin E is a fat-soluble molecule and a chiral compound with eight stereoisomers of which α-tocopherol is the most bioactive for humans. Due of its hydrophobic nature, E vitamin is mostly involved in cellular membrane protection against lipid peroxidation [57]. Moreover, it seems to have beneficial effects on vascular dysfunction, promoting the release of prostacyclin as a potent vasodilator and inhibitor of platelets aggregation [63].

Vitamin A converted to retinol, *lycopene* as well as flavonoids, omega-3 and omega-6 fatty acids, coenzyme Q-10, melatonin and iron-copper binding proteins represent other

important molecules with antioxidants activity and a central role in biological systems protection from oxidative damages and cell apoptosis [57].

ROS/RNS Physiological Involvement

Physiological moderate ROS/RNS levels are crucial for the regulation of several signal transduction pathways (as described in figure 10), resulting in a mechanism for cellular adaptation to environmental stimulus (growth factors, cytokines, stress signals) and changes [65]. Particularly, ROS/RNS signaling is involved in the modulation of different cellular functions as metabolic processes, vascular tone, oxygen sensing, growth-factor and cytokine genes expression, cell cycle progression, cytoskeletal organization, antigen processing, cell proliferation, differentiation, migration and apoptosis and inflammatory response [62,66]. ROS/RNS signaling is based on the reversible oxidation/nitration-mediated activity regulation of redox-sensitive and catalytic enzymes, intracellular effectors of signal transduction factors and transcription modulators [62].

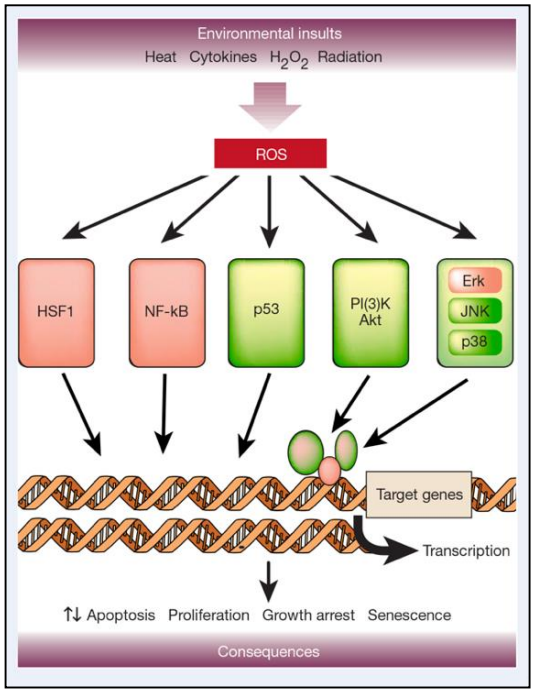

Fig. 10 ROS redox signaling [69]

ROS and Hypoxia-inducible Factors (HIFs)

HIFs are regulated by oxygen concentration and cellular ROS levels [64]. HIF-1, one of the major O_2 homeostasis regulator, is a heterodimeric molecule composed of two subunits: HIF-1α and HIF-1β. Under normoxic conditions, HIF-1α is degraded by the proteasome whereas under hypoxic conditions HIF-1α is stabilized leading to the formation of a stable heterodimer. It is able to translocate into the nucleus, modulating expression of central genes for cellular adaptation to lower O_2 concentrations (as transcriptional activation of erythropoietin, vascular endothelial growth factor, glucose transporter 1 and glycolytic enzymes) [65].

ROS and Nf-kB Signaling Pathway

Nf-Kb is an eukaryotic transcription factor involved in the regulation of immune and inflammatory responses, cellular adhesion, proliferation and differentiation, anti-apoptotic response and also in anti-inflammatory mechanisms. Recently, many studies underlined the double cytoprotective and cytotoxic role of Nf-kB in acute and chronic pathological heart remodeling, triggered by hypoxia or ischemic myocardial injury [70]. Microbial products, stress or pro-inflammatory cytokines (IL-1β and TNF) [64] can induce canonical Nf-kB activation by inhibitory subunit IkBα phosphorylation and ubiquitination. Nf-kB moves into the nucleus where it activates the transcription of target genes [71]. Oxidative stress condition can alter Nf-kB activation: indeed, ROS-mediated phosphorylation of Nf-kB inhibitory subunit IkB, phosphorylation of IkB-kinase (IKK) or abnormalities in ubiquitination and degradation of IkB may induce Nf-kB activation [71]. Increased tissue levels of enzyme COX-2, IL-1β, TNF-α) and other inflammatory mediators are observed in response to ROS-mediated Nf-kB activation.

ROS and Phosphoinositide-3-kinase- (PI3K) Akt Signaling Pathway [71]

PI3K-Akt pathway has a central role in several cellular functions, particularly protein synthesis, cell cycle progression, proliferation, apoptosis, autophagy and drug resistance. Growth factors (Epithelial Growth Factor-EGF, Platelet-Derived Growth Factor-PDGF, Nerve Growth Factor-NGF and Vascular Endothelial Growth factor-VEGF), hormones (prostaglandin, PGE_2) and cytokines (IL-17, IL-2, IL-2) are some of the principal trigger factors for the PI3K-Akt signaling pathway activation and transcription of their target genes (Glycogen Synthase Kinase 3-GSK3, Forkhead Box Protein-FOXO, Bcl2 Associated Agonist of Cell Death-BAD, Mammaliann Target of Rapamicin-mTOR1 and p53). In this context, ROS show a double role in PI3K-Akt pathway regulation: it is directly involved both in PI3K activation band inactivation. Indeed, ROS avoid the synthesis of phosphatidylinositol 3,4,5 triphosphate (PIP3) and also inhibit the activation of Akt oxidizing cysteine residues in the enzymatic active center.

ROS and MAPKs (Mitogen-Activated Protein Kinase) Signaling Pathway

The MAPK cascades is composed by Extracellular Signal-related Kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinases (JNK), p38 kinases (p38) and MAP Kinase 1 (BMK1/ERK5) pathways. MAPK pathways is central in several processes as cellular growth, differentiation, cell cycle, survival and cell death. Their signaling cascades are regulated by phosphorylation/dephosphorilation of serine and/or threonine amino residues on tyrosine kinases receptor as well as cytokines and growth factors receptors [62,64] In this context, oxidative stress can influence the physiological effects of these signaling-cascade pathways [64]. Particularly, ROS are able to stimulate EGF and PDGF receptors, which can activate Ras and the related ERK pathway. Similarly, also JNK, p53 and p38 pathways are susceptible to oxidative stress signals besides inflammatory cytokines, leading to the activation of all signal cascade [71]. In general ERK, PI(3)K/Akt

and Nf-kB signaling pathways show a pro-survival effect during oxidative injury whereas activation of p53, p38 and JNK are more commonly associated with apoptosis [69].

ROS and Kealch-like ECH-associated protein 1 (Keap1), Nuclear Factor Erythroid 2-related Factor 2 (Nrf2) and Antioxidant Response Elements (ARE) Signaling Pathway

Keap1, Nrf2 and ARE compose a complex signal pathway involved in the maintenance of cellular redox balance and adaptive response to oxidative stress. Moreover, it can lead to several inflammatory disorders as cancer, Alzheimer's disease, Parkinson's disease and diabetes [71]. If Nrf2 is physiologically inhibited by the association with Keap1, higher ROS levels can induce Keap1-Nrf2 dissociation, promoting Nfr2 translocation into the cellular nucleus and its binding to ARE. Antioxidant enzymes gene expression is promoted [71].

ROS and Mitochondrial Permeability Transition Pore (mPTP)

Several data in literature describe the role of mPTP, an unspecific channel on internal and external mitochondrial membrane, in permeability changes that influence mitochondrial-driven health. In this context, ROS can modulate mPTP opening by oxidation of different cysteine (Cys) amino residues but also increasing mitochondrial Ca^{2+} concentration [71].

ROS and Protein Kinase [71]

Several protein kinases, including protein kinase A (PKA), protein kinase C (PKC), protein kinase D (PKD), receptor tyrosine kinase (RTK) and Ca/calmodulin independent protein kinase II (CaMKII), represent molecular target for ROS that modulate enzymatic activation state by oxidizing sulfhydryl (SH) groups of cysteine amino residues. The

activated protein kinases then phosphorylate their target proteins involved in different cellular signaling mechanisms.

However, not only ROS may influence PKA phosphorylation, but also phosphorylated PKA shows an important effect on ROS homeostasis. Indeed, cAMP/PKA signaling pathway modulates the expression, assembly and catalytic activity of mitochondrial respiratory chain complex I, a physiological site of ROS production.

Referring to PKC and PKD activity, ROS acts influencing catalytic enzyme activation. Particularly, cysteine-rich regions in regulatory and catalytic domains of PKC are susceptible to ROS-mediated oxidation: accordingly, ROS stimulate PKC activity at moderate levels whereas higher levels of oxidants result in enzymatic inhibition.

ROS and Ubiquitination/proteasome System (UPS) [71]

Ubiquitination/Proteasome System (UPS) is composed by four components including proteasome, ubiquitin, the ubiquitination machinery and the deubiquitinase and it is central in different biological processes as cell cycle regulation, inflammatory and immune response, protein misfolding and endoplasmic reticulum-associated degradation of proteins. As many authors reported in their studies, an interaction between ROS and UPS seems to exist. Firstly, UPP is sensible to oxidative stress due to the presence of cysteine amino residues in E1, E2, E3 enzyme subunits that can be oxidized leading to mixed disulfide bonds which blocks their binding to ubiquitin. Furthermore, UPS can regulate cellular redox status by inducing Nrf2 degradation and Nf-kB activation, both involving in the regulation of ROS levels and also by modulating mitochondrial processes (oxidative phosphorylation, tricarboxylic acid (TCA) cycle and mitochondrial dynamics).

ROS/RNS Harmful Effects

The increase in ROS levels leads to an imbalance with antioxidant defense mechanisms, inducing oxidative stress condition generally associated with molecular damages on lipids, proteins and nucleic acids (figure 11) [66]. This evidence suggests oxidative stress pathophysiological involvement in several disorders as inflammation and tumors, cardiovascular and autoimmune diseases, neurodegeneration and in some female reproductive system disorders in addition to aging [69,72]. Moreover, the increase in ROS content is also associated with several cardiovascular risk factors as hypertension, hypercholesterolemia, diabetes mellitus, cigarette smoking as well as cardiovascular disease itself [66].

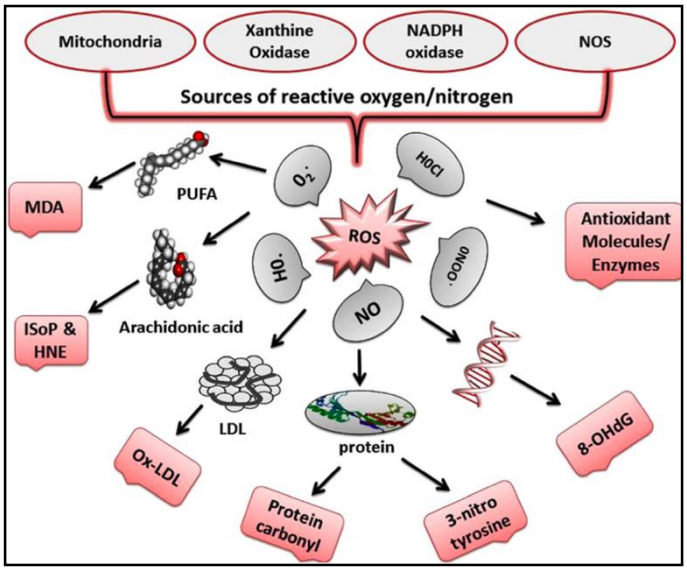

Fig.11 ROS harmful effects [121]

ROS and Ca^{2+} Signaling Systems

Ca^{2+} is an important signal molecule involved in regulating and controlling several cellular processes and functions in eukaryotic cells, as contraction and secretion mechanisms, metabolism, gene expression, cell survival and death [71]. Cytosolic Ca^{2+} is determined by a dynamic balance between import mechanisms as Ca^{2+} influx from

extracellular compartment or endoplasmic reticulum (ER) / sarcoplasmic reticulum (SR), resulting in higher cytosolic Ca^{2+} concentration and export mechanisms, as Ca^{2+} efflux across plasma membrane and sequestration into the mitochondria.

ROS and Ca^{2+} signaling pathways are strongly related, influencing each other in multiple ways. Accordingly, Ca^{2+} levels can regulate both ROS production and clearance processes, affecting cellular redox status. The principal Ca^{2+} mediated effects on ROS physiology are reported below [71]:

- ATP synthesis and mitochondria ROS generation triggering Krebs cycle enzymes and oxidative phosphorylation;
- Activity modulation of several ROS generating enzymes, as NOX and NOS, in pathological/physiological conditions;
- Regulation of antioxidant defense mechanisms for ROS clearance. Particularly, Ca^{2+} can directly activate antioxidants (CAT, glutathione reductase), increase SOD levels and induce mitochondrial GSH release. Moreover, Ca^{2+} can act indirectly by calmodulin (CAM)-mediated activation of catalase and downregulation of hydrogen peroxide levels.

On the other hand, ROS play a central role in altering intracellular Ca^{2+} homeostasis. The oxidation of free cysteine residues in several membrane-bound Ca^{2+} channels and receptors (SERCA pump or others sarco/endoplasmatic reticulum calcium ATPase enzymes) compromise their activity, resulting in a decreased Ca^{2+} influx from cytoplasm to ER and SR [56, 71]. The increased cytosolic Ca^{2+} levels are involved in the activation of several calcium sensitive signaling pathways but also it can affect chaperone activity, leading to the accumulation of misfolded proteins and further generation of ROS. Misfolding is strictly connected with the stimulation of unfolded protein response (UPR) that can represent a source of cell damage and apoptosis [56].

Higher Ca^{2+} cytosolic content can also affect mitochondrial function due to oxidative damages of membrane lipids and proteins altering membrane permeability. It results in mitochondrial membrane potential and ATP synthesis affection leading to lower ATP levels, loss of ionic homeostasis and consequently cell apoptosis [56].

DNA oxidation

Among biological molecules, DNA is one of the ROS molecular target. Several modifications such as degradation of bases, single/double stranded DNA breaks, purine, pyrimidine or sugar-bound alterations, mutations, deletions or translocations and also protein cross-links can be identified, suggesting the oxidative stress involvement in aging, cardiovascular, neurodegenerative and autoimmune disorders and also in carcinogenesis [60].

DNA is vulnerable to $OH^{\cdot}$ activity, generating several products that can be measured biochemically as biomarkers of oxidative stress. $OH^{\cdot}$ can react with guanine to produce **8-hydroxy-2'-deoxyguanosine** (8-OH-G), an important marker of DNA oxidative damage detectable immunohistochemically in several biological samples [56]. For example, many studies revealed increased concentration of 8-OH-G in follicular fluid of unfertile women describing a condition of altered redox homeostasis in the follicular fluid microenvironment of those patients and suggesting cell apoptosis [73]. DNA oxidation, involving DNA bases or deoxyribose sugars, can result in nucleic acids strand breaks: chromatin structure, DNA repair and transcription mechanisms can be affected as well as mutations and alterations in gene expression can be identified [56]. Indeed, 8-OH-G is relevant in promoter regions of genes containing consensus sequences for transcription factors GC-rich. Oxidation of guanosine can alter the mechanism of binding of transcription factor affecting related genes expression [60]. Moreover, oxidative stress

can compromise DNA methylation, an important epigenetic mechanism in regulating gene expression, with harmful effects on DNA repair activity [60].

Mitochondrial DNA seems to be more susceptible to oxidative damage than nuclear DNA. Indeed, mitochondria are one of the major sources of ROS and the lack of histone protection as well as lower DNA repair systems, may legitimate the higher rate of mutations (5-10 fold higher) found in mitochondrial DNA than in nuclear one, affecting essential proteins for mitochondrial activity and electrons transport chain [56,69,74]. Alterations in mitochondrial integrity and function further stimulate ROS production, causing oxidative damages on to the principal cellular structures and cell apoptosis [69].

Lipid peroxidation

Plasma and membrane lipids, especially poly-unsatured fatty acids (PUFA), are vulnerable to free radicals derived oxidation. Lipid peroxidation is strictly associated with fluidity and permeability membrane alterations, inhibition of membrane-bound enzymes and receptors and activation of apoptotic cascade, suggesting a pathophysiological involvement of the process in several disorders as inflammation, cardiovascular and neurodegenerative diseases, autoimmunity, female reproductive system disorders as well as aging [58,74].

As shown in figure 12, lipid peroxidation is generally triggered by free radical attack and abstraction of hydrogen from the hydrocarbon side-chain of a fatty acid, yielding to a carbon-centered lipid radical ($L^{\cdot}$) as reported below.

$$\mathbf{L\text{-}H + OH^{\cdot} \rightarrow H_2O + L^{\cdot}}$$

Lipid radical interaction with O_2 produces a *lipid peroxyl radical* (LOO·), able to react with an adjacent fatty acid propagating the process and its internal molecular rearrangements with conjugated dienes and hydroperoxides generation [56,58,75,76].

The entire process ends with the formation of more stable lipid peroxidation products as **malondialdehyde (MDA)**, **4-hydroxy-2-nonenal (HNE)**, **isoprostanes**. In the last years a growing interest on biochemically and immunochemically detection of these molecules in several biological samples (plasma, urine, follicular/peritoneal/seminal fluid) developed, appearing useful biomarkers for the evaluation of disease progression, effectiveness of therapeutics supplementation with antioxidants [77].

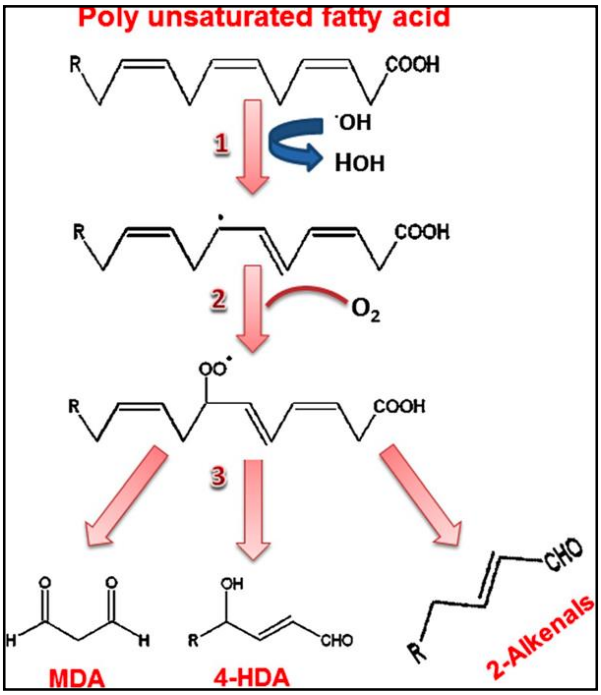

Fig.12 Lipid peroxidation process [121]

Moreover, lipid peroxidation products can also react with proteins, DNA and phospholipids generating end products involved in cellular dysfunction and disorders development. Particularly, the interaction of lipid peroxidation products with amino residues can result in protein oxidation affecting its structural and functional features [74,77].

Introduction

Malondialdehyde

MDA is physiologic ketoaldehyde and one of the most common products of unsatured lipids oxidation. At abundant levels, it is able to interact with specific amino residues including Lys, generating MDA-modified protein characterized by a great immunogenic potential. As well as ox-LDL, MDA plays a central role in inflammatory process and atherosclerosis, suggesting a pathophysiologically involvement in coronary artery disease and stroke [74].

Despite criticisms, MDA and TBARS (Thiobarbituric Reactive Substances) Assays are the most used methods for lipid peroxidation products detection in biological samples [75,77,78].

4-hydroxy-2-nonenal

Among lipid peroxidation products, also HNE plays an important role in biological processes. At moderate and physiological levels, this molecule is involved in the regulation of oxidative stress-induced apoptosis, cellular proliferation and signaling pathways. On the contrary, excessive content of HNE can be detected biochemically as a marker of redox status alterations [74].

Isoprostanes

Arachidonic acid independent cyclooxygenase oxidation generates isoprostanes, prostaglandin-like compounds and clinically reliable indicators of oxidative stress in several pathological acute and chronic conditions [74,77].

Protein Oxidation

Among post-translational modifications, amino acids oxidation represents a cellular response mechanism to environmental changes. Several ROS/RNS as $O_2^{\cdot -}$, $OH^{\cdot}$, H_2O_2, $^1O_2^-$, HOCl and ONOO- can mediate reversible and irreversible protein oxidative modifications [56,58,79]. Many studies described the harmful effects induced by protein oxidative damages. Oxidation of amino residue side chains, peptide bonds cleavage and formation of protein-protein cross linkages may cause protein structural and functional features affection, alterations in enzyme activity, receptors and transport proteins function, becoming central in aging and diseases, particularly cardiovascular disorders and neurodegeneration [58,79-81]. However, the physiological role of ROS/RNS as secondary messengers also suggests beneficial effects of protein oxidation for several cellular functions. Indeed, in many cases variations of redox homeostasis represent positive stress conditions that stimulate and reprogram cell survival mechanisms [79].

Several factors are involved in determining the extent of damage to biological targets, such as the concentration and location of molecular target, the nature of oxidative reactions and its side effects and the presence of antioxidant defense mechanisms [82].

Based on this evidence, ROS/RNS-mediated hydrogen atom abstraction from the α-carbon of amino acids represents the first step in protein oxidation mechanism (figure 13). Under normoxia, the generated radical is rapidly converted to peroxyl radical able to react with amino residues of the same/different polypeptide chain, leading to peptide bond cleavage. In case of hypoxia, the α carbon-centered radical can react with another carbon-centered radical producing protein-protein cross linkages [81,83].

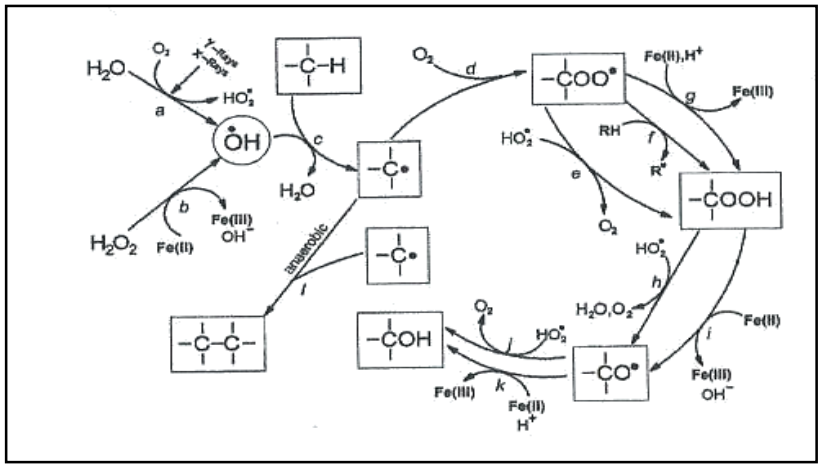

Fig.13 Free radical-mediated oxidation of the protein polypeptide backbone [81]

Protein oxidation can be mediated by several mechanism and it can occur on several different amino acyl side chains. Protein oxidative modification subset is various and parallel and some post-translational protein modifications could diverge in the target oxidized amino residue and in the generated product. On the contrary, others oxidative modifications can affect multiple residues and may lead to several end products [84]. In order to evaluate oxidative stress status in healthy subjects and patients, protein oxidation products are considered good biomarkers due to its stability [58,84].

The principal protein oxidative modifications are reported below.

Protein Carbonylation

Oxidation of several amino acids residues as cysteine and methionine, lysine, arginine, proline and threonine leads to the formation of carbonyl derivatives, one of the major used biomarkers for oxidative stress assessment in aging and pathological conditions [79]. Carbonyl groups generation can be induced by different ROS/RNS or by reactions with lipid peroxidation products (aldehydes) or derivatives of lysine glycation/glycoxidation reactions and it can occur at different sites and by different mechanisms [83]. Carbonylated amino acid residues, except proline, show higher hydrophobicity, resulting

in protein aggregation and cell death if not eliminated and parallel, this change in biochemical features causes a reduced interaction with chaperons [72].

Carbonylation may induce protein structural alterations and alter biological activity leading to enzyme inactivation and abnormalities in binding activities. Moreover, changes in protein susceptibility to proteolytic degradation can be considered among side effects of carbonyl groups introduction in protein side chains. The presence of carbonyl groups on amino residues (as proline and arginine) located in the cleavage site of thrombin or peptide bonds by oxidation of glutamyl residues has been found in several pathological conditions including post-AMI and BS patients, where a reduced fibrin clotting ability and a decreased fibrin susceptibility to plasmin digestion was described [1,85].

In redox status evaluation, the use of protein carbonyl group as a biomarker of oxidative stress is largely spread and advantageous because of its early formation and stability in comparison with lipid peroxidation products; several fluorometric and spettrophotometric assays are available at the moment [72,74,84].

Oxidation of cysteine and methionine

Cysteine residues play a central role in buffering and sensing systemic redox homeostasis variations due to the presence of reactive thiol group as an electron donor. Cysteine is crucial in regulating protein activity: indeed, it often characterizes the catalytic and regulatory site of several enzymes as well as actors and modulators of several signaling pathways [62]. Cysteine oxidation products can be summarized in disulfide formation (S-S), S-glutathionylation (protein-SSG), S-nitrosylation (-SNO), sulfenic acid formation (-SOH, or S-sulfenation) with effects on protein functions [79]. Both harmful and beneficial side effects of cysteine oxidation have been described: these oxidative modifications protect target proteins from further modifications and regulate redox signaling cascades inducing cellular responses to environmental changes [62,79].

Introduction

As well as cysteine, also methionine shows an antioxidant role and acts regulating cellular metabolism. ROS/RNS-induced methionine oxidation results in methionine sulfoxide, a reversible modification central in modulating biological activity of protein especially in vascular physiology [81,86]. Methionine sulfoxide can also undergo a second oxidation generating methionine sulfone, an irreversible post-translational modification. Methionine oxidation appears as a regulation mechanism of protein interactions, enzyme activity and cellular function. Many studies revealed the presence of methionine residues in several proteins involved in haemostatic system, suggesting a central role of methionine oxidation in vascular biology as well as in pathogenesis of vascular diseases due to some evidence of protein loss of function as a consequence of specific protein methionine residues oxidation [86]. Particularly, CaMKII, apolipoprotein A-I, thrombomodulin, von Willebrand factor (vWF) expose some methionine residues susceptible to oxidation. Among plasma proteins, fibrinogen contains three methionine residues (Met78, Met367, Met476) sensible to ROS-mediated oxidation (especially by HOCl), resulting in fibrin structural and dynamic features affection [87] and parallel, plasminogen activator inhibitor-1 (PAI-1), coagulation factor VII, antithrombin and α-2-antiplasmin can be regulated in their biological function by methionine residues oxidation.

S-nitrosylation, S-glutathionylation and S-persufidation

Both $NO^{\bullet}$ and $ONOO^{-}$ can react with proteins causing S-nitrosylation (formation of –SNO group) on cysteine residues, due to the nucleophilic –SH thiol group but also on tyrosine, serine or threonine residues. S-nitrosylation is functionally similar to protein phosphorylation/dephosphorylation; a proper enzymatic system of nitrosylation and denitrosylation is lacking though the existence of denitrosylases have been reported [62,79]. Cysteine nitrosylation is implicated in the regulation of several physiological and

pathophysiological processes affecting protein activity, translocation and function [70,79].

S-glutathionylation, the formation of mixed disulfide with GSH, also occurs in proteins and peptides especially on cysteine residues [62,74]. As S-nitrosylation, this oxidative modification acts in regulating protein stability and activity, cytoskeletal remodeling, energy metabolism and redox homeostasis [62]. Protein S-S-G is associated with harmful effects on protein function; however, it can also prevent irreversible damages on target proteins, suggesting the importance of modulating protein activity as a consequence of redox homeostasis changes under physiological and pathological conditions [79].

Cysteine oxidation can also result in S-persulfidation (sulfhydration) where –SH group is converted to persulfide (-SSH) through interaction with hydrogen sulfide H_2S. Together with S-nitrosylation, protein phosphorylation and tyrosine nitration reactions, S-persulfidation is a relevant protein oxidative modification, able to influence cellular metabolism regulation, cell survival (or apoptosis), proliferation and differentiation, mitochondrial bioenergetics and blood pressure [62]. R-SSH has stronger nucleophilic and reductant properties than a thiol group, so its presence is associated to the increase in chemical reactivity of protein cysteine residues [62].

Oxidation and Nitration of Tyrosine and Tryptophan

Tyrosine residue is susceptible to oxidation generating 3-hydroxytyrosine, 3-nitrotyrosine, halogenated tyrosine and tyrosine cross-links as principal derivatives with different effects on cellular functions [62].

The mechanism of dityrosine formation is shown in figure 14. Particularly, ROS-mediated tyrosine attack generates tyrosyl radical, able to react with other tyrosine residue and promotes tyrosine dimerization and formation of 3,3-dityrosine, leading to inter-intra molecular protein cross links [62,89]. 3,3-dityrosine can be found as a product of UV

irradiation, γ-irradiation, aging, oxidative and nitrative stress, detectable by fluorometric assays or mass-spectrometry (LC/MS/MS) approach [89-91]. Based on this evidence, dityrosine can be used as a reliable biomarker of redox homeostasis variations.

Tryptophan is also susceptible to ROS-induced oxidation, especially by hydroxyl radical, producing tryptophanyl radicals. Moreover, both tyrosine and tryptophan residues can undergo nitration by $^{\cdot}NO^2$ and $ONOO^-$, forming 3-nitrotyrosine and 6-nitrotryptophan respectively. Particularly, tryptophan is characterized by multiple reactive carbons than tyrosine, leading to more possible oxidative modifications [62].

Several proteins, as mitochondrial proteins and redox signaling enzymes, are vulnerable to nitration, resulting in changes of structural and functional protein features and alterations of cell signaling pathways.

Finally, as several others oxidative stress bio-products, also oxidized tyrosine seems to be associated with diseases progression due to the presence of 3-nitrotyrosine in human atherosclerotic lesions and plaques [62,90].

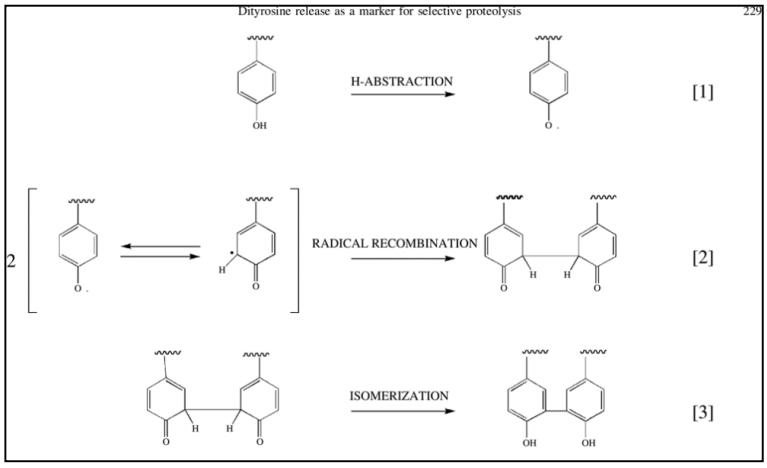

Fig.14 Mechanism of dityrosine formation [89]

Introduction

Protein Disulfides

Under physiological conditions, intra polypeptide chain disulfide bond formation is needed for protein folding and catalyzed by disulfide isomerase into the endoplasmic reticulum and the mitochondrial intermembrane space [79]. Oxidative stress can induce disulfide bonds formation between free cysteine residues, changing protein structural and functional characteristics and regulating its stability [79]. Disulfide bonds can also occur between two different polypeptides: this represents a molecular mechanism for protein-protein cross linkage as well as the direct interaction among two carbon-centered radicals or two tyrosine radicals and the interactions of carbonyl groups or lipid peroxidation products with amino acid residues of different proteins [81].

Chlorination reactions

Under oxidative stress conditions, MPO-derived HOCl, is implicated in protein chlorination as an additional protein post-translational modification. Particularly, HOCl is considered a strong oxidant molecule whose side effects on amino residues can be summarized below: methionine oxidation and tyrosine chlorination, chloramine derivatives of lysine residues formation, sulfhydrilic groups oxidation to sulfenic acid derivatives and lysine residues oxidation to carbonyl derivatives [81].

Endogenous proteins are highly susceptible to ROS and RNS induced oxidation. This process generally results in the loss of protein biological activity, characterizing the development and progression of aging and several disorders as neurodegenerative/cardiovascular/autoimmune diseases as well as cancer. However, protein oxidation also represents a mechanism to induce cell signaling reprogramming in response to environmental changes [80-83].

Introduction

In order to preserve a moderate intracellular level of oxidized proteins, antioxidant molecules and also a proper system of protein degradation are crucial. However, in aging and pathological conditions a decrease in protein turnover modulation by proteasome system is described, inducing oxidized proteins accumulation and tissue physiology affection [80-83].

The Role of Fibrinogen in Haemostasis

Fibrinogen is a soluble blood plasma protein synthesized primarily in the hepatocytes. It is a dimeric glycoprotein that represents the third most abundant protein in plasma with an average concentration of 150-400 mg/dl. Fibrinogen plays an essential role in blood coagulation, being a critical molecule for clot formation and fibrinolysis (figure 15), but also in inflammatory response (as an acute phase protein), cellular and matrix interactions, wound healing and neoplasia. Fibrinogen involvement in several pathophysiological processes is due to the presence of interactive sites on protein, generally available as a consequence of intramolecular rearrangements during fibrinogen polymerization to fibrin [92,94]. Fibrinogen polypeptide chains are encoded in three genes. Despite molecular processes for the regulation of genes expression are still not completely determined, single nucleotide polymorphisms in fibrinogen genes seem to be involved in alterations of gene expression and signaling pathways [94].

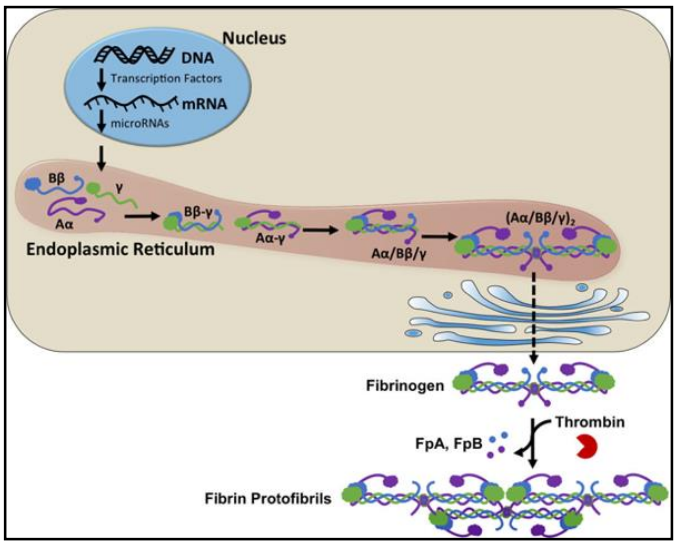

Fig 15. Fibrinogen synthesis and expression [94]

Fibrinogen Structural and Functional Features

Thanks to the X-ray crystallography, several details about fibrinogen molecular structure and variants as well as interactions that occur during fibrin formation have been displayed [95,96].

Fibrinogen is a 340 kDa molecule with an α-helix secondary structure and a length of 45 nm. As shown in figure 16 (panel A and B), two external D domains, together with a central E domain to which they are joined by a coiled-coil segment, describe the biochemical organization of the protein. Fibrinogen molecules are characterized by two sets of three polypeptide chains termed Aα, Bβ and γ and connected together by 29 symmetrical disulphide bridges. Particularly, the Aα chain is composed by 610, the Bβ chain by 461 and the major form of the γ chain, γA, by 411 amino residues. γ' is an important γ chain variant that exists in a smaller percentage (about 8% of the total fibrinogen γ chain population) and it is due to alternative processing of the primary mRNA transcript. 427 amino residues describe γ' chains and the presence of an anionic 20 amino acid sequence, including two sulphated tyrosines instead of the γA chains four ultimate C-terminal γA residues, AGDV411, represents the principal distinctive trait between the two variants of fibrinogen γ chains. In plasma, the heterodimeric fibrinogen molecules γ'/γA describe about the 15% of all circulating fibrinogen molecules and they differ from homodimeric γA/γA ones. On the contrary, about 1% of fibrinogen is represented by the homodimeric γ'/γ' one [94,97].

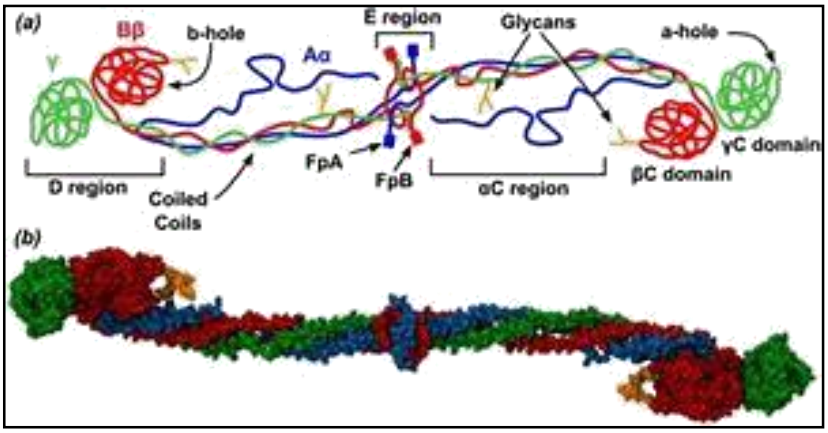

*Fig 16. Fibrinogen molecular structure [98]. Fibrinogen molecule representation (**a**):the three Fg chains Aα, Bβ and γ are represented in blue, red and green respectively. Fibrinogen crystallographic representation (**b**).*

Thrombin-Induced Fibrin Polymerization Mechanism

Fibrin polymerization is a process within the enzymatic cascade of blood clotting and it occurs, together with platelet adhesion and aggregation, during physiological mechanisms of stopping bleeding at the site of vascular injury (haemostasis) as well as during pathological haemostatic system disorders (thrombosis) [99]. Fibrin polymerization is described by a cascade of enzymatic events catalyzed by thrombin, a serine protease enzyme produced by the cleavage of two sites on prothrombin mediated by activated Factor X (Xa). Prothrombin is synthetized in the liver and co-translationally modified in a K vitamin -dependent reaction that converts 10-12 glutamic acids located on the N terminus of the molecule to gamma-carboxyglutamic acid (Gla). In the presence of calcium, the Gla residues promote prothrombin binding to phospholipid bilayers. K vitamin deficiency or anticoagulant warfarin administration inhibits the production of gamma-carboxyglutamic acid residues, slowing the coagulation cascade activation.

The molecular mechanism of fibrin polymerization is displayed in figure 17.

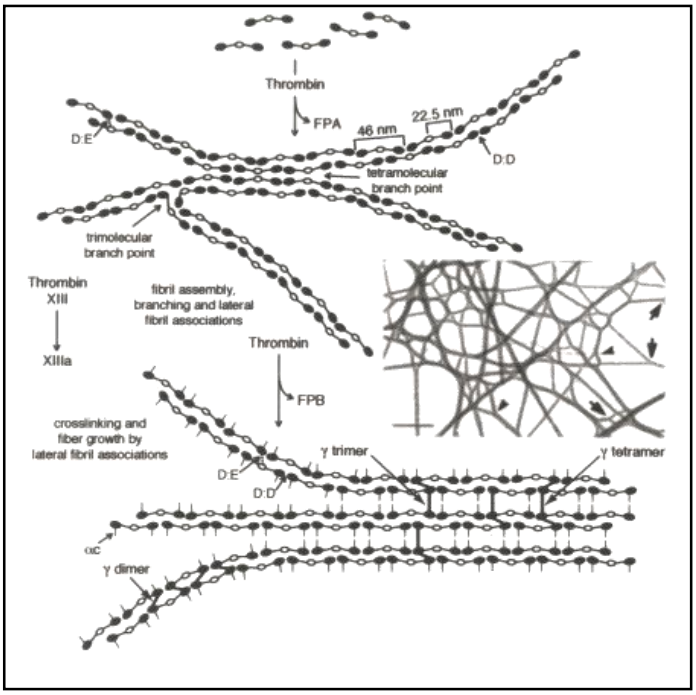

Fig 17. Fibrin polymerization process [93]

Fibrin polymerization is triggered by the thrombin-induced proteolytic cleavage of fibrinopeptides A (FpA) and B (FpB) from the N-terminal subunits of fibrinogen Aα and Bβ-chains, producing fibrin monomers $(\alpha, \beta \text{ and } \gamma)_2$. FpA and FpB cleavage does not occur simultaneously. Indeed, FpA release (through the proteolytic cleavage between Arg16 and Gly17 amino residues) is earlier and promotes the beginning of fibrin assembly process [99] by exposing an N-terminal α-chain motif Gly-Pro-Arg called E_A polymerization site. E_A interacts with a complementary binding site (D_A), located between γ337 and γ379 within the D domain of neighbouring fibrinogen molecules. The $E_A : D_A$ interaction promotes fibrin molecules overlapping in an end-to-middle domain arrangement way producing double-stranded twisting fibrils [97]. Dimer and trimer can be increased by the longitudinally addition of fibrin monomers, becoming larger oligomers and then protofibrils, defined as oligomers with the potential of lateral aggregation and fibers assembly [99]. Indeed, fibrils are involved in lateral and intermolecular associations needed for the three-dimensional fiber network.

The FpB is cleaved off more slowly than FpA and its release seems to be essential for protofibrils lateral aggregation. Accordingly, several data displayed that clots composed of thinner fibers are associated with the release of FpA in absence of FpB cleavage instead of what happens after the proteolytic elimination of both fibrinopeptides. This suggests the central role of B:b interactions in the lateral aggregation of protofibrils [99].

Thrombin-induced FpB release (through the proteolytic cleavage between Arg14 and Gly15 amino residues) exposes an N-terminal β-chain motif Gly-His-Arg-Pro called E_B polymerization site, which complementary combines to the binding site D_B on the D domain of the same or different protofibrils β-chains [97]. However, also αC regions are important during polymerization process: particularly, αC polymers produced by the plasma transglutaminase factor XIIIa, contribute to fibrils lateral aggregation [99].

Among factors able to influence fibrin polymerization, calcium ions are central for fibrinogen stability and clot assembly, but just a moderate effect on thrombin-catalyzed fibrinopeptides release is observed. Ca^{2+} promotes fibrils lateral aggregation, indeed higher Ca^{2+} levels are associated with thicker fibrin fibers [99].

Fibrinogen shows calcium binding sites located on β and γ-nodules of polypeptide chains and low/high affinity calcium binding residues can be displayed. If low-affinity binding residues substitutions do not have important effects on fibrin polymerization, changes in high-affinity ones can affect protofibril formation and fibrin features [99].

Moreover, branching process is essential for clot three-dimensional architecture during fibrin polymerization. Two types of branching can be described for the elongation and thickening of fibrin fibers [93, 99]. *"Tetramolecular or bilateral branch point"* is observed when two protofibrils undergo lateral aggregation to form a 4-stranded fibril and then diverge again in two separate protofibrils, promoting the strength and rigidity of fibers network [99]. *"Equilateral branch point"* occurs when three fibrin molecules, that

connect three double-stranded fibrils of equal widths, join together inducing clot elasticity [93]. However, lateral aggregation and branching are in competition: lateral aggregation is involved in generation of clots with thick fibers and few branch points, whereas inhibition of lateral aggregation is associated with clots composed of thinner fibers and more branch points [99].

During and after fibrin polymerization, factor XIIIa, activated by thrombin and calcium levels, acts stabilizing fibrin clot that acquires irreversible characteristics [99]. Coagulation factor XIIIa catalyzes the introduction of intermolecular ε-(γ-glutamyl) lysine bridges between the lysine at γ406 of the C-terminal portion of a fibrin γ chain and a glutamine at γ398/399 of another one [91,96]. The same interactions are evident between C-terminal portions of fibrin α-chains and cross-linking also occurs between α and γ chains [91, 96, 97]. Factor XIIIa polymorphisms, as 34Val to 34Leu, can alter cross-linking process affecting fiber features, fibrin clot structure and permeability [99].

Plasmin-Induced Fibrinolysis Mechanism

Under physiological conditions, a balance between thrombotic and fibrinolytic mechanisms at the site of vascular injury characterizes haemostatic system. After endothelial tissue repair, the activation of fibrinolysis is central for fibrin clot removal. Fibrinolysis is promoted by plasmin, a serine-protease enzyme derived from tissue-type plasminogen activator (tPA)-mediated proteolytic activation of *plasminogen* that is produced by vascular endothelial cells and found in blood flow. tPA-mediated plasminogen induction is increased by fibrin, due to the role of fibrin polymers and cross-linked fibrinogen polymers as trigger factors for that [92,97,100].

Specific high-affinity plasminogen and tPA binding sites were found in the distal portion of each fibrinogen αC-domain and genetic mutations at that level may affect fibrinolysis

and plasminogen availability for interacting with fibrin [92]. Physiologically, a ternary complex between tPA, fibrin and plasminogen is obtained, being a crucial mechanism for plasminogen activation to plasmin. Plasmin cleaves off fibrin, making additional lysine-binding sites available in order to substance fibrinolysis [92]. Particularly, Aα148-160 and γ312-324 are the two principal fibrinogen binding sites involved in plasminogen activation. They are cryptic on fibrinogen, but intermolecular D:E domains interaction and conformational changes in D region occurring during fibrin polymerization, can induce their exposure. When the ternary complex dissociates, those binding-sites return in their cryptical status [92,93]. Plasmin proteolytic activity on fibrin molecules produces a moderate percentage of large fragments and a group of smaller peptides detectable in blood flow (figure 18). Particularly, the cleavage of αC-domain is the first step of fibrinolysis process, followed by the removal of β chains N-terminal portions. Plasmin enzyme can also act on the coil-coiled segments that connect D domains to the E one, forming an **E fragment** and a **D-dimer** as products of plasmin fibrinolytic activity. The biochemical detection of these products can give clinicians important information not only about fibrinolytic process but also on coagulation activity.

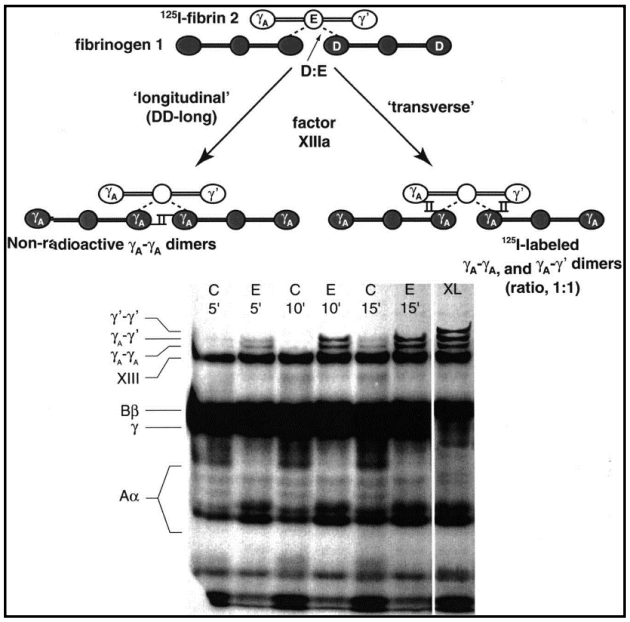

Fig 18. Fibrin degradation products [93]

Several factors could be involved in the alterations of plasmin-induced fibrin lysis, including interferences in plasminogen interaction with fibrinogen, genetic/acquired fibrin structural and dynamic features alterations, environmental components, etc. Among plasma proteins able to bind fibrinogen/fibrin affecting fibrinolysis process, α_2-antiplasmin plays a central role in fibrin resistance to plasmin digestion. Indeed, its cross-linkage on Aα303 residues of fibrin α chains prevents plasminogen interaction with fibrin and its activation to plasmin. However, a covalent interaction between α_2-antiplasmin and fibrinogen has been also observed under physiological condition [93].

Plasminogen activator inhibitor-2 (PAI-2), a serine protease that inactivates tPA and urokinase, can be involved in reducing fibrin susceptibility to plasmin digestion due to its bonds on several sites of fibrin αC-domain [92].

Moreover, histidine-rich glycoprotein (HRGP) is a plasma and platelet protein with a great binding affinity for fibrinogen and fibrin. It circulates in association with plasminogen (mediated by lysine binding) and prevents its binding to fibrin affecting fibrinolysis. Another protein associated with fibrinolysis impairment is Lp(a), that is formed by the complex between apoLP(a) and apoLP B-100 and represents a new risk factor for atherosclerotic disorders. LP(a) has revealed a specific affinity for αC-domain binding sites on fibrinogen/fibrin via a lysine-independent mechanism. In the presence of factor XIII, LP(a) can cross-link to fibrinogen, displaying a competition with plasminogen for the same binding sites on the target protein. Fibrinolysis results inhibited and LP(a) accumulation instead of fibrin deposition is observed at the site of endothelial injury or atherosclerotic lesions [92].

Fibrin Clot Structure and Stability

Clot formation, structure and stability are determined by several factors that occur during fibrin polymerization and that are essential for its functions and behavior (figure 19). A lot of determinants in (patho)physiological conditions including pro-coagulants and anticoagulants concentration, vascular cells and cell-derived microvesicles, blood flow, fibrinogen-binding proteins and metal ions, fibrinogen and thrombin concentration can affect structural and dynamic features of clot [94,101]. Indeed, fiber pore sizes may affect clot permeability, fiber density and fibrin network architecture may modulate the rate of lytic enzymes that interact with clots and their distribution over it [100]. Based on this evidence, haemostasis and thrombosis seem to be actively influenced by fibrin structure, as a critical factor in haemostatic balance. Accordingly, fibrin networks composed of thinner/compact fibers have been found in pathological conditions associated with pro-thrombotic complications, whereas the increased bleeding risk seem to be related with networks of thicker/thinner fibers [94,102].

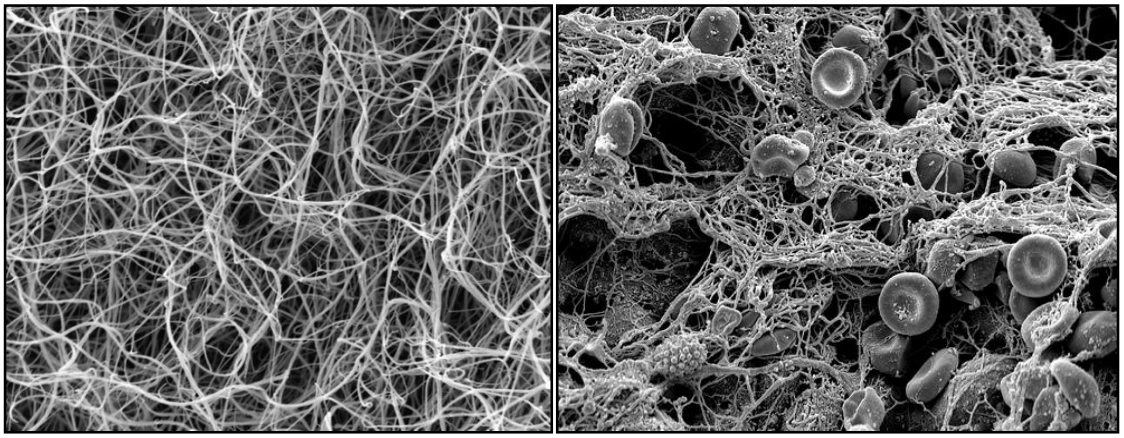

Fig 19. Scanning electron microscopy of fibrin clots [103]

Some of clot structure determinants are listed below.

Hereditary and acquired fibrinogen variations

Fibrinogen splice variants, genetic mutations and single-nucleotide polymorphisms may affect fibrin network structure. Among multiple-spliced forms of fibrinogen, γ' is one of the most interesting. It differs from the native γ chain because of 20 amino acids instead of 4 terminal amino residues physiologically located in the polypeptide chain [94,99].

Epidemiologic studies displayed the association of circulating levels of γA/γ' heterodimeric fibrinogen with both arterial and venous thrombosis: accordingly, ncreased γA/γ' fibrinogen appears related to higher incidence of CAD, cardiovascular risk and heart failure as well as ischemic stroke. On the contrary, γA/γ' fibrinogen may prevent venous thrombosis but investigations are still in progress [94].

Moreover, *in vitro* studies revealed the involvement of γA/γ' fibrinogen isoform in thinner fibrin fibers generation, resulting in clots with a reduced susceptibility to enzymatic lysis [94,104].

Several investigations also explored the role of two important fibrinogen polymorphisms in fibrin clot mechanical/structural characteristics and thrombotic risk. Particularly, **AαThr312Ala** polymorphism is associated with higher factor XIIIa cross-linking and thicker fibers, but no difference in clot permeability has been found in association with the Thr312 variant clots. On the contrary, **BβaRG448Lys** polymorphism may induce thinner fibrin fibers and clots with smaller pores if compared to the Arg448 variant [100].

As mentioned before, post-translational modifications of fibrinogen as ROS/RNS-induced oxidation, glycation, phosphorylation, tyrosine sulfation, proline hydroxylation, methionine oxidation, asparagine or glutamine deamidation, glutamine cyclization, acetylation and homocysteinylation can induce alterations in fibrinogen structural features, affecting fibrin clotting ability and its susceptibility to plasmin digestion [94,105,106]. Accordingly, several recent *in vitro* and *in vivo* studies displayed the

relationship between oxidative stress and fibrinogen oxidation with fibrin clot structure abnormalities and fibrinolysis alterations in haemostatic system disorders as pulmonary hypertension, thrombosis, myocardial infarction, autoimmune disorders, cirrhosis etc [1,85,107-109].

Environmental determinants

Metal ions, pH value, several plasma proteins as albumin, fibronectin, lipoprotein(a) etc can influence clot forming. However, this process seems to be also determined by thrombin concentration. High thrombin levels are associated with fibrin clots characterized by thin fibers, many branch-points and small pores, resulting in more resistant to plasmin digestion clots. On the contrary, low thrombin levels induce fibrin clots with thicker and unbranched fibers, suggesting an increased susceptibility to fibrinolysis [94,99].

Cells and cellular components effects on fibrin clot forming

Cellular effects on fibrin clot assembly can be generally summarized in the modulation of clot viscoelastic properties, permeability, stability and resistance to plasmin-induced lysis. Intra-extra vascular cells as leukocytes, endothelial cells and fibroblasts can influence clot structure and stability by physically interacting with fibrin and by releasing several molecules with pro-thrombotic effects [99].

Platelets are involved in the physiological mechanisms of thrombus formation, being the principal actors of platelet phase during the coagulation cascade. However, clot structure can be compromised by platelet polyphosphate and factor 4 release and also by clot retraction. This is a central phenomenon in blood coagulation, based on fibrinogen interaction with platelets via $\alpha_{IIb}\beta_3$ glycoprotein and where both fibrinogen and platelet concentrations are relevant modulation factors [94]. Clot retraction causes the decrease

in clot permeability to proteolytic enzymes, suggesting its pathophysiological involvement in thrombosis risk [94].

Moreover, many studies also reported Red Blood Cells (RBCs) and NETs effects in clot architecture and dynamic features. RBCs act in coagulation and their increased levels are associated with blood viscosity and higher risk of venous thrombosis. RBCs can also interact with platelets and endothelial cells as well as with fibrinogen via two receptors expressed on their surface, β3 or β3-like molecule and the integrin associated CD47, showing RBCs active role in thrombus formation [110]. The presence of RBCs during clot formation can contribute to fibrin network heterogeneity, but their influence on fibrin fibers thickness it is not completely defined yet [94]. However, several data in literature underlined the role of RBCs in altering clot viscoelastic features and in promoting resistance to fibrinolysis via decreasing plasminogen activation [94].

NETs, composed of DNA, histones and antimicrobial proteins and produced by activated neutrophils during NETosis process [111], are an immune system response to pathogens. In the last years, literature largely revealed how NETs could represent a fundamental link between inflammation and thrombosis [1,2,112]. Indeed, NETs can interact with cells and coagulation factors and are included in the clot structure. NETs components interfere in the intrinsic pathway of coagulation and in platelets activation (via toll-like receptors-2 and -4-dependent mechanisms) contributing to thrombin production. Parallel, histones contribute to the generation and activation of C protein by thrombin/thrombomodulin mechanism observed both in vitro and in mice. Altering local thrombin levels, NETs may indirectly influence fibrin assembly and its properties [94,113,114].

Hydrodynamic Flow

Fibrin network architecture and dynamic features can be also affected by blood flow. It can alter local thrombin concentration, changing pro-coagulant proteins and active

enzymes levels and it is involved in fibrinogen, platelets, leukocytes and RBCs transport to the site of clot assembly. Blood flow seems to be central in fibers orientation affecting clot mechanical features and susceptibility to fibrinolysis and it can modulate fibrin deposition in several regions of a clot, changing its viscoelasticity and its stability and increasing the risk of embolization [94,99].

Stability refers to the resistance of the clot to the mechanical stress and fibrinolytic dissolution and it is generally investigated by measuring clotting times, clot properties (thromboelastography or similar techniques) or the rate of fibrinolysis [115].

In parallel, elasticity indicates reversible clot mechanical deformation while viscosity is associated with irreversible deformation induced by force [101]. These viscoelastic features characterize not only the whole fibrin but also each fiber and several environmental factors together with other physical and biochemical ones can modify and compromise them. Structural fibrin changes as unfolded regions, lack of secondary α-structure can occur at fibrin molecular level during deformation. These alterations may affect fibrin viscoelasticity resulting in changes of clot behavior under (patho)physiological conditions. [101,115].

Molecular and Cellular Binding Interactions of Fibrinogen

Fibrinogen Integrin Binding Sites

Fibrinogen exposes two integrin binding sequences at Aα95-98 containing Arg-Gly-Asp-Ser (RGDF) and at Aα572-575 containing Arg-Gly-Asp-Ser (RGDS) involved in several cellular interactions. RGD sequences play a central role in fibrinogen interaction with platelet $\alpha_{IIb}\beta_3$ glycoprotein [116], $\alpha_v\beta_3$ integrins on endothelial cells, melanoma and fibroblast cells [93].

Particularly, platelet interaction with fibrinogen specific RGD sequences or C-terminal portion of γ chain results in platelets activation, aggregation and in clot retraction as a

consequence of fibrin-platelet interaction [116]. Further, past investigations revealed the central role of peptide β15-42 on the amino terminal portion of fibrinogen β chain, released as a consequence of FBp thrombin-induced cleavage, in platelets spreading on a surface of polymerized fibrin [116]. β15-42 is involved in heparin binding and cellular matrix interactions as well as in platelets and endothelial cells spreading, fibroblast proliferation, proliferation and capillary tube formation and release of vWF [93].

Accordingly, fibrin β chain seems to play an important role in stimulating vascular cell response via vWF release from endothelial cells but also in spreading of both endothelial cells and platelets. Cell proliferation is crucial during vascular repair and fibrin is involved in the modulation of this process, showing how specific proteolytic modification of adhesive protein substrates at sites of tissue injury may act in regulating cell adhesion and growth processes [117].

Fibrinogen is also molecular related to leukocytes and involved in the regulation of their immune response. Indeed, stimulated monocytes and neutrophils exposes $\alpha_M\beta_2$ integrin (Mac-1) on their surface. Mac-1 is a high affinity receptor for D domain fibrinogen specific sequences, promoting fibrinogen-leukocyte interaction and consequently affecting inflammatory response [92,93].

Proteins, growth factors and cytokines binding to fibrinogen

Fibrinogen interaction with several other proteins and biological molecules may affect their function and involvement in different physiological pathways.

Plasma fibronectin binding to fibrinogen on its C-terminal region of Aα chain is mediated by factor XIIIa, involving multiple fibrinogen lysine residues and Gln-3 of fibronectin. This interaction is central for fibrinogen incorporation in cellular matrix [93].

Fibroblast growth factor-2 (FGF-2, bFGF) and VEGF binding to fibrinogen avoid their proteolytic degradation and induces endothelial cell proliferation, whereas fibrinogen

cross-linkage with insulin-like growth factor-1 (IGF-1) is involved in stromal cells function and proliferation, inducing wound healing process [92,93].

Furthermore, fibrinogen can interact with several cytokines as IL-1 promoting its stimulatory activity on endothelial cells [92].

Moreover, thrombin concentration, lipoprotein(a) and anticoagulants drugs as well as statins seem to be associated with alterations in fibrin structure and function but molecular mechanisms are still unclear in several cases [118].

Oxidative Stress and SLE

Inflammation and Oxidative Stress: General Details

Free radical generation is traditionally accepted as a mechanism of action during inflammatory response. Immune system activation is associated with increased phagocytosis, NETs releasing as well as pro-inflammatory cytokines, chemokines and ROS/RNS production. Free radicals are great instruments to kill bacteria but they are also crucial in the stimulation of adaptive immune system T cells and B-cells, supporting inflammation. Excessive ROS/RNS levels are associated with oxidative stress, causing tissue injury and consequently maintaining the inflammatory status. These elements have been found also in autoimmune disorders including SLE disease, where immune system de-regulation and autoantibodies overproduction are involved in the development of a chronic inflammatory status and in the clinical features of the pathology. However, low ROS production is implicated in higher susceptibility to microbial infections as well as immune tolerance affection, suggesting that physiological ROS levels are needed to promote health and reduce the possibility of autoimmune disorders onset [119].

Despite the evidence of a redox imbalance as a consequence of autoimmunity and inflammation, several studies underlined the involvement of oxidative stress in the pathogenesis of lupus disorder. Higher ROS levels, mediated by free radical overproduction, mitochondrial dysfunction or antioxidant genes polymorphisms, can alter apoptotic pathways, resulting in growing apoptosis and decreased clearance of apoptotic debris and promoting their interaction with ROS [120,121]. The generation of new epitopes is observed, inducing immune system reactivity and autoantibodies production followed by inflammation and tissue damages [122,123].

Introduction

Several investigators reported that oxidative stress is increased in SLE. In this regard, markers of lipids, proteins or DNA oxidation, biochemically detected, could be useful indicators of disease activity and progression, driving therapeutic supplementation with antioxidants in order to enhance SLE patients' redox status. Indeed, if a decreased antioxidant capacity (as intracellular GSH) seems to be associated with nephritis, CNS and endothelial activation in patients affected by SLE, treatment with antioxidant agents can reduce organ damages [121].

A large amount of data about oxidative stress assessment in SLE is reported in literature. Someone supported the idea of oxidative biomarkers increasing during active disease, especially in presence of renal failure and antiphospholipid antibodies and of their relation to the severity of symptoms with particular reference to fatigue [123]. Higher 8-OHdG plasma levels and alterations in cytokines/chemokines production/elimination were found significantly correlated with SLEDAI Index in SLE patients as well as increased lipid peroxidation products, oxidized/nitrosylated proteins and lower antioxidants levels [120,124]. Among oxidative stress biomarkers, protein oxidation seems to be associated with new antigens generation, immune profiles (with particular reference to Th1-Th17 immune shift), auto-immune response and severity of the disorder [125]. Redox imbalance has a crucial role in cardiovascular manifestations of SLE and in this context, lipoproteins reveal structural and functional differences in SLE patients when compared to them of healthy subjects; particularly an increased susceptibility to de novo oxidation and fragmentation is described [126].

Among immune-deregulation and inflammatory conditions, one of the principal actor in free radical generation is represented by neutrophils, that are central in redox imbalance amply described in SLE.

The Role of Neutrophils in SLE

Among immune system cellular mediators, polymorphonuclear neutrophils are active in organizing defensive responses against bacteria and fungi mediated by proteases and bactericidal peptides release, phagocytosis and NETosis, together with the production of pro-inflammatory cytokines and ROS. Physiologically, neutrophils have a half-life of 4-10 hours, but during inflammation they could be found in blood flow for 1-2 days.

Quantitative and qualitative neutrophils abnormalities are identified in lupus disorder. Neutropenia is one of the first hematological alterations detectable in SLE patients, but several data also suggest genetic and epigenetic modifications on neutrophils, involved in the pathogenesis of SLE. Indeed, patients display neutrophils with altered phagocytic capacities and oxidative ability as well as increased production of pro-inflammatory cytokines as IFN-1. Moreover, a subtype of neutrophils, lupus low-density granulocytes (LDGs), have been found in SLE. They are characterized by a pro-inflammatory phenotype and a great susceptibility to undergo NETosis [127]. Parallel, they represent a way to externalize autoantigens and enhance immune-stimulation, inducing break self-tolerance in autoimmune disorders.

Polymorphonuclear neutrophils play also a central role in adaptive immunity, contributing to T and B cells activation via pro-inflammatory molecular mediators.

NADPH Oxidase

Neutrophils and also monocytes host one of the principal sources of free radicals called NADPH oxidase (or NOX), a membrane bound enzyme that catalyzes the reduction of extracellular O_2 in $O_2^{\cdot-}$, using cytosolic NADPH as electrons donor. It exists in four isoforms as NOX1, NOX2, NOX3, and NOX4 and it is located into the plasma membrane as well as into the membranes of phagosomes used by neutrophils to engulf microorganisms [61].

Others redox reactions can be triggered downstream of NADPH oxidase including the generation of ONOO- (in endothelium) and hydrogen peroxide (due to the reaction between superoxide anion and protons), the last one potentially used by MPO for the production of HOCl. If ROS show both beneficial effects in immune defense from infections and harmful ones due to inflammation and tissue injury in case of free radical overproduction, a great system of NADPH oxidase activity regulation is needed. The phosphorylation of all the NADPH oxidase subunits, the activation of Rac2 and the cytosolic proteins transfer to the membrane are the principal mechanisms that influence inactivated/activated enzyme state [128].

As shown in figure 20, NADPH oxidase is a multi-component enzyme characterized by several proteins in the active site. The cytochrome b558 is a heterodimer composed by the two transmembrane proteins gp91phox (phox: phagocyte oxidase) and p22phox and by proteins p47phox p67phox p40phox and Rac2 (in neutrophils) originally located in cellular cytosol.

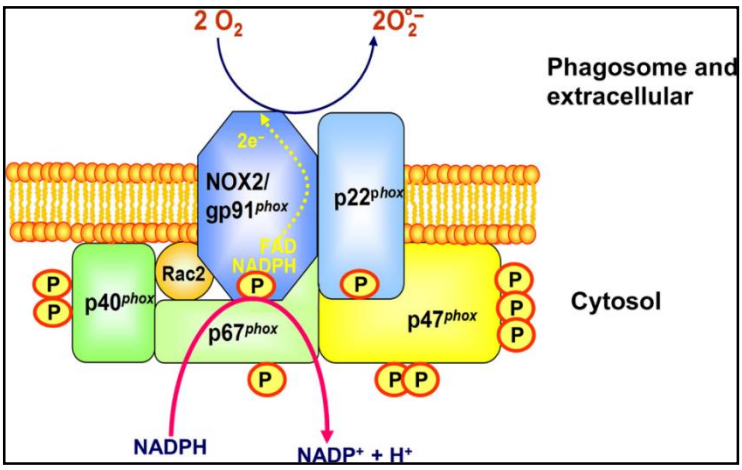

Fig 20. NADPH oxidase structure [128]

Four different states of NADPH oxidase activation have been identified: a resting state, a primed state, a fully activated state and a hyper-active state. The first one characterizes the inactivated enzyme, the priming state is a consequent response to stimuli from

bacterial LPS or pro-inflammatory cytokines as TNFalpha, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and IL-8. Enzyme activation is due to mobilization and phosphorylation of granular and cytosolic components of NOX to the phagosomal membrane, promoting the formation of a complex with gp 91^{phox} able to oxidize NADPH and reduce O_2 into $O_2^{\cdot-}$ [61,128].

NOX enzymes can be found in different cells and tissues displaying specific roles. NADPH oxidase is associated with cytoskeleton in endothelium, adventitia and cardiomyocytes and the production of superoxide anion and hydrogen peroxide in response to different factors, including thrombin, angiotensin II, endothelin-1 and mechanical forces, is involved in regulating the expression of several genes as PAI-1, ICAM-1, VEGF. Moreover, NOX-mediated ROS are implicated in the proliferation and migration of vascular smooth cells as well as in the regulation of MMPs enzyme, affecting vascular remodeling [129].

As just anticipated in the section of Oxidative Stress of this draft, NOX2 is considered “the primary-source of immune-active ROS in neutrophils”. Producing ROS, releasing proteases and triggering NETosis process, NOX2 plays a crucial role in protecting organism against infections. However, it is also able to control immune response and derived inflammation by modulating several signaling pathways associated with innate/adaptive immune cells recruitment/elimination and by regulating specific transcriptional factors as Nf-kB [130]. Its involvement in several pro-inflammatory pathways can explain the potential implication of the enzyme in inflammation and injury enhancement [130].

NETs and Thrombosis

As reported for the first time in 2004 by Brinkmann et al. [111], NETs are complexes of chromatin and proteins (histones, MPO and other antimicrobial proteins) able to degrade

virulence factors and bacterial compounds. They are released in the extracellular space as a consequence of infections or inflammation-induced neutrophil activation (figures 21 and 22). In addition to phagocytosis, proteolytic enzymes, pro-inflammatory cytokines and ROS release, NETs represent a further new immune defense mechanism against Gram-positive and Gram- negative bacteria. They originate from NETosis process, a particular cellular death pathway where neutrophils stimulation by ROS, LPS or cytokines and chromatin unfolding are the principal trigger events [131].

NETs can also act avoiding the spread of bacteria and protecting tissues from inflammation-mediated injury: indeed, they can block the release of several bacterial proteases in the neighboring sites, preventing their damage. Moreover, NETs might also be involved in autoimmunity onset including SLE, due to the exposure of chromatin and protein complexes that can trigger immune system activation [111].

However, data in the literature have underlined the role of NETs also in thrombosis. Both platelets and neutrophils are active protagonists in thrombus formation and progression, suggesting how pro-thrombotic alterations of haemostatic system could be associated not only to coagulation/fibrinolytic system factors abnormalities, but also to inflammation. Particularly, the pathogenetic involvement of neutrophils in thrombosis is due to the release of NETs, implicated in deep vein thrombosis (DVT) as demonstrated by several studies on animal models of DVT and by NET plasma biomarkers, reflecting disease activity [132,133].

Importantly, NETs have been described to form scaffolds in circulation that promote thrombus formation by interacting with the endothelium, platelets, coagulation factors and red blood cells. Consistently, depletion of neutrophils or injection of exogenous DNase I have been shown to prevent thrombus formation in mouse models and humans. IL-8 and ROS released from endothelial cells can recruit and trigger neutrophils to form NETs, which in return activate and damage the endothelium by

binding of histones to endothelial membranes. The release of Weibel–Palade bodies from the endothelium and deposition of fibrin and vWF promote blood coagulation by formation of thrombus scaffolds. vWF and fibrin have a high affinity for histones and therefore readily bind to NETs. Furthermore, histones have been shown to inhibit anticoagulants in the plasma, thereby further promoting thrombus formation.

Based on this evidence, systemic inflammation more than usual thrombophilic factors is thought to be one of the principal trigger factor of thrombosis in chronic inflammatory and autoimmune disorders as SLE and seems to be mainly mediated by T lymphocytes, monocytes, neutrophils and pro-inflammatory cytokines along with endothelial cell dysfunction [112-114].

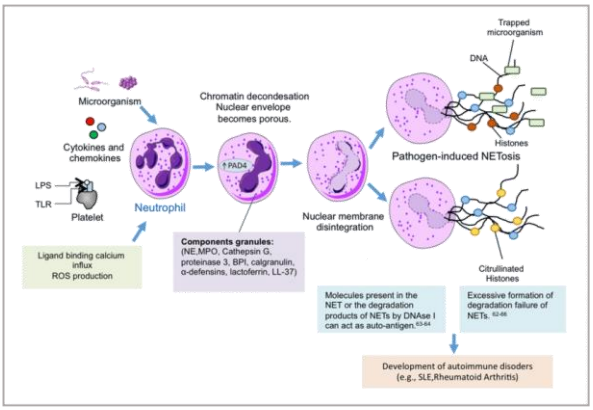

Fig 21. NETosis process [134]

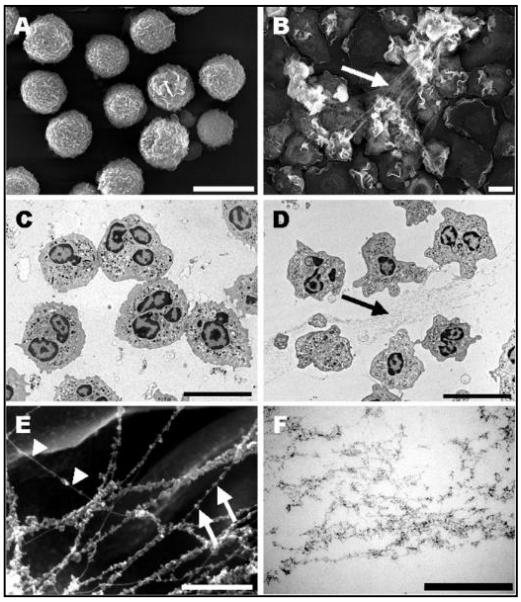

Fig 22. Electron microscopical analysis of activated neutrophils and NETs release [111]

Aim of the Study

Fibrinogen plays an essential role in inflammatory response and in blood coagulation, being a critical molecule for clot formation. Modifications in fibrinogen structure and dynamic features may influence fibrin network assembly, promoting haemostatic system alterations. During several pathological and chronic-inflammatory disorders, oxidative stress can lead to oxidation of plasma proteins including fibrinogen, that is 20x more susceptible to oxidative damage than albumin, as our group recently described [85]. We observed in BS, myocardial infarction and pulmonary hypertension and patients that fibrinogen oxidative modifications, especially increased fibrinogen carbonyl content, were significantly associated to structural and functional alterations of the protein [1,85,107]. Particularly, a loss of α-helix secondary structure as well as a reduced clotting ability and a decreased susceptibility to plasmin-induced lysis were found on purified fibrinogen from those patients.

Based on this background, being SLE an autoimmune and chronic inflammatory disorder where the main cause of mortality is represented by cardiovascular manifestations in patients with long duration of disease [34], it can represent an excellent experimental model for our investigations. In particular, in fibrinogen purified from SLE patients, the assessment of oxidative-dependent structural and functional modifications could shed light on the pathogenetic mechanisms involved in the increased cardiovascular risk in these patients.

Materials and Methods

The study sample included 144 patients with SLE (90% female) who attended the Florence Lupus Clinic (SOD Medicina Interna Interdisciplinare, AOUC Careggi) and 90 age-matched healthy control subjects. The characteristics of patients are reported in Table 8.

All patients were diagnosed as having SLE disease according to SLICC 2012 criteria [28,47]. Patients with other autoimmune diseases, active infections or neoplastic conditions were excluded. Blood samples were collected from patients under immunosuppressive therapy with glucocorticoids.

The study protocol was submitted by the local Ethics committees and informed written consent was obtained from all the subjects before entering the study.

Patients (n)	**144**
Age (yrs, mean ± SD)	**50.3 (±14.9; min 21 max 89)**
Gender (n)	
Female	**128 (89%)**
Male	**16 (11%)**
Clinical Manifestations	
Articular	**122 (85%)**
Cutaneous	**83 (57.5%)**
Haematological	**76 (52.5%)**
Renal	**50 (35%)**
Serological	**27 (19%)**
Neuropsychiatric	**25 (17.5%)**
Cardiac	**9 (6.5%)**

Gastrointestinal	**1.9 (1.3%)**
CV risk factors	
BMI	**24.1 (± 4.3); min 16 max 42.2)**
Hypertension (ESC Clinical Practice Guidelines)	**59 (41.2%)**
Smoke	**41 (28.8%)**
Dyslipidemia	**23 (16.3%)**
History of CV events	**13 (8.8%)**
Diabetes	**9 (6.3%)**
aPLs (antiphospholipid antibodies)	**42 (29%)**
Atherothrombotic events (n)	**32 (22.5%)**
Arterial	**16**
Venous	**12**
Mixed	**4**

Table 8. Clinical characteristics of SLE patients enrolled in the study.

Samples Collection

Blood samples were collected in Vacutainer tubes containing 0.109 mol/L buffered trisodium citrate (1:10) or EDTA (0.17 mol/L). After centrifugation (1500g for 15 minutes at 4°C), aliquots of sodium citrate plasma were used for experiments, fibrinogen purification or stored at -80°C for further analyses.

Fibrinogen Purification

Fibrinogen was purified using the previously described ethanol precipitation method [1,85,107]. After the purification process, fibrinogen concentration was determined by

ultraviolet spectroscopy (ONDA UV-20) at a wavelength of 280 nm, assuming an extinction coefficient of 1.51 mg/mL. The amount of purified fibrinogen was not statistically different between patients and controls (data not shown). The purity of purified fibrinogen (from 10 mL of citrated plasma) was assessed performing fibrinogen electrophoresis under reducing conditions and then densitometric analysis of Coomassie-stained polyacrylamide gels. No significant statistical difference between controls and patients was observed at the end of purification (data not shown).

Blood Leukocytes Intracellular ROS Levels Assessment

As described in others work of our group [1,135,136], after collection 100 µl of EDTA-anticoagulated blood samples was resuspended in 2 mL of BD FACS Lysing Solution (Becton Dickinson Biosciences, San Jose, CA, USA), gently mixed and incubated at room temperature in the dark for 15 minutes. Next, cells were centrifuged (700g for 7 minutes at 20°C), the supernatant was discarded and cells were washed twice in PBS. The evaluation of intracellular ROS levels was performed incubating cells with H_2DCF-DA (2.5 µM) (Invitrogen, Carlsbad, CA, USA) in RPMI medium without serum and phenol red for 30 min at 37°C. H_2DCF-DA is a chemically reduced form of fluorescein used as a ROS indicator in cells. Indeed, the 2',7'-dichlorofluorescein (DCF) fluorescent probe is particularly sensitive to several oxygen radical species as hydrogen peroxide, peroxynitrite, hydroxyl radicals and also by superoxide anions and if compared to more specialized ROS probes in development, H_2DCF-DA represents the most versatile indicator of cellular oxidative stress and the gold standard for ROS measurement [137]. After labelling, cells were washed and resuspended in PBS and analysed immediately using FACSCanto flow cytometer (Becton-Dickinson, San Jose, CA, USA). The sample flow rate was adjusted to about 1000 cells/s. For a single analysis, the fluorescence properties of at least 20,000 events were collected per sample. The individual cell

subpopulations were gated using their distinctive forward-scatter and side-scatter properties. Moreover, the viability of the cells was controlled by flow cytometry with propidium iodide staining, and was found to exceed 95 %. Data was analysed using BD FACSDiva software (Becton-Dickinson, San Jose, CA, USA).

Plasma Lipid Peroxidation Assessment (Thiobarbituric Acid Reactive Substances Assay, TBARS ASSAY)

Plasma Thiobarbituric Acid Reactive Substances (TBARS) levels were estimated using a TBARS Assay Kit (TBARS-Cayman) following the manufacturer's sheet. It is based on the thiobarbituric acid as an optimal reagent able to react with lipoperoxidation products (after 1h at 95°C), leading to the formation of a chromophore adduct measured spectrofluorometrically with excitation at 530 nm and emission at 550 nm in a Microplate Fluorometer (Biotek Synergy H1). Results were expressed in terms of malondialdehyde, MDA (nmol/mL) [1,85,135,138].

Plasma Total Antioxidant Capacity Estimation (Oxygen Radical Absorbance Capacity Assay, ORAC ASSAY)

The ORAC method (Oxygen Radical Absorbance Capacity) is based on the fluorescence decay of a fluorescent probe, fluorescein, consequent to its oxidation by free radical species (particularly peroxyl radical) generated after the thermal decomposition of azo compounds as 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). A fluorescein solution (6 nM) prepared daily from a 4 µM stock in 75 mM sodium phosphate buffer (pH 7.4), was used. Trolox (250 µM final concentration), a water-soluble analogue of E vitamin, was used as a standard. 70 µl of each sample was pre-incubated for 30 min at 37°C in each well with 100 µl of fluorescein and then AAPH solution (19 mM final

concentration) was added starting the reaction. Fluorescence was measured with excitation at 485 nm and emission was measured at 537 nm in a Microplate Fluorometer (Biotek Synergy H1). Results were expressed as Trolox Equivalents (μM) and then normalized for protein concentration [1,85,135,138,139].

Assessment of Dityrosine Content in Purified Fibrinogen

Dityrosine content was evaluated on purified fibrinogen fractions by fluorometric measurements using a Jasco Fluorimeter (Jasco 810). Samples were dialyzed in 5 liters of PBS pH 7.4 and then Dityrosine Fluorescence Spectra were recorded at 25 °C in a 1 cm quartz cuvette with an excitation wavelength of 316 nm and maximum emission of 367 nm. Three spectra for each sample were acquired and then normalized for protein concentration (mg/ml) of each related sample [89-91].

Evaluation of Thrombin-Catalyzed Fibrin Polymerization

For functional analysis, purified fibrinogen fractions stored at -80°C and not previously thawed were used. The reaction was performed in duplicate using 40 μg of fibrinogen and PBS with Ca^{2+}-Mg^{2+} (starting volume of 100 μl) to which 100 μl of thrombin at the final concentration of 0.20 U/ml (in PBS with Ca^{2+}-Mg^{2+}) were added, starting the process. Fibrin polymerization was monitored at 405 nm in a 96-well microtiter plate reader (Biotek Synergy H1) for 120 minutes at 25°C. The procedure was performed in accordance to the protocol previously described in our recent studies [1,85].

Absorbance curves were characterized using the following parameters:

1. The *maximum slope* (Vmax), calculated as the slope of the steepest part of the polymerization curve (using 10 points), indicates the rate of lateral protofibril association;

2. The *lag phase*, measured as the time elapsed until an increase in absorbance was seen, represents the time to the start of lateral fibril aggregation;
3. *Maximum absorbance* (Max Abs) of the growing clot, recorded 60 min after polymerization was initiated, which reflects an average fibrin fiber size and the number of protofibrils per fiber.

Evaluation of Fibrin Susceptibility to Plasmin-Induced Lysis

According to our protocol performed during other studies [1,85,107], 10 µg of purified fibrinogen were incubated with bovine thrombin (50 U/ml final concentration) in 20 µl of Buffer (50mM Tris, 100mM NaCl, 20mM EDTA, pH 7,4) [140] for 1h at 25°C in microcentrifuge tubes. After, plasmin was added (5 µl of 50 U/ml final concentration) and the fibrin clots were digested over a period of 6h at 37°C. 10 µl of Lithium Dodecyl Sulfate (LDS) Gel Electrophoresis Sample Buffer (50mM Dithiothreitol, DTT and LDS 4X) was used to terminate fibrin clots degradation. The same lot of thrombin and of plasmin were used for all experiments. Samples were incubated at 90°C for 15 minutes under reducing conditions and then loaded in 4-12% Bis-Tris gels. After electrophoresis, gels were stained with Coomassie Blue and band intensities of stained gels were quantified by Image J Software. Data were expressed as the ratio between the densitometric reading of the purified protein at a given digestion time and that at the undigested protein (time 0 for incubation with plasmin).

Circular Dichroism (CD) Spectra of Purified Fibrinogen

CD Spectra of purified fibrinogen 1 mg/ml were recorded on a Jasco Fluorimeter (Jasco 810) at 25 °C in 0.2 cm quartz cell from 250 to 195 nm (far UV). Samples were filtered through 0.22 µM filters and three spectra recorded for each sample. Protein ellipticity at

208 and 222nm has been evaluated to investigate fibrinogen α-helix secondary structure [1,85]. Molar ellipticity values [q] were calculated according to the equation: [θ] (deg-cm^2 $dmol^{-1}$) = [θ (MRW)]/[10(l)(c)], where ϕ is the displacement from the basaline value X to the full range in degrees; MRW is the mean residue weight of the aminoacids; l is the path length of the cell (cm) and c is the protein concentration (g/ml).

Intrinsic Fluorescence Spectra of Purified Fibrinogen

In order to provide information on conformational changes of purified fibrinogen, we performed Intrinsic Fluorescent Spectra of the protein deriving from the naturally fluorescent amino acid tryptophan and to a lesser extent from tyrosine. Spectra were recorded on a Jasco Fluorimeter (Jasco 810) at 25 °C in a 1 cm quartz cells with an excitation wavelength of 280 nm and maximum emission of 352 nm. Three spectra were acquired for each sample and then normalized for protein concentration (mg/ml) of each related sample [141,142].

Statistical Analysis

All the experiments were performed in triplicate and data were expressed as the overall mean of the means of the 3 experiments ± Standard deviation (SD), after testing the low intra-experiment and inter-experiment variability and the reproducibility of measures using ANOVA Bonferroni Test. All data presented a normal distribution and were considered statistically significant with a value of $p < 0.05$. All statistical operations data were processed using the Graph Pad Prism 5 Software.

Results

According to the aim of this study, in 144 SLE patients and 90 sex and age-matched controls blood systemic redox status and signs of fibrinogen oxidation were evaluated. Assessment of structural and functional features of purified fibrinogen fractions were performed and finally, correlation analyses between the considered redox markers and the functional fibrinogen parameters were executed.

Blood Leukocytes Intracellular ROS Levels

The evaluation of blood systemic redox status was performed in SLE patients and healthy subjects by the assessment of intracellular ROS levels in blood leukocyte subpopulations of lymphocytes, monocytes and granulocytes.

As reported in figure 23 (panel B, C and D), SLE patients showed a significant increase in ROS levels in all the three leukocyte fractions, if compared to controls ($p < 0.001$).

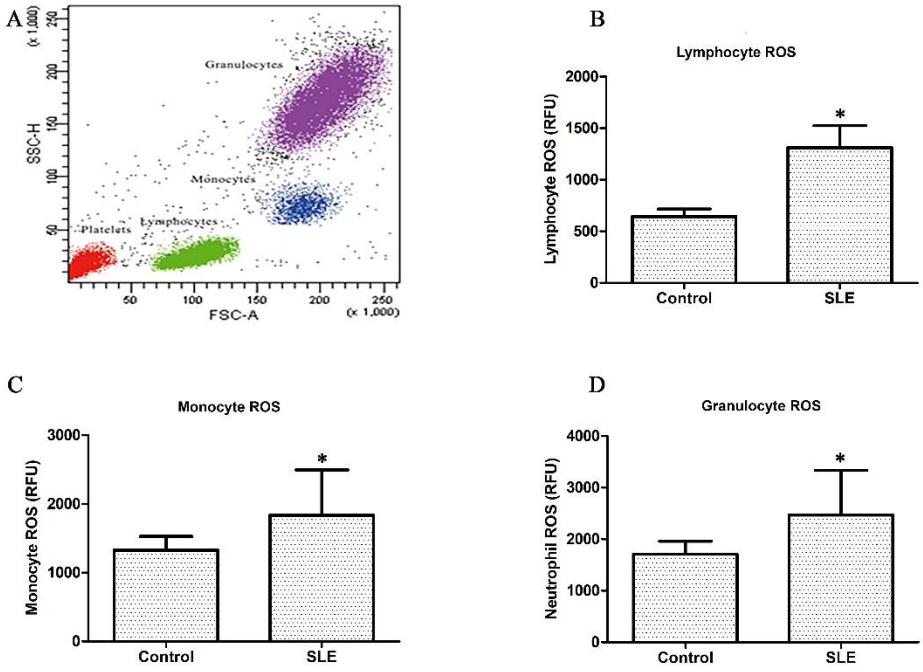

Fig 23. Blood leukocytes ROS production in SLE patients and controls.
*FACS representation of blood leukocyte subpopulations using their distinctive forward-scatter and side-scatter properties (**A**). Lymphocyte, monocyte and neutrophil ROS production (**B, C, D**) in SLE patients (n=144) and controls (n=90). * indicates that differences are significant at the p< 0.05 level by ANOVA Bonferroni Test.*

Oxidative Stress Assessment in Plasma Samples and in Purified Fibrinogen

In SLE patients, plasma samples oxidative stress monitoring revealed a significantly higher lipid peroxidation (0.39 ± 0.08 vs 1.42 ± 0.49, p<0.0001) and a lower total antioxidant capacity (TAC) (21.33 ± 3.10 vs 16.12 ± 3.78, p < 0.0001) as shown in figure 24 (panel A and B). Moreover, signs of fibrinogen oxidation were found. Particularly, data in figure 24 (panel C) show a significant increase in dityrosine content on purified fibrinogen from SLE (151 ± 33 vs 297 ± 78, p < 0.0001).

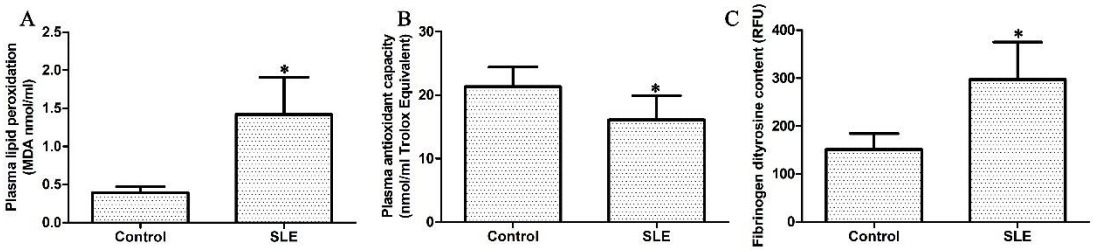

Fig 24. Plasma oxidative stress marker in SLE patients and controls.
*Plasma lipid peroxidation (**A**), plasma antioxidant capacity (**B**) and dityrosine content on purified fibrinogen fractions(**C**) in SLE patients (n=144) and controls (n=90). * indicates that differences are significant at the p < 0.05 level by ANOVA Bonferroni Test.*

Thrombin-Catalyzed Fibrin Polymerization

Fibrin assembly mechanism has been investigated performing kinetics of fibrin polymerization during the time. Representative curves of thrombin-induced fibrin polymerization are reported in figure 25, displaying quantitative but particularly qualitative differences in the process between SLE patients and controls.

Indeed, a reduced fibrinogen clotting ability is shown in SLE. This is in accordance with the significant differences between the principal parameters of fibrin polymerization process (Lag Phase, Vmax and Max Abs, as described in Materials and Methods section), suggesting abnormalities in clot structural architecture. Particularly, in SLE Lag phase

value is increased (3.9 ± 0.8 vs 8.6 ± 4.6, $p < 0.0001$) whereas a reduction in Vmax (0.011330 ± 0.001120 vs 0.002293 ± 0.001704, $p < 0.0001$) and Max Abs (0.211 ± 0.019 vs 0.115 ± 0.043, $p < 0.0001$) values are observed (figure 26 panel A, B and C).

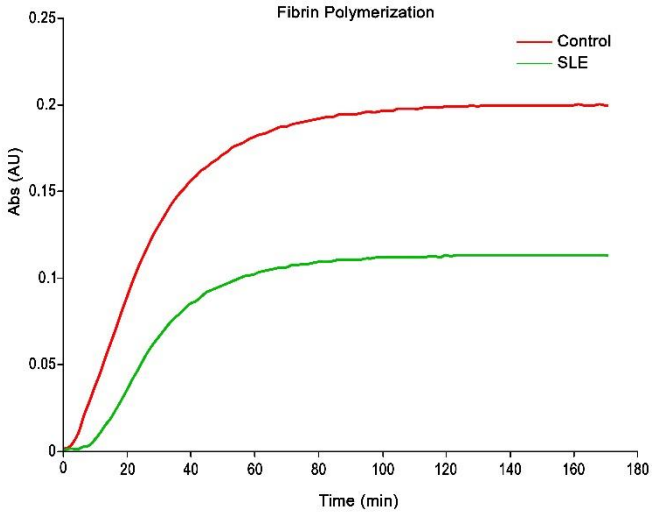

Fig 25. Representative curves of fibrin polymerization in SLE patients and controls

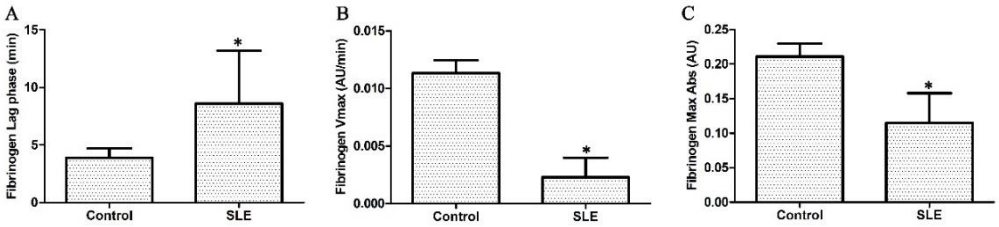

*Fig 26. Thrombin-catalyzed fibrin polymerization and corresponding Lag phase (**A**), Vmax (**B**) and Max Abs (**C**) in fibrinogen purified from SLE patients (n=144) and controls (n=90).*
** indicates that differences are significant at the $p < 0.05$ level by ANOVA Bonferroni Test.*

Plasmin-Induced Fibrinolysis

Purified fibrinogen functional features were also investigated, evaluating fibrin susceptibility to plasmin-induced lysis. Particularly, we focused on the degradation rate of the fibrin ß chain that has been monitored before and after 3 hours and 6 hours of plasmin digestion, as shown in figure 27 (panel A).

The quantification of residual fibrin β chain after 6 hours of plasmin digestion revealed a significantly different and higher content of this parameter in SLE than in controls (23 ± 6 vs 63.7 ± 21.8, $p < 0.0001$), showing a fibrin resistance to plasmin induced lysis in SLE (figure 27 panel B).

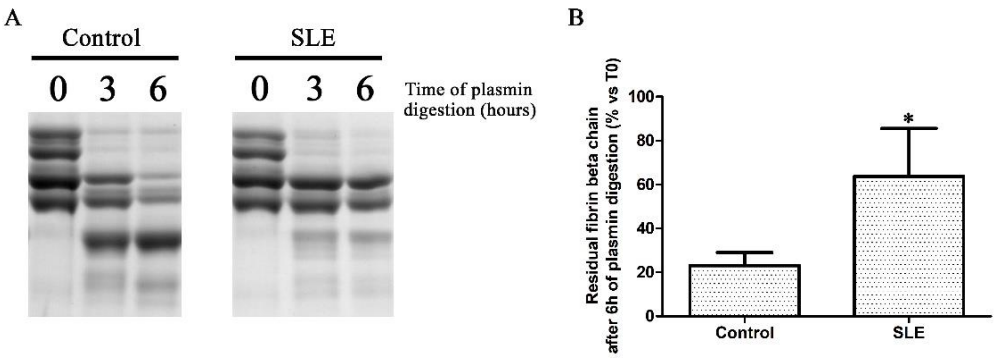

*Fig 27. Representative gel of fibrin lysis after 0-6h of plasmin incubation with fibrinogen purified from SLE patients and controls (**A**). Quantification of residual fibrin β chain after 6h of plasmin digestion in SLE patients (n=144) and controls (n=90) (**B**).*
** indicates that differences are significant at the p < 0.05 level by ANOVA Bonferroni Test.*

Analysis of Fibrinogen Structural Features

Circular Dichroism Spectroscopy (CD)

Fibrinogen secondary structure is defined by α-helices and β-pleated sheets and it strongly influence protein biological activity.

In this study, fibrinogen secondary structure was firstly evaluated using far-UV Circular Dichroism Spectroscopy. Comparing CD spectra of purified fibrinogen from patients and healthy subjects, differences in protein structure were evident. Particularly, if fibrinogen purified from controls shows a typical alpha-helix secondary structure with minima at 208 nm and at 222nm, a decreased negative peak in the 215 to 225v nm region is observed in SLE, suggesting a reduction in α-helical content (figure 28).

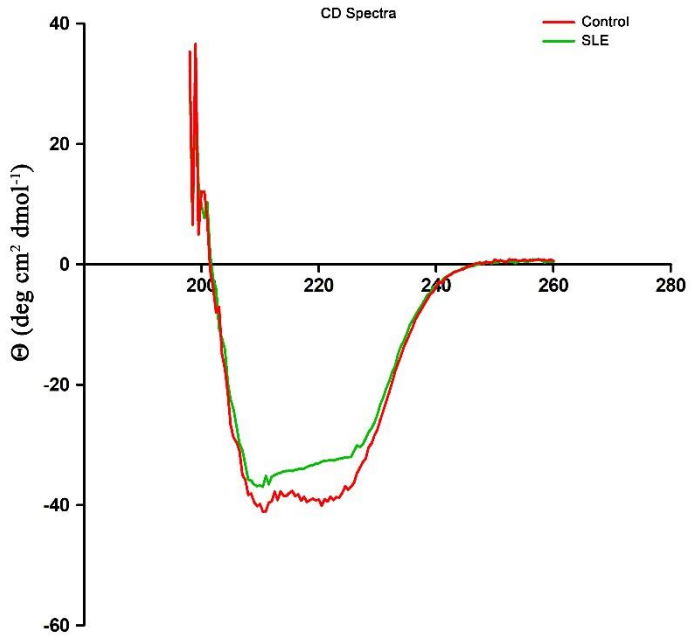

Fig 28. Representative CD spectra of purified fibrinogen from SLE patients and controls.

Intrinsic Fluorescence Spectra Analysis

Further investigations on fibrinogen structural features were performed analyzing the intrinsic fluorescence properties of protein samples, determined by the different exposure of hydrophobic amino residues to the solvent. Evaluating intrinsic fluorescence spectra of purified fibrinogen from patients and healthy subjects, an inverse correlation between the maximum fluorescence emission at 352 nm and fibrinogen structure was observed (figure 29 panel A). Particularly, as shown in figure 29 (panel B), the intrinsic fibrinogen fluorescence intensity was significantly different and higher in SLE patients than in controls, suggesting protein conformational alterations according to the CD results (406 ± 66 vs 185 ± 128, $p < 0.0001$).

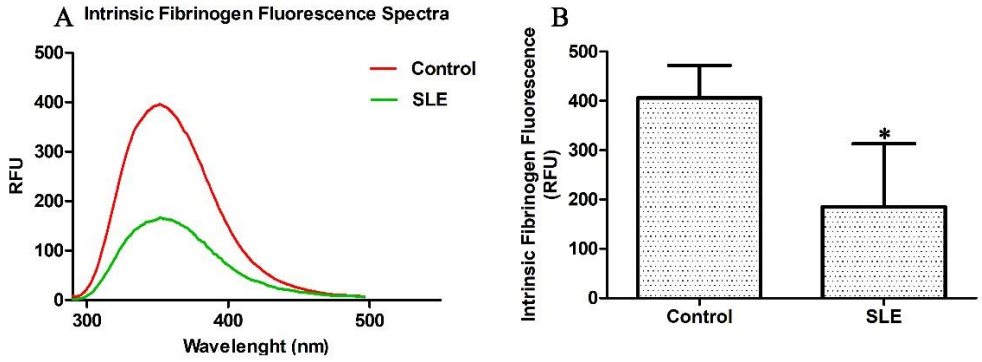

*Fig 29. Representative intrinsic protein fluorescence spectra of purified fibrinogen (**A**); quantification of intrinsic fibrinogen fluorescence in in SLE patients (n=144) and controls (n=90) (**B**).* indicates that differences are significant at the $p < 0.05$ level by ANOVA Bonferroni Test.*

Correlation Analysis

At the end of the study, correlation analysis between fibrinogen functional and structural features and the considered redox parameters were also performed.

As shown in figure 30 (panel A, B, C, F, G, H and I), residual fibrin β chain after 6h of plasmin digestion significantly correlated with lymphocyte ROS (r^2 = 0.03623, p = 0.0223), monocyte ROS (r^2 = 0.03750, p = 0.0200), neutrophil ROS (r^2 = 0.04586, p = 0.0100), fibrinogen dityrosine content (r^2 = 0.1164, p < 0.0001) and fibrin polymerization parameters of Lag phase (r^2 = 0.6564, p < 0.0001), Vmax (r^2 = 0.2835, p < 0.0001) and Max Abs (r^2 = 0.2360, p < 0.0001). Conversely, no significant correlations have been found with plasma lipid peroxidation (r^2 = 0.02077, p = 0.0848) and plasma antioxidant capacity (r^2 = 0.01380, p = 0.1609), as reported in figure 30 (panel D and E).

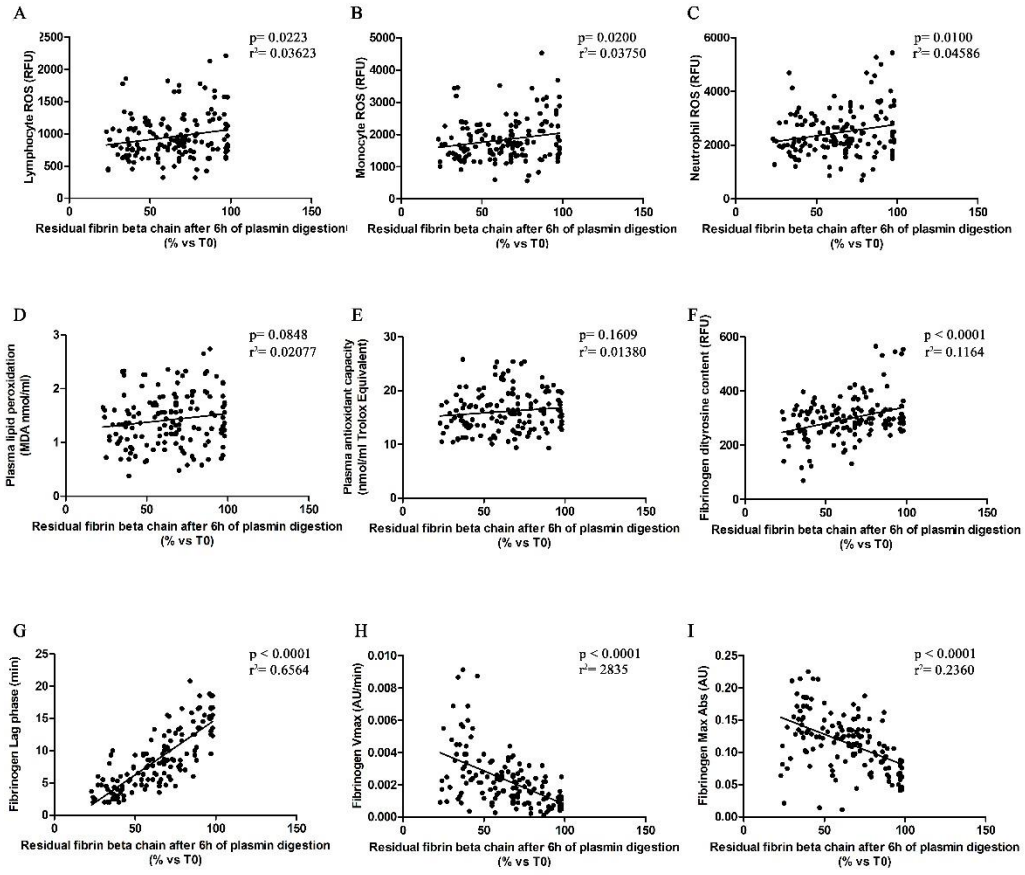

*Fig 30. Correlation analysis comparing residual fibrin β chain after 6h of plasmin digestion with leukocyte subpopulations intracellular ROS production (**A, B, C**), plasma oxidative stress markers (**D, E**), fibrinogen dytyrosine content (**F**) and fibrin polymerization parameters (**G, H, I**) in SLE patients (n=144). Statistical significance was considered at the p < 0.05 level by ANOVA Bonferroni Test.*

Results

Figure 31 (panel A, B and C) shows that fibrinogen dityrosine content significantly correlates with all fibrin polymerization parameters of Lag phase (r^2 = 0.06945, p = 0.0014), Vmax (r^2 = 0.03207, p = 0.0317) and Max Abs (r^2 = 0.02761, p = 0.0466).

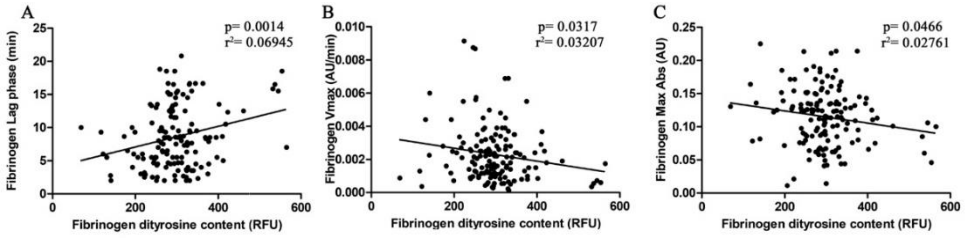

*Fig. 31. Correlation analysis comparing purified fibrinogen dityrosine content with fibrinogen polymerization parameters of Lag phase (**A**), Vmax (**B**) and Max Abs (**C**) in SLE patients (n=144). Statistical significance was considered at the p < 0.05 levell by ANOVA Bonferroni Test.*

Moreover, significant correlations have been found between the intrinsic fibrinogen fluorescence and fibrin polymerization parameters of Lag phase (r^2 = 0.1556, p < 0.0001), Vmax (r^2 = 0.06002, p= 0.00031) and Max Abs (r^2 = 0.08406, p = 0.0004), residual fibrin β chain after 6h of plasmin digestion (r^2 = 0.2850, p < 0.0001) and fibrinogen dityrosine content (r^2 = 0.1871, p < 0.0001), as shown in figure 32 (panel A, B, C, D and E).

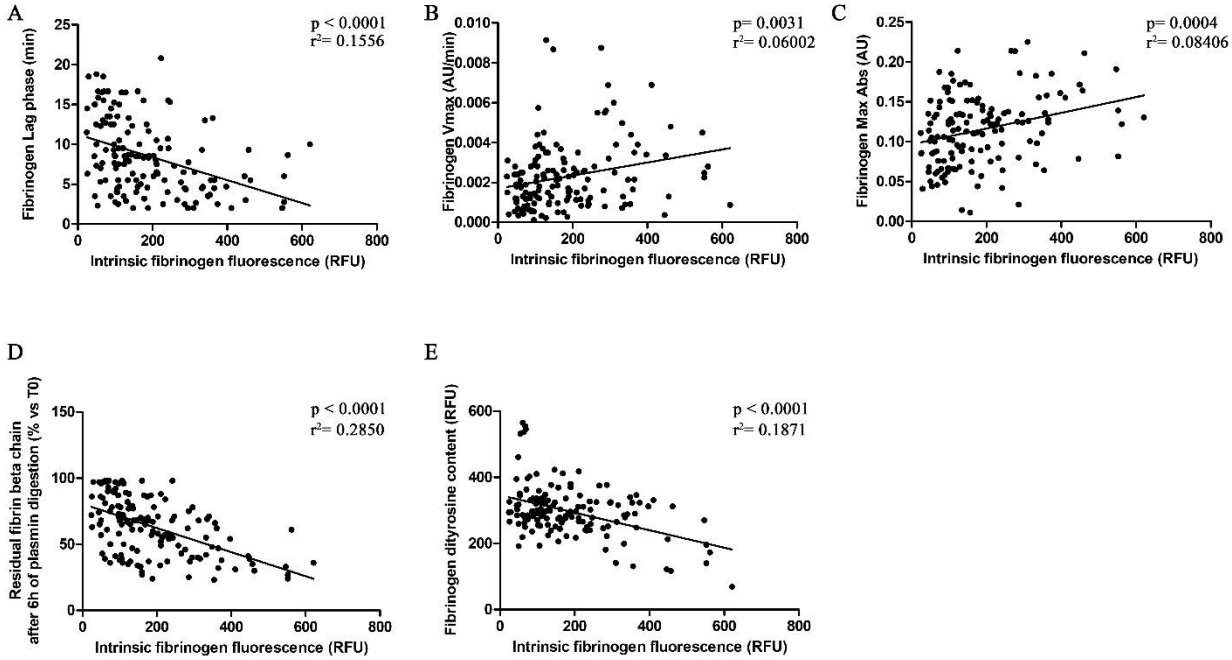

*Fig. 32. Correlation analysis comparing intrinsic purified fibrinogen fluorescence with fibrinogen polymerization parameters (**A, B, C**), residual fibrin β chain after 6h of plasmin digestion (**D**) and fibrinogen dityrosine content (**E**) in SLE patients (n=144).*
Statistical significance was considered at the p < 0.05 level by ANOVA Bonferroni Test.

Discussion

Cardiovascular events represent one of the main causes of mortality in developed countries. In addition to alterations of haemostatic processes, including platelet activation and aggregation, coagulation and fibrinolysis, over the last years a growing literature has underlined the pivotal role of oxidative stress in vascular health affection. Oxidative stress, traditionally defined as an imbalance between ROS/RNS production and antioxidant defense mechanisms, is strongly related to several acute and chronic disorders [143]. Both ROS and RNS are considered crucial mediators of inflammation, together with pro-inflammatory cytokines, chemokines and adhesion molecules, growth factors and proteases. Free radicals are also able to substantiate pro-inflammatory responses due to tissue injury via cellular components (lipids, proteins and DNA) oxidation [143]. Based on this evidence, a strong correlation between oxidative stress, vascular inflammation and endothelial dysfunction can be observed. Indeed, an increase in pro-inflammatory cytokines (as IL-1, IL-6, IFN, TNF-α) production and in adhesion molecule (as VCAM-1, ICAM-1) expression, promoting leukocytes migration and infiltration as well as platelet and coagulation factors recruitment has been described in this context [2]. Moreover, higher levels of proteases (as MMP-2, MMP-9 in smooth muscle cells) and reduced NO amounts are involved, together with the before reported factors, in a pro-thrombotic and pro-atherogenic endothelial phenotype [63,144].

Recent studies observed vascular complications and higher risk of thrombosis also in autoimmunity, suggesting a crucial association between systemic inflammation and thrombotic events [2]. Accordingly, autoimmune disorders are chronic inflammatory pathological conditions where immune system dysfunctions and autoantibodies overproduction, due to the loss of self-tolerance mechanisms, may induce the clinical manifestations via progressive tissue and organ injury. Moreover, if a physiological interaction between components of coagulation and immune system (as leukocytes and

macrophages) exists, based on the inflammatory properties of vascular insult that induces coagulation cascade activation, inflammation could be also considered a trigger factor of haemostatic system alterations and thrombosis in chronic disorders [2].

Many studies in literature proposed BS as a model of inflammation-induced thrombosis. Accordingly, therapeutic treatment with glucocorticoids and immunosuppressants produced a reduction of patients' mortality due to arterial/venous thrombosis unlike anticoagulant drugs [145]. Inflammation and oxidative stress in BS have been also supported by our recent investigations, showing blood redox status alterations (described by the significant increase in plasma oxidative stress markers and in leukocytes intracellular ROS levels) and an hyperactivation of leukocytes in patients than in healthy subjects. Interestingly, the considered redox parameters were significantly correlated with functional and structural alterations of fibrinogen (purified from plasma fractions of patients and controls), underlining a new pathogenic mechanism in the relation between immune system and thrombogenesis [1].

Among autoimmune disorders, SLE is characterized by an increased cardiovascular risk that represents one of the main causes of mortality in patients with a long-standing disease [34, 35]. Traditional Framingham factors as well as early atherosclerosis and disease-related factors are included among pathogenetic mechanisms of thrombosis and cardiovascular manifestations in SLE, but further investigations are needed.

Based on this evidence, the aim of our study was to evaluate the effects of oxidative stress on structural and functional features of fibrinogen purified from plasma of SLE patients, in comparison with healthy subjects. Indeed, fibrinogen is a plasma glycoprotein with a central role in inflammation, being an acute phase protein, but also in coagulation, being one of the main actors of fibrin clot assembly. Changes in its structure and conformation have been associated to pro-thrombotic alterations of haemostatic system.

Discussion

As a chronic inflammatory and autoimmune disease, redox status alterations were largely observed in plasma, serum or urinary samples of SLE patients [123]. Higher levels of urinary F_2 isoprostane excretion, an increased serum levels of nitrotyrosine and anti-MDA/anti-HNE protein adduct antibodies as well as higher oxidized low-density lipoproteins have been biochemically assessed in SLE patients. In addition, a lower content of SOD was found in serum of patients than in controls. All these data seem to be potentially associated with disease activity, fatigue, organ damage and comorbidities [123,124,126], suggesting the need of further studies devoted to improve the quality of life and clinical manifestations by the use of new therapeutic approaches. In this context, our biochemical assessment of oxidative stress both in plasma samples and blood leukocytes of SLE patients could supply precious information about the systemic redox status in these patients.

In 144 SLE patients and 90 sex-age matched healthy subjects, ROS production in blood leukocyte subpopulations of lymphocytes, monocytes and granulocytes was detected by FACS analysis and resulted significantly higher in patients than in controls. Our recent investigations showed higher intracellular ROS levels also in other pathological conditions as BS [1], non-ST elevation myocardial infarction (NSTEMI) [136] and Retinal Vein Occlusion (RVO) [146]. This evidence supports that leukocytes are an important model to study variations of redox homeostasis in biological systems. Plasma oxidative stress status was evaluated by the assessment of TBARS levels, as an index of lipid peroxidation and Total Antioxidant Capacity (TAC) content. It is traditionally accepted that ROS-induced plasma lipoproteins oxidation is associated with the pathogenic mechanisms of atherosclerosis, being one of the main actors in the atherosclerotic plaque formation. Autoimmune disorders are characterized by inflammation, redox status alterations and, consequently, increased ox-LDL [126]. All these features have been also reported in SLE, suggesting an accelerated atherosclerosis

and an increased CVD risk [122]. Accordingly, our data showed a significant increase in plasma lipid peroxidation products from patients compared to healthy subjects. These results are in line with other reports in pathological conditions such as BS, post-myocardial infarction, and Marfan Syndrome [1,85,148], underlining the relevance of lipid peroxidation products as stable biomarkers to assess sample redox status. However, lipid peroxidation involvement in cardiovascular risk is not only due to the triggering of atherosclerosis and atherosclerotic plaque formation, but also to alterations of dynamic characteristics of cellular membrane in specific blood cellular subsets. Indeed, two recent papers performed by our group reported that higher intracellular ROS levels and membrane lipid oxidation in erythrocytes were positively correlated with higher blood viscosity and reduced membrane fluidity and deformability, leading to the thrombotic clinical manifestations of RVO and Sudden Sensorineural Hearing Loss (SSHL) [146,149].

In our cohort of patients, oxidative stress status was also confirmed by a significant decrease in plasma TAC levels. Together with leukocyte ROS production and lipid peroxidation, TAC levels, even if representing an indirect indicator of redox balance, give an important contribution to oxidative status evaluation in SLE. Indeed, in the early phase of oxidative stress, ROS induce antioxidant gene expression, whereas during prolonged phase of oxidative stress TAC decreases as an effect of antioxidant consumption.

Several biological molecules are susceptible to ROS-mediated oxidation. Especially, protein oxidative modifications may induce polypeptide structural changes, causing protein dysfunction and its involvement in the pathogenic mechanisms of several disorders such as neurodegenerative, cardiovascular diseases and tumors. Among plasma proteins, fibrinogen results 20x more susceptible to oxidative modifications than albumin [85], according to several studies that described it as an important target of oxidation with potential effects on its structural and functional characteristics [150]. Fibrinogen

polypeptide chains expose several amino residues to oxidative attack including arginine, proline, histidine and lysine, tyrosine, methionine and cysteine located in the specific thrombin or plasmin binding sites. It has been reported that methionine oxidation plays a pivotal role in vascular biology, affecting several proteins of haemostatic system and particularly fibrinogen [86]. As reported by Weigandt et al. [87], the oxidation of one or all the three fibrinogen methionine residues Met78, Met367 and Met476 may alter fibrin clot polymerization and delays fibrinolysis. This may be potentially due to a reduction in protofibrils lateral aggregation, leading to fibrin clots with smaller pore sizes [105]. Abnormalities in structural and viscoelastic features of fibrin clots have also been found as consequences of fibrinogen nitration [151] or carbonylation. Carbonyl groups are considered one of the main biomarkers of protein oxidation, causing an increase in protein hydrophobicity, aggregation and dysfunction [84,152].

Oxidation of the above mentioned amino acids may lead to the formation of cross links, nitration derivatives or carbonyl compounds that can induce protein intramolecular rearrangements and changes leading to fibrinogen biological activity alterations [150]. This evidence may legitimate our analysis, evaluating signs of oxidative stress markers on fibrinogen purified from plasma of SLE patients and in healthy subjects. Particularly, dityrosine content was fluorometrically assessed on fibrinogen fractions of patients and controls. After normalization for protein concentration, our data show a significant increase in fibrinogen dityrosine content in SLE patients compared to healthy subjects, suggesting further investigations aimed at evaluating the potential relationship between fibrinogen oxidation and the increased cardiovascular risk in SLE. Several data in the literature suggest that fibrinogen oxidative modifications may alter clot structural architecture and consequently clot dynamic, leading to thrombosis and thromboembolism [150].

Discussion

When we assessed thrombin-induced fibrin polymerization process, a reduced clotting ability was observed in SLE patients, as demonstrated by the significant increase in Lag phase value and the reduction of Vmax and Max Abs values if compared with controls. Moreover, Lag phase value appears significantly and positively correlated with fibrinogen dityrosine content whereas Vmax and Max Abs values are significantly and inversely associated with the considered marker of fibrinogen oxidation. In 1995, Shacter et al. [153] reported preliminary evidence of a decreased clot formation after fibrinogen oxidation and later, several other studies confirmed this data. Recently, our group demonstrated the association of oxidative modification of fibrinogen with structural and functional alterations both in BS and in subacute phase of myocardial infarction and in [1, 85], showing the thrombogenic phenotype of fibrinogen in that pathological context. Similarly, the central role of fibrinogen oxidation in other thrombotic disorders has been revealed, including cirrhosis [109] and coagulopathy after traumatic injury [154].

Our investigations on fibrinogen functional features in SLE were extended to the evaluation of fibrin susceptibility to plasmin-induced lysis. A reduced fibrinolysis has already been found by our group in several pro-thrombotic or chronic inflammatory disorders as BS [1], post-acute myocardial infarction [85] and pulmonary hypertension [107]. However, this evidence can be found in other pathological conditions characterized by thrombotic events, suggesting alterations in fibrin clot structure/function as a transversal mechanism involved in thrombogenesis. Indeed, fibrin resistance to lysis has been also described in deep vein thrombosis and pulmonary embolism [102], in idiopathic venous thromboembolism [108], chronic thromboembolic pulmonary hypertension [140] and in acute coronary syndrome [155].

When we analyzed SLE patients' fibrin susceptibility to lysis after 6 hours of fibrin incubation with plasmin, a marked resistance to the enzyme catalytic activity was observed in comparison with healthy subjects. This was confirmed by the significant

increase in residual fibrin β chain after 6 hours of plasmin digestion in SLE patients. In accordance to our investigations performed in BS and in the subacute phase of myocardial infarction [1, 85] where fibrin resistance to lysis was significantly related to fibrinogen carbonyl content, a significant and positive correlation between the residual fibrin β chain after 6 hours of plasmin digestion and the considered redox parameters of blood leukocyte ROS production and fibrinogen dityrosine content has been also found in our cohort of SLE patients. Interestingly, another main result is the significant correlation among the residual fibrin β chain after 6 hours of plasmin digestion and Lag phase, Vmax and Max Abs values, supporting that fibrinogen oxidation can alter fibrin clot architecture and influence fibrin susceptibility to plasmin activity.

Accordingly, a reduced protofibrils lateral aggregation and stiffer clots can be observed in pro-thrombotic pathological conditions, where thinner fibrin fibers and smaller pore sizes result in clot with higher stability and less permeability to plasmin, as largely reported by Undas et al in 2008 [155] but also by Mills et al. in 2002 [156] and Collet et al 2006 [157]. On the contrary, clots composed by thick fibrin fibers and larger pore sizes are found in healthy subjects, suggesting a higher permeability to plasmin and an accelerated fibrinolysis [1].

Based on the data here reported, our main future perspective will be represented by an in-detail clot structure analysis by Transmission Electron Microscopy and/or DIC analyses- in order to characterize the structural features of fibrin network in SLE patients.

Several factors may influence fibrin clot conformational and mechanical features as well as stability, including blood flow and cells, environment, thrombin and fibrinogen concentration, fibrinogen genetic variants, intrafibrillar structure and protofibril packaging [158,159]. However, in haemostatic system disorders, a great relevance in modulating fibrin network assembly is also represented by oxidative stress, a consequence of systemic response to vascular injury. Fibrinogen oxidation is associated

with alterations in clot structure/function, resulting in a fibrin thrombogenic phenotype [1,85,106]. Based on this evidence, in the last part of this study fibrinogen structure was also investigated. Using far-UV Circular Dichroism Spectroscopy (CD), fibrinogen secondary structure was analyzed. Comparing CD spectra of fibrinogen purified from patients and controls, a reducing in α-helical content was observed in SLE patients. Fibrinogen structure was further explored by assessing intrinsic protein fluorescence. Our results revealed a different exposure of fibrinogen hydrophobic amino residues to the solvent in patients, confirming fibrinogen conformational changes in SLE.

Interestingly, a significant correlation was found between the intrinsic fibrinogen fluorescence and both fibrinogen dityrosine content and fibrinogen functional features parameters (the residual fibrin β chain after 6 hours of plasmin digestion and Lag phase, Vmax and Max Abs values).

The findings here reported show a systemic redox imbalance in SLE patients and mostly important, the observed fibrinogen oxidative modifications seem to be associated with alterations in fibrinogen secondary structure, affecting fibrin clot assembly and its dynamic features. Moreover, experiments performed *in vitro* support this evidence. In literature, several reports described that *in vitro* fibrinogen oxidation with HOCl promoted the formation of Advanced Oxidation Protein Products (AOPP), leading to changes in its structural domains and consequently in its biological activity (a reduced clotting time) [160]. In addition, Nowak et al [161] confirmed in his study that peroxynitrite-induced oxidative/nitrative modifications of fibrinogen may be related to functional consequences including a reduced fibrin clotting ability and a decreased fibrin susceptibility to plasmin-induced lysis. Particularly, AαC fibrinogen domain is largely involved in protofibrils lateral aggregation and results the most susceptible domain to oxidation, causing abnormalities in fibrin polymerization process as well as in clot structure. Indeed, clots with higher number of fibrin fibers as well as thinner fibers are

observed after fibrinogen oxidation. However, also our group evaluated fibrinogen structural and functional effects after *in vitro* oxidation using 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) as a free radical generator. A great correspondence with our data obtained *in vivo* was described. Indeed, a significant increase in carbonyl content and dityrosine formation was observed in AAPH treated samples compared to controls. Thrombin-induced fibrin polymerization and plasmin catalyzed fibrinolysis were assessed, showing a reduced clotting ability and a fibrin resistance to lysis in AAPH treated samples [85]. Moreover, fibrinogen structural alterations were discovered: a reduction in α-helical content (as suggested by circular dichroism spectroscopy) and an alteration in tertiary structure (detected by Thioflavin T and ANS assays) in AAPH treated samples were evident. These results were confirmed by FTIR analysis, which showed marked structural modifications in treated samples. Moreover, AFM analysis revealed that AAPH treatment induces changes in fibrinogen morphology and aggregates formation (unpublished data).

Altogether, the data presented in this study provide evidence for fibrinogen oxidative modifications as a new risk factor for thrombotic events in SLE patients. The association of fibrinogen oxidative modifications with its structural and functional alterations and prothrombotic phenotype suggests a potential role of oxidative stress in the pathogenetic mechanisms underlying the increased cardiovascular risk in autoimmunity and particularly in SLE.

Therapeutic approaches based on antioxidant supplementation could be useful to improve SLE patient redox status and parallelly to reduce fibrinogen oxidative–mediated structural and functional alterations ultimately decreasing SLE mortality for CVD.

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