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Study of structural and functional fibrinogen modifications in patients with Eosinophilic granulomatosis with polyangiitis (EGPA)

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Summary

Introduction

Vasculitis: an overview

Vasculitis is a general term to indicate the inflammation of blood vessel walls, whose structure and function are disrupted. Consequently, these alterations can lead to tissue and organ damage. The different forms of vasculitis can be classified according to specific features: etiology, pathogenesis, type of vessel affected, type of inflammation, organ distribution, clinical manifestations, genetic predispositions, and demographic characteristics. Disease categorization based on etiology is often a preferred method but it is unfeasible for most vasculitides because the etiology is still unknown.

Vasculitides can be broadly classified into infectious vasculitis (caused by direct invasion of pathogens in vessel walls) versus non infectious vasculitis.

Chapel Hill Consensus Conference 2012 (CHCC2012) subdivides non infectious vasculitis by integrating knowledge about etiopathogenesis, pathology, demographics, and clinical manifestations. In the first place, the vasculitides are classified based on the predominant type of vessels involved, i.e., large vessel vasculitis, medium vessel vasculitis, and small vessel vasculitis (Figure 1). This classification considered not only the size of vessels, but also their structural and functional features [1,2,3,4].

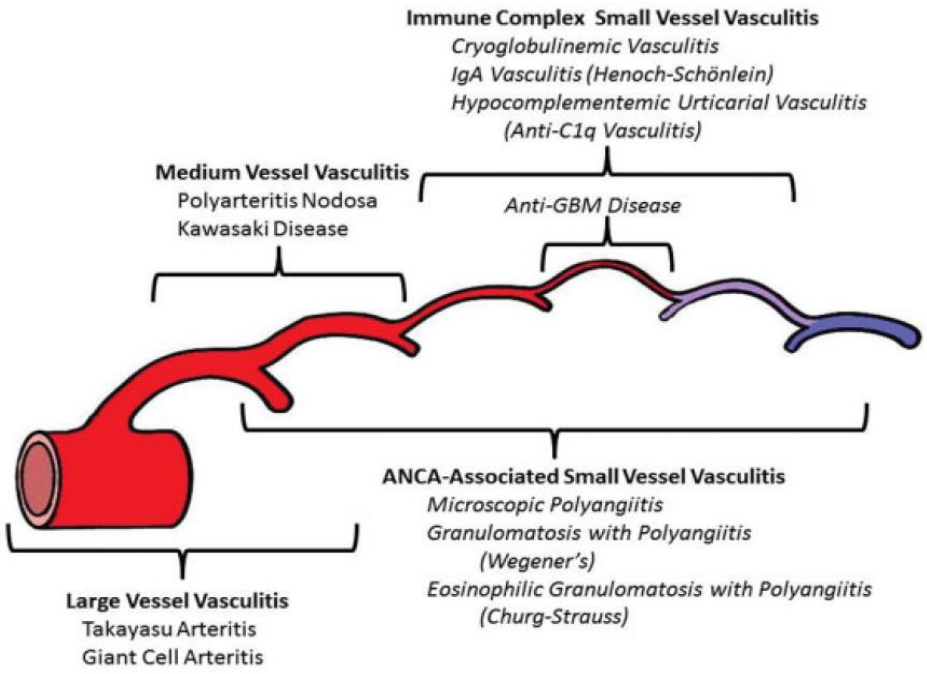

Figure 1: Distribution of vessel involvement by large vessel vasculitis, medium vessel vasculitis, and small vessel vasculitis. The diagram illustrates (from left to right) aorta, large artery, medium artery, small artery/arteriole, capillary, venule, and vein [1].

Large vessel vasculitis (LVV): LVV is vasculitis that affects large arteries more often than do other vasculitides. The two major categories of LVV are Takayasu arteritis (TA) and Temporal arteritis (also named giant cell arteritis - GCA) [1,2].

Medium vessel vasculitis (MVV): MVV is vasculitis predominantly affecting medium arteries and, specifically the main visceral arteries and their branches. Any size artery may be affected. The two main vasculitis of this group are Polyarteritis nodosa (PAN) and Kawasaki disease (KD). The occurrence of inflammation in MVV is more acute and necrotizing than in LVV [1,2].

Small vessel vasculitis (SVV): SVV is vasculitis predominantly affecting small vessels, defined as small intraparenchymal arteries, arterioles, capillaries, and venules. Medium arteries and veins may be affected.

SVV was sub-divided into:

- *ANCA-associated vasculitis (AAV)*: a group of pauci-immune necrotizing vasculitides associated with ANCA specific for myeloperoxidase (MPO-ANCA) or proteinase 3 (PR3-ANCA);
- *Immune complex small vessel vasculitis*: vasculitis with moderate to marked vessel wall deposits of immunoglobulin and/or complement, predominantly affecting small vessels;

The major clinicopathologic variants of AAV are microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA-Wegener's), **eosinophilic granulomatosis with polyangiitis (EGPA or Churg-Strauss syndrome)**, and single-organ AAV (for example, renal-limited AAV) [1,2,3,4]

Variable vessel vasculitis (VVV): VVV is vasculitis with no predominant type of vessel involved that can affect vessels of any size and type. Behçet's disease (BD) and Cogan's syndrome are the 2 examples included in these category. [1,2]

Single-organ vasculitis (SOV): SOV is vasculitis in arteries or veins of any size in a single organ, with no features that indicate that it is a limited expression of a systemic vasculitis. The involved organ and vessel type should be included in the name (e.g., cutaneous small vessel vasculitis, testicular vasculitis, CNS vasculitis). Vasculitis distribution may be unifocal or multifocal within an organ or organ system. [1,2]

Eosinophilic granulomatosis with polyangiitis

Eosinophilic granulomatosis with polyangiitis (EGPA), formerly called Churg–Strauss syndrome (CSS), was first described in 1951 by Jacob Churg and Lotte Strauss. It is a systemic necrotizing vasculitis of small- and medium-size vessels, which distinguishes itself from other small-vessel vasculitides by the presence of severe asthma, and blood and tissue eosinophilia. Both vessel inflammation and eosinophilic proliferation are thought to contribute to organ damage, but the clinical presentations are heterogeneous, and the respective roles of vasculitis and hypereosinophilia in the disease process are not well understood [5-6].

The knowledge of EGPA has recently evolved. Antineutrophil cytoplasmic antibodies (ANCA) have been found among 30-40% of EGPA patients. Therefore, EGPA has been included in the spectrum of AAV together with GPA and MPA [7].
EGPA is commonly divided into two phenotypes depending on ANCA status:

I. ANCA-positive patients: more frequently show vasculitis phenotype, with peripheral neuropathy, purpura and renal involvement;
II. ANCA–negative patients: show lung infiltrates and especially higher risk of cardiac involvement.

Pathogenesis and roles of ANCA in EGPA are still largely unknown, and there is no standard therapy for EGPA based on accumulation of clinical results [5-10].

Classification criteria for EGPA

There are no accepted diagnostic criteria for EGPA but these have been adjusted over the years. In 1984, Lanham et al. proposed that patients with EGPA should have asthma, eosinophilia, and vasculitic involvement of two or more organs (Table 1). In 1990, the American College of Rheumatology (ACR) developed a set of classification criteria for several types of vasculitis.

ACR identified six criteria for EGPA: asthma, eosinophilia >10%, neuropathy, non-fixed lung infiltrates, paranasal sinus abnormalities and extravascular eosinophils on biopsy. When these criteria are met, vasculitis can be classified as EGPA with a sensitivity of 85% and a specificity of 99.7%.
In 1994, the Chapel Hill consensus conference established mutually exclusive clinico-pathological definitions for primary vasculitides. (Table 1) [7].

Lanham et al. (13)
Asthma
Eosinophilia >1.5 × 10^9/l
Clinical or pathological evidence of vasculitis involving at least two organs
ACR 1990* (14)
Asthma
Eosinophilia >10%
Neuropathy (mono- or poly-neuropathy)
Non-fixed pulmonary infiltrates
Paranasal sinus abnormalities
Extravascular eosinophil infiltration on biopsy
Chapel Hill Consensus Conference 1994 (4)
Eosinophil-rich and granulomatous inflammation involving the respiratory tract, necrotizing vasculitis affecting small to medium-sized vessels, and associated with asthma and eosinophilia.
*At least four of the six ACR criteria are required to classify vasculitis as EGPA.

Table 1: Classification criteria and definitions commonly used for EGPA[7]

In 2007, the European Medicine Agency introduced an algorithm for the classification of AAV that allowed the application of ACR criteria in patients with a clinical diagnosis of vasculitis, based on results of clinical, laboratory and imaging investigations and not only on histological findings. Currently, most EGPA cases are diagnosed and classified according to the clinical presentation and the results of non-invasive tests [10].

Epidemiology

EGPA is a rare disease and one of the less common vasculitides. This syndrome may occur at all ages, with a mean age at onset of 38 to 54 years. Its prevalence has been estimated at 2-22 cases per million people, with an annual incidence of 0.5-3.7 cases per million. Childhood-onset EGPA is very rare and characterized by predominant pulmonary and cardiac involvements and high mortality. No gender predominance, familiar clustering and ethnic predisposition has been clearly demonstrated in EGPA [5-10].

Etiopathogenesis

EGPA pathogenesis remains unknown, with many factors contributing to its pathophysiology: genetic predisposition, environmental factors and immune dysregulation seem to be implicated [11-14]

Genetic and environmental factors

Immunogenetic factors may confer susceptibility to EGPA. The HLA-DRB1*04 and *07 alleles and the related HLADRB4 gene are associated with an increased risk of developing

EGPA, whereas the HLA-DRB1*13 and the related HLA-DRB3 gene are protective [13]. The finding of a skewed HLA repertoire, as in other autoimmune conditions, supports the hypothesis of an antigen-driven disease.

Interestingly, the ANCA-negative subset of EGPA was found to be associated with the IL10.2 haplotype of the IL10 gene promoter, which functionally translates into an increased IL-10 expression. IL10 is a Th2 cytokine, thus a genetically determined increase in its production may be relevant for Th2-mediated diseases like CSS [12].

Recently, Lyons et al. performed a first genome-wide association study (GWAS) with 684 EGPA patients16 and found that the risk genes were separated by the ANCA status.

As with MPA and other autoimmune diseases, HLA-DQ was detected as a risk allele in MPO ANCA- positive EGPA.

Variants at GATA3, TSLP, LPP, and BACH2, which may contribute to eosinophilic inflammation, were detected in all EGPA patients.

On the other hand, variants at IRF1/IL5 and GPA33 were associated with MPO-ANCA negative patients. GPA33 gene encodes a cell surface glycoprotein that potentially has a role in maintaining barrier function in the intestinal epithelium and also in bronchial tissue. This fact may give a clue why ANCA status strongly correlates with specific organ manifestations.

EGPA was long suspected to be triggered by exogenous factors including environmental agents (such as dark diesel fumes, grain dust, cereal dust, dust and silica), infections, vaccinations, and drugs.

The role of infections and vaccinations in EGPA is still elusive, and no single infectious agent or type of vaccination proved to have a causal role. Peptides from *Staphylococcus aureus* have strong homology with peptides from complementary PR3, which could underlie the development of PR3-ANCA.

Drugs such as cocaine, levamisole and propylthiouracil are well-known as triggers of AAV. Recent studies have revealed that propylthiouracil induces abnormal conformation of neutrophil extracellular traps (NETs), resulting in the resistance to the degradation by DNase I and subsequent development of MPO-ANCA production and vasculitis [8]. Numerous studies have described the development of EGPA following treatment with different drugs, such as macrolide antibiotics and mainly leukotriene-receptor antagonists (LTRAs) or, more recently, anti-IgE antibodies(e.g., omalizumab), but the reason remains unclear. According to the most reliable hypotheses, both LTRA and anti-IgE antibody may be involved in EGPA pathogenesis simply unmasking the disease, due to the delayed use of steroids [7,8,10,11]

Pathogenesis

Although the events starting the disease process are poorly understood, recent research improved our understanding of the pathophysiology of the eosinophilic and vasculitic responses in EGPA. The principal players are eosinophils, T and B lymphocytes.

The asthmatic and eosinophilic components clearly suggest an activated and skewed T-cell balance. Indeed, T-cell activation and (oligo-)clonal expansion was identified in active EGPA. Specifically, EGPA is classically considered as a disease with a prevalent activation of the Th2-pathway.

Peripheral T-cell lines from EGPA patients can produce Th2-associated cytokines (e.g., IL-4, IL-13 and IL-5), which enhance eosinophil maturation in bone marrow and peripheral activation. Tissue recruitment of Th2 cells is likely to be mediated by specific chemokines such as CCL17, most likely produced by dendritic cells. Interestingly, CCL17 serum levels strongly correlate with peripheral eosinophil counts.

However, the clinical phenotype of EGPA cannot be explained by an exaggerated Th2 response alone. There is evidence of involvement of Th1 and also Th17 cells secreting high amounts of IL-17A in the late EGPA phases. Moreover, reduced levels of regulatory CD4+ T-cells (Tregs) have been discovered in active EGPA and also quiescent phase. Tregs physiologically have a protective role toward the development of autoimmune disorders [7,13].

Eosinophils are multifunctional granular leukocytes that are implicated in the pathogenesis of a wide variety of disorders, including asthma, helminth infection, and rare hypereosinophilic syndromes. Peripheral blood eosinophils are terminally differentiated myeloid cells that develop in the bone marrow under the influence of a number of transcription factors, including GATA-binding factor 1 (GATA-1), and cytokines, including IL-3, IL-5 and granulocyte-macrophage colony- stimulating factor (GM-CSF). Although IL-5 is the key cytokine mediating the release of eosinophils into the bloodstream and recent data suggests that local secretion of IL-5 by type 2 innate lymphoid cells in the tissues might have an important role in tissue accumulation of eosinophils, various chemokines and other soluble mediators have been implicated in directing circulating eosinophils to bind with selectins and integrins and thereby traffic into tissues. In healthy individuals, the majority of eosinophils (>90%) reside in the tissues, where they can be found in the gastrointestinal tract, spleen, lymph nodes, thymus, mammary glands and uterus. In disease states, however, eosinophils can move along chemokine gradients produced on epithelial surfaces or vascular endothelium to sites of inflammation in many different tissues. First described by Ehrlich in 1879, the

eosinophil was so named because of the exuberant staining of its secondary granules with the red dye eosin. These eosinophil-specific granules contain cationic proteins including major basic protein (MBP), eosinophilic cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil derived neurotoxin (EDN), as well as a wide variety of preformed cytokines, growth factors and enzymes, all of which can be selectively released upon cell activation.

Eosinophils are also characterized by primary granules, which contain Charcot–Leyden crystal proteins, and lipid bodies, which are complex inducible organelles that are the site of eicosanoid synthesis and release during eosinophil activation (Figure 2) [12,13,15-17].

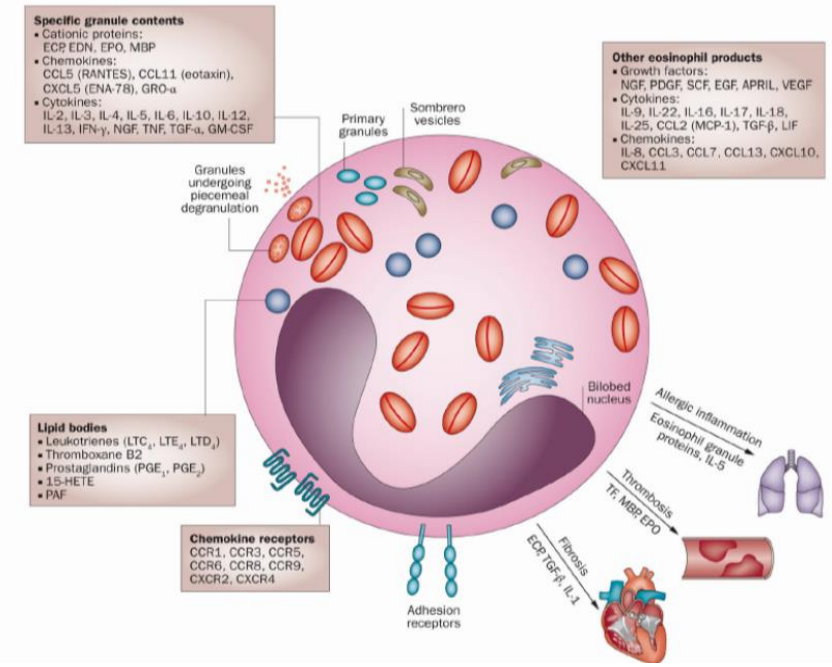

Figure 2: Characteristics of eosinophils [16]

Eosinophils are thought to be key players in EGPA pathogenesis with abnormal eosinophilic proliferation, impaired apoptosis, and elevated tissue toxicity attributed to eosinophil products. Different studies have demonstrated the cytotoxic and pro-coagulant properties of this cell type, which may result in the development of cardiovascular and cerebrovascular complications in patients with any type of hypereosinophilic syndromes including EGPA[12].

Eosinophil proliferation seems to be due to defective CD95 (Apo-1/Fas)-mediated apoptosis. Circulating eosinophils in patients with active EGPA express less primary proapoptotic genes (e.g., CASP2 [caspase-2], CARD4 [caspase recruitment domain family member-4], BLC2L13 [mitochondrially localized protein with conserved B cell lymphoma-2 homology motifs]) involved in NF-κB (nuclear factor-kappa B) regulation.

Eosinophils are abundant both in the periphery and in EGPA lesions. Eotaxin-3, produced by epithelial and endothelial cells, might contribute to tissue influx of eosinophils. Activated tissue eosinophils secrete considerable amounts of eosinophil cytotoxic granule proteins thereby contributing to tissue damage [12,13,15,16].

Although eosinophils are usually considered to be effector cells, they may act as immunoregulatory cells. A cross-talk between T-lymphocytes and eosinophils has been pointed out. Moreover, they can also induce cell apoptosis and present antigens to Th cells, thus sustaining a vicious circle [13-14].

Granuloma formation is mediated by interferon-ϒ and represents an attempt to hold back eosinophil toxic products. Vasculitic lesions frequently show neutrophils. Of note, IL-17, which stimulates neutrophil recruitment and activation, is increased in active EGPA [10].

Aggression of respiratory epithelial cells following exposure to the triggering factors could lead to the production of cytokines, such as IL-25. Elevated concentrations of IL-25 are found in EGPA patient's blood, which may drive CD4+ T-lymphocytes toward a skewed T-helper cell type 2 (Th2) phenotype and then maintains eosinophil proliferation via cytokines synthesized by Th2 cells. EGPA patients' sera, urine, and tissues also contain high levels of eosinophilic cytotoxic proteins that are directly implicated in tissue damage.

Recent evidence points to B cells and the humoral response as further contributors to EGPA pathogenesis. EGPA patients often show an abnormal humoral response. The first evidence came from the observation of ANCA, mainly with a perinuclear pattern (P-ANCA) and directed against MPO, in ~40% of the patients. Their pathogenetic role and their potential harmful effect is still matter of debate.
Emerging clinical and in vivo (animal model) observations provide compelling evidence that ANCAs are primarily and directly involved in the pathogenesis of ANCA-associated systemic vasculitis (AASVs). They are capable of activating neutrophils in numerous ways resulting in the release of reactive oxygen species (ROS), granule proteins, cytokines, chemokines, and adhesion molecules. ANCA-activated leucocytes adhere to the endothelium and cause endothelial damage [6].

Further, strong elevation of IgE levels is common in EGPA, and recently, a dramatic increase in serum IgG4 in active EGPA became evident. Patients with frequently relapsing EGPA had higher percentages of CD27+, CD80+, or CD95+ B cells and lower rates of CD19+ B cells.

During EGPA flares, the IgG4 level, an indirect surrogate sign of B-lymphocyte activation, can be elevated [11]. The switch towards IgG4 production is related to the inflammatory milieu conditioning B-cell maturation, and particularly to the presence of Th2 cytokines such as IL4, IL5 and IL13. However, other cytokines such as the immunoregulatory IL10 and the Th1-linked IL12 contribute to such responses [12].

In a recent analysis of 46 EGPA patients, IgG4 levels correlated with the number of disease manifestations and the Birmingham vasculitis activity score (BVAS). Furthermore, serum IgG4 levels paralleled the disease course as they normalized during remission [13]

Possible mechanisms underlying eosinophilic inflammation and ANCA-mediated vasculitis in EGPA were summarised in figure 3.

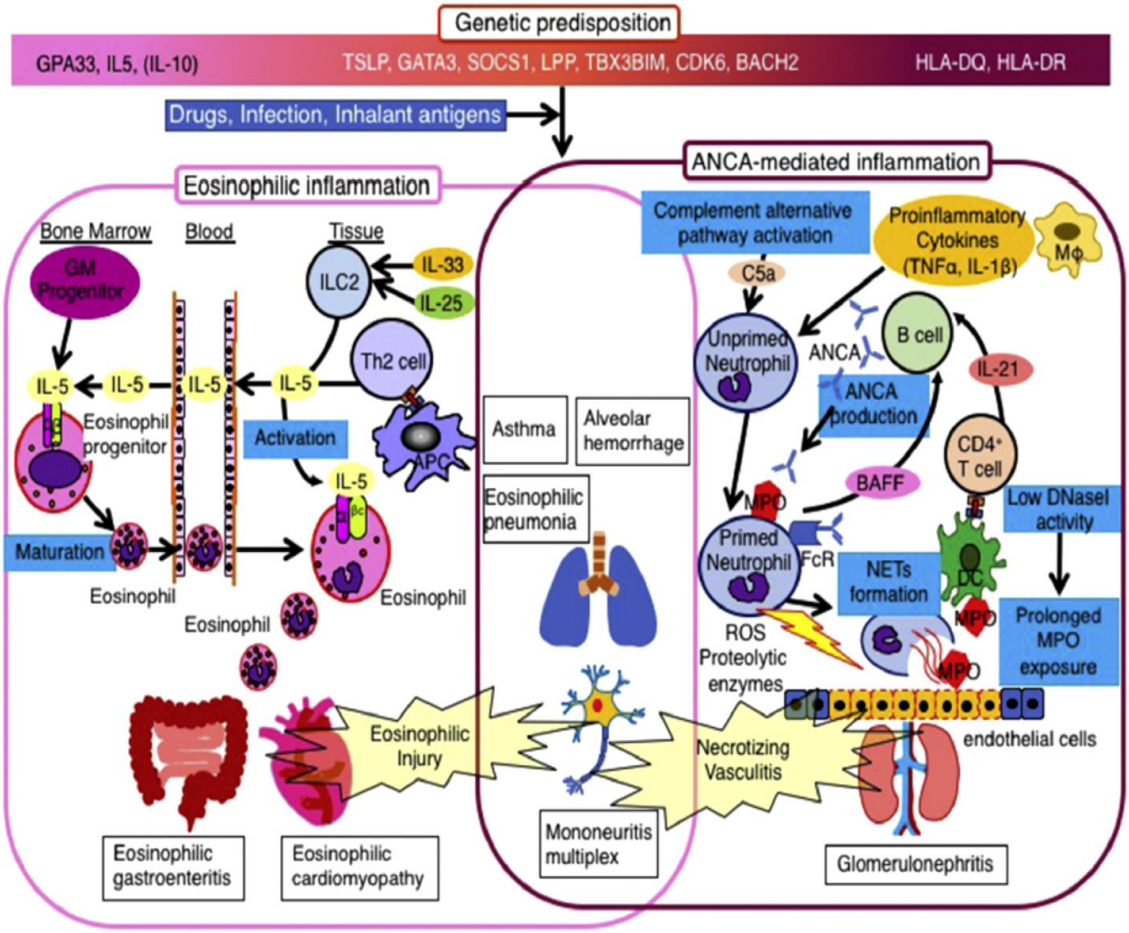

Figure 3: Overview of the genetic predisposition, eosinophilic inflammation, and ANCA-mediated inflammation putatively involved in the development of EGPA [5].

Symptomatology

EGPA is a systemic disorder that impacts on several organs. The typical clinical picture is the presence of asthma in an adult with chronic rhinosinusitis in the presence of more than 10% eosinophilia and pulmonary infiltrates. Three distinct phases are well recognized: allergic or prodromal, eosinophilic, and vasculitic (Figure 4) [12-14]. These phases partially overlap and may not appear in such a defined order, although asthma and rhino-sinusitis only rarely arise after the vasculitic manifestations [7]. Constitutional symptoms are common before systemic manifestations. Notably, 38.9% of our EGPA patient series had fever and myalgias, 29.8% had arthralgias, and 50% had lost weight [12].

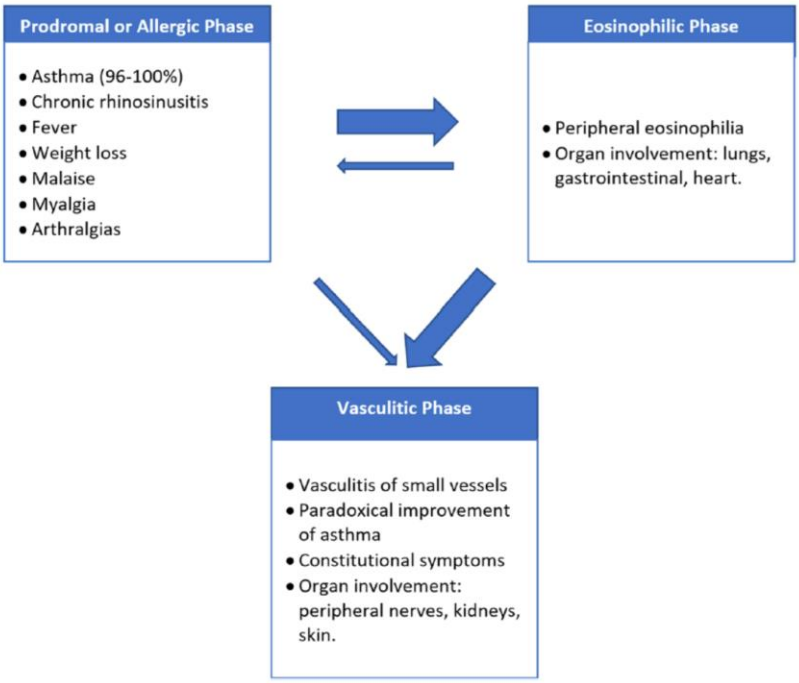

Figure 4: EGPA phases [15].

Pulmonary Manifestations

Asthma is the major EGPA characteristic, affecting 96 to 100% of the patients during prodromal phase. Indeed, asthma is one of the ACR classification criteria. Asthma often begins at approximately 30 to 40 years of age but can start during childhood. Asthma is generally severe and often glucocorticoid dependent. In a retrospective study on 157 EGPA patients, asthma was mild, moderate, or severe in 17, 26, or 57%, respectively. Asthma severity tended to increase 3 to 6 months before systemic disease became apparent. Compared with MPA, alveolar haemorrhage, resulting from pulmonary vasculitis, is rarer (4%) but can sometimes be severe with massive haemoptysis (often associated with renal involvement). However, it is usually mild in most patients, with only bloody sputum or diagnosed by BAL.

Eosinophilic exudative pleural effusions have been described, with pleural biopsy, rarely obtained, showing signs of vasculitis with eosinophilic infiltrates. Lung infiltrates are common (38.6%), and can be uni- or bilateral, transitory, and often disappear after a few days of glucocorticoids. Phrenic paralysis has been described but is extremely rare [11].

Ear, Nose, and Throat Manifestations

Maxillary sinusitis is common and is one of the ACR classification criteria defining EGPA. Nasal polyposis, allergic rhinitis and/or chronic sinusitis also hallmark the prodromic EGPA phases. Nasal polyposis are found in 50% of EGPA patients and systematically recur after surgery in patients not receiving immunosuppressive therapy. Although they are considered EGPA symptoms, we think they represent the underlying predisposition to the disease, rendering patients more susceptible to developing vasculitis symptoms. Once a vasculitis flare has been successfully treated, most patients have polyps and symptoms at some time during the disease, which supports the hypothesis described earlier [11]. Secretory otitis media, chronic ear drainage, sensorineural hearing loss, and facial nerve paralysis may also occur, especially in children [7,14].

Neurological Manifestations

Peripheral neuropathies, mainly mononeuritis multiplex, are frequent, affecting 46 to 75% of EGPA patients, especially during the vasculitis phase. The most frequently involved nerves, in decreasing order of frequency, are the common peroneal, internal popliteal, then those of the arm (radial, cubital, and/or median), and, lastly, cranial nerves. The most common pattern is mononeuritis multiplex, often complicated by asymmetric foot or wrist drop, but it may also evolve as a symmetric or asymmetric polyneuropathy [7,14].

Paraesthesia or sometimes painful hyperaesthesia may occur before the onset of motor or sensory deficiencies. When moderate, only superficial sensitivity is affected. Peripheral neuropathy is not a Five-Factor Score (FFS)- defined poor-prognosis factor associated with mortality, but sequelae may worsen the functional prognosis. Indeed, recovery is long and unpredictable. Regression of motor symptoms with recovery of function is more frequent, but sensory symptoms can persist definitively, and functional recovery is usually partial and unpredictable. Pain, paraesthesia, and hypoesthesia can become major difficulties for patients, thereby necessitating therapeutic escalation.

Central nervous system (CNS) involvement is uncommon and reflects cerebral vasculitis. Clinical manifestations are non-specific: seizures, hemiplegia, coma, brain haemorrhage [7,11].

Skin Manifestations

Cutaneous involvement, frequency ranges from 40 to 70% of EGPA patients, are also a prominent feature of the vasculitis phase. Particularly purpura occurs in 25% cases and usually involves the lower limbs. Subcutaneous nodules, which are often symmetrical, red, and predominantly affecting fingers, elbows, and upper limbs, occur in 10 to 30% of EGPA patients. Skin nodules can also be seen on the forehead. EGPA patients can also have Raynaud's phenomenon, livedo reticularis, and urticarial or gangrenous necrotic lesions [7,11].

Gastrointestinal Manifestations

Gastro-intestinal involvement is also often due to eosinophilic infiltration of the gastrointestinal mucosa and more frequently affects the small bowel. Gastrointestinal bleeding with a high possibility of perforation, and small bowel involvement may occur. Rare cases of cholecystitis have been reported [6,7,10,11,14].

Cardiac Manifestations

Symptomatic cardiac involvement occurs in as many as 27–47% of EGPA cases and represents the major cause of early death and poor long-term prognosis. A significant proportion of patients with cardiac involvement is asymptomatic. In the absence of symptoms and major ECG abnormalities, cardiac involvement may be detected in nearly 40% of the patients. Patients are more frequently ANCA, have mild to severe left ventricular dysfunction and intraventricular thrombi due to eosinophil-related endocardial damage.

Cardiac specific involvement (CSI) may occur at several levels in the heart, including the myocardium, epicardium, endocardium, conductive tissue, coronary arteries, valves and pericardium, and may have important prognostic implications in patients with EGPA. CSI associated with high peak of eosinophilic count. Endomyocarditis, was associated with impaired cardiac function and occasionally intra-cardiac thrombus formation Additionally, patients with EGPA are at increased risk of venous thrombo-embolic events, such as deep venous thrombosis and/or pulmonary embolism.

Thrombosis is a manifestation of a variety of eosinophilic disorders, and its pathophysiology appears secondary to initiation of the coagulation cascade due to tissue factors released during degranulation, endothelial dysfunction, inhibition of the endothelial vascular thrombomodulin by MBP, and platelet activation by MBP and EPO [6,7,21-26].

Myocardial injury in EGPA is mainly caused by eosinophilic cytotoxicity, as the histologic picture seen in this disease is similar to the one observed in other HES. Microscopic examination of the cardiac tissue reveals endomyocardial fibrosis, myocardial lymphocytic infiltration, and marked eosinophilia. Immunohistochemistry staining shows the presence of eosinophil-derived cationic proteins within the inflammatory lesions [21-26].

Renal Manifestations

Kidneys are affected in 25% of cases, but it is clinically less severe than in other types of AAV. It may occur as isolated abnormalities (e.g. isolated mild proteinuria and microhaematuria) in the urinary sediment to rapid progressive glomerulonephritis, and more rarely some patients may exhibit chronic kidney disease at presentation. The most typical picture is pauci-immune focal and segmental necrotizing glomerulonephritis, with or without crescents, which usually involve less than 50% of the glomeruli. Tubulo-interstitial nephritis with eosinophilic predominance is found occasionally. A few patients have mesangial glomerulonephritis or focal segmental sclerosis. Ureteral stenosis has been described rarely In EGPA patients, glomerulonephritis is usually associated with anti-MPO ANCA positivity [6,7,10,11,14].

Ophthalmological Manifestations

Ophthalmological ischemic vasculitis, uveitis, episcleritis, and/or orbital inflammatory pseudotumor with conjunctival involvement are rare signs that have been reported [11].

Histopathology

EGPA was originally described as a pathological triad consisting of eosinophilic infiltration, necrotizing vasculitis and extravascular granuloma formation. These characteristics were mostly observed on autopsy. The early phase of the syndrome is characterized by extra vascular tissue infiltration by eosinophils of virtually any organ.

Once the disease progresses to the “vasculitis” phase, pathologic signs of inflammation are observed in small to medium-sized vessel walls. Vasculitis presents fibrinoid necrosis in the wall of the vessels and rupture of internal elastic lamina. Granulomas often involve the

arteries, but the more EGPA-specific lesion is the extravascular granuloma, which consists of a core of necrotic eosinophilic material surrounded by palisading lymphocytes and epithelioid and multinucleated giant cells [7,8].

Typical pathohistological presentations of EGPA are different among the affected organs. In purpura of the skin, a wide range of conditions between eosinophilic vasculitis and leukocytoclastic vasculitis without eosinophilic infiltration is observed. Necrotising vasculitis and eosinophilic granulomas are seen in pneumonitis and lung nodules. Cardiac involvement shows mixture of eosinophilic infiltration in myocardium and endocardium causing myocarditis and endocarditis, and small vessel vasculitis. Eosinophilic infiltration is rarely observed in peripheral nerve and kidney involvements. Renal histology is characterized by pauciimmune necrotizing crescentic glomerulonephritis. Eosinophilic infiltration is sometimes observed in the interstitium of the kidney. Interestingly, peripheral neuropathy and glomerulonephritis are the organ involvements associated with ANCA-positive patients [7,8].

Key histopathological features of EGPA are shown in figure. Arrows, in the figure 5A, indicate the presence of many eosinophils in inflammatory infiltrate. In this tissue biopsy, admixed lymphocytes is seen in the media of this small submucosal artery. In addition to great eosinophilic vasculitis, the arrows, in the figure 5B, show this vessel exhibits striking fibrinoid necrosis of its inner wall [6].

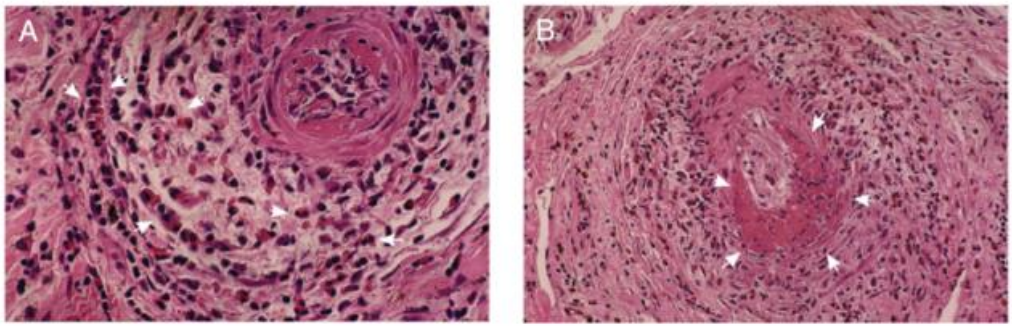

Figure 5: Tissue biopsy specimens from Churg–Strauss patients [6].

Diagnosis and laboratory findings

A gold standard test to establish a diagnosis of EGPA is not available. Diagnosis is based on the clinical characteristics found and the application of the criteria of CHCC2012. The presence of peripheral eosinophilia, at least 10% or more than 1500 cells/μL, elevated ESR and CRP, are of relevance to establish a diagnosis of EGPA. Degree of eosinophilia, as well as CRP, correlates well with disease activity.

ANCA, predominantly P-ANCA of anti-MPO specificity, are present in close to 40% of EGPA patients but some can be antiproteinase 3 (PR3). ANCA titers do not correlate with disease evolution characteristics. On the other hand, ANCA positivity or negativity does not influence a final diagnosis.

It is essential, however, to confirm diagnosis by biopsy findings. Imaging studies, from conventional radiographs to CT scan and cardiac MRI of different involved organs are very helpful to establish degree and severity of involvement in affected organs. Pulmonary function tests, bronchoscopy, and echocardiographic studies will also provide useful information [8,11,14,19].

Although there are no validated diagnostic tests for EGPA, new biomarkers are emerging. A recent study demonstrated a good diagnostic performance of eotaxin-3, an eosinophil chemoattracant. Its serum levels were significantly higher in active EGPA than in a control groups including patients with different eosinophilic and immunemediated disorders. Importantly, all patients with HES investigated in this study had active disease at the time of blood sampling. At a cut-off level of 80 pg/ml, the sensitivity and specificity of eotaxin-3 for the diagnosis of active EGPA were, respectively, 87.5% and 98.6%. This biomarker needs diagnostic validation but is likely to enter routine clinical practice [6,7,10].

Differential diagnosis

The differential diagnosis of EGPA essentially includes eosinophilic and vasculitic disorders. Idiopathic hyper-eosinophilic syndrome (HES) is defined as a sustained peripheral blood eosinophilia of unknown origin where eosinophils exceed 1500 cells/mm^3 for more than six consecutive months and are responsible for the development of organ dysfunction and/or damage. Although the organs involved in the two syndromes are similar, HES is characterized by the histological evidence of tissue infiltration by eosinophils, the absence of asthma and vasculitis on biopsy specimens, and ANCA-negative serum [6].

The differentiation between ANCA-negative EGPA and HES is particularly difficult. In a recent study comparing patients with ANCA-negative EGPA and FIP1L1-PDGFRA-negative HES, none of the tested serum biomarkers (such as IL2R, IL-5, IL- 6, IL-8, IL-10, CCL17, eotaxin-1) could differentiate the two patient groups. As cited above, we found that eotaxin-3 might indeed differentiate active EGPA from various forms of HES as well as other allergic or immune-mediated diseases associated with eosinophilia.

Before vasculitis onset, concomitant eosinophilia, asthma and lung infiltrates may resemble certain parasitic infections, like helminthiases, or allergic bronchopulmonary aspergillosis (ABPA). ABPA may mimic a respiratory tract-limited EGPA but the isolation of *Aspergillus*

spp on BAL or sputum and high serum levels of *Aspergillus fumigatus*-specific IgE are diagnostic of ABPA. Acute eosinophilic pneumonia is also hallmarked by pulmonary infiltrates and a eosinophil-rich BAL fluid, but usually presents as an acute febrile illness with respiratory failure, lacks peripheral eosinophilia and other organ manifestations [7].

Once vasculitis becomes predominant in the clinical picture, the main differential diagnoses of EGPA are other systemic vasculitides, especially GPA and PAN. In general, the distinction between EGPA and the other primary systemic vasculitis can be made on the basis of the distinct association of EGPA with asthma and marked blood hypereosinophilia.

GPA may indeed overlap with EGPA, particularly in cases with eosinophilia: differential features include ANCA specificity (C-ANCA/ proteinase-3 ANCA being more frequent in GPA) and the presence, in GPA, of lung cavitated nodules, nasal crusts and nasal and paranasal sinus bone erosions. MPA, although usually associated with P-ANCA/MPO-ANCA as is EGPA, rarely shows pronounced eosinophilia and upper airway tract involvement, whereas its renal complications are often more severe than those of EGPA [7,8].

Prognosis

EGPA prognosis is generally good, even though historically, before the advent of therapy, it was almost always fatal. Following timely detection and treatment, CSS has a favourable prognosis with a 5-year survival of 90%. The relapse rate is estimated at approximately 20% to 30% and it is often minor with fever, joint pain, and constitutional symptoms.

Certain risk factors for relapse include:

1. The sudden rise in eosinophil count;
2. Persistent ANCA positivity;
3. Gastrointestinal tract (GI) involvement;
4. The rise in ANCA titers;

Just as peripheral eosinophilia is a hallmark of diagnosis, there is an association between the degree of eosinophilia and the extent of vasculitis disease. A sudden rise in eosinophil count also precedes a relapse of vasculitis [13-15].

The French Vasculitis Study Group has identified five prognostic factors, together called the five-factor score (FFS), in patients with necrotizing vasculitis:

1. Elevated serum creatinine levels (>1.58 mg/dl);

2. Proteinuria (>1 g per day);
3. GI involvement;
4. Cardiomyopathy;
5. Central nervous system involvement.

The absence of any of the five factors carries a good prognosis and the presence of 2 or more increases the risk of mortality. Cardiac involvement is the most frequent cause of mortality in patients with poor response to therapy [21-26].

Treatment

There is no consensus regarding the use of a staged, remission-induction and remission-maintenance approach in EGPA. A practical way of making a decision regarding the therapy to follow is to stratify the patient according to their prognostic factors at the time of their initial diagnosis [14]. Patients with an FFS ≥ 1 have a worse prognosis and are usually treated with glucocorticoids and immunosuppressants, whereas glucocorticoid treatment alone is recommended in those with FFS = 0 [6,10,12].

The important role of eosinophils in EGPA and recent development of effective agents to treat other eosinophil-related diseases (e.g., asthma, hypereosinophilic syndrome) have created new therapeutic possibilities for EGPA [9].

Glucocorticoids (CS) remain the cornerstone of EGPA treatment, and can be used as monotherapy or in combination with other immunosuppressive agents depending on the severity of organ involvement. Their use to treat vasculitides since the 1950s has been associated with notably improved remission rates and sharply reduced disease-related mortality [27,28].

CS have multiple targets in the immune and hematopoietic systems, notably eosinophils. It is well-known that these drugs can normalize the eosinophilia within a few days. The mechanisms underlying CS efficacy comprise pleiotropic effects at genomic (gene expression suppression/ activation) and non-genomic levels. CS can induce T-cell suppression, with subsequently fewer inflammatory vasculitis/granulomatous processes, and ‘‘shutdown’’ the Th2-cytokines (IL-4, IL-13, IL-5) implicated in eosinophil migration and tissue recruitment. CS also target eosinophils directly, through an apoptotic process resulting in eosinopenia. In addition, CS can control histamine release by basophils and mast cells implicated in asthmatic and allergic ENT manifestations. Overall, these properties explain the rapid and remarkable CS efficacy against EGPA asthma and systemic manifestations.

The recommended CS daily dosage at induction is 1 mg/kg of prednisone-equivalent to obtain remission of organ/life-threatening disease. This dosage is followed by gradual reduction until withdrawal or ≤ 7.5 mg/d to limit adverse effects. Instead, during severe EGPA flares, pulse methylprednisolone (7.5–15 mg/kg/day) can be infused over 60 min and repeated at 24-h intervals for 1–3 days.

Frequent CS adverse events (AEs) include hypokalemia, high blood pressure and insomnia. However, severe AEs, albeit rare, are sudden death, cardiac arrhythmia, myocardial infarction, gastrointestinal bleeding, seizures and manic episode [9].

Immunosuppressive agents

ISs are commonly prescribed in combination with CS to treat severe EGPA. The drugs and/or doses differ depending on the treatment phase, i.e., induction or maintenance regimen. Cyclophosphamide (CYC), the most frequently prescribed immunosuppressant to control severe vasculitis, is an alkylating agent whose metabolites disrupt DNA function, causing cell death. In inflammatory conditions, like active autoimmune disease, it mainly blocks B- and T-cell replications and probably that of eosinophils through indirect effects on bone-marrow precursors.

Courses of 6–12 intravenous pulses (15 mg/kg) led to remission in ≥ 85% cases, but the risk of relapse is lower with prolonged treatment. Because of CYC relevant toxicity, patients ≥ 65 years may benefit from 'lighter' regimens with reduced CYC dose (500 mg-fixed dose at each infusion) and rapid steroid tapering. Young and adult males receiving CYC can undergo semen cryopreservation, while women of child-bearing potential must be treated with GnRH analogues [10].
It is thus a non-specific IS, with several AEs (haemorrhagic cystitis, bone-marrow suppression, ovarian failure and increased risk of cancer) [9].

Biological agents

The therapeutic array for EGPA has been expanded with the introduction of monoclonal antibodies used for diseases with similar pathogenesis. However, no single agent can allow a complete control of the disease, but the choice should be made according to patient characteristics. Biological agents can be used in patients' refractory to conventional therapy, or with severe organ involvement [10]. Figure 6 summarizes the new biological therapies for EGPA patients.

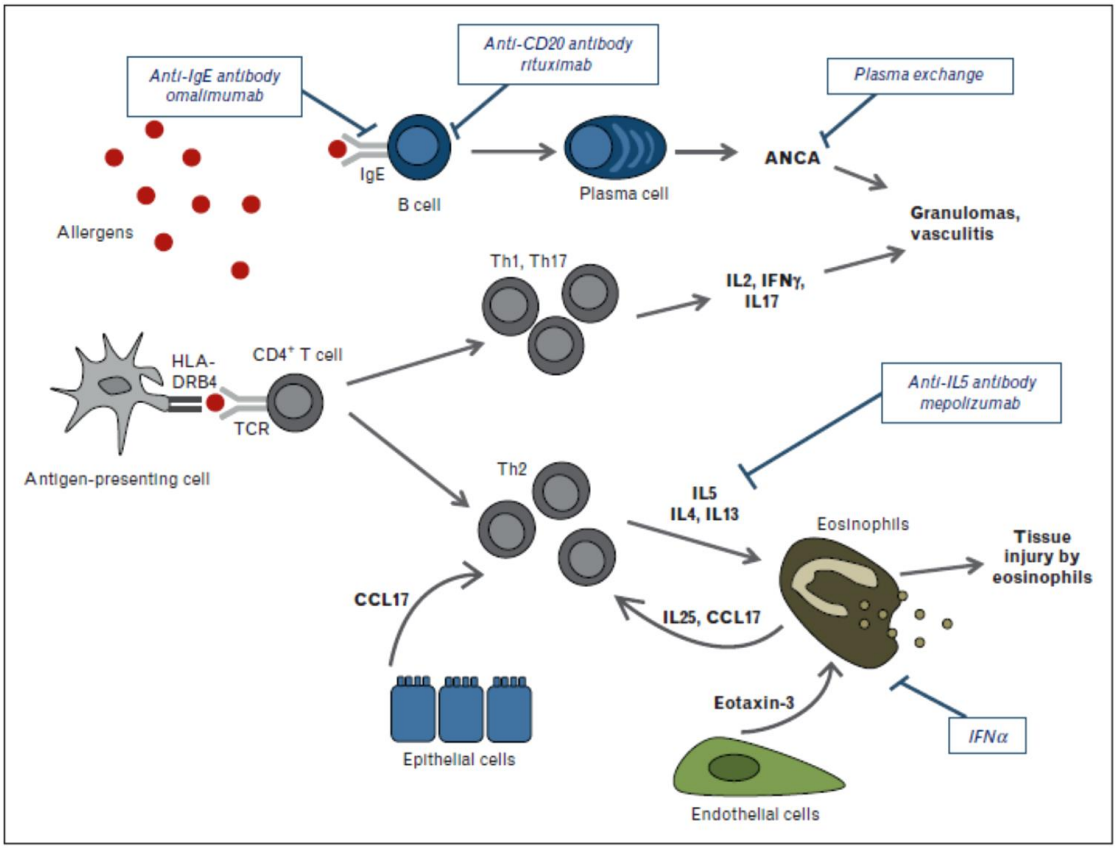

Figure 6: The main therapies for EGPA patients [12].

Rituximab

This biotherapy has become one of the first-line treatment options for AAV-remission–induction and–maintenance regimens. Rituximab is an anti-CD20 chimeric mouse-human monoclonal IgG antibody. It induces B-cell depletion for a mean of 6–9 months with a high variability for normal B-cell count recovery, usually between 12 and 24 months. It was initially developed to treat B-cell lymphomas but also proved useful against several autoimmune diseases, even though the exact mechanism(s) underlying its efficacy remain unclear. Currently it is licensed in Europe for two autoimmune diseases: rheumatoid arthritis, and severe AAVs in adults (e.g. GPA and MPA). While ongoing randomized controlled trials (RCTs) are evaluating rituximab for EGPA patients the information now available relies on case reports and open-label studies. The REOVAS study in progress is a phase 3, and it represents the first large randomized study that evaluates the use of rituximab in patients with EGPA. Rituximab also leads to a reduction in prednisolone requirement, but asthma and upper airway involvement relapse rates are high despite continued treatment. In addition, the ANCA-positive subgroup exhibits a more sustained response [14]. Despite their low level of evidence in this setting, rituximab globally provided potential benefit against asthma and/or ENT symptoms and EGPA-vasculitis–induced organ impairment [9,20].

Mepolizumab (Anti-IL-5)

IL-5 is the major cytokine responsible for eosinophil activation, chemoattraction and survival. The findings of several reports indicated elevated IL-5 levels in EGPA patients, positioning this cytokine as a potential treatment. Mepolizumab is the first anti-IL-5 developed and approved to treat severe eosinophilic asthma. Mepolizumab, a humanized monoclonal IgG1 antibody, binds free IL-5 and thereby prevents its binding to the a-subunit of the IL-5 receptor that is predominantly expressed on human eosinophils. It thereby inhibits IL-5 signalling in eosinophils, and blocks their activation and tissue accumulation [9]. Thus, the efficacy of Mepolizumab, was evaluated in patients with EGPA. Results of the double-blind, randomised placebo controlled trial (MIRRA) were reported in 2017. In the MIRRA trial, relapsing or refractory EGPA patients receiving 7.5 mg/day or more of prednisolone were randomly assigned to receive Mepolizumab or placebo in addition to standard care. In this trial, Mepolizumab led to significantly more accrued weeks of remission than placebo and a higher percentage of participants in remission at both week 36 and week 48. The efficacy of Mepolizumab with minimal adverse effects was clearly shown, and Mepolizumab was approved for EGPA [8,14].

Omalizumab (Anti IgE)

Omalizumab, a monoclonal IgG antibody that recognizes free circulating IgE, prevents its binding to its specific high-affinity receptor FceRI that is constitutively expressed on basophils and mastocytes, which ultimately blocks the allergic cascade, notably cell degranulation (histamine); it also causes transient lowering of eosinophilia. This biotherapy was first approved to treat severe asthma with elevated plasma IgE levels, and then for chronic spontaneous urticaria. Its potential against chronic rhinosinusitis with nasal polyposis is also being examined. Omalizumab is injected subcutaneously, once or twice a month, at a dose dependent on the patient's circulating IgE levels and weight.

So far, Omalizumab use has been reported almost exclusively as an add-on therapy for severe CS-resistant EGPA-associated asthma, in several case reports. Its efficacy in that context was apparently satisfactory but two patients suffered severe asthma exacerbations. Notably, information on its potential efficacy against EGPA systemic complications is lacking because few treated patients corresponded to this scenario. Overall, these observations support a potential Omalizumab benefit for EGPA patients with severe asthma and/or allergic ENT manifestations [9].

Interferon-α (IFN-α)

IFN-α, a known immunomodulatory, also has cytoreductive properties during myeloproliferative neoplasms. Both of those mechanisms could explain the observed reduction of eosinophil numbers in patients with eosinophilic disorders or EGPA. IFN-α treatment of EGPA was reported in several case reports and case series but its efficacy remains difficult to assess without a control group for comparison [9,20].

Other treatments

Other types of treatment such as mycophenolate mofetil, intravenous immunoglobulins, TNFα-blockers and other immunosuppressants, may be considered in specific populations. These agents have been reported to be beneficial in CSS patients, but no clear evidence yet supports their use.

Finally, plasma exchange is also one of the most widely used treatments for AAV, particularly in cases with lifethreatening manifestations such as alveolar haemorrhage or rapidly progressive glomerulonephritis [9,10,12].

Redox homeostasis

Oxygen, is a molecule often referred to as *Janus* gas [32], has played a pivotal role in life mechanisms since the advent in the atmosphere. However, oxygen has both positive effects and potentially damaging side-effects on biological systems. Oxygen participates in high-energy electron transfers, and hence supports the formation of large amounts of adenosine-5-triphosphate (ATP) through oxidative phosphorylation [29-32].

Redox homeostasis is central to life. Oxidation–reduction (redox) processes involve practically all fundamental processes, from bioenergetics to metabolism and life functions. In various compartments within and outside the cells, a given physiological or pathophysiological situation is characterized by hugely different set points of redox systems operating concurrently. More pronounced variations, such as an excessive production of oxidants, may ultimately cause damage to biomolecules and can modulate, and even disrupt, physiological redox signaling [29-32]. In order to deal with oxidative attacks, human body has evolved a complex system of antioxidant defence that generally hold in balance the oxidants species production. This condition is called ***oxidative eustress*** and is essential for governing life processes through redox signaling. On occasions, however, this balance can be perturbed, leading to a condition known as ***oxidative stress*** [29-32,34-37].

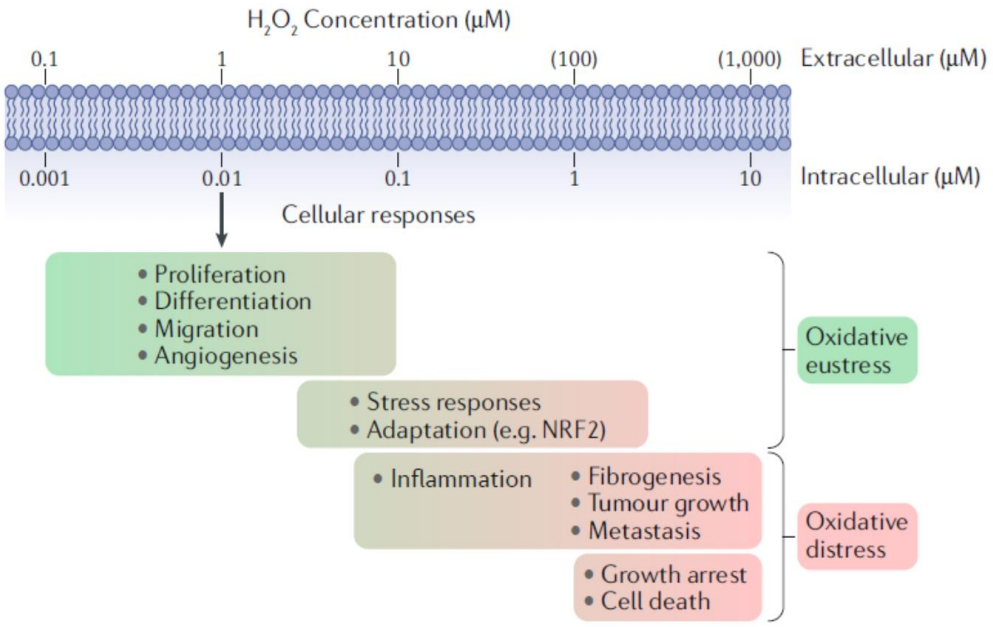

Figure 7: Oxidative eustress and oxidative distress [29].

Oxidative stress

The early use of the term seems to begin with rubber chemistry in 1956. In 1970, "cells were subjected to oxidative stress" was used to describe the addition of H_2O_2 to erythrocytes. Since 1985, the term denoted oxidative damage to cells and organs. However, the concept of oxidative stress was first formulated in the introductory chapter of the 1985 book entitled Oxidative Stress as "a disturbance in the prooxidant-antioxidant balance in favor of the former". This process is initially responsible for an adaptive response consisting of the induction of an antioxidant response, and following antioxidant depletion, cellular injury and dysfunction [29-32].

ROS are a double-edged sword: at physiological levels they serve as key signal molecules in biological processes but at higher levels, they have a role in most pathological processes. In fact, ROS have been implicated in several disorders including inflammation, atherosclerosis and cardiovascular disease, infertility, autoimmune disorders, metabolic disease, respiratory disease, neurological disease and cancer [29-38].

In the recent years, numerous biomarkers of oxidative stress have been employed, and the clinical relevance of such biomarkers has been examined in several disease, as show in figure 8 [29,31].

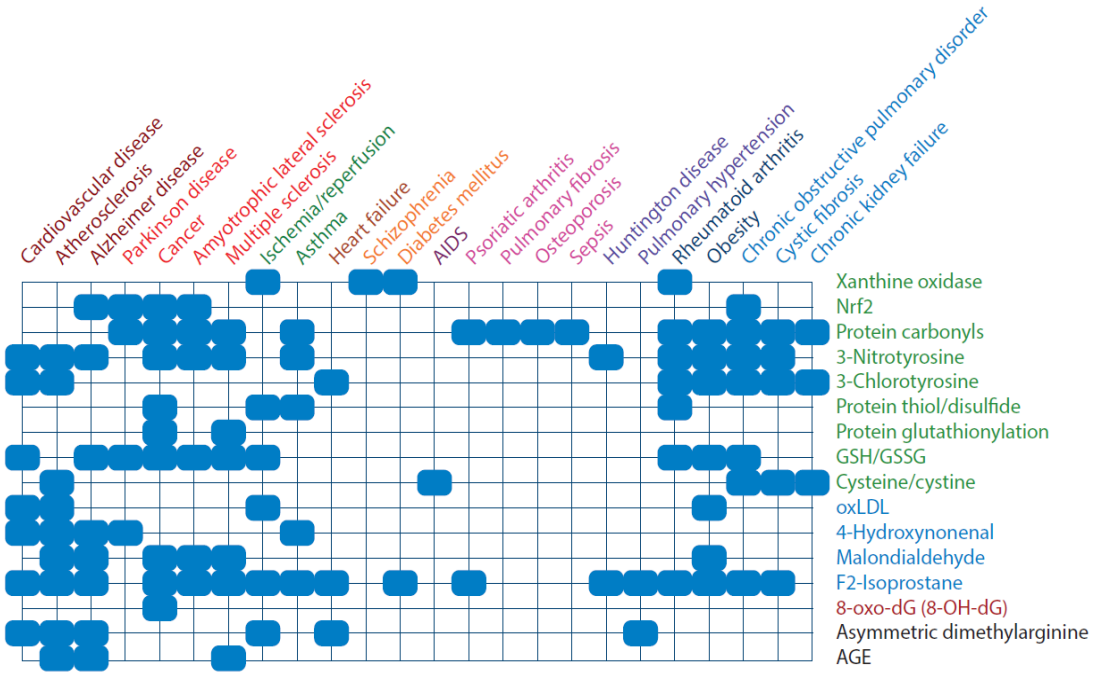

Figure 8: Clinical relevance of biomarkers of oxidative stress. Protein (*green*), lipid (*blue*), and DNA (*red*) biomarkers were analyzed in various diseases [31].

Free radicals

Free radicals are defined as unstable, highly reactive and with a very short half-life species. This reactivity arises from the presence of one or more unpaired electrons in the outer shell of an atom. They become stable by acquiring electrons from nucleic acids, lipids, proteins, carbohydrates or any nearby molecule causing a cascade of chain reactions resulting in cellular damage. The several types of "reactive species" are named according to the nature of the reactive atom (i.e., oxygen, sulphur, carbon, selenium) where oxygen and nitrogen are the most important, producing reactive oxygen species (ROS) and reactive nitrogen species (RNS) respectively [30,32].

Oxidants are generated by various sources. To maintain their levels at physiological concentrations, various mechanisms to control their production and availability, including localized and compartmentalized generation as well as engagement of detoxifying factors and redox relays, are in place. The reactive species are a result of both endogenous and exogenous pathways. Endogenous production of free radicals take place during the mitochondrial electron transport chain (ETC) or it can be mediated by enzymatic production such as xanthine oxidase (XO), nitric oxide synthases (NOS), NADPH oxidase (NOX) cytochrome p450, cyclooxgenase, lipoxygenase and myeloperoxidase, instead exogenous production requires the exposure to damaging agents (i.e., environmental pollutants, radiations, tobacco, smog and drugs) [39].

The formation of reactive species may involve three main mechanisms:

- homolytic cleavage of covalent bonds;
- donation of one electron from a donor molecule to an acceptor molecule;
- redox reactions which involve transition metals (Fe and Cu), such as via Fenton reaction;

Reactive oxygen species (ROS)

ROS are generated during crucial processes of oxygen (O_2) consumption. They can be divided into non- radical and free radical (with at least one unpaired electron) species. The chemical reactivity of the various ROS molecules is vastly different, spanning up to 11 orders of magnitude in their respective second-order rate constants with specific targets. The three main types of ROS are: superoxide anion (O_2^{-}•), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•) [29-38].

In physiological conditions, the most common oxygen free radical is the **superoxide anion** ($O_2^{-}\bullet$), which is mainly generated from electron leakages at the level of the mitochondria during oxidative phosphorylation. Mitochondria produce superoxide and hydrogen peroxide in two major categories: mitochondrial inner membrane complexes I, II, and III and the mitochondrial matrix and/or inner membrane–bound dehydrogenases. About twelve mitochondrial sources of $O_2^{-}\bullet/H_2O_2$ have been identified, as depicted in figure 9 [30-32,35-37,44].

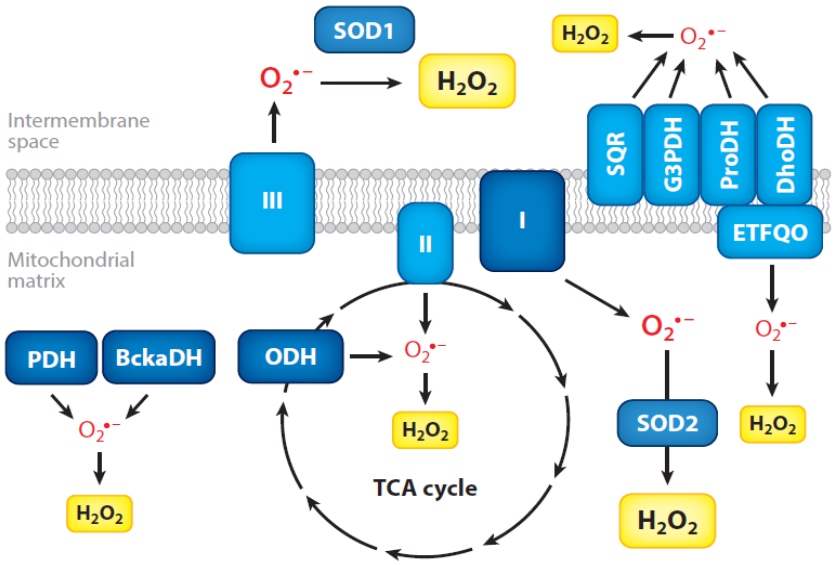

Figure 9: Mitochondrial $O_2\bullet$- and H_2O_2 sources [30].

Under normal conditions, 2% of oxygen consumed is converted to $O_2^{-}\bullet$ while the rate of formation is increased in peculiar states, such as hyperoxia and a rising of glucose levels. Paradoxically, the formation of superoxide anion is increased in conditions of hypoxia, when the reduced availability of oxygen to act as the final electron acceptor for complex IV causes electrons to accumulate.

Similarly, superoxide can also be generated through leakage of electrons from the shorter electron transport chain within the ER. The formation of disulphide bonds during protein folding is an oxidative process, and about 25% of $O_2^{-}\bullet$ within cells is generated within the ER. This can increase in cells with a high secretory output, and also under conditions of ER stress when repeated attempts to refold misfolded proteins may take place [31,34].

Because of its charge, $O_2^{-}\bullet$ is membrane impermeable, and so remains within the mitochondrial matrix where it can be converted to hydrogen peroxide (H_2O_2) by specific enzymes, such as SOD, otherwise it undergoes spontaneous dismutation.

SOD: $O_2^{-}\bullet + O_2^{-}\bullet + 2H^{+} \rightarrow H_2O_2 + O_2$

Other sources of superoxide under physiological conditions include the enzymes nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, cytochrome P450, and other oxidoreductases[30-37].

Hydrogen peroxide (H_2O_2), is not a free radical, consequently it is less reactive than $O_2^{-}\bullet$. Hydrogen peroxide is produced from O_2 mainly by NADPH oxidase in conjunction with SOD, by the mitochondrial electron transport chain and by other enzymes. As it is non-polar, it is able to diffuse through cells and organelle membranes through specific aquaporins (referred to as peroxiporins) and hence acts widely as a second messenger in signal transduction pathways. Hydrogen peroxide can be considered as the major redox metabolite with a role in redox sensing, signaling and redox regulation. As a messenger molecule, H_2O_2 diffuses through cells and tissues to initiate immediate cellular effects, such as cell shape changes, initiation of proliferation and recruitment of immune cells. It became clear that H_2O_2 has a fundamental role in regulation of metabolism in addition to its role as damage signal [29-34].

Hydrogen peroxide is in turn detoxified to water by the enzymes catalase and glutathione peroxidase[5]. It is important that the antioxidant enzymes act in concert, because an imbalance in the concentrations of hydrogen peroxide (H_2O_2) and $O_2^{-}\bullet$ can result in the formation of the much more dangerous hydroxyl radical (OH•) by via Fenton and Haber-Weiss reactions.

Hydroxyl radical (OH•) is the most harmful ROS and has an estimated life of 10^{-9}s. It is mainly generated via Haber-Weiss and Fenton reactions. The Haber-Weiss reaction, given below, is the major mechanism which can generate more toxic radicals through interactions between the superoxide anion and hydrogen peroxide.

$$O_2^{-}\bullet + \mathbf{H_2O_2} \rightarrow \mathbf{OH\cdot} + \mathbf{OH^{-}} + \mathbf{O_2}$$

Fenton reaction, which consists of two reactions, involves the use of a metal ion (such as copper and iron $Fe^{2+/3+}$) catalyst in order to generate OH·, as shown below:

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

The hydroxyl radical reacts with any neighboring biological molecule (i.e, DNA, proteins, lipids) in a diffusion-limited manner. Since it is so highly reactive, there is no known scavenger of OH•.

Singlet molecular oxygen ($^1O_2$) is an electronically high excited, meta-stable state of molecular oxygen and it is a toxic highly reactive species. Its generation by photoexcitation makes $^1O_2$ particularly important in light-exposed tissues such as the skin and the eye. $^1O_2$ is a highly reactive molecule that mediates the photoaging-associated mitochondrial common deletion.

It is produced in vivo by the activation of neutrophils and eosinophils. It is also formed by some of the enzymatic reactions catalyzed by enzymes such as lipoxygenases, dioxygenases, and lactoperoxidase. It is a highly potent oxidizing agent that can cause DNA damage and tissue damage [29,38].

Ozone (O_3) is a powerful oxidant which may be produced in vivo by antibody catalyzed water oxidation pathway which plays an important role in inflammation. It can form free radicals and other reactive intermediates by oxidizing biological molecules. It can cause lipid peroxidation and oxidize different functional groups (amine, alcohol, aldehyde and sulphydryl) in proteins and nucleic acids. It can also cause chromosomal aberrations which may be due to direct attack by O_3 or by the free radicals generated by it [38].

Hypochlorous Acid and hypobromous acid (HClO - HBrO) are produced from H_2O_2 by myeloperoxidase (MPO) in the phagocytic vacuole in neutrophils for pathogen defence. MPO is unique among mammalian peroxidases in having a sufficiently high reduction potential (1.16 V) to oxidize chloride to hypochlorous acid. HClO is a strong reactive species involved in oxidation and chlorination reactions. It can oxidize thiols and other biological molecules including ascorbate, urate, pyridine nucleotides, and tryptophan. HClO chlorinates several compounds, such as amines to give chloramines; tyrosyl residues to give ring chlorinated products, cholesterol and unsaturated lipids to give chlorohydrins, and it can also chlorinate DNA. Furthermore, the production of HClO, by MPO, is a critical step in the production of Neutrophil Extracellular Traps (NETs) during inflammatory response [38].

Reactive nitrogen species (RNS)

RNS include nitric oxide (NO) and nitrogen dioxide (NO_2) in addition to non-reactive species such as peroxynitrite ($ONOO^-$) and nitrosamines. In mammals, RNS are mainly derived from NO, which is formed from O_2 and L-arginine, and its reaction with the superoxide anion, which forms peroxynitrite (Figure 10). An excessive production of RNS can affect protein

structure and function inducing changes in catalytic enzyme activity, cytoskeletal organization and cell signal transduction [46-48].

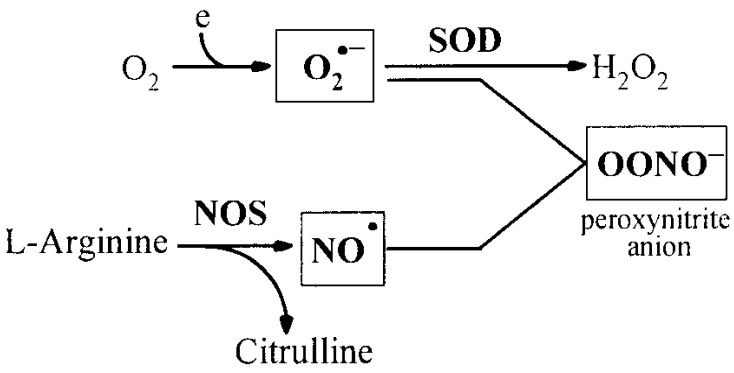

Figure 10: Formation of NO and ONOO−.

Nitric oxide (NO) is synthesized during the enzymatic conversion of L-arginine to L citrulline by nitric oxide synthase (NOS) using NADPH as an electron donor. There are 3 types of nitric oxide synthase (NOS) isoenzymes in mammals involving endothelial NO synthase (NO synthase 3), neuronal NO synthase (NO synthase 1) and inducible NO synthase (NO synthase 2). First two isoforms are constitutive NO synthases, and responsible for the continuous basal release of NO.

Inducible NO synthase (iNOS) occurs in mononuclear phagocytes (monocytes and macrophages) and produces a large amount of NO. This is expressed in response to pro inflammatory cytokines and lipopolysaccharides.

With an unpaired electron, NO•, a highly reactive free radical against proteins, carbohydrates, nucleotides and lipids can mediate cellular and tissue damages. It is an important cellular signaling molecule involved in many physiological and pathological processes. NO has vasodilatory properties, acting on arterial and venous smooth muscles and inhibiting platelet aggregation and adhesion. These evidences suggest a therapeutic approach of NO [29-32].

Peroxynitrite (ONOO− is a powerful pro-oxidant. As it is capable of diffusing up to 5 μm, it may affect neighboring cells[5]. The peroxynitrite is generated in presence of excessive amount of superoxide that reacts rapidly with nitric oxide (NO•). Peroxynitrite is able to induce lipid peroxidation and nitrosation of many tyrosine molecules that normally act as mediators of enzyme function and signal transduction. Peroxynitrite acts as a biological oxidant, affecting mitochondrial functions and triggering cell death via oxidation and nitration reactions [29-32].

Antioxidant defenses

Antioxidants are molecules involved in the maintenance of cellular redox balance detoxifying biological systems from an excessive presence of reactive species. Antioxidants are defined as "any substance that delays, prevents or removes oxidative damage to a target molecule". Thus, effective antioxidants have the ability to delay oxidation reaction or obstruct the development of free radicals or break the generation of the autoxidation chain reaction that generates free radicals/oxidants.

Cellular antioxidant defense mechanisms are multiple and compartmentalized. These are expressed both within the mitochondrial compartment (uncoupling proteins, thioredoxins, glutathione peroxidase, and superoxide dismutase), in nucleus and cytosol [55]. There are two main types of antioxidants: enzymatic and non-enzymatic molecules (Figure 11) [29-32,35,38,44,53].

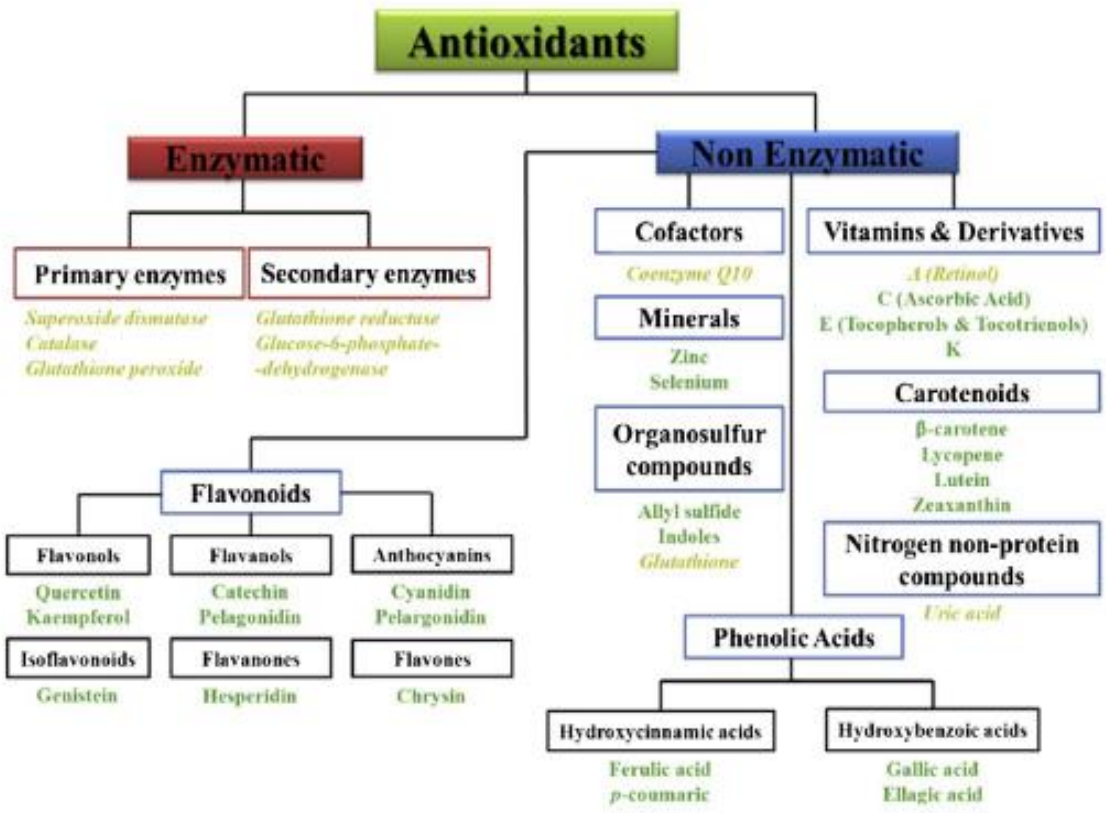

Figure 11: Different classes of antioxidants [53].

Enzymatic antioxidants

Enzymatic antioxidants, also known as natural antioxidants, show a transition metal at their core, with a central role in detoxification processes. They neutralize excessive ROS levels and prevent damages to cellular structures. Endogenous antioxidants include SOD, catalase, glutathione peroxidase, and glutathione oxidase which also causes reduction of hydrogen peroxide to water and alcohol [53,55].

Superoxide dismutase (SOD) exists as three isoenzymes: SOD 1, SOD 2, and SOD 3. SOD 1 contains copper (Cu) and zinc (Zn) as metal co-factors and is located in the cytosol. SOD 2 is a mitochondrial isoform containing manganese (Mn), and SOD 3 encodes the extracellular form. Dismutation of the superoxide anion to H_2O_2 by SOD is fundamental to anti-oxidative reactions. SOD prevent the accumulation of superoxide, which can damage and inactivate proteins containing iron–sulfur cluster. This enzyme converts superoxide radical in the following reaction: **SOD:** $\mathbf{2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2}$

Catalase (CAT) is a tetramer of four polypeptide chains composed by four iron containing heme-groups that are central in the enzyme reaction with H_2O_2. Catalase is one the most efficient enzymes found in the cells and it is principally located in peroxisomes. Catalase catalyzes a dismutation reaction of H_2O_2 to water as reported below .

Catalase catalyzes the following reaction: **CAT:** $\mathbf{2\ H_2O_2 \rightleftarrows O_2 + 2\ H_2O}$

The **glutathione** (GSH) **family** of enzymes includes **GPx** (glutathione peroxidase), **GST** (glutathione S-transferase) and **GSH reductase** (see the figure below).

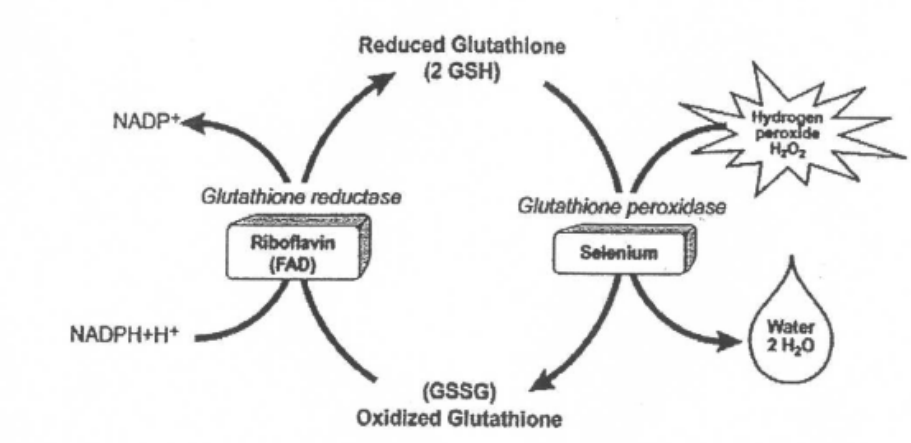

Figure 12: The glutathione oxidation reduction cycle.

Glutathione peroxidase (GPx) is a selenium-containing enzyme that catalyzes the reduction of hydrogen peroxide to water in the presence of GSH, which is converted to GSSG[1,17,25]. GPx exists as five isoforms in the body from GPx1 to GPx5. GPx1 is the cytosolic isoform that is widely distributed in tissues, while GPx2 encodes a gastrointestinal form with no specific function. GPx3 is present in plasma and epididymal fluid and GPx4 specifically detoxifies phospholipid hydroperoxide within biological membranes. Finally, Gpx5 is found in the epididymis.

Glutathione reductase reduces GSSG in the presence of nicotinamide adenine dinucleotide phosphate [NAD(P)H], which is generated mainly in the pentose phosphate pathway.

Glutathione S-transferase (GST) catalyzes the association of the reduced glutathione (GSH) to xenobiotic substrates for the purpose of detoxification.

Non-enzymatic antioxidants

Non-enzymatic antioxidants are also known as synthetic antioxidants or dietary supplements. The body's complex antioxidant system is influenced by dietary intake of antioxidant vitamins and minerals such as vitamin C, vitamin E, selenium, zinc, taurine, hypotaurine, glutathione, β-carotene, and carotene [53,55].

There are two main categories of non-enzymatic antioxidants:

- Hydrophobic antioxidants (such as vitamins A, E, carotenoids, ubiquinones, etc.);
- Water-soluble antioxidants (such as ascorbic acid, uric acid, taurine, etc.);

Glutathione (GSH), is a tripeptide thiol and the major non-protein sulfhydryl compound in mammalian cells, known to have numerous biological functions. It is the major endogenous antioxidants produced of non-enzymatic systems. This thiol plays a prominent role in detoxification and antioxidation of exogenous and endogenous compounds, and it maintains the intracellular redox status (for example it keeps vitamins C and E in their reduced forms). It is formed in the cytosol from a combination of cysteine, glycine and glutamine. Its levels are regulated through its formation de-novo, which is catalyzed by the enzymes γ-glutamylcysteine synthetase and glutathione synthetase. Glutathione is a natural reservoir of reducing power, which can be quickly used by the cells as defense against oxidative stress. Depletion of GSH results in DNA damage and increased H_2O_2 concentrations. Glutathione exists in two forms: the reduced form (GSH) and the oxidized form (GSSG). The protective

action of glutathione against ROS is facilitated by the interactions with its associated enzymes, such as glutathione peroxidase and glutathione reductase.

Glutathione levels can be improved and optimized by consuming sulfur-rich foods such as garlic, onions, cabbage, cauliflower, broccoli, etc., and by consuming bioactive whey proteins found in non-pasteurized and non-industrially produced milk, which is a great source of cysteine and the amino acid building blocks for glutathione synthesis. Also physical exercise and antioxidant supplementation with C and E vitamin can be useful to recycle glutathione.

Non-enzymatic antioxidant defence include **ascorbate** (vitamin C) and **α-tocopherol** (vitamin E), that acts in concert. **Vitamin C** is a known redox catalyst that can reduce and neutralize ROS. It is a chain breaking antioxidant that stops the propagation of the peroxidative process. Its reduced form is maintained through reactions with GSH and can be catalyzed by protein disulfide isomerase and glutaredoxins. **Vitamin E** (α-tocopherol) is a lipid soluble vitamin with antioxidant activity. It consists of eight tocopherols and tocotrienols and plays a major role in antioxidant activities because of its reaction with lipid radicals derived from lipid peroxidation. This reaction produces oxidized α-tocopheroxyl radicals that can be transformed back to the active reduced form by reacting with other antioxidants like ascorbate, retinol, or ubiquinol.

The hormone **melatonin** is an antioxidant that, unlike vitamins C and E, is produced by the human body. In contrast to other antioxidants, however, melatonin cannot undergo redox cycling; once oxidized, melatonin is unable to return to its reduced state because it produces stable end-products after the reaction occurs.
In addition, thiol compounds, such a **thioredoxin** (Trx), are capable of detoxifying hydrogen peroxide, but in turn require converting back to the reduced form by thioredoxin reductase[2]. The thioredoxin system regulates gene functions and coordinates various enzyme activities. Normally, Trx is bound to apoptosis-regulating signal kinase (ASK) 1, rendering it inactive. However, when the thiol group of Trx is oxidized by the superoxide anion, ASK1 detaches from Trx and becomes active leading to enhanced apoptosis. ASK1 can also be activated by exposure to H_2O_2 or hypoxia reoxygenation, and inhibited by vitamins C and E.

Ceruloplasmin and **transferrin** play important roles in the inhibition of Fenton reaction and OH• production by sequestering free iron ions.

Transferrin and **ferritin**, both iron-binding proteins, play a role in antioxidant defense by preventing the catalyzation of free radicals through chelation.
Nutrients such as **Se**, **Cu**, and **Zn** are required for the activity of some antioxidant enzymes, although they have no antioxidant action themselves.

Biological actions of ROS

At homeostatic levels, ROS have different actions on cell function, including activation of redox sensitive transcription factors and activation of protein kinases. ROS/RNS signaling is involved in the modulation of different cellular functions as metabolic processes, vascular tone, cytokine and growth-factor genes expression, cell cycle progression, cytoskeletal organization, antigen processing, cell proliferation and differentiation, apoptosis and inflammatory response. 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.

At pathological levels, ROS are associated with several molecular damages such as opening of ion channels, lipid peroxidation, protein modifications and DNA oxidation, suggesting its involvement in the pathogenesis of several disorders [29-33,39-41,48-53].

Activation of redox-sensitive transcription factors

Activation of redox-sensitive transcription factors, such as AP-1, p53, Nrf2/Keap1 and NF-κB (Nuclear factor κB) regulate the expression of pro-inflammatory and other cytokines, cell differentiation and apoptosis.
Under physiological conditions, **NF-kB** is held inactive by the binding of its inhibitory sub-unit IkB. However, due to oxidation by H_2O_2, IkB becomes phosphorylated and dissociates from NF-kB, which then translocates to the nucleus and activates expression of genes involved in inflammatory, immune and acute phase responses. Activation of the pathway is associated with increased tissue levels of the proinflammatory enzyme COX-2, interleukin 1ß, increased secretion of TNF-α, and activation of the apoptotic cascade as evinced by cleavage of caspase 3. All these effects can be blocked by the addition of vitamins C and E or sulfasalazine, an inhibitor of NF-kB activation [29-32].

Nrf2 (nuclear factor-E2-related factor 2) belongs to a small family of transcription factors inducing a set of antioxidant enzymes. Keap1 (Kelch-like ECH-associated protein 1) subjects Nrf2 to rapid ubiquitination and degradation and thus suppresses the transcriptional activity of

Nrf2 under physiological conditions. Under oxidative or electrophilic stress, specific cysteinyl residues of Keap1 are modified and Keap1 loses its ability to ubiquitinate Nrf2, allowing it to accumulate in the nucleus to induce expression of its target genes.

Interestingly, the selenoprotein thioredoxin reductase 1 (TrxR1) has been proposed as a potent regulator of both above-cited transcription factors. Activity is stimulated by nuclear Trx1 and inhibited by increased nuclear H_2O_2 production. The following figure 13 illustrates mechanisms of action of Nrf2/Keap1 and NF-κB in physiological condition or under oxidative stress [29-32].

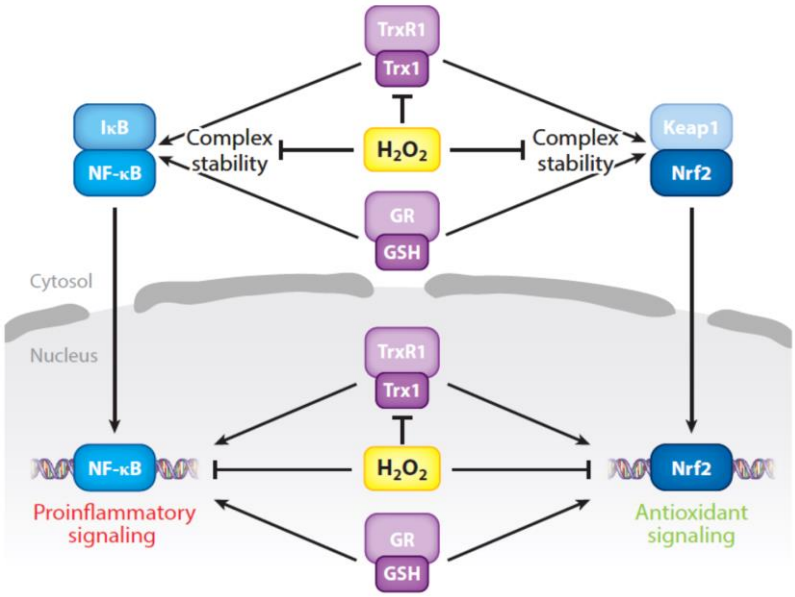

Figure 13: Molecular mechanisms of redox-sensitive transcription factors [30].

Activation of protein kinases

With the activation of protein kinases, cells respond to a variety of extracellular signals and stress through a family of mitogen-activated protein kinases (MAPK). Within this family, ROS-induced activation of extracellularly regulated kinases (ERK1/2) generally promotes cell survival and proliferation, whereas stimulation of p38MAPK (p38) and stress activated protein kinase–c-Jun amino terminal kinases (SAPK–JNK) mostly induces apoptosis. p38 and SAPK–JNK are activated by phosphorylation through an upstream kinase, apoptosis-regulating signal kinase 1 (ASK1). Under normal conditions ASK1 is held inactive by binding to thioredoxin, but O_2^{-}• is capable of oxidizing the thiol groups in the latter, leading to a conformational change and its release [29-32].

Opening of ion channels

Imbalances of ROS lead to the loss of intracellular Ca^{2+} homeostasis, releasing Ca^{2+} ions from the endoplasmic reticulum (ER). The calcium concentration within the ER lumen is much higher than in the cytosol reaching millimolar levels. This concentration is maintained by pumps belonging to the sarco/endoplasmic reticulum calcium ATPase family, and is necessary for the correct functioning of the protein-folding machinery. ROS are able to activate calcium release channels in the ER membrane, which include the inositol-1,4,5,triphosphate receptor (IP3R) and the ryanodine receptor. The resultant release of Ca^{2+} from the ER will activate diverse Ca^{2+}-sensitive processes within the cell, including many of the signaling pathways above.

In addition,the release of Ca^{2+} from ER adversely affects mitochondrial function, including an increase in their own production of ROS and the opening of the permeability transition pore. Consequently, the mitochondrial membrane potential becomes unstable and ATP production decreases. Opening of the membrane permeability transition pore is promoted synergistically by increased Ca^{2+} ions and oxidation of the thiol groups of proteins located in the inner mitochondrial membrane. If mitochondria throughout the cell are affected, ATP concentration falls suddenly and sharply, ionic homeostasis is lost and the cell undergoes primary necrosis [29-32].

Lipid peroxidation

The membrane lipids, especially the polyunsaturated fatty acids (PUFAs) residues of phospholipids, are more susceptible to oxidation by free radicals. Lipid peroxidation can be described generally as a process under which oxidants such as free radicals or non-radical species attack lipids containing carbon-carbon double bond(s), especially PUFAs. Among free radicals, hydroxyl radicals are strongly involved in oxidation of plasma and membrane lipid resulting in several lipid peroxidation products detected biochemically [29-32,39,40].

The overall process of lipid peroxidation consists of three main steps: initiation, propagation, and termination (Figure 14).

1. **Initiation**: the first step of this process is a ROS dependent abstraction of a hydrogen atom from the PUFA to form a carbon-centred fatty acid free radical (C•);

2. **Propagation**: If oxygen is present, this may react with the fatty acid radical to form a peroxyl radical (-C-O-O•), which in turn is capable of abstracting hydrogen from an adjacent fatty acid, therefore causing the propagation of the reaction.
3. **The final step or termination**: is the formation of a non-radical species from the combination of two radicals.

Because vitamin E is lipid-soluble and possesses a hydrophobic tail, it tends to accumulate within the interior of lipid membranes. Here, it acts as the most important chain-breaker, as it reacts with lipid peroxyl radicals about four times faster than they can react with adjacent fatty acid side chains.

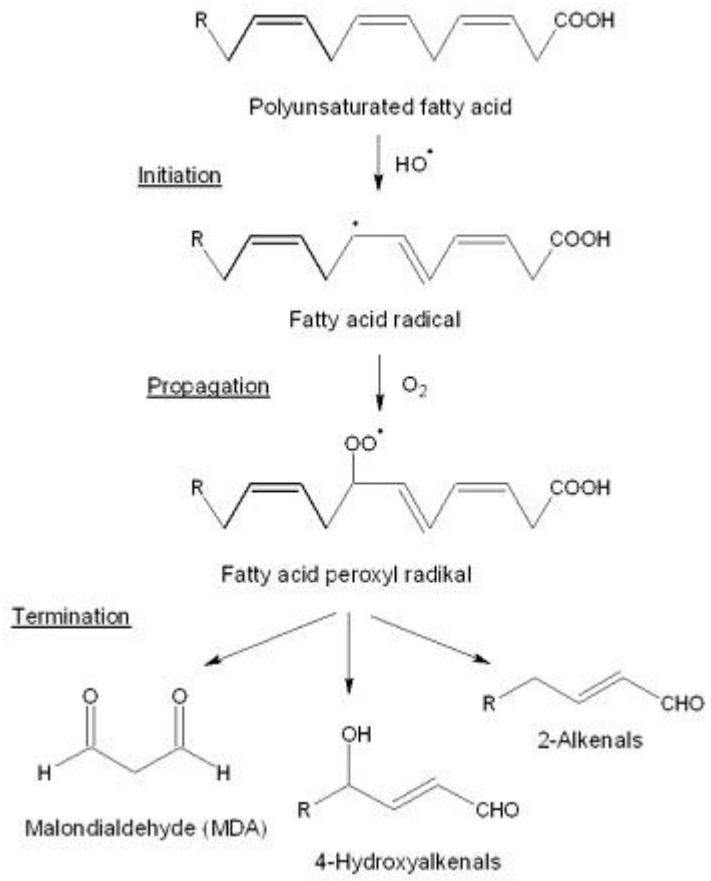

Figure 14: Chain reaction of lipid peroxidation.

Lipid peroxidation produces a wide variety of oxidation products. The main products of lipid peroxidation are lipid hydroperoxides (LOOH), lipid hydroxides and epoxides (including cholesterol oxidation products), isoprostanes, malondialdehyde (MDA) and other aldehydes and ketones. Among the many different aldehydes which can be formed as secondary products during lipid peroxidation, MDA, propanal, hexanal, and 4-hydroxynonenal (4-HNE) have been extensively studied.

MDA appears to be the most mutagenic product of lipid peroxidation, whereas 4-HNE is the most toxic due to its rapid reactions with thiols and amino groups. High levels of lipid

radicals are associated with loss of membrane fluidity and function, and activation of the apoptotic cascade. [32]. MDA and 4- HNE adducts play a critical role in multiple cellular processes and can participate in secondary deleterious reactions (e.g., crosslinking) by promoting intramolecular or intermolecular protein/DNA crosslinking that may induce profound alteration in the biochemical properties of biomolecules, which may facilitate development of various pathological states [39,40].

Nucleic acids oxidation

DNA oxidation, along with DNA hydrolysis and DNA methylation, is a major contributor to instability and decay of the genome. DNA is principally attacked by OH• radicals, and a variety of products can be generated through reactions with either the DNA bases or the deoxyribose sugars. Among the DNA bases, guanine is the most susceptible to oxidative damage. The major mutagenic lesion is 8-oxo-7,8-dihydroguanine (also called 8-oxoguanine or 8-hydroxyguanine), which base pairs with adenine rather than with cytosine, thus generating transversion mutations after replication. In addition, its accumulation leads to mitochondrial dysfunction.

Attacks on the sugar moieties may cause double/single strand breakages, whereas those on histone proteins may lead to cross-linkages that interfere with chromatin folding, DNA repair and transcription. Mutation or aberrant gene expression may therefore result.

Mitochondrial DNA is particularly vulnerable to ROS attack because it is located in close proximity to the ROS generating site (ETC), for the lack of histone protection and the minimal repair mechanisms that exist. As mitochondrial DNA encodes several proteins, including enzymes of the electron transport chain, mutations may lead to impaired energy production and the risk of extensive electron leakage, compounding the original stress.

On the other hand, also RNS contribute to DNA damage. Peroxynitrite interacts with guanine to produce nitrative and oxidative DNA lesions such as 8-nitroguanine and 8-oxodeoxyguanosine respectively. 8-nitroguanine formed is unstable and can be spontaneously removed, resulting in the formation of an apurininc site. Conversely adenine can be paired with 8-nitroguanine during DNA synthesis resulting in a G-T transversions [38]. Evidence is also accumulating for oxidative damage to RNA, but the potential functional impact has not yet been fully elucidated. The RNA is more prone to oxidative damage than DNA, due to its single stranded nature, lack of an active repair mechanism for oxidized RNA, less protection by proteins than DNA. 7, 8-dihydro-8-oxoguanosine (8-oxoG) is the most extensively studied RNA damage product and its levels are raised in various pathological conditions like

Alzheimer's disease, Parkinson's disease and atherosclerosis [38]. However, non-coding RNA or microRNAs have been shown to be redox sensitive and for this reason were termed "redoximiRs"[29,30].

Protein modifications

Exposure of amino acids, both free or linked within proteins, to ROS/RNS may virtually alter any level of protein structure, from primary to quaternary. Protein oxidation can be mediated by several mechanism and can involve several different side chains [51]. Individual proteins may display different susceptibility to ROS/RNS depending on oxidant types and other factors such as amount and distribution of thiol groups, presence of Fe-S clusters, transition-metal ions (Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, etc.) as prosthetic groups, short linear motifs (SLiMs), and residues exposed on molecular surface of proteins. Moreover, chemical modifications in proteins may occur either non-specifically at multiple side-chain and backbone sites or may be selective to a residue type and its location. From a chemical point of view, oxidative protein modification requires a compound with electrophilic properties, an oxidant, that accepts one or two electrons to become reduced. This may also include covalent bond formation between an electrophile and a nucleophile [53].

Oxidation of different amino acids present in the proteins, can lead to the formation of protein–protein cross linkages that results in the denaturation and loss of functional proteins, such as enzymes, receptors and transport proteins[38,41]. Histidine, proline, arginine, lysine, methionine, and cysteine residues are particularly sensitive to site-specific metal-catalyzed oxidation. The sulfur containing amino acids such as methionine and cysteine are more susceptible to oxidation by ROS and are converted to disulfides and methionine sulfoxide, respectively. However, in biological systems only these two oxidized forms of proteins can be converted back to their native form by two different enzymes, namely disulfide reductase and methionine sulfoxide reductase, respectively [38,41,43,52,53]. The main oxidative modifications are reported below.

Protein carbonylation

Protein carbonylation is generally defined as an irreversible post-translational modification (PTM) that yields a reactive carbonyl moiety in a protein, such as an aldehyde, ketone, or lactam. Historically, metal-catalyzed oxidation (MCO) was first identified as a source of protein-bound carbonyls. MCO results as a consequence of the Fenton reaction, when transition metal ions are reduced in the presence of hydrogen peroxide with the generation of

highly reactive hydroxyl radicals. These radical species oxidize amino acid side chains or cleave the protein backbone, leading to numerous modifications, including reactive carbonyls. For example, the oxidation of proline and arginine residues yields glutamic semialdehyde, whereas lysine is oxidized to aminoadipic semialdehyde and threonine to 2-amino-3-ketobutyric acid. Besides this, direct oxidation of other amino acid residues can also lead to protein-bound carbonyls [50-52].

Another important source of protein-bound carbonyls is reactive lipid peroxidation products (LPP). LPP are strong electrophiles and thus readily react with nucleophilic groups in proteins, such as the side chains of lysine, cysteine, and histidine, predominantly forming Schiff bases or Michael adducts. Recently, 122 LPP capable of modifying proteins by oxidation of phosphatidylcholine lipids were identified [52].

Protein carbonylation can also occur via glycoxidation. Glycation, defined as the non-catalyzed reaction of reducing sugars, such as glucose or fructose, with the side chains of lysine and arginine residues, leads to the formation of Amadori and/or Hynes products. These glycated residues can be further decomposed by ROS into advanced glycation end products (AGE) carrying carbonylated moieties. Reactive α-carbonyls formed during glycoxidation, such as glyoxal, methylglyoxal and 3-deoxyglucosone, can modify the basic residues Lys and Arg to yield, for example, pyrralines and imidazolones [50-52].

The main pathways of protein carbonylation are summarized in figure 15.

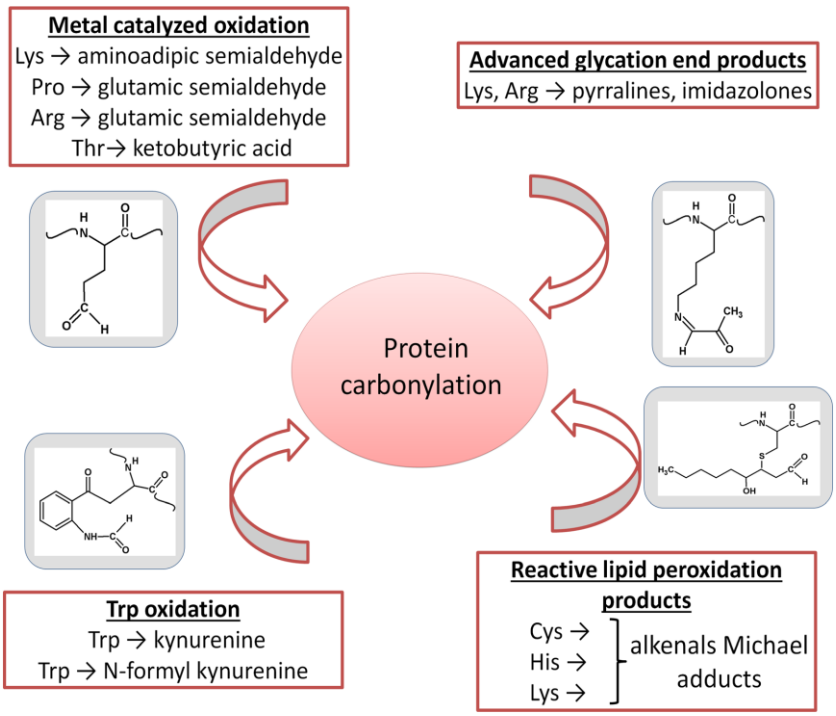

Figure 15: Main pathways of protein carbonylation [52]

As carbonylated proteins cannot be repaired by cellular enzymes, they tend to aggregate and, if not eliminated, result in cell death. Basal levels of cellular carbonylation are around 2 mmol/g protein, whereas severe oxidative conditions can increase this level up to threefold.

The carbonyl products are stable and their detection is the most commonly used method to assay protein oxidation. The biochemical and analytical methods used to identify and quantify reactive carbonyls can be classified into three categories:

1. Biochemical and immunological techniques (such as immunoblotting and ELISA) which provide global information on the modified proteins and carbonylation levels;
2. Spectrophotometric and chromatographic assays to determine the total protein carbonyl content;
3. Mass spectrometry (MS) for identification of the modified proteins, including modification sites, and relative quantification of protein-bound carbonyls [50-52].

Oxidation of cysteine and methionine

The hydroperoxides can contribute to oxidative modification of sulfur-containing amino acids, Cys and Met. The direct oxidation of Cys and Met belongs to major types of protein oxidative modifications, Cys residue being a key redox sensitive residue due to the presence of chemically reactive thiol group (-SH), which provides high structural and functional flexibility of proteins. Cys residue may be often found in catalytic and regulatory sites of various effector proteins, including enzymes, intracellular effectors of signaling pathways, transcription factors and their modulators; this dictates the importance of thiol group oxidative modification in protein activity regulation [53]. 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.

The sulfur atom of Met has been also shown to undergo a $ONOO^-$- dependent one-electron or two-electron oxidation, leading to the formation of methionine sulfoxide or ethylene, respectively. The main oxidation product of Met, methionine sulfoxide, is formed by addition of an oxygen atom to the sulfur atom. Methionine sulfoxide can be reduced back to Met by the activity of methionine sulfoxide reductase (Msr), a Trx-dependent enzyme. Furthermore, HClO has been shown to react rapidly in vitro with Met at physiological pH values, while H_2O_2 was less efficient in Met oxidation [53]. 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 [50].

Oxidation and nitration of Tyrosine and Tryptophan

Tyrosine residues in proteins typically constitute in average 3–4 % of the amino acid residue in a protein and are located in different regions due to the presence of the relatively large phenolic amphipathic sidechain, capable to interact with water, participate in hydrogen bond formation and undergo cation-π and non-polar interactions. Typically, both solvent-exposed and buried residues coexist within a protein; on average, only 15% of tyrosine residues in proteins are at least 95% buried [48,49].

One of the most important oxidative tyrosine modifications is the formation of **dityrosine**. Dityrosine was first recognized over 40 years ago. It is an unusual amino acid that is characterized by an intense 420-nm fluorescence emission, measurable upon excitation at either 315 nm (alkaline solutions) or 284 nm (acidic solutions) [51].

The mechanism of dityrosine formation begins with the generation of a tyrosyl radical, followed by a diradical reaction, and finally enolization (Figure 16). Once the tyrosyl radical is formed, the production of dityrosine involves two monomeric molecules of tyrosine-containing proteins, joined by intermolecular crosslinking, thus resulting in the formation of protein dimers [51]

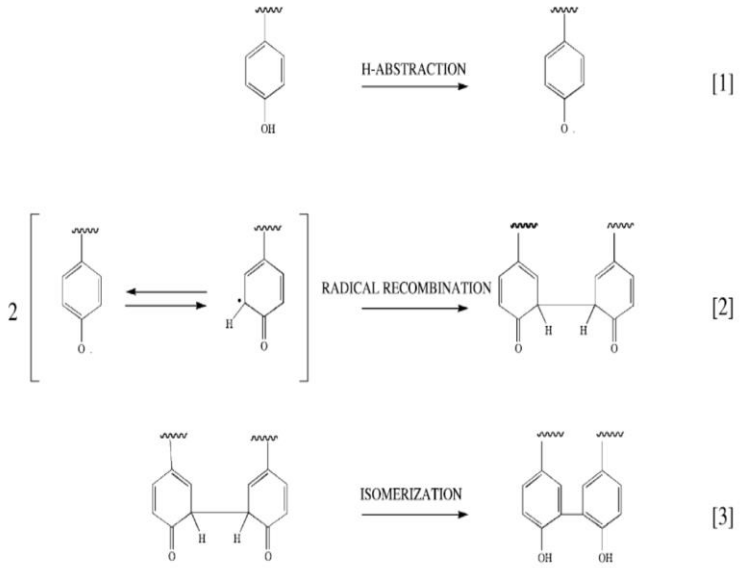

Figure 16: Mechanism of dityrosine formation.

Several reports have shown the formation of 3,3'-Dityrosine in different proteins exposed to a variety of oxidants (UV light, γ-irradiation, oxygen radicals), supporting a wider use of dityrosine release as a clinical marker for oxidative stress. In this regard, dityrosine

concentrations were found 100-fold higher in low-density lipoproteins isolated from atherosclerotic lesions than in normal ones [51].

The nitration of protein tyrosine residues to **3-nitrotyrosine** represents an oxidative post translational modification that unveils the disruption of nitric oxide (•NO) signaling and metabolism towards pro-oxidant processes. Thus, protein 3-nitrotyrosine has been established as a biomarker of cell, tissue and systemic "nitroxidative stress". Moreover, tyrosine nitration modifies key properties of the amino acid (i.e. phenol group pK_a, redox potential, hydrophobicity and volume). Thus, the incorporation of a nitro group ($-NO_2$) to protein tyrosines can lead to structural and functional changes, some of which contribute to altered cell and tissue homeostasis [48,49].

The main mechanisms of protein tyrosine nitration mediated by free radical are summarized in figure 17. This process implies the formation of a Tyr• intermediate, and subsequent reactions with either •NO or $•NO_2$. Depending on the predominant nitrating species, mechanism and milieu, relevant one electron oxidants are carbonate radicals ($CO_3^{•-}$), oxo-metal complexes (e.g. MPO compound I, hemin-Fe^{4+}) and even lipid peroxyl radicals (LOO•) [48,49].

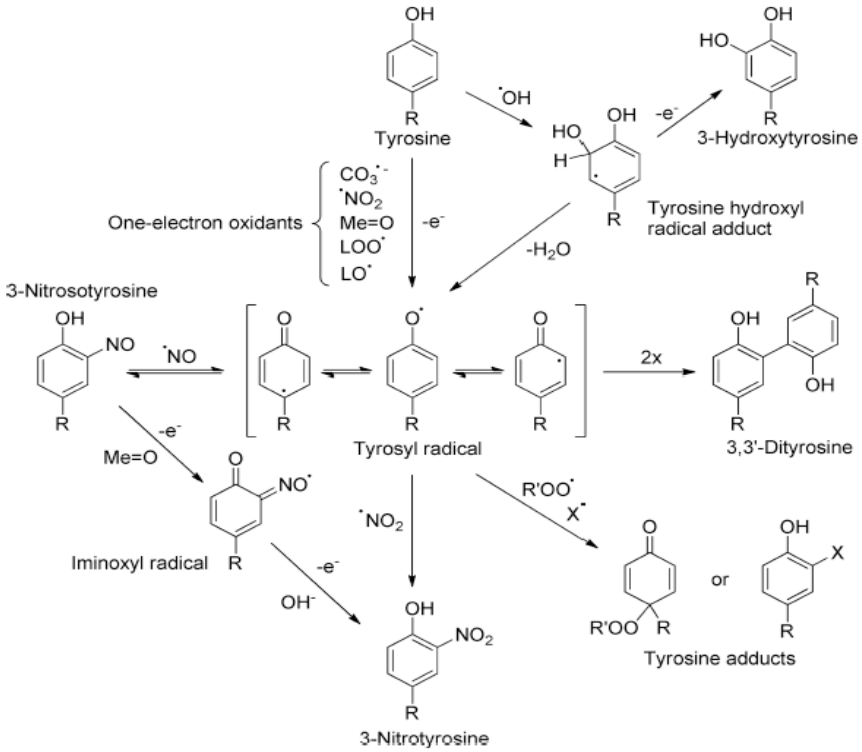

Figure 17: Free radical mechanisms of nitration and alternative pathways of the tyrosyl radical

Tyrosine nitrated proteins have been detected in a large variety of disease conditions. Immunochemical-based studies using antibodies against protein 3-nitrotyrosine have been

useful for detection and cellular/tissue distribution of nitrated proteins, proteomic-based analyses have been fundamental to identify preferential protein targets of nitration. Possible biochemical consequences of protein tyrosine nitration involve changes in activity (loss or gain of function), eliciting of immunogenic responses, interference in tyrosine-kinase-dependent pathways, alteration of protein assembly and polymerization, facilitation of protein degradation (turnover) and participation in the creation of proteasome-resistant protein aggregates [48,49].

Protein disulfides

Under physiological conditions, the formation of specific disulfide bonds within a polypeptide chain is needed for a correct folding process and is catalyzed by disulfide isomerase inside the endoplasmic reticulum or the mitochondrial intermembrane space. Oxidative stress can induce disulfide bonds formation between any free cysteine residues, leading to scrambled non-native bonds and changing protein structural and functional characteristics. Moreover, the formation of disulfide bonds can affect protein folding causing protein loss of function, but also protein aggregation and cell death [29-34].

Oxidative stress and EGPA

Free radical generation is traditionally accepted as a mechanism of action during inflammatory response. Oxidative mechanisms are important in immune system function, not only in protecting against infectious agents but also in critical stepwise functions required for complex processes, such as would healing. However excessive reactive species levels are associated with oxidative stress, causing tissue injury and consequently maintaining the inflammatory status. In this context, phagocytes, neutrophils in particular, are major sources of $O_2^{\cdot -}$ and H_2O_2 [29,46,50,55,57-59].

Oxidative stress has been widely implicated in several pathological conditions such as degenerative diseases, autoimmune diseases, chronic inflammatory disorders, cardiovascular disease and also in atherogenesis and thrombosis. Vasculitis comprises a spectrum of disorders in which blood vessels are destroyed by inflammation. In anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis, neutrophils are both the direct target of auto-antibodies and the main protagonist of vascular damage through the production of ROS. Auto-antibodies are produced against neutrophil granule proteins MPO or proteinase-3 (PR3), with anti-MPO ANCAs being most commonly associated with microscopic polyangiitis and anti-PR3 ANCAs being present in >90% of patients with granulomatosis with polyangiitis. Anti-MPO ANCAs stimulate the production of ROS by unprimed, healthy control neutrophils, and anti-PR3 ANCAs stimulate the production of ROS by vasculitis neutrophils, with levels of ROS produced correlating with the NET production. Recent *in vitro* studies have shown that autoantibodies and serum immune complexes obtained from patients with ANCA-associated vasculitis are capable of inducing NETosis [66].

Activation of ROS by ANCAs is enhanced by the presence of high mobility group box-1 (HMGB1), an inflammatory protein which is elevated in the serum of vasculitis patients and which is associated with increased levels of neutrophil migration and vascular damage. Increased production of MPO-containing NETs by vasculitis neutrophils and low-density granulocytes may exacerbate damage to vascular tissue [61,73-76].

NADPH OXIDASE: Nicotinamide Adenine Dinucleotide Phosphate-oxidase

The NADPH oxidase (or NOX) system is the main source of ROS in the vessel wall and is present in endothelial cells, VSMC, adventitial fibroblasts, and infiltrating monocytes/macrophages. NADPH oxidase isoforms in mammals have a catalytic subunit called NOX (NOX-1-5) or DUOX (DUOX-1-2) and up to seven regulatory subunits, leading

to the formation of seven NADPH oxidase isoforms. NOX-1, NOX-2, NOX-4 and NOX-5 are expressed in the cardiovascular system. Upon activation, neutrophils produce large amounts of superoxide anion via NADPH oxidase (NOX2) as the electron donor and flavin-adenine dinucleotide (FAD), catalyzes the monovalent reduction of oxygen (Figure 18). Vascular production of O_2^- increases as a consequence of risk factors for atherosclerosis. Atherosclerotic lesions contain abundant p22phox and NOX-2 that correlated with the severity of atherosclerosis [72].

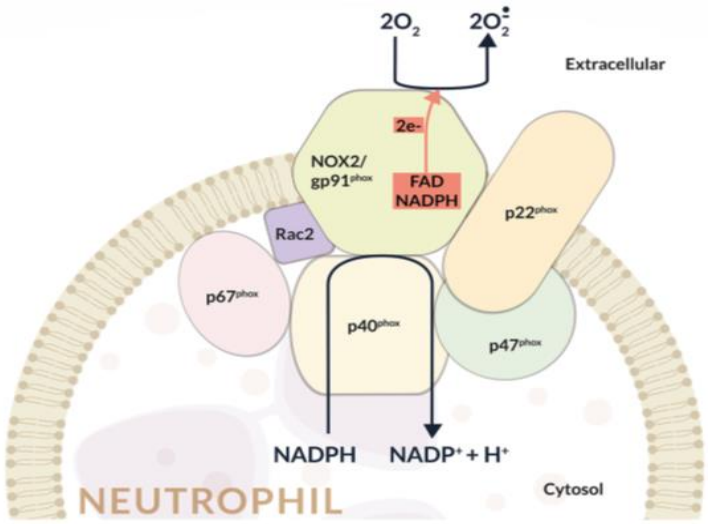

Figure 18: NADPH oxidase structure [74].

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 lipolysaccaride (LPS) or pro-inflammatory cytokines as TNFα, 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 gp91phox able to oxidize NADPH and reduce molecular oxygen. Priming followed by activation can contribute to a rapid and efficient elimination of the microbes, but as it induces the generation of large quantities of toxic ROS by hyper-activation of the NADPH oxidase, it can also damage surrounding tissues and participate in uncontrolled inflammation. [61,62].

Neutrophil extracellular traps (NETs)

Neutrophils also contribute to inflammation and tissue damage in inflammatory disease, if they become inappropriately activated by cytokines, chemokines and auto-antibodies. Auto-

immune neutrophils function in a multitude of ways to direct the inflammatory response, including release of proteases which damage host tissue and activate soluble proteins, secretion of cytokines and chemokines which direct both the innate and adaptive immune responses, shedding of receptors such as the interleukin-6 receptor to initiate trans-signalling, release of neutrophil extracellular traps (NETs) providing a source of auto-antigens, and production of ROS [60-63,65-67,73,74].

A key mechanism by which neutrophils contribute to thrombus composition involves generation of NETs. These were first described in 2004 as a means for neutrophils to trap and kill bacteria and are released as a result of a programmed cell death mechanism referred to as NETosis. NETs are neutrophil-derived structures mainly composed of DNA and histones, microbicidal proteins and various proteins with proinflammatory characteristics, such as histones, high-mobility group box 1 (HMGB1), LL37, neutrophil elastase (NE), calprotectin and, interestingly, MPO and PR3. NETosis has been shown to depend on NADPH oxidase and ROS production as well as on autophagy and histone citrullination. ROS regulate several processes of NET formation, from the induction of morphological changes to the increase in membrane permeability and the release of neutrophil elastase from granules, thus inducing the degradation of histones and chromatin decondensation [64-67,75].

NET have previously been described as double-edged swords of innate immunity, considering that they are involved in both fighting pathogens and in contributing to autoinflammatory and autoimmune conditions. NETs contribute to vessel inflammation directly by damaging endothelial cells and by activating the complement system, which plays an important role in amplifying the inflammatory process in AAV, and indirectly by acting as a link between the innate and adaptive immune system through the generation of PR3-ANCA and MPO-ANCA. This can lead to a vicious circle because ANCAs can activate neutrophils [65-67].

The role of NETs in AAV and the complex relation between ANCAs and NETs is shown on figure 19. The various letters describe the different steps of the process:

I. Pathogenic ANCAs reacting with PR3 and MPO on the surface of neutrophils cause ROS production and the release of NETs (A).

II. NETs contribute to vessel inflammation and also promote thrombosis (B-C);

III. NETs can also act as a link between the innate and adaptive immune system through the generation of ANCAs (D).

IV. ANCAs seem to belong to repertoire of “natural” antibodies, indicating that not all ANCAs are pathogenic, and it has been proposed that ANCAs can aid in clearance of circulating NET remnants (E).

V. However, under unfavorable circumstances, pathogenic ANCAs are produced, creating a vicious circle that promotes inflammation.

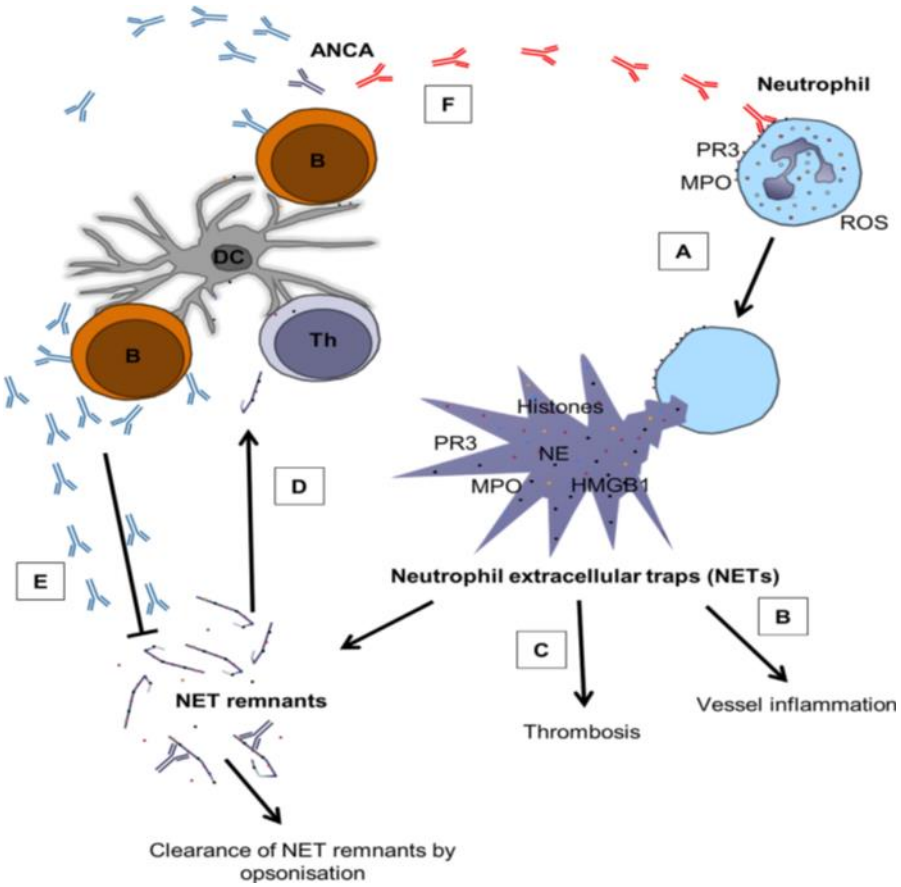

Figure 19: NETs and AAV [66].

However, data in the literature have underlined the role of NETs also in thrombosis. The presence of histones in NETs can contribute to thrombus formation and the presence of tissue factor in NETs can contribute to the generation of thrombin [65-67].

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 [61-63]. In 2009, Kessenbrock et al. showed that NETs were present in the glomeruli in kidney biopsies from AAV patients. They reported the presence of NETs as co-localizations of DNA, histones, and the granule proteins PR3, LL37, NE, and MPO in various combinations. In-vitro studies on neutrophils from AAV patients show that they are more prone to undergo spontaneous NETosis and are more responsive to NET-inducing stimuli. Neutrophil

extracellular traps have also been shown to be present in skin lesions and thrombi from AAV patients. In addition to the presence of NETs in various lesions from AAV patients, it has been shown that these patients also have elevated levels of NETs in the circulation [65-67].

Role of oxidative modifications in atherosclerosis.

Oxidised lipoproteins, associated with an elevated risk of atherosclerosis in vasculitis patients, are elevated in vasculitis serum and may form as a result of increased production of MPO-derived oxidants such as HClO. As well as inducing damage to the endothelial layer itself, neutrophil-derived ROS enhance activation and ligand-binding activity of αMβ2 integrin and increase interactions of neutrophils with both platelets and endothelial cells.

The vascular endothelium is pivotal in maintaining vascular function, and endothelial dysfunction is a major initial cause of cardiovascular disease. Regulation of endothelial function has an important redox component, and oxidative stress and inflammation are major contributors to cardiovascular disease. Atherothrombosis is a very complex pathology that involves, among many other processes, lipid deposition, oxidative stress, inflammatory cell recruitment and platelet activation (Figure 20) [29,68-73].

Extensive earlier research also documented the contribution of ROS to cardiovascular disease as key mediators of atherosclerotic plaque formation by promoting oxidation of low-density lipoprotein (LDL), generating oxidized LDL (ox-LDL).

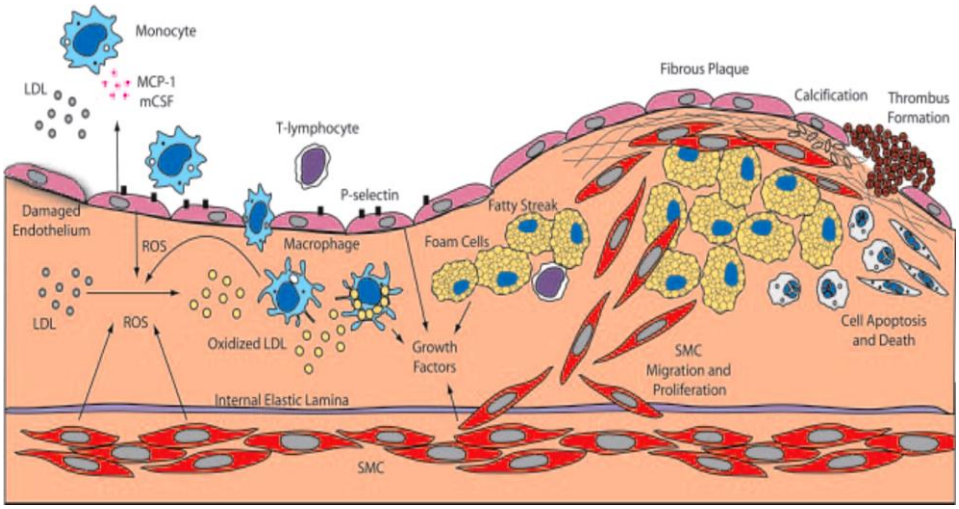

Figure 20: ROS as mediators of atherosclerotic plaque formation

Modified LDL induces endothelial injury, increases the expression of adhesion molecules, favouring monocyte adhesion and its differentiation to macrophages. Generation of ROS occurs at the sites of endothelial damage, which are caused by ox-LDL itself as well as by

physical or chemical forces due to disturbed flow and infection, with endothelial cells, macrophages and smooth muscle cells contributing to the ROS load [68-73].

Ox-LDL then damages endothelial cells, causing inflammation that leads to the recruitment of macrophages, which then take up ox-LDL and transform into plaque-forming foam cells. Rupture of fibrous plaque leads to thrombus formation and occlusion of the vessel [29].
Swets et al. measured anti ox-LDL antibodies in 25 patients ANCA associated vasculitis and 42 healthy controls using an ELISA assay. Their data showed higher levels of ox-LDL antibodies in patients with active vasculitis suggesting as reactive species are responsible for oxidation of LDL which may lead to accelerated atherosclerosis in these patients [115].
Oxidized LDL is immunogenic and the oxidative modifications of apolipoprotein B-100 resulted in the formation of neoepitopes. Oxidation-specific epitopes (OSE) may be indirectly reflected by the presence of circulating antibodies and immune complexes. These are often measured as IgG and IgM autoantibodies to MDA-LDL and apoB-immune complexes. These biomarkers can predict CVD and associated events. In atherosclerotic CVD, IgG and IgM titters to OSE such as MDA-LDL, were predictive of recurrent events in a prospective study with a 15-year follow-up [72].

Fibrinogen

Based on its wide angle X-ray diffraction pattern, the fibrinogen was first classified as a fibrous protein due to its α-helical coiled-coil structure. Human fibrinogen is a large glycoprotein synthesized in hepatocytes and normally present in human blood plasma at a concentration of about 1.5–4 g/L with a half-life of about 3 days [76-86].

Fibrinogen is a multifunctional plasma protein: it is essential for haemostasis, wound healing, inflammation, angiogenesis, and several other biological process (Figure 21). These functions are regulated by interactive sites on fibrin(ogen), some of which are masked or otherwise not available on fibrinogen, and they commonly evolve as a consequence of fibrin formation or fibrinogen–surface interactions [76,77]. Binding sites have been identified on different parts of the protein that are uniquely required for its binding to different receptors or adhesion molecules, expressed on the surface of hematopoietic cells, immune and nervous systems [78].

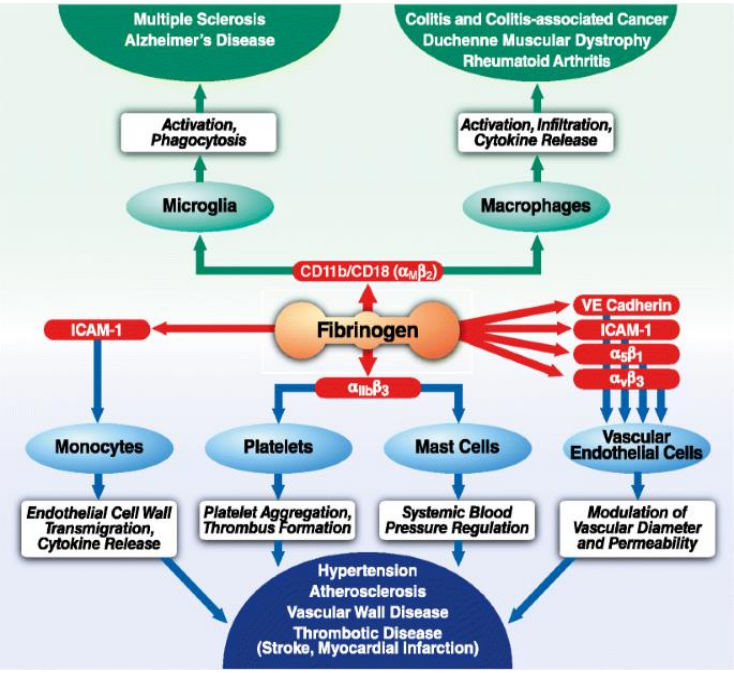

Figure 21: The multifaceted role of fibrinogen in tissue injury and inflammation [78].

Fibrinogen is a soluble macromolecule, but forms an insoluble clot or gel on conversion to fibrin by the action of thrombin, which is activated by a cascade of enzymatic reactions triggered by vessel wall injury, activated blood cells or a foreign surface. Fibrin clots are dissolved by the fibrinolytic system, acting in a series of enzymatic reactions with positive and negative feedback [76-80].

Under physiological condition, clotting and fibrinolysis are perfectly balanced. Imbalance in one direction (prevalence of fibrinolysis) can lead to bleeding, while the opposite imbalance (prevalence of clotting) can lead to thrombus formation (Figure 22). Thrombosis, arising from atherosclerosis or other pathological processes, is the most common cause of myocardial infarction, ischemic stroke, deep vein thrombosis, and other cardiovascular diseases. Thrombotic disorders are often associated with inflammation and the production of oxidants [85,86,89,90,119,120,133].

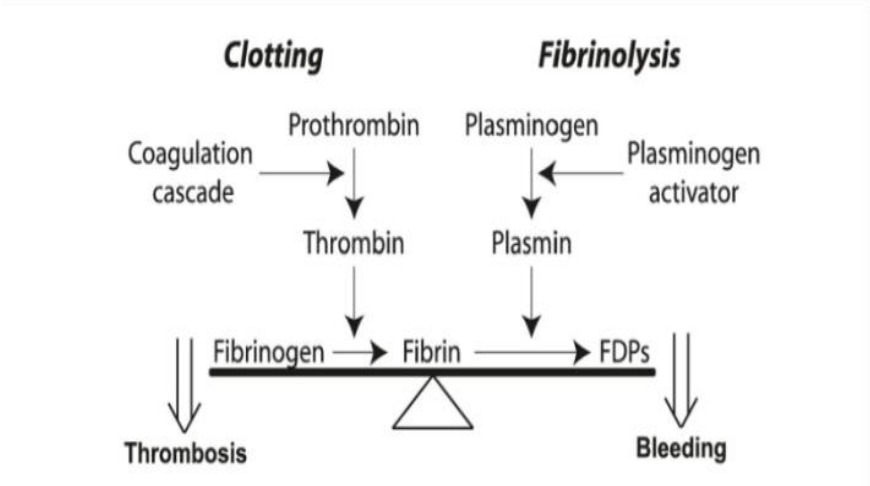

Figure 22: Scheme of fibrin clot formation and fibrinolysis and the balance between these processes [80].

In addition to fibrin clot formation, fibrinogen is also necessary for an earlier step in haemostasis (called "primary hemostasis"), the aggregation of platelets leading to formation of a platelet "plug" at the site of vessel wall injury. The C terminus of fibrinogen's γ-chain binds to a site on the αIIbβ3 integrin receptor on the platelet surface, thereby mediating the formation of bridges between platelets and facilitating their aggregation [78-82].

Fibrinogen structure

Human fibrinogen is made up of three pairs of polypeptide chains, designated Aα, Bβ and γ, with molecular masses of 66,500, 52,000, and 46,500 Da, respectively (Figure 23). Two copies of each polypeptide chain assemble in a stepwise manner to form a 340 kDa hexamer $(A\alpha B\beta\gamma)_2$ held together by 29 disulfide bonds. There are 8, 11, and 10 cysteine residues in the Aα, Bβ, and γ chains, respectively, and the amino termini of all the six chains are held together by disulfide bonds in the central nodule [76-85].

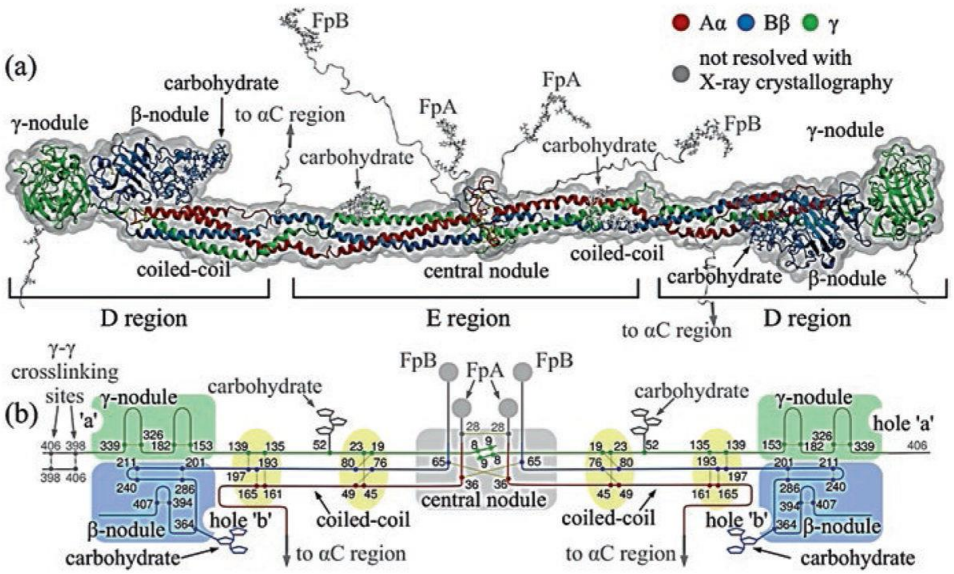

Figure 23: Fibrinogen structure [79].

By means of transmission electron microscopy, atomic force microscopy, and X-ray crystallographic data, it has been shown that the fibrinogen molecule has an elongated shape, 45 nm in length and ~2–5 nm in diameter. The atomic resolution structure of more than two-thirds of fibrinogen has been accomplished through X-ray crystallography. The unresolved portions have been reconstructed computationally to gain a complete molecular structure of fibrinogen. Unlike other crystallographically unresolved parts of fibrinogen, the 390 residue-long C-termini of the Aα chains (residues 221–610), named the αC regions, were visualized using transmission electron microscopy and atomic force microscopy. The αC regions fold back from the distal ends of the triple coiled-coils to form a fourth strand and then extend outward via a flexible connector to relatively compact C-terminal domains that interact with the central nodule of fibrinogen. Truncation of the αC regions affects substantially the hydrodynamic behaviour of fibrinogen [79].

Fibrinogen is organized into domains, or independently folded structural units [80]. From the X-ray crystallographic studies of fibrinogen, the central E region has four domains and each D region comprises seven domains. The E region is composed of two symmetrical parts, in which the C-terminal parts of the Aα, Bβ and γ chains form a coiled-coil-E domain comprising a triple α-helical structure. The N-terminal parts of two γ chains form an asymmetric γN-domain domain in the center of the E region. The globular part of the E region without the coiled-coil-E domains is often called the central nodule. In the lateral D region, the N-terminal parts of all the Aα, Bβ and γ chains form a coiled-coil-D domain comprising a triple α-helical structure. The C-terminal parts of the β and γ chains make up the β-nodule and

γ-nodule, respectively. They are also named β- or γ-modules and each is made of three domains [79-82].

α-Helical Coiled-Coils of Fibrinogen

The central nodule and end globular parts of fibrin(ogen) are joined together by 17-nm-long triple (and partially quadruple) α-helical coiled-coil connectors formed by 111 or 112 amino acid residues from each of the Aα, Bβ and γ chains that are bordered by "disulfide rings" (Fig. 13.2). In the α-helical coiled-coil, three right-handed α-helices wind around each other to form a left-handed supercoil. The bent portion of the coiled-coil exposes the cleavage sites that can be hydrolyzed by plasmin and other proteases followed by degradation of fibrinogen into proteolytic fragments. The functional role of the coiled-coil region has been recently ascribed to the tensile deformation of fibrin fibers, namely to the ability to undergo partial unraveling and spring-like reversible extension-contraction [79]

Ca^{2+}-Binding Sites in Fibrinogen

Fibrinogen has both strong and weak binding sites for calcium ions, which are important for its functions, including fibrin polymerization, and lytic stability. High-affinity binding sites (named γ1) for calcium ions are present in the γ chains and are associated with four coordinating amino acid residues and two strongly bound water molecules. Other high-affinity Ca^{2+}-binding sites (named β1) are located in the β-nodules in loop β381–385, each of which has one coordinating water molecule.

Two other Ca^{2+}-binding sites named γ2 and β2 have much lower affinities. It is likely that the γ2 sites are formed as a result of molecular rearrangements induced by crystal packing. The β2 sites anchor the β-nodules to the coiled-coil connector and were shown to be involved in the lateral aggregation of protofibrils. It has been proposed that the β2 sites regulate accessibility of the tissue plasminogen activator (t-PA)-binding site in fibrin(ogen) [79,82].

Carbohydrate Moieties of Fibrinogen

Four oligosaccharide chains are linked to each molecule of fibrinogen by way of N-glycosidic bonds: two are connected to the BβAsn364 residues in the β-nodule and the other two are connected to γAsn52 in the coiled-coils. These carbohydrate attachment sites contain the classic NXS or NXT sequences that are typical of N-glycosylation. Variable desialylation or removal of the terminal N-acetylneuraminic acid residue, are responsible for part of the heterogeneity of circulating fibrinogen.

The carbohydrate portion of fibrinogen affects fibrin polymerization and clot structure. Patients with cirrhosis and other liver diseases have fibrinogen with high levels of sialyation, resulting in fibrin networks containing thinner fibers with a higher density of branch points. Contrary, removal of carbohydrate has remarkable effects on clot structure with very thick fibers. These data highlight that both the charge and mass of the carbohydrate help to modulate the extent of lateral aggregation and that the carbohydrate moieties significantly enhance the solubility of fibrinogen [79].

Biosynthesis of Fibrinogen

Fibrinogen is the product of three closely linked genes, FGA, FGB, and FGG, each specifying the primary structure of one of its three polypeptide chains, Aα, Bβ, and γ, respectively. The fibrinogen genes encoding for these chains are found in a cluster of 65 kilo bases on human chromosome 4 (4q23-q32) and they translate into nascent polypeptides of pre-pro-Aα chain (644 amino acid residues), pre-pro-Bβ chain (491 residues), and pre-pro-γ chain (437 residues) [88]. The alternative splicing of a γ chain mRNA produces 8–15 % of plasma fibrinogen molecules in which the γ chain C-terminal 400–411 dodecapeptide (γA chain) is altered by adding new amino acids from 408 to 427 (γ′ chain).

Before fibrinogen is secreted in the bloodstream, it undergoes different steps of assembly of the polypeptide chains. Following translation of each of the chains, the interactions of the chains with chaperone proteins and quality control mechanisms distinguish properly assembled fibrinogen from unassembled forms that are degraded intracellularly [78,79,81].

During translocation of the single polypeptides into the lumen of the endoplasmic reticulum, a signal peptide is cleaved from each chain. After full processing and assembly in the endoplasmic reticulum and Golgi organelles, there are 610, 461, and 411 amino acids in each of the common final forms of the human Aα, Bβ and γ chains, respectively, remaining from the corresponding nascent pre-pro-polypeptides [79].

In the endoplasmic reticulum there is a progression from single chains to two-chain complexes to trimeric half molecules. Assembly of the six chains takes place in a stepwise manner in which single chains assemble first into Aα-γ and Bβ-γ complexes, then into Aα/Bβ/γ half-molecules, and finally into hexameric complexes $(Aα/Bβ/γ)_2$[88]. Although the distal clusters of interchain disulfide bonds (named “disulfide rings”) are not necessary for assembly of the two half molecules, they are necessary for secretion. In addition to assembly and formation of disulfide bonds, before secretion fibrinogen undergoes a number of co-translational and post-translational modifications (Figure 24).

The structure of fibrinogen can be modified even after it has been secreted into the blood, for example by limited proteolysis, oxidation and glycation [79].

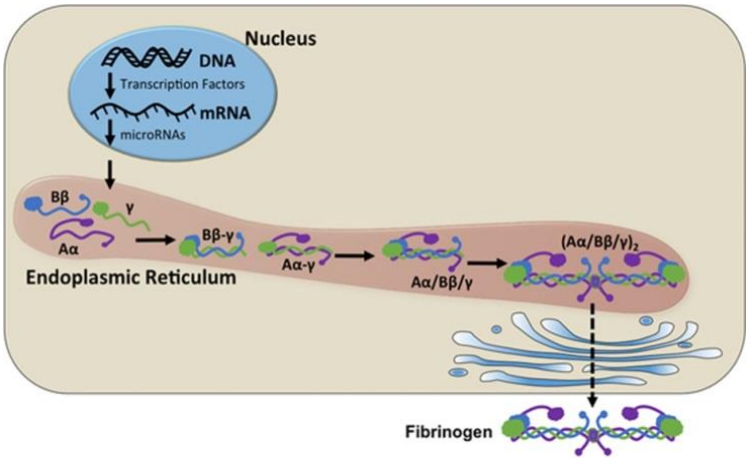

Figure 24: Fibrinogen synthesis [89].

Fibrinogen Metabolism

The liver is the primary source of plasma fibrinogen, with a steady state rate of synthesis of 1.7–5 g per day and a large intracellular reserve. Three quarters of human fibrinogen is present in the plasma but it is also in platelets, lymph and interstitial fluid. The normal half-life of fibrinogen is 3–5 days, but despite the numerous studies on the distribution of iodine-labeled fibrinogen, its physiological catabolic pathway is largely unknown. Coagulation and fibrinolysis accounts for only 2–3 % of fibrinogen loss in healthy individuals [79,83,84].

Fibrinogen is one of the acute phase proteins, which are up-regulated in response to injury and inflammation, followed by an up to ten-fold increase in its concentration in the blood. The up-regulation is mediated largely by IL-6 and perhaps other pro-inflammatory mediators that trigger intracellular signaling pathways in hepatocytes and modulate gene expression via various transcription factors [78,85,86].

Although the liver is the primary source of plasma fibrinogen, the protein is also synthesized in some extra-hepatic tissues. Only fibrinogen γ chain gene expression has been shown in vivo in bone marrow, brain, and lung. Epithelial cells from lung and intestine secrete small amounts of fibrinogen in a polarized manner from their basolateral face. It is possible that lung epithelium secretes fibrinogen and incorporates it into the extracellular matrix under certain pathological conditions, contributing to fibrotic lung disease.

Synthesis of fibrinogen by cultured granulosa cells may reflect a possible function for it in ovulation. Taken together, the normal biological relevance of the synthesis of fibrinogen in extra-hepatic tissues is unclear, but it may become important under certain pathological circumstances [79].

Molecular Mechanisms of the conversion of fibrinogen to fibrin: Polymerization

Conversion of fibrinogen to fibrin is one of the major consequences of the enzymatic cascade of blood coagulation that is essential for stopping bleeding, as well as in vascular obstruction or thrombosis. This process occurs in two major steps: **enzymatic** and **non-enzymatic** (Figure 25) [77,79,82].

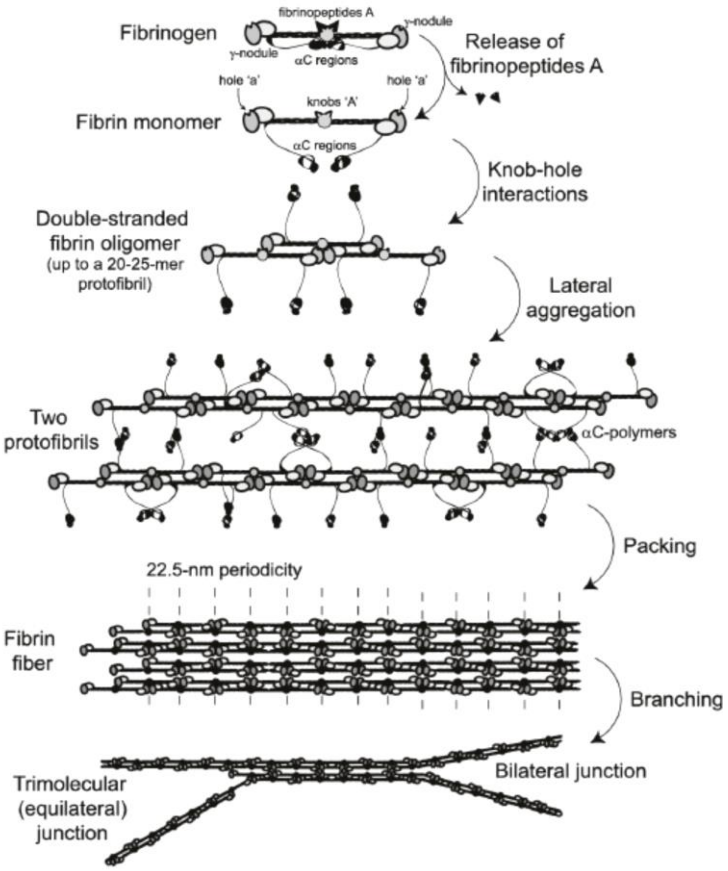

Figure 25: Schematic diagram of fibrin polymerization [82]

At the enzymatic step: there is thrombin-catalyzed cleavage of the fibrinopeptides (Fp) of fibrinogen to form the fibrin monomer. Thrombin is a highly specific serine protease upon activation of its zymogen, prothrombin, normally present in the blood.

Fibrin polymerization is triggered when thrombin cleaves FpA (residues 1-16) and FpB (residues 1-14) from the N-terminal portions of the Aα and Bβ chains of fibrinogen, respectively, producing monomeric fibrin [82].

With polymerization, the rate of release of FpBs increases, reaching maximum when polymerization is almost complete, indicating that they are preferentially released from fibrin polymer. In surface-attached fibrinogen, unlike in solution, FpBs are cleaved at a faster rate than FpAs, depending on fibrinogen surface density and orientation, indicating that the conformation of fibrinogen determines the ability of thrombin to access and cleave FpAs and FpBs differentially. This distinction in the cleavage rate of FpA and FpB is based on the spatial restrictions of the binding of thrombin to fibrinogen, implying that the N-termini of the Aα chain containing FpA are more accessible to the active site of thrombin. When thrombin cleavage sites are mutated at positions AαArg16 or BβArg14 (such as in dysfibrinogenemias) the release of FpA or FpB is precluded, leading to impaired fibrin formation [82-85].

At the non-enzymatic stage: the monomeric fibrin self-assembles spontaneously to yield fibrin oligomers that lengthen to make two-stranded protofibrils. Protofibrils aggregate both laterally and longitudinally to form fibers that branch to yield a three-dimensional gelled network called a clot [82].

After cleavage of FpAs, the α chains have new N-terminal sequences Gly-Pro-Arg-(GPR) named knobs 'A'. During fibrin polymerization, knobs 'A' interact with holes or pockets 'a' that are always open in the γ-nodules of the interacting fibrin molecules: the binding of knobs 'A' to holes 'a' is called the 'A-a' interaction. The 'A-a' interactions have been characterized biophysically at the single-molecule level as quite strong, highly specific, and stable intermolecular associations [82-85].

Structures of fragment D (corresponding to the lateral D regions containing the γ-nodule) co-crystallized with the peptide GPRP. The hot spots of holes 'a' directly involved in the binding of the GPRP peptide were identified as residues γTrp315-Trp330, γTrp335-Asn365, and γPhe295-Thr305. The existence of constitutively open holes 'a' in fibrinogen and fibrin is also essential for fibrin polymerization. If the holes 'a' are obstructed by the GPRP peptide or compromised, fibrin polymerization is abrogated. Overall, these and other data suggest that fibrin polymerization and clot formation are driven by the 'A-a' interactions [82-84].

Fibrin polymerization begins when two fibrin monomer molecules interact to form a half-staggered dimer in which knob 'A' binds hole 'a', and there are two 'A-a' knob-hole

interactions holding the two monomers together. A third fibrin molecule added to a half-staggered dimer forms an end-to-end connection where the two adjacent molecules touch each other and the lateral D regions of two molecules form the D:D interface that comprises the junction between monomers in each of two strands in fibrin oligomers. The D:D interface comprises residues γ275–309 and is very weak, because it yields first upon forced stretching of fibrin(ogen) oligomers. It was found that the D:D interactions are essential for elongation of fibrin strands. Fibrin monomers can add longitudinally to form longer two-stranded fibrin oligomers of varying length. Lateral interactions between two strands of fibrin oligomers are mediated by the central E region of one fibrin molecule and two lateral D regions of two other molecules. The D-E-D complex is held together mainly by the 'A-a' knob-hole bonds and by additional interactions at the D:E and D:D interfaces [82].

Fibrin monomers continue to add longitudinally to the oligomers, which lengthen further to make two-stranded protofibrils, a critically important intermediate product of fibrin polymerization. Protofibrils are usually about 0.5–0.6 μm in length, which corresponds to ~20–25 monomers, and they are long enough to self-interact and aggregate laterally.

Protofibrils aggregate laterally to form more or less thick fibers, in which the half-staggered molecular packing gives rise to a 22.5-nm periodicity corresponding to half the length of the fibrin molecule. Importantly, protofibrils self-associate laterally only after they reach a certain threshold length, implying that the bonds mediating the interactions between protofibrils are weak and cooperative along the axis of the protofibril [82-85].

When FpB is cleaved off, the β chain acquires a new N-terminal sequence Gly-His-Arg-Pro (GHRP), comprising knob 'B' capable of binding to hole 'b' located in the β-nodule. The peptide GHRP, which reproduces the structure of knob 'B', has a much lower equilibrium binding affinity for fibrinogen (Kd = 140 μM) compared to the peptide GPRP (Kd = 25 μM). Despite direct evidence for the existence of 'B-b' interactions the role of the 'B-b' interactions in fibrin formation is not quite clear because it is supported only indirectly.

During fibrin polymerization, the protruding and flexible αC regions can self-interact both within and between protofibrils; these αC-αC interactions can lead to formation of αC polymers that are reinforced with additional crosslinking by Factor XIIIa. The αC-αC polymerization involves two mechanisms:

1. Self-association of the αC-domains that occurs via their N-terminal subdomains by β-hairpin swapping;

2. The interaction of the C-terminal subdomain with the αC-connector.

Fibrinogen lacking the αC regions forms clots with thinner fibers and a higher density of branch points than clots made of full-length fibrinogen.

As fibrin fibers thicken by lateral aggregation and grow in length, they also branch, yielding a space-filling 3D network. Electron microscopy revealed at least two different molecular mechanisms by which branch points may form (Figure 26):

1. One of them known as a **"bilateral junction"**, originates from two protofibrils that undergo incomplete lateral aggregation but diverge into two separate protofibrils, each of them giving rise to a new fiber.
2. The second type of branchpoint, called a **"trimolecular junction" or "equilateral junction"**, forms when a fibrin monomer is attached to the end of a protofibril via only one 'A-a' bond (or one γ-nodule), such that both the monomeric molecule and the protofibril to which it is bound can grow independently, forming two strands each.

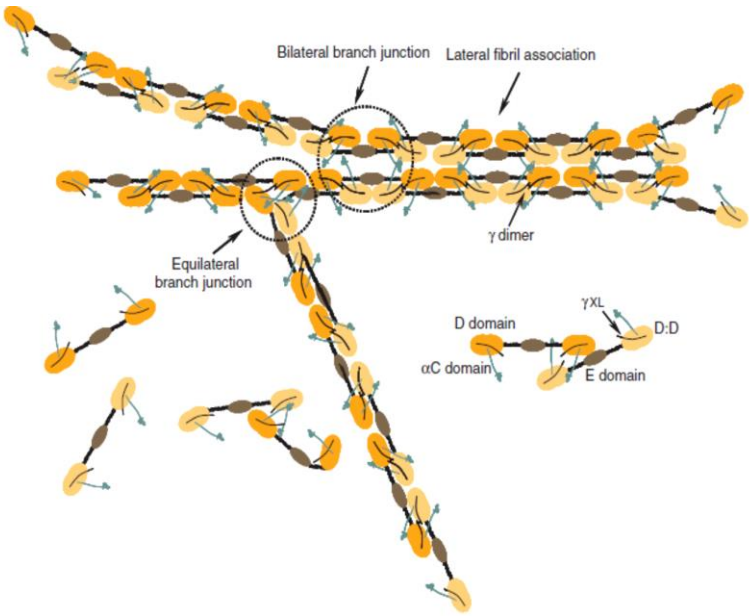

Figure 26. Schematic diagram of fibrin assembly, branching, lateral fibril association, and c-chain cross-linking [76].

In either case, most of branch points in clots consist of three fibers of about the same diameters joined together, suggesting that the type of initial branchpoint does not affect much the final network structure. The structure of fibrin networks can be determined and quantified using scanning electron microscopy by a number of parameters, such as the fiber diameter, fiber density, number of branch points, fiber length, and the size of the pores. All of these parameters are variable and depend on the kinetics of fibrin polymerization [76,77,79,80].

Finally, to stabilize the clot against proteolytic and mechanical insults, fibrin is covalently crosslinked by the plasma transglutaminase, Factor XIIIa, an active from of Factor XIII zymogen activated by thrombin in the presence of Ca^{2+}. The C-terminal ends of the γ chains of fibrin(ogen) have amino acid residues comprising a crosslinking site for two end-to-end interacting molecules that form covalent isopeptide ε-(γ-glutamyl)-lysyl bonds between the γLys406 of one molecule and γGln398/399 of another molecule. After crosslinking within and between protofibrils, polymerization becomes irreversible, and the clot is more stable, mechanically strong, and resistant to fibrinolysis. The crosslinked fibrin can be dissolved either by reduction of disulphide bonds that hold polypeptide chains together or by chemical/enzymatic hydrolysis of peptide bonds [76,77].

Fibrinolysis

After a clot has formed in vivo and fulfilled its haemostatic function, it is normally dissolved by the fibrinolytic system, to restore the impaired blood flow [91]. Coagulation and fibrinolysis are precisely regulated by the measured participation of substrates, activators, inhibitors, cofactors and receptors. Molecular links between these systems permit localized, timely removal of ongoing or acutely induced fibrin deposits.

Plasmin is the major fibrinolytic protease. Plasminogen (PLG), a circulating plasma zymogen, can be converted to plasmin by both tissue PLG activator (tPA) as well as by urokinase (uPA). Through a positive feedback mechanism, plasmin cleaves both tPA and uPA, transforming them from single chain (sc-tPA or sc-uPA) to more active two-chain polypeptides (tc-tPA or tc-uPA) [79] .

Plasmin also cleaves several substrates, including extracellular matrix proteins, and activates some proteases and growth factors. So, PLG and plasmin are involved in several other pathophysiological processes, such as wound healing, inflammation, cell migration, angiogenesis, embryogenesis, ovulation, tumorigenesis, and atherosclerosis [90]. Diverse cell types promote plasmin generation through their expression of cell surface receptors. Endothelial cells, monocytes, macrophages, neutrophils and some tumour cells, all bind PLG, as well as tPA and/or uPA. Their receptors localize cell surface fibrinolytic activity, serve as cofactors in acute or ongoing plasmin generation, and provide specialized environments that are protected from circulating inhibitors [79].

Fibrin, the major plasmin substrate, regulates its own degradation by binding both PLG and tPA on its surface, thereby localizing and enhancing plasmin generation. Once formed,

plasmin cleaves fibrin, generating soluble degradation products, and exposing carboxy-terminal lysine (Lys) residues. Endogenous inhibitors of fibrinolysis target both tPA and plasmin, and include plasminogen activator inhibitor-1 (PAI-1), α_2-antiplasmin (α_2-PI), and thrombin-activatable fibrinolysis inhibitor (TAFI). The figure 27 depicts the fibrinolytic process [77,79].

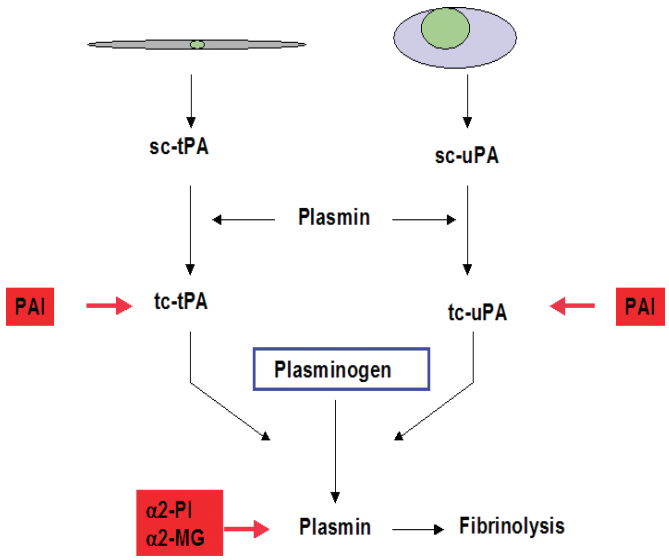

Figure 27: Overview of the fibrinolytic system. Inhibitors are indicated by red boxes [90].

Fibrinogen possesses distinct proteolytic cleavage sites for plasmin, which give rise to fragments [Aa, Bb and fragment fibrinopeptide B (FPB)] from the C- and N-termini of fibrinogen's three polypeptide chains. The resulting Mr c. 250 000 molecule is termed fragment X and represents a clottable form of fibrinogen. Subsequently, plasmin cleaves the three polypeptide chains that connect the D- and E-domains, giving rise to fragments D, Y and E. Some of these fragments inhibit the spontaneous polymerization of fibrinogen [77,79].

Enzymatic lysis of fibrin results in formation of soluble degradation products. The fibrin, cross-linked by factor XIII, is initially cleaved by the plasmin at the level of the C-terminal region of the a- and b-chains within the D-domain. Subsequently, some of the connecting regions between the D- and E-domains are severed. Fibrin is ultimately solubilized upon hydrolysis of additional peptide bonds within the central portions of the coiled-coil connectors, giving rise to fibrin degradation products. The smallest crosslinked fibrin degradation product is D-dimer formed of two D regions of the adjacent fibrin molecules connected by a γ-γ bond (Figure 28) .

Because the only source of D-dimer is crosslinked fibrin, a high level of D-dimer-containing products in blood is widely used by clinicians as a laboratory sign of intravascular fibrin deposition occurring during local thrombosis or disseminated intravascular coagulation [91].

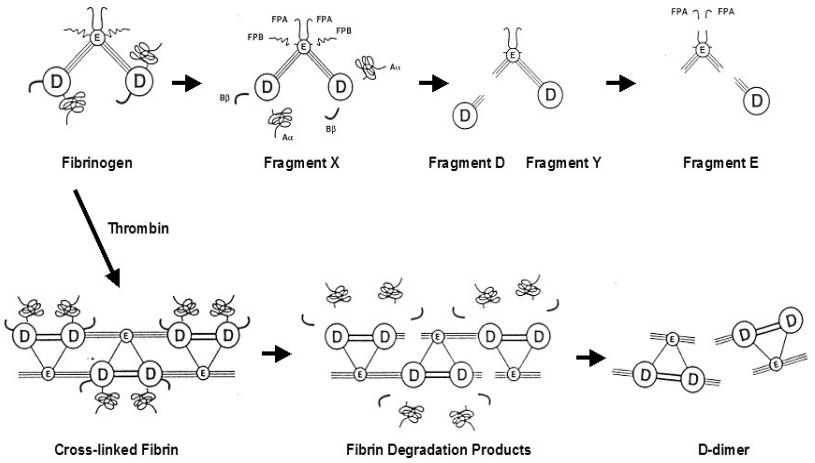

Figure 28: Degradation of fibrinogen and cross-linked fibrin by plasmin.

Variations and modulation of fibrinogen structure and properties

Fibrinogen heterogeneity is the result of several types of variation: genetic polymorphisms, alternative mRNA processing, proteolytic cleavage, environmental factors, and post-translational modifications of fibrinogen. The different combinations of these determinants lead to more than a million forms of fibrinogen within a healthy individual [88]. Therefore, some of these alterations can also affect the function of fibrinogen, consequently influencing clot formation, clot structure and susceptibility to clot lysis. These effects could have consequences in the occurrence and progression of thrombotic diseases [88,93-95].

Genetic Polymorphisms of Fibrinogen

Variants of fibrinogen are present in the blood as a result of several common polymorphisms or normal alternative primary structures. The most abundant fibrinogen variants contain two types of γ chains, γA and γ ′, that result from an alternative polyadenylation signal in intron 9 of the FGG gene. About 8–15 % of total fibrinogen contains the γ′ chain, of which the majority is in the heterodimeric γA/γ′ form with a homodimer γ′/γ′ comprising only about 1 %. The presence of γ′ chains was shown to slow down lateral aggregation of protofibrils and alter fibrin formation and structure. An increase in plasma fibrinogen γ′ concentration is associated with the risk of myocardial infarction and other thrombotic states.

Fibrinogen polymorphisms with functional consequences can occur in the polypeptide chains other than the γ chain. αE fibrinogen has a reduced rate of fibrin polymerization and forms

thinner and more branched fibers than fibrin networks containing the α chains. The fibrinogen Aα chain gene FGA polymorphism 2224G/A has been associated with reduced clot permeability, while the Ala312 allele of FGA 6534/Thr312Ala was associated with increased clot stiffness, and the Lys448 allele of the fibrinogen Bβ chain gene (FGB) polymorphism BβArg448Lys resulted in a compact fibrin network structure resistant to lysis. The other most widespread polymorphisms in the fibrinogen genes occur in the noncoding regions and can result in changes in plasma fibrinogen levels [82-88].

Post-translational Modifications of Fibrinogen

There are many molecular forms of fibrinogen present in blood, as detected from variations in biochemical properties and gel electrophoretic behaviour. Several of these variations arise by post-translational heterogeneities originating from multiple biochemical reactions that accompany various physiological and especially pathological conditions, such as inflammation or ischemia.

These reactions can modify the fibrinogen molecule in many ways, such as phosphorylation at specific seryl and threonyl sites, prolyl hydroxylation, tyrosyl sulfation, asparaginyl or glutaminyl deamidation, N-terminal pyroglutamate formation from glutaminyl precursors, oxidation of methionine, histidine and tryptophan residues, tyrosine nitration, modifications of cysteine residues, formation of dityrosine and carbonyl groups [87,88].

Oxidative stress has been widely implicated in physiological processes such as aging, in various disease pathogenesis, including arterial and venous thrombosis. Proteins are the main targets for ROS that may alter every level of their structure from primary to quaternary, causing physical changes. Compared with other plasma proteins, fibrinogen is more susceptible to oxidation than most other plasma proteins; in particular, fibrinogen is 20X more susceptible to oxidation than albumin [72,73,78,96,97]. For this reason, oxidation is the most studied post-translational modification of fibrinogen. Most studies used (human) fibrinogen and added a compound or condition that oxidizes fibrinogen (reactive oxygen species, ozone, or illumination). However, the conditions used *in vitro* show a lot of variation. A few studies used fibrinogen from patients with diseases known to increase oxidative stress, and therefore the level of fibrinogen oxidation is increased. Overall, oxidation seems to decrease the rate of clotting and results in more dense fibrin clots with thinner fibers which are less permeable [81,88,93-95].

A post-translational modification that is relatively similar to oxidation and often studied in combination with oxidation is nitration of fibrinogen. Nitration of fibrinogen mainly affects tyrosine residues, resulting in the formation of 3-nitrotyrosine. Also cysteine residues can be affected, resulting in the formation of 3-nitrocysteine. Nitrated fibrinogen, (primarily tyrosine nitration within the β chain of the molecule) was initially identified in the plasma of subjects with several diseases including acute respiratory distress syndrome, venous thromboembolism (VTE), lung cancer, end stage renal disease. Tobacco smokers tend to have nitration of tyrosine and oxidation of methionine, histidine or tryptophan residues. Many of these chemically modified forms are associated with differences in functional and structural properties of fibrinogen and fibrin, including a thrombogenic phenotype associated with increased risk of arterial and venous thrombosis [81,88,92]

N-glycosylation can be another source of fibrinogen heterogeneity because it may result in formation of oligosaccharides with a variable structure. Acquired abnormal fibrinogen variants occur in patients with several conditions via non-enzymatic reactions, including glycation of lysine residues in uncompensated diabetes mellitus or homocysteinylation in hyperhomocysteinemia [88].

Another modification is the acetylation that occurs in vivo at N-termini of polypeptide chains by N-acetyltransferases or at the ε-amino group of lysine residues by lysine acetyltransferases. Acetylation can also be caused by the intake of aspirin, which is known to exert its beneficial effects in cardiovascular disease by acetylating serine residues in the enzyme platelet cyclooxygenase. In fibrinogen and other coagulation proteins, aspirin can acetylate lysine residues, affecting their functions. Data from several studies show that acetylation of fibrinogen decreases fibrin polymerization and increases permeability and susceptibility to fibrinolysis [88].

In recent years, the effect of phosphorylation or dephosphorylation of fibrinogen on clotting have been studied. In fibrinogen, phosphorylation occurs mainly on serine and threonine located in the Aα chain. It is shown that fetal fibrinogen is phosphorylated to a higher degree than adult fibrinogen, suggesting a functional importance of phosphorylation. In addition, phosphorylation of fibrinogen increases after surgery, potentially contributing to prevention of bleeding. *Ex vivo* studies have highlighted that increased phosphorylation of fibrinogen results in increased clot turbidity and fiber diameters, although this was only investigated by one study using few patients [88].

The figure below summarized the post-translational modifications and its effects on functions of fibrinogen (Figure 29).

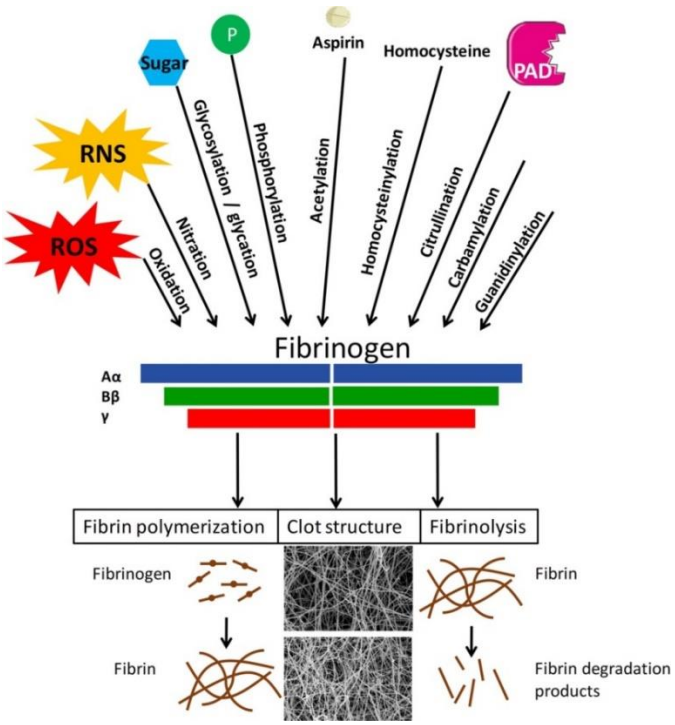

Figure 29: Post-translational modifications of fibrinogen influence the occurrence and progression of thrombotic diseases [88].

Aim of study

EGPA is a complex systemic necrotizing vasculitis with a heterogeneous clinical presentation, often complicated by cardiovascular events. Cardiovascular system is involved in 30% of EGPA patients (especially during active phase of disease) and it is a major cause of morbidity and mortality [5-26]. Many factors contribute to its pathophysiology: genetic predisposition, environmental factors and immune dysregulation, but the pathogenesis are not well elucidated [5-20]. Vessel inflammation and eosinophilic proliferation contribute to organ damage that can be compounded by excessive oxidants production. It is well known that immune system, inflammation and haemostasis are closely linked and play a key role in the thrombotic process [15,16]. Oxidative stress has been widely implicated in several pathological conditions such as diabetes, degenerative diseases, autoimmune diseases, chronic inflammatory disorders, cardiovascular disease and in the etiology of arterial and venous thrombosis [29,35-38].

Many studies support oxidative stress and inflammation as interconnected processes that co-exist in the inflamed milieu. Low levels of ROS are essential for the regulation of multiple cellular processes and the signalling pathways, whereas uncontrolled reactive species production leads to oxidative stress that damages all cellular components [29-34]. Fibrinogen plays a key role in blood clotting, fibrinolysis, cellular and matrix interactions, in the inflammatory response and wound healing [82-85]. Compared with other plasma proteins, fibrinogen is especially susceptible to oxidation. Consequences of proteins oxidation are the formation of carbonyl groups and the modification of amino acids that could be the cause of alterations of polypeptide chain. Post-translational modifications also affect the function of fibrinogen, consequently influencing clot formation, clot structure and susceptibility to clot lysis. These effects could have consequences in the occurrence and progression of thrombotic diseases [87-89].

We observed that in myocardial infarction (MI), pulmonary hypertension and Behçet Syndrome patients that fibrinogen oxidative modifications, especially increased fibrinogen carbonyl content, were significantly associated to structural and functional alterations of the protein [96,98-101].

Here, we investigated fibrinogen structure and function, plasma redox status and leukocyte ROS production in 35 EGPA patients and 35 age-matched healthy controls, in order to highlight the mechanisms of inflammation-induced thrombosis.

Materials and Methods

The study sample included 35 patients with EGPA who attended to SOD Medicina Interna Interdisciplinare, AOUC Careggi and 35 age-matched healthy control subjects. Demographic and clinical characteristics of the population studied are summarized in Table 2. All patients were diagnosed as having EGPA disease according to ACR criteria.

Patients	**35**
Age (yrs, means ± SD)	**60 (46-74)**
Gender (n)	
Female	**22 (63%)**
Male	**13 (37%)**
Positive ANCA (MPO o PR3)	**11 (31%)**
General manifestations	**17 (49%)**
Cutaneous manifestations	**9 (26%)**
ENT manifestations	**32 (91%)**
Pulmonary manifestations	**35 (100%)**
Cardiac manifestations	**11 (31%)**
Thrombotic events	**6 (17%)**
TIA	*1 (17%)*
STROKE	*1 (17%)*
DVT	*1 (17%)*
AMI	*2 (33%)*
PRINZMETAL ANGINA	*1 (17%)*
Digestives manifestations	**8 (23%)**
Renal manifestations	**2 (6%)**
Neurological manifestations	**22 (63%)**
BVAS at diagnosis (means ± SD)	12,5 ± 5.1
ANCA+	10 (29%)
p-ANCA+	9 (26%)
c-ANCA+	0
Anti-MPO+	9 (26%)
Anti-PR3+	2 (6%)
Eosinophils count (/mm3)	6944 ± 8824
Treatments	
Corticosteroids	32 (91%)
Cyclosporine	8 (23%)
Methotrexate	6 (17,5%)
Mycophenolate/MMF	8 (23%)
Azathioprine	12 (34%)
Cyclophosphamide (Cyc)	4 (11%)
Intravenous Ig	8 (23%)
Rituximab	15 (43%)
Infliximab	0
Omalizumab	4 (11%)
Others (immunosuppressants)	1 (3%)

Table 2: Clinical characteristics of EGPA patients enrolled in the study.

Patients with other autoimmune diseases, active infections or neoplastic conditions were excluded. The study protocol was approved by local Ethical Committee and informed consent was obtained from all subject enrolled.

Sample 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 (1500 x g for 15 min at 4ºC), the top yellow plasma layer was collected without disturbing the white buffy layer. The aliquots of plasma were used for experiments or stored at -80ºC for further analysis. Another aliquot of sodium citrate plasma was used for fibrinogen purification.

Fibrinogen Purification

Fibrinogen (from 10 ml of citrated plasma) was purified using the previously described ethanol precipitation method [96,97,99,101].

After the purification procedure, control and patient fibrinogen samples were extensively dialyzed against 100 mmol/L Tris/HCl buffer, pH 7.4. Fibrinogen concentration was determined by ultraviolet spectroscopy (ONDA UV-20) at a wavelenght of 280 nm, assuming an extinction coefficient of 1.51 $(mg/ml)^{-1}$.

The purity of the fibrinogen preparations was assessed by densitometry of Coomassie-stained polyacrylamide gels after electrophoresis under reducing conditions. The yield of purified fibrinogen was not statistically different between patients and controls.

ROS assessment in leukocytes (lymphocytes, monocytes, granulocytes and eosinophils) by Flow Cytometry Analysis

Flow cytometry is a vital tool in biological research and clinical diagnostic. This instrument is capable of rapid and highly quantitative interrogation of individual cells for the presence or absence of a wide range of fluorescence and light scattering signals that correlate to cell morphology, surface and intracellular protein expression, gene expression, and cellular physiology. A variety of fluorescent reagents are utilized in flow cytometry, that includes fluorescently conjugated antibodies, DNA binding dyes, viability dyes, ion indicator dyes and fluorescent expression proteins [102-105].

Flow cytometer (Figure 30) is characterized by five main components: a flow chamber, a measuring system, a detector, an amplification system, and a computer (to output the data to a form that can be analyzed by a researcher).

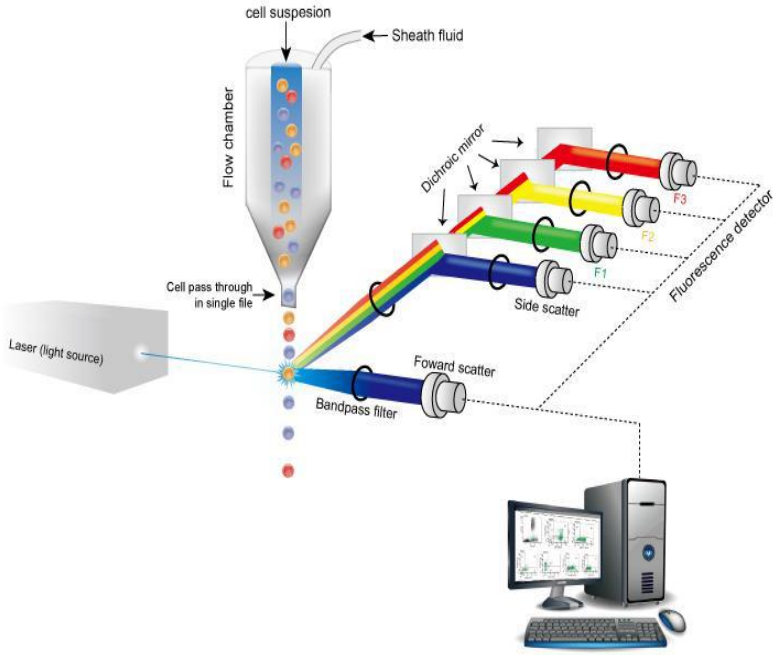

Figure 30: Main components of flow cytometer.

In all cytometers, cells or particles that need to be analysed, are in suspension and pressurized into a directed fluid stream.

To ensure that particles emerge in single file for analysis, the sample is injected as a core stream into a larger, surrounding column of flowing sheath fluid and under conditions of laminar flow, it becomes hydronamically focused within the sheath stream. Air or another gas pressurizes both streams, and the differential pressure between the streams controls sample injection rate. Cells or particles must fall in a range of 1-30 μm in diameter.

This stream containing particles sequentially intersects one or more laser beams placed orthogonal to the flow of fluid. The laser beams are focused such that they only illuminate a single particle at any given time. Lasers used in cytometers are represented by gas lasers (e.g., argon ion, krypton ion, or helium–neon lasers) or solid state lasers (e.g., blue solid state laser, red or green diode lasers, violet or near-UV diode lasers, or the relatively new orange fiber laser). Wavelengths of the light from a given laser are defined and inflexible, based on the characteristics of the lasing medium.

The most common laser is an argon ion laser that produces a turquoise light (488 nm) used to excite several dyes including dyes, phycoerythrin, propidium iodide, peridinin chlorophyll protein (PerCP).

If the given cell or particle contains a fluorescent tag that is excitable by the laser, a measurable pulse of photons of a specific wavelength will be emitted. Signals are collected by an array of photo-detectors and optical filters, processed by specialized electronics, and stored

on a computer. The function of the flow cytometer electronics system is twofold: it converts light signals into electronic signals (voltages) and it performs data analysis.
At constant laser power, the intensity of emission will be dependent on the number of present fluorophores, thereby making flow cytometry both a qualitative and highly quantitative analysis tool. By using multiple lasers with different excitation wavelengths, the potential number of excitable fluorophores is increased, as fluorescence signals are emitted sequentially from the same particle intersecting each laser. The produced signals are then processed using appropriate cytometer calibration and displayed to correspond to an individual particle.
In addition to fluorescence signals, incident laser light is also scattered from the flowing cells. Light scattered in the forward direction is proportional to the size of the particle, while light perpendicularly scattered (orthogonal to the incident laser light) correlates with intracellular granularity. Accordingly, granulocytes show higher side scatter (SSC) values than lymphocytes because of their irregular nuclei. Thus, as a result of light scattering data alone, useful information regarding cell morphology could be obtained [102-103].

Many fluorescent probes for the detection of reactive species have been developed in the last years, with a different degree of specificity and sensitivity. In the following experiments, the dichlorofluorescein diacetate H_2DCFDA probe (Invitrogen, CA, USA) is used for the evaluation of intracellular ROS. This probe is the most versatile indicator of cellular oxidative stress and represents the gold standard for ROS measurement. H_2DCF-DA (2',7'-dichlorofluorescein diacetate) is a cell-permeable peroxide-sensitive fluorescent probe used for intracellular free radicals (hydrogen peroxide, peroxynitrite, and hydroxyl radicals) detection. Once H_2DCF-DA enters into the cells, intracellular esterases cleave the probe at the two ester bonds, producing a relatively polar and cell membrane-impermeable product, DCFH. This non-fluorescent molecule accumulates intracellularly and subsequent oxidation yields the highly green fluorescent product 2',7-dichlorofluorescein (DCF) measured by flow cytometer (Figure 31) [101-105].

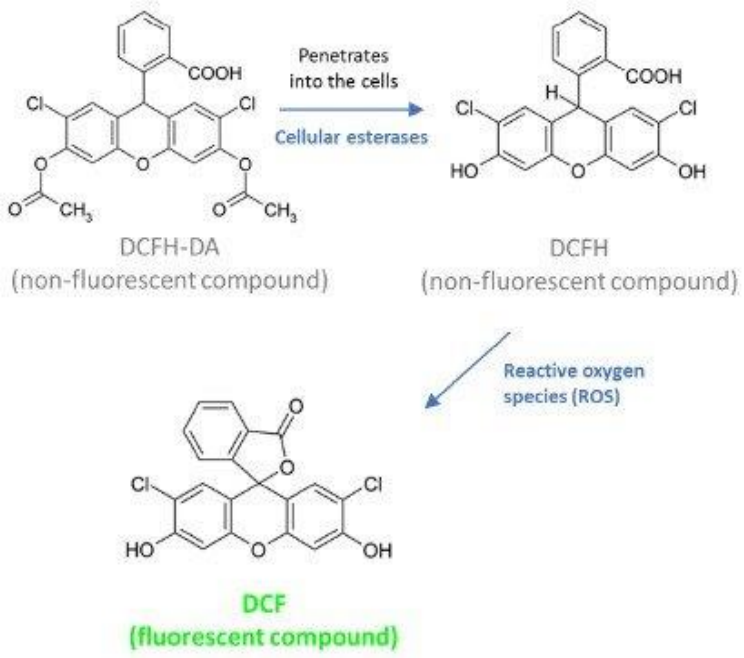

Figure 31: The generally formation mechanism of DCF in cells.

Protocol

- After collection, 100 µl EDTA-anticoagulated blood samples was re-suspended in 2,5 mL of BD FACS Lysing Solution 10X (Becton Dickinson Biosciences, San Jose, CA), gently mixed, and incubated at room temperature in the dark for 15 minutes;
- After incubation cells were centrifuged (20°, 700 x g for 7 minutes) and the supernatant was discarded;
- To determine the level of intracellular ROS generation, cells were incubated with 250 µl H_2DCF-DA (2.5 µmol/L; Invitrogen, Carlsbad, CA) for 30 minutes at 37°;
- After incubation, samples were immediately analyzed using a FACSCanto flow cytometer (Becton-Dickinson, San Jose, CA).
- Data were analyzed using BD FACSDiva software (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 20.000 leukocytes were collected. The respective gates were defined using the distinctive forward-scatter and side-scatter properties of the individual cell populations. Forward and side scatter were used for identification of leukocyte subpopulations (lymphocytes, monocytes and neutrophils) and were also used to exclude debris and dead cells. Moreover, the viability of the cells was controlled by flow cytometry with propidium

iodide staining and was found to exceed 95%. Data were analyzed using BD FACSDiva software (Becton-Dickinson, San Jose, CA).

Plasma Lipid Peroxidation Estimation (ALDetect Lipid Peroxidation assay)

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues. ALDetect Lipid Peroxidation assay is a method of choice for screening and monitoring lipid peroxidation in several types of samples including human and animal tissues, biological fluids (such as plasma, serum, urine), drugs and food products.

Plasma lipid peroxides levels were estimated using a ALDetect Lipid Peroxidation assay (BML-AK170-Enzo Life) following the manufacturer's sheet. The BML-AK170 assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA and 4-hydroxyalkenals to yield a stable chromophore with maximal absorbance at 586 nm. One molecule of either MDA or 4-hydroxyalkenal reacts with 2 molecules of N-methyl-2-phenylindole to yield a stable chromophore (after 4h at 45°C) as reported below.

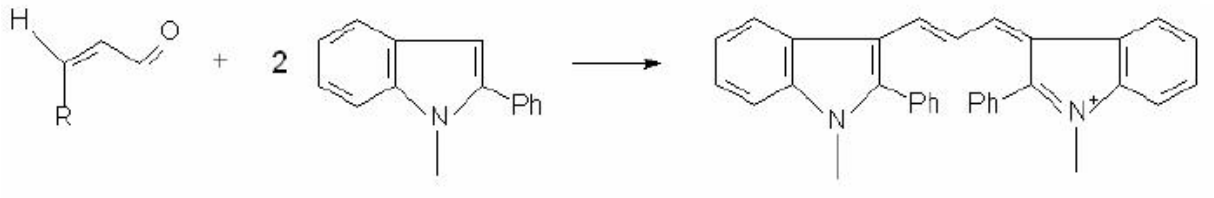

Figure 32: The reaction of a chromogenic reagent with MDA and 4-hydroxyalkenals.

The adduct can be measured in colorimetric determination at 586 nm in a Microplate Fluorometer (Biotek Synergy H1). In this assay, an MDA standard is used for the elaboration of a standard curve against which unknown samples can be plotted. Results are expresses as equivalent of MDA (nmol/ml).

Procedure

- The frozen aliquots were thawed at room temperature;
- Label each disposable test tube with the standard number or sample identification;
- Add 200 µl plasma sample or MDA standard, in duplicate, to appropriately labelled tube. The scheme for the preparation of MDA standard curve is in the instruction manual of kit.

- Add 450 µl R1 Reagent (N-methyl-2-phenylindole, in acetonitrile) to each tube and swirl to mix.
- Add 100 µl R2 Reagent drop by drop in each tube.
- Close each tube and incubate at 45º C for 4 hours.
- Remove from incubation and cool to room temperature in an ice bath to stop reaction. Incubate on ice for 10 minutes.
- Centrifuge samples for 20 min at 4°C at 15000 x g.
- Transfer the clear supernatant to another test tube with the standard number or sample identification;
- Centrifuge samples for 10 min at 4°C at 15000 x g.
- Remove supernatant from samples and load 150µl (in duplicate) from each tube to either the plate.
- Read the absorbance at 586 nm in a microplate reader.

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

The ORAC (oxygen radical absorbance capacity) assay is a simple, sensitive, and reliable method to determine the antioxidant capacity of biological fluids. It can also be used to assay the antioxidant activity of naturally occurring or synthetic compounds suggested as dietary supplements or therapeutics. This kinetic assay detects the oxidative degradation of a fluorescent probe after adding AAPH (2,2'-azobis-2-methyl-propanimidamide), a thermal decomposed azo-radical generator (Figure 33). ROS-mediated (especially peroxyl radical) fluorescein oxidation results in the intensity fluorescence decay of the probe. Antioxidants are considered in order to protect the fluorescent molecule from the oxidative degeneration.

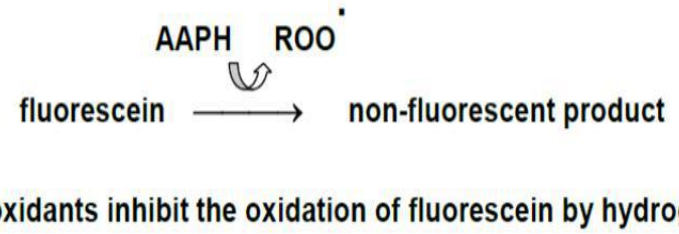

Figure 33: the reaction between AAPH and Fluorescein

In this assay Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue, was used as a standard molecule to quantify the antioxidant capacity of each tested sample.

All ORAC analyses were performed with ORAC ASSAY KIT (Oxford Biomedical Reasearch).

Preparation of reagents:

- Fluorescein solution 10 nM was prepared daily from a 1mM stock in DMSO;
- AAPH solution 400 mM in PBS;
- Trolox 200 µM final concentration was prepared daily from a 20 mM stock in ethanol; Trolox 200 µM was used to construct a standard curve (Table n°4) against which unknown samples can be plotted.

Procedure

- The frozen aliquots were thawed at room temperature.
- Samples were diluted 1:200 with PBS in labelled eppendorf with the standard number or sample identification.
- Standard curve preparation: the amount of Trolox 200µM and PBS were added to each of the inner well of a 96 MW in duplicate as described in Table n°4.

Standard curve	Trolox 200 µM	PBS
0	0 µl	70 µl
10	3,5 µl	66,5 µl
25	8,75 µl	61,25 µl
50	17,5 µl	52,5 µl
75	26,25 µl	43,75 µl
100	35 µl	35 µl
150	52,5 µl	17,5 µl
200	70 µl	0 µl

Table n°4: Trolox standard curve.

- Add 70 µl of each diluted plasma sample to a 96 MW in duplicate;
- Add 100 µl of fluorescein to each of the inner well and pre-incubated the plate for 30 min at 37°C;
- 50 µl of AAPH solution were rapidly added in each well;
- Fluorescence was measured at 37°C in a Biotek Synergy H1 Microplate Reader with excitation wavelength of 485 nm and maximum emission wavelength of at 537 nm. Probe fluorescent intensity is typically measured every 30 seconds, for 4 hours, after the addition of AAPH.

The loss of fluorescence intensity is an indication of the extent oxidative damage but the presence of antioxidants slows the fluorescence decay. Curves of intensity fluorescence decay (fluorescence intensity vs. time) are recorded at the end of experiment and the respective AUC (area under the curve) are measured. TAC of each sample is calculated using the standard curve based on Trolox concentration. Results are expressed as Trolox equivalents (μM) and then normalized for protein concentration.

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 Di-Tyrosine Fluorescence Spectra were recorded at 25°C in a 1 cm quartz cells 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.

Evaluation of Thrombin-Catalyzed Fibrin Polymerization

An in vitro analysis that assesses fibrinogen function is thrombin-catalyzed fibrin polymerization assay. Before the polymerization assay, fibrinogen samples were diluted to a final concentration of 40μg/ml in PBS with Ca^{2+}-Mg^{2+}.The polymerization reaction was started by adding 100 μl thrombin (at a final concentration of 0.2 U/ml in PBS with Ca^{2+}-Mg^{2+}). This kinetics process was monitored at 405 nm in a 96-well microtiter plate reader (Synergy H1 Hybrid Multi-Mode Reader, BioTek Instruments Inc., Winooski, VT, USA) at 25°C for 3 hours. The procedure was performed in accordance to the protocol previously described in our recent studies [96-101].
Absorbance curves were characterized using the following parameters:

I. The lag phase, measured as the time elapsed from t0 until an increase in absorbance was observed, which reflects the time to the start of lateral fibril aggregation;
II. The maximum slope (Vmax), calculated as the slope of the steepest part of the polymerization curve, which represents the rate of lateral protofibril association;
III. The maximum absorbance (MaxAbs) of the growing clot, i.e. the maximum value of absorbance recorded at the end of the kinetic polymerization. The value of MaxAbs reflects the average fibrin fiber size and the number of protofibrils per fiber.

Evaluation of Fibrin Susceptibility to Plasmin-Induced Lysis

Another functional analysis is the fibrin digestion with plasmin and the next electrophoretic analysis of plasmin digests. According to our protocol performed during other studies [96-101], fibrin clots was prepared in 1,5 ml microcentrifuge tubes by incubating bovine thrombin (50 U/ml final concentration) with fibrinogen (10 μg/ml final concentration) in 20 μl of 100 mM Tris/HCl, 5 mM $CaCl_2$, pH 7.4, for 1 h at 25 °C.

Plasmin was added (5 μl of 50U/ml), and the fibrin clots was digested over a period of 6 h at 37°C. The digestion reaction was terminated by adding 10μl of Lithium Dodecyl Sulfate (LDS) Gel Electrophoresis Sample Buffer (50mM Dithiothreitol, DTT and LDS 4X).The same lot of thrombin and of plasmin were used for all experiments.

Samples was heated at 90 °C for 15 min under reducing conditions. Then, aliquots from each digest were loaded onto 4–12% Criterion XT Bis-Tris Protein Gel. 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).

Analysis of Fibrinogen Structural Features

Circular Dichroism (CD) Spectra of Purified Fibrinogen

Circular dichroism (CD), defined as the unequal absorption of left-handed and right-handed circularly polarized light, is an excellent tool for rapid determination of the secondary structure, folding and binding properties of proteins [106]. CD spectra were recorded on a Jasco 810 Fluorimeter at 25 °C from 350 to 180 nm (far UV), using a purified fibrinogen concentration 1 mg/ml. CD spectra are collected in high transparency quartz cells of 0.2 cm. 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. Molar ellipticity values [q] were calculated according to the equation: [θ] (deg-cm2 dmol-1) = [θ (MRW)]/[10(l)(c)], where θ is the displacement from the baseline value X to the full range in degrees; MRW is the mean residue weight of the amino acids; (l) is the path length of the cell (cm); and (c) is protein concentration (g/ml) [97,99,101].

Intrinsic Fluorescence Spectra of Purified Fibrinogen Spectra

In order to provide information on fibrinogen tertiary structure we performed intrinsic fluorescent spectra. The intrinsic fluorescence of proteins is caused by three amino acid residues with aromatic side chains: tryptophan and to a lesser extent from tyrosine and phenylalanine. Tryptophan residues, which are naturally present in most proteins, are intrinsically fluorescent. Furthermore, the use of intrinsic tryptophan fluorescence offers the possibility to study structure and function of proteins without the need to modify the protein [107]. Spectra Intrinsic fibrinogen fluorescence has detected spectrophotometrically in a 1 cm quartz cells on a Jasco Fluorimeter (Jasco 810) at 25 °C by exciting the protein with 280 nm ultraviolet light and an emission wavelength from 290 to 500 nm. Three spectra were acquired for each sample and then normalized for protein concentration (mg/ml).

Statistical Analysis

All 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. The statistical analysis *t-test* was used for all experiments 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 GraphPad Prism software version 6.01 (GraphPad Software, San Diego, California, USA).

Results

Based on the aim of the study, we assessed, in 35 EGPA patients and in 35 healthy subjects, leukocyte ROS production, plasma oxidative stress markers and signs of fibrinogen oxidation to better understand the relationship among oxidative stress, inflammation and thrombosis in Churg-Strauss syndrome patients.

Blood Leukocytes Intracellular ROS Levels

Intracellular ROS levels have been assessed in leukocyte subpopulations of lymphocytes, monocytes, neutrophils and eosinophils by FACSCanto analysis as shown in material and methods section. Data reported in Figure 34 show the significant increase in ROS production in EGPA leukocyte subsets. Particularly, in EGPA patients, compared to controls, lymphocytes intracellular ROS levels value result significantly increased (1324 ± 264 vs 693 ± 169, $p < 0.05$) as well as in monocytes (2788 ± 613 vs 1258 ± 211, $p < 0.05$), neutrophils (2989 ± 663 vs 1921 ± 344, $p < 0.05$) and eosinophils (41263 ± 16241 vs 26794 ± 7797, $p < 0.05$).

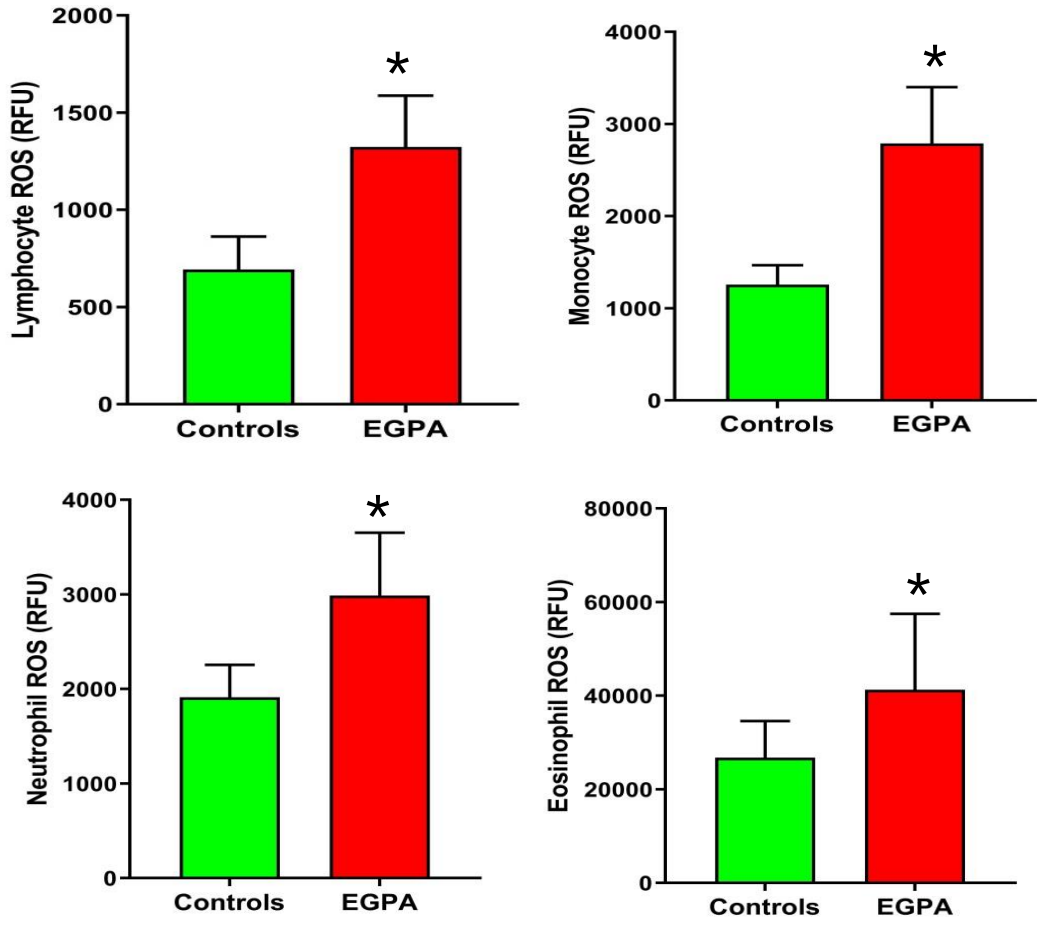

Figure 34: Leukocyte ROS production in EGPA patients (n =35) and controls (n =35). Statistical significance was considered at the $p< 0.05$ level.

Assessment of plasma lipid peroxidation by ALDetect Lipid Peroxidation assay.

MDA and 4-hydroxyalkenals are able to react with N-methyl-2-phenylindole generating an adduct complex measured in absorbance, according to Materials and Methods. In EGPA patients, plasma oxidative stress monitoring revealed signs of lipid peroxidation if compared to healthy subjects (4.15 ± 1.31 vs 2.72 ± 0.72, $p < 0.05$) as shown in figure 35.

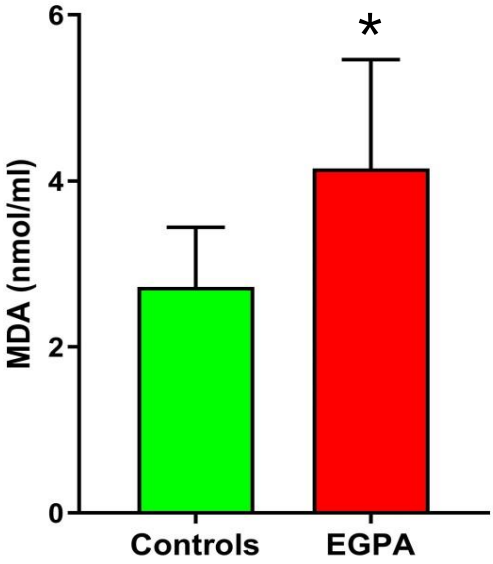

Figure 35: Levels of lipid peroxidation in plasma of EGPA patients and in controls. * indicates that differences are statistically significant at the $p < 0.05$ level.

Assessment of plasma Total Antioxidant Capacity (TAC)

TAC is an indirect marker of redox status. Indeed, it increases in the early phase of oxidative stress, as a consequence of ROS-mediated stimulation of antioxidant genes expression, but it strongly decreases by consumption in prolonged redox status alterations.

The results shown in Figure 36 indicate that plasma antioxidant capacity is significantly decreased in EGPA patients (17.44 ± 2.41 vs 22.33± 3.12, $p < 0.05$) if compared to controls.

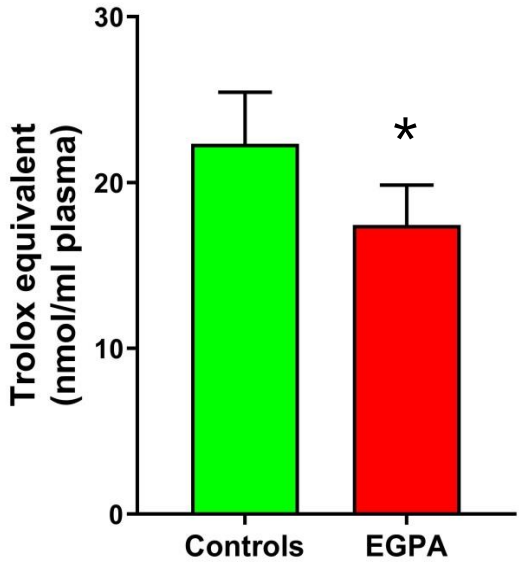

Figure 36: Evaluation of TAC in plasma in EGPA patients and healthy control subjects. Statistically significant difference is indicated (*p< 0.05).

Oxidative stress in purified fibrinogen fractions: assessment of dityrosine content

Oxidative damage to proteins has been evoked in several disease. For this reason, the determination of di-tyrosine has been widely used as a biomarker for oxidative stress. In Fig 37 a significant increase in dityrosine content on purified fibrinogen from EGPA patients respect to controls (365.5 ± 121.9 vs 179.2 ± 37.90, p<0.05) is shown.

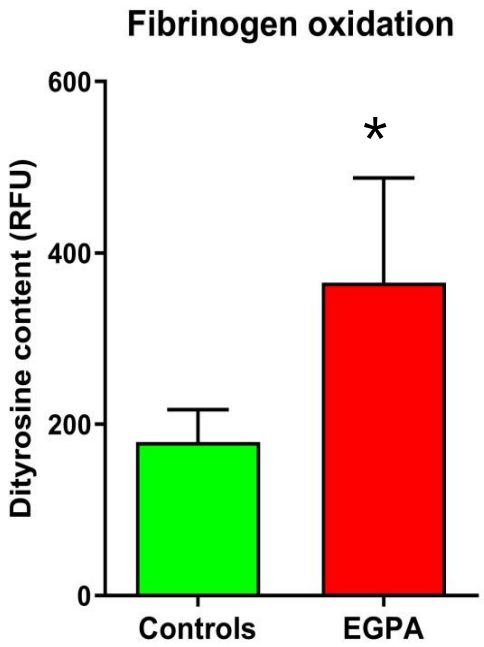

Figure 37: Dityrosine content in purified fibrinogen fractions in patients and controls. * indicates that differences are significant at the p < 0.05 level.

Thrombin-catalyzed Fibrin Polymerization

The kinetics of fibrinogen polymerization, as an index of its clotting function, have been assessed. In Churg-Strauss syndrome patients, the ability of fibrinogen to undergo polymerization was diminished compared to controls, as indicated by significant changes ($P<0.05$) in lag time (9.41 ± 1.12 vs 5.22 ± 0.45), Vmax (0.0082 ±0.0019 vs 0.0171 ± 0.0032) and Max Abs (0.136± 0.0431 vs 0.341 ±0.0342) suggesting a different clot structure (Figure 39).

Representative curves of thrombin-induced fibrin polymerization are reported in Fig 38.

The different kinetics are indicative of qualitative, rather than quantitative, differences in purified fibrinogen fractions from EGPA patients and controls.

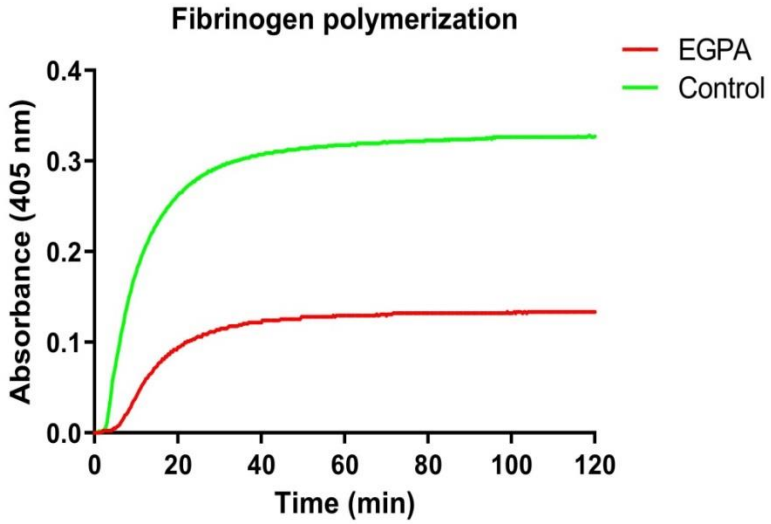

Figure 38: Representative curves of fibrin polymerization in EGPA patients and controls.

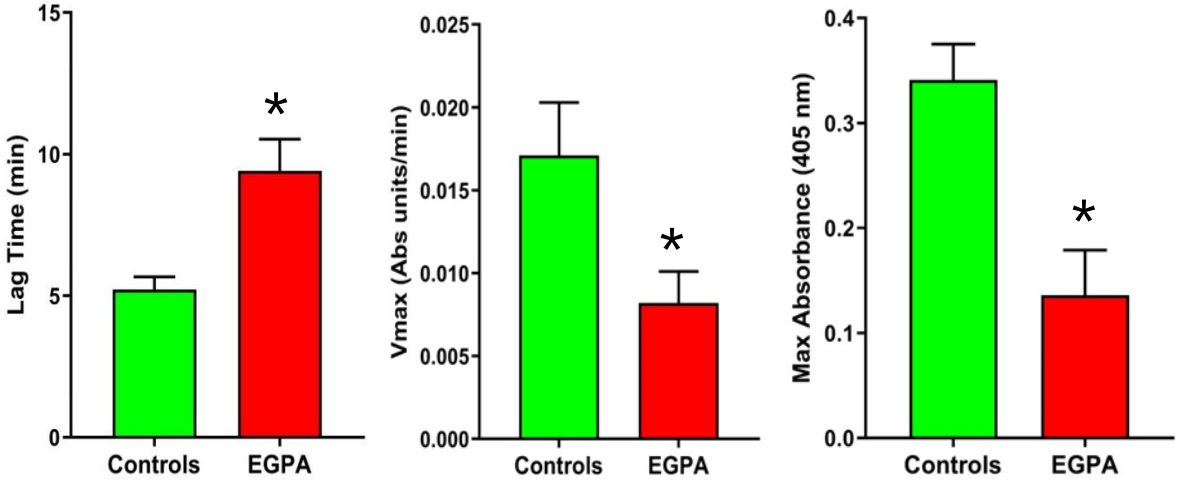

Figure 39: Thrombin-catalyzed fibrin polymerization and corresponding Lag phase, Vmax and Max Abs in fibrinogen purified from EGPA patients (n=35) and controls (n=35).* indicates that differences are significant at the $p<0.05$ level.

Plasmin-induced Fibrinolysis

Purified fibrinogen functional features, evaluating fibrin susceptibility to plasmin-induced lysis, were also investigated. Particularly, we focused on the degradation rate of the fibrin ß chain that was monitored before and after 3 hours and 6 hours of plasmin digestion, as indicated by the electrophoretic analysis in Fig 40 . In EGPA patients, the relative band intensity at each considered time of plasmin digestion was significantly higher than controls (60 ± 17 vs 26 ± 4, $p < 0.05$), showing a fibrin resistance to plasmin-induced lysis in EGPA patients (Fig 40).

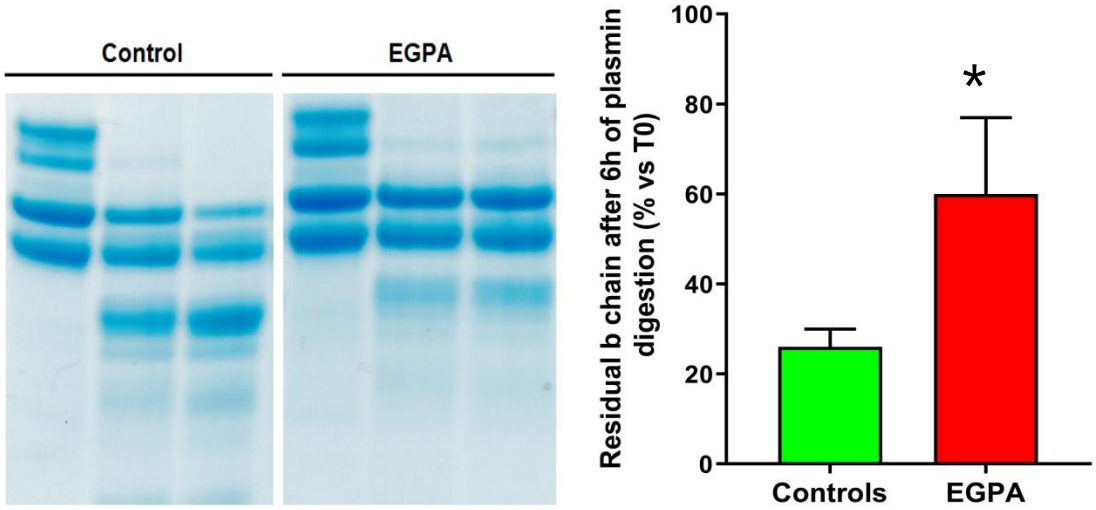

Figure 40: Representative gel of fibrin lysis after 0-6h of plasmin incubation with fibrinogen purified from patients and controls. Quantification of residual fibrin β chain after 6h of plasmin digestion in EGPA patients (n=35) and controls (n=35). * indicates that differences are significant at the $p < 0.05$ level

Circular Dichroism Spectroscopy (CD)

Secondary protein structure was analyzed by far-UV circular dichroism (CD) spectroscopy. CD spectrum is a first-class method for secondary structure determination and results from electronic transition between molecular orbitals in ground and excited states of proteins [97,107]. The two negative peaks at 208 and 222 nm are typical of protein α-helix structure. Comparing CD spectra of purified fibrinogen from patients and healthy subjects, differences in protein structure were evident. In controls, the observed fibrinogen spectrum was indicative of a typically alpha-helical structure. An altered CD spectrum, mainly consisting of a decrease in the negative peak in the 215–225 nm region, was observed in fibrinogen from EGPA subjects, suggesting a decrease in alpha-helical content (Figure 41).

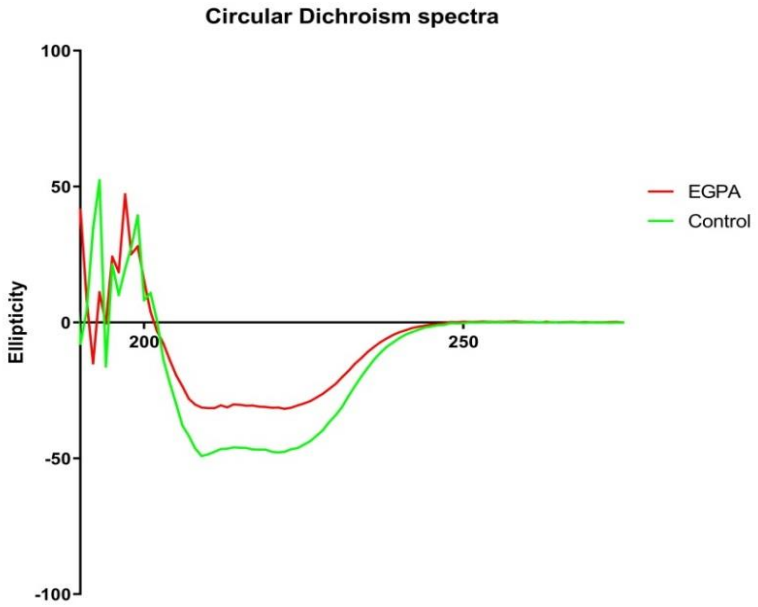

Figure 41: Representative CD spectra of purified fibrinogen from EGPA patients and controls.

Intrinsic Fluorescence Spectra Analysis

Intrinsic emission fluorescence spectroscopy can be used to investigate several parameters: changes in protein tertiary structure, protein microenvironment and the amount of tryptophan residues. Evaluating intrinsic fluorescence spectra of purified fibrinogen from patients and healthy subjects, differences in the maximum fluorescence emission at 352 nm of fibrinogen were observed between patients and controls (Figure 42). Fibrinogen from EGPA patients showed lower intrinsic fluorescence intensity values than fibrinogen purified from controls (932 ± 54 vs 725 ± 73.69, $p < 0.05$), indicating changes in protein tertiary structure (Figure 43). These results, similar to data obtained with CD spectra, suggest fibrinogen conformational alterations.

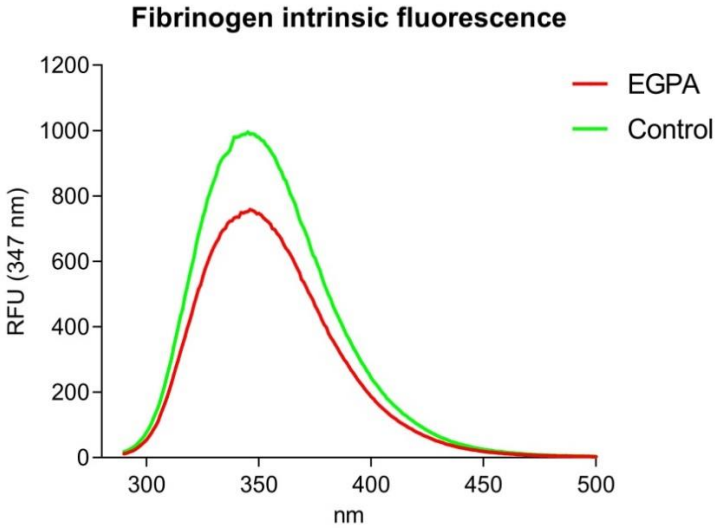

Figure 42: Representative intrinsic protein fluorescence spectra of purified fibrinogen.

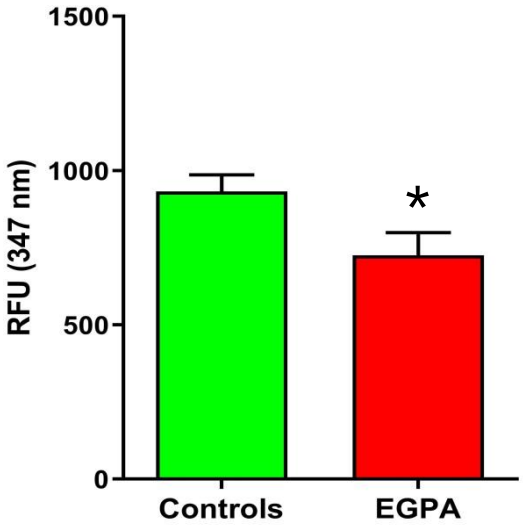

Figure 43: Quantification of intrinsic fibrinogen fluorescence in EGPA patients (n=35) and controls (n=35).* indicates that differences are significant at the $p < 0.05$ level.

Discussion

EGPA is a rare systemic vasculitis characterised by peripheral eosinophilia, eosinophil tissue infiltration and disseminated necrotizing vasculitis with extravascular granulomas [5-11]. The pathogenesis of this disease remains largely unknown, but recent studies have suggested the involvement of oxidative stress. ROS likely originate from inflammatory cells (eosinophils, neutrophils and macrophages) and their deleterious activity can result in lipid peroxidation, modified proteins and oxidative DNA damage [12-16].

Many studies supported oxidative stress and inflammation as interconnected processes that co-exist in the inflamed milieu. In addition, inflammation-induced endothelial injury has emerged as a key factor connecting chronic inflammation and thrombosis. Collectively, it is now widely accepted that a strict relationship among inflammation, endothelial dysfunction and oxidative stress exists [72,73,98].

Cardiovascular involvement has important prognostic implications in patients with EGPA and is a major determinant of mortality [21-26]. An increased frequency of vascular events due to the involvement of medium-sized and large vessels has been reported consistently in AAV. Recent evidence indicates an association between disease activity and thrombotic risk in AAV regardless of clinical diagnosis or ANCA specificity, suggesting that vascular inflammation *per se* is a major determinant of thrombosis. In particular, neutrophils enhance the increased risk, severity and adverse outcome of thrombosis acting as modulator of several processes: causing the rupture of atherosclerotic plaque, inducing platelet activation, possible tissue factor carriage, altering the antithrombotic function of the endothelium and inhibiting response to fibrinolytic agents [60,61,63-66,72-75,129].

Nonetheless, myocardial eosinophilic infiltration followed by granulomatous formation, tissue damage and replacement fibrosis are key events in the progression of cardiac involvement. Eosinophils contain toxic inflammatory mediators which are stored in cytoplasmic granules and can be released upon activation, thereby causing inflammatory reactions and necrosis [24,25,110]. Besides, in the presence of antineutrophil cytoplasmic autoantibodies (ANCA) and/or through the release of specific eosinophil cytokines, the endothelium may shift from an antiadhesive and antithrombotic state to a proadhesive and prothrombotic state. The above-mentioned concept is supported by studies where eosinophil and endothelial activation markers were measured: MBP concentration was found increased in plasma and vessels of patients with eosinophilic vasculitis including EGPA, plasma concentrations of von Willebrand factor and thrombomodulin were also elevated, the latter in parallel with ECP in active disease and vascular endothelial growth factor was strongly expressed in infiltrating

eosinophils and its high plasma concentration positively correlated with peripheral blood eosinophilia [109].

In this context fibrinogen, a plasma protein particularly susceptible to oxidation, plays key roles. The ability of fibrinogen to contribute to the inflammatory response rely on its specific interaction with integrins which are leukocyte cell surface adhesion receptors expressed on neutrophils, monocytes, macrophages, and several subsets of lymphocytes [73-75,77,78]. Post-translational modifications of fibrinogen influence the occurrence and progression of thrombotic diseases [81,82,86-90].

In this context, our group recently showed in Behçet disease that neutrophil activation contributes to thrombus formation through NADPH oxidase derived-ROS [74,75,99] which are able to induce fibrin modification which induce a less susceptibility to plasmin-induced lysis [62,74,75,99].

Eosinophils produce ROS upon exposure, for example, to viruses and allergens and by the concomitant release of eosinophil peroxidase (EPO) are able to brominate the amino acid tyrosine. Furthermore, eosinophils produce a wide range of inflammatory cytokines with the potential of modulating the immune response [24,25,110].

Inflammation-induced thrombosis is by now considered a feature not only of autoimmune rheumatic diseases, but also of systemic vasculitis such as Behcet's syndrome, ANCA-associated vasculitis or giant cells arteritis, especially during active disease [98].

A systematic retrospective study on 1130 patients conducted by Allenbach et al. confirmed high frequency of venous thromboembolic events in EGPA (8.2% of patients), Wegener's granulomatosis and microscopic polyangiitis. For WG, MPA or CCS, the VTE frequency did not differ significantly between patients who were ANCA positive and ANCA negative patients [127].

The findings of Zampieri et al. revealed that 1 out of 4 EGPA patients had evidence of cardiovascular involvement associated with high peak eosinophilic count. Although rarely severe and life-threatening, CSI often required long-term cardioactive treatment and follow-up [24]. The results of the study conducted by Bettiol et al. show a higher risk of both acute VTE events in patients with EGPA as compared to a reference population, particularly around the time of EGPA diagnosis and in those without immunosuppressive treatments. Notably, the occurrence of venous events was stably high throughout the whole duration of the disease, suggesting the need of a close and long-term monitoring particularly for such events [25].

Peripheral leukocytes represent a reliable model for studying oxidative stress-mediated homeostasis variations, which can be responsible for cell dysfunction and cell injury [33,57].

We showed that leukocyte ROS production is significantly enhanced in EGPA patients respect to controls. In this regard, the study of Rubattu et al. underlined that the redox status of PBMCs closely reflect the systemic cardiovascular oxidative stress condition and also with the presence and progression of CVD. Indeed, it has been suggested that an altered redox status of circulating leukocytes can act as an amplifier of oxidative stress at tissue level, worsening the evolution of the disease [57]. Similar results have been obtained in the study of Straface et al. [135].

In particular, in EGPA patients it has been observed that eosinophils play a key role in promoting vascular injury via release of preformed granules during active disease. Accordingly, our study shows a higher ROS production also in the eosinophils population in EGPA patients compared to controls. These data are in agreement with a previous study showing upregulated oxidative metabolism in eosinophils obtained from allergic patients compared to controls [112].

The role of oxidative stress in the pathogenesis of vascular diseases has been widely reported [54-56]. Our data indicate increased levels of oxidative biomarkers and decreased antioxidant activity in the circulating blood of EGPA patients providing further evidence for enhanced oxidative stress in these subjects. Recently, our group found signs of oxidative stress also in patients affected by Horton Arteritis, a systemic inflammatory vasculitis, confirming the key role of oxidative stress in inflammatory diseases [108]. Moreover, the research group by Tisma et al. showed that systemic redox homeostasis and iron metabolism are altered in small vessel vasculitis **(**SVV) patients. Further, the results of their study demonstrate that SVV is not only associated with systemic oxidative stress but also with tissue-specific oxidative stress that promotes acrolein formation and protein modification correlating with the severity of cutaneous vasculitis [113].

The study of Rajesh et al. highlights a significant increase in the expression of iNOS, as well as 3-nitrotyrosine (3NTYR) accumulation, diminished SOD activity, elevated lipid peroxides, iron, copper and decreased zinc content in patients with Eales disease (ED), an idiopathic retinal vasculitis condition. The elevated levels of ROS and RNS products correlated with diminished antioxidant status in patients with ED. In addition, a strong immunoreactivity for iNOS and (3NTYR) was observed in inflammatory cells and endothelial cells in epiretinal membrane obtained from patients with ED [130]. Moreover, protein carbonyl content increased with disease severity and correlated with diminished antioxidant status [131]. High levels of oxidative stress in vasculitis were confirmed by Yahata et al. in inflammation-based Kawasaki disease [127]. In addition, the study of Ece et al. assessed the role of oxidative

stress in the pathogenesis of Henoch–Schönlein purpura (HSP) vasculitis. The researchers measured TAS (total antioxidant status) and the activities of catalase (CAT), arylesterase (ARYL), and paraoxonase (PON) that showed significantly lower activities of antioxidant enzymes and TAS in the active stage of HSP. In addition, higher MDA levels were observed in patients [132]. Similar results have been obtained in many other studies [134,135].

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, metabolic, cardiovascular diseases and tumors [38,35,46,55,59,61]. Consequences of protein oxidation are the formation of carbonyl groups and the modification of amino acids (e.g., methionine is converted into methionine sulphoxide and tyrosine into dityrosine)[41,48-53]. Compared with other plasma proteins, fibrinogen is especially susceptible to oxidation with potential effects on its structural and functional characteristics. It was shown that fibrinogen is 20 times more susceptible to oxidation than albumin, the most abundant plasma protein [93,95,88,99].

The risk of thromboembolism is increased in a range of disorders associated with marked peripheral eosinophilia, including eosinophilic myeloproliferative disorders, idiopathic hypereosinophilic syndrome, and eosinophilic vasculitis [15,16,21-26]. Although the mechanism of eosinophil-induced hypercoagulability is not entirely clear, contributing factors probably include initiation of the clotting cascade by tissue factor (released during eosinophil degranulation), inhibition of vascular endothelial thrombomodulin (a potent anticoagulant) by MBP and activation of platelets by MBP and EPO. Xu and Hakansson showed that stimulated eosinophils incubated with fibrinogen released large amounts of eosinophilic cationic protein (ECP), which indicates that adhesion to fibrinogen and fibrin may play a role in eosinophil degranulation and release of ECP [117].

This evidence led us to evaluate signs of oxidative stress markers, specifically dityrosine content, on fibrinogen purified from plasma of EGPA patients and in healthy subjects. Our data show a significant increase in fibrinogen dityrosine content in EGPA patients compared to healthy subjects, suggesting further investigations aimed at evaluating the potential relationship between fibrinogen oxidation and the increased cardiovascular risk. As previously reported in several studies, oxidative alterations can have a deep impact on fibrinogen function, ultimately producing prothrombotic clots [96,99,101,111,119-122].

For this reason, we investigated functional features of purified fibrinogen by assessing thrombin-catalyzed fibrin formation and fibrin susceptibility to plasmin-induced lysis. Both analyses were significantly impaired in EGPA patients. When we assessed, in EGPA patients, fibrin polymerization, a slower rate and turbidity was observed compared with healthy controls, as indicated by decreased Vmax and Max abs values. When we analysed EGPA 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 EGPA patients. A reduced fibrinolysis has already been found by our group in several pro-thrombotic or chronic inflammatory disorders as BD [96,99], post-acute myocardial infarction [101], cirrhosis [97] and pulmonary hypertension [100].

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 [132,133]. Susceptibility to fibrinolysis is highly influenced by clot structure [119-126]. Thicker fibers, reduced branching, and larger pores increase the permeability and susceptibility to fibrinolysis, which is considered anti-thrombotic. However, thinner fibers, more branching, and smaller pores make the clot less permeable and more resistant to lysis by plasmin (prothrombotic) [88-90]. For the first time, the study of Becatti et al. reveals that clot structure, obtained by stimulated emission depletion (STED) super-resolution microscopy, indicates modifications in fiber diameter and in clot porosity in cirrhotic patients [97].

The study of Mastalerz et al. show that EGPA is associated with prothrombotic plasma fibrin clot phenotype, which may contribute to thromboembolic manifestations. Specifically, compared with controls, patients with EGPA were characterized by denser fiber clots, faster fibrin polymerization using calibrated automated thrombography (CAT), thicker fibrin fibers, higher maximum levels of D-dimer released from clots and prolonged clot lysis time. Scanning electron microscopy images confirmed denser plasma fibrin networks composed of thinner fibers formed in EGPA. They demonstrated that EGPA in clinical remission is associated with faster formation of significantly less permeable and poorly lysable fibrin clots, similarly to other diseases leading to thrombotic complications [116]. In addition, the study conducted by Undas et. al showed altered fibrin clot structure and function (lower clot permeability, lower compaction, higher maximum clot absorbancy and prolonged clot lysis time) in patients with idiopathic venous thromboembolism [122-124].

A systematic review of 105 research articles dealing with the effects of post-translational modifications of fibrinogen conclude that fibrinogen oxidation decreased polymerization rate,

which results in more dense fibrin clots with thinner fibers and lower permeability. This results in clots that are more resistant to fibrinolysis, and therefore, prothrombotic [88].

The mechanism by which oxidation affects clotting is thought to be methionine residues oxidation in the αC region, which is involved in lateral aggregation. The αC region is proven to be most vulnerable to oxidation by ozone and hypochlorite. Weigandt et al. identified methionine residue at position 476 in the αC region to be oxidized by hypochlorite. Conversion of Met476 into methionine sulfoxide was shown to impair dimerization of αC domains, thereby inhibiting lateral aggregation [88,118].

In the second part of the study, fibrinogen secondary structure was investigated by far-UV circular dichroism spectroscopy, a reduction in α-helical content was detected in EGPA patients. Considering that protein functionality largely depends on its secondary structure, this finding suggests that oxidative modification induce the formation of a species less rich in α-helix, which may specifically affect the biological activity of fibrinogen. Similar results have already been found by our group in BD and cirrhotic patients [97,99].

Finally, 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 EGPA.

In conclusion, the observed differences between EGPA patients and healthy subjects suggest the involvement of oxidative stress in the pathogenesis of EGPA. In particular, the data presented in this study suggest that fibrinogen oxidative modifications can represent a new risk factor for thrombotic events in EGPA patients and a potential target for tailored therapeutic treatments.

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