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TESI DI LAUREA

Performance of the 2023 ACR/EULAR antiphospholipid syndrome classification criteria: long-term outcomes in primary and secondary APS

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Abstract

Background: The 2023 ACR/EULAR antiphospholipid syndrome (APS) classification criteria distinguish between anticardiolipin (aCL) or anti-β2-glycoprotein I (aβ2GPI) IgG vs. IgM isotypes and define aCL and aβ2GPI thresholds based on fixed cut-off values established only through ELISA solid-phase essay. Low weight is attributed to Isolate IgM positivity, insufficient for APS classification. In addition, the new criteria differentiate venous and arterial thrombosis depending on patient's venous thromboembolism and cardiovascular profile risk, assigning lower significance to thrombosis occurred within the context of high-risk VTE or CVD profile. We assessed the performance of the 2023 ACR/EULAR APS classification criteria in a cohort of primary vascular APS patients (PAPS) and in a cohort of secondary vascular APS (SAPS), previously classified according to the Sydney criteria.

Methods: PAPS and SAPS patients meeting the Sydney classification criteria with previous arterial, venous, or small-vessel manifestations followed between 1980 and 2023 were re-evaluated to identify cases that would not be classified as PAPS based on the 2023 ACR/EULAR criteria. Sensitivity and specificity were estimated exclusively in SAPS cohort using clinical judgment as gold standard.

Results: Our cohort included 205 PAPS patients and 57 SAPS patients. 170 out of 205 were confirmed as PAPS by the new ACR/EULAR criteria, while 35 (17.1%) were not, 32 due to insufficient score in laboratory domain, 1 due to insufficient score in clinical domain, 2 due to insufficient score in both domains. On the other hand, 50 out of 57 patients were confirmed as SAPS, while 7 (12.2%) were not, 1 due to insufficient score in laboratory domain, 6 due to insufficient score in clinical domain. Notably, 9 out of 35 (25.7%) patients not confirmed as PAPS and 1 out of 7 (14.3%) patients not confirmed as SAPS had a thrombotic relapse during the follow-up, confirming a pro-thrombotic profile. ACR/EULAR APS classification criteria sensitivity and specificity in a cohort of aPL positive patients (57 SAPS and 49 aPL carriers) with SLE were 82% and 100%, respectively.

Conclusion: In this report, 17.1% of PAPS patients and 12.2% of SAPS patients classified as APS by the Sydney criteria would not meet the 2023 ACR/EULAR criteria. In clinical practice, inappropriately using these criteria as

diagnostic, could result in the lack of adequate antithrombotic therapy, exposing these patients to the risk of a new thrombotic event.

Chapter 1 – Systemic Lupus Erythematosus (SLE)

1.1 – Definition and epidemiology

Systemic lupus erythematosus (SLE) is a multifactorial autoimmune disease that is part of the wider group of connective tissue diseases characterized by the inflammation of the connective tissues. Unregulated B cell and T cell responses along with loss of immune tolerance against self-antigens give SLE the propensity to affect every organ and tissue of the body. Its pattern of clinical manifestations is extremely heterogeneous ranging from mild fatigue and joint pain to severe, life-threatening organ damage (1).

The global incidence and prevalence of SLE is estimated to be 5.14 per 100.000 person-years (0.40 million every year) and 43.7 per 100.000 (3.41 million people), respectively. Both disease indicators vary broadly depending on the geographical area, from the lowest incidence in central Asia with 1.18 per 100.000 person-years to the highest in Central Europe with 13.74 per 100.000 person-years. Prevalence instead changes from 15.9 per 100.000 persons in southern Asia to 110.85 in tropical Latin America (2).

It is widely known that women are more likely to be diagnosed of SLE than men with a global incidence and prevalence of 8.82 per 100.000 person-years and 78.73 per 100.000 persons against the men equivalent of 1.53 per 100.000 person-years and 9.26 per 100.000 persons. In other words, SLE affects women more than men with a 6:1 ratio. Furthermore, SLE is an autoimmune disease typical although not exclusive of the adulthood. Incidence in women reaches its peak between the third and fifth decade of life, while prevalence peak sits later between the fifth and the seventh decade of life. Both age-related disease indicators peaks are one-decade-delayed in men (2,3).

Another crucial epidemiological characteristic regarding SLE is its different frequency in various ethnicities. Both prevalence and incidence are the highest in Afro-Caribbean people and the lowest in Caucasians with Hispanic and Asian ethnic groups in between. Despite this data, incidence and prevalence of Sub-Saharan Africa are unknown or uncertain, with the majority of SLE studies on ethnic differences conducted on inhabitants from high income countries (3,4).

In Italy, prevalence and incidence of SLE are among the lowest in Europe. An observational study published in 2024 and conducted on the entire Italian

population over a period of 5 years between 2017 and 2022 estimated that the incidence and prevalence of SLE are increasing over time, in line with the rest of the world. In 2022 the incidence rate was 6.51 per 100.000 person-years and the prevalence rate 60.57 per 100.000 persons with a female to male ratio of 5 to 1. Incidence showed a geographical gradient being the highest in the North (44.74% of cases) and the lowest in the South and the Islands (20.53%) (5). Interestingly, incidence in the Veneto region sits only at 2.2 per 100.000 person-years, while prevalence with 70.6 per 100.000 persons is one of highest within the country (6).

1.2 – Etiology

Even though black women at childbearing age are the population with the highest incidence and prevalence of SLE, geographical differences are seen within this group. In fact, SLE has a multifactorial etiology, with genetic, epigenetic, and environmental factors contributing to the development of the disease. This concept is further corroborated by twin and family linkage studies. In fact, while it's true that siblings of affected individuals have 20 to 30-fold increase in risk of development of lupus, there is an up to 75% rate of SLE discordance in monozygotic twins, percentage that reaches 95% in dizygotic ones (4,7).

1.2.1 – Genetic factors

Genome-wide association studies found almost 90 different loci that play a role in the pathogenesis of SLE, but only for around 20 of them there are consolidated evidence. These genes are involved in related cellular pathways such as B and T cells activation and proliferation, leukocytes vascular adhesion and extravasation, immune complex clearance and cytokines production. The three main categories of genes associated with SLE are determinant of HLA haplotypes, type-I interferon alpha (IFN-α) pathway and integrin-ICAM-mediated adhesion pathway. Although in a minority of patients the disease is caused by a single gene mutation such as the one that cause complement component 1q deficiency, most of the times SLE develops because of a mix of multiple gene variants effects (8–10) .

Two single nucleotide polymorphisms (SNPs) located in the HLA region and identified in the two haplotypes HLA-DR2 and HLA-DR3 are both strongly related to SLE susceptibility. Other significant genes are IRF5, IRF7 and IRF8, transcription factors which stimulate the transcription of IFN-α. Gain of function variants of these genes are responsible of a higher interferon serum level. Not only

higher IFN-α sera levels are present in patients affected by SLE compared to the general population, but there is also a great correlation between SLE-related autoantibodies titers and high interferon alpha (IFN-α) serum levels. However, it is important to note that a portion of SLE patients has a low IFN-α serum level, suggesting that what makes a difference is the interferon degree of activity rather than its absolute concentration. In favor of this theory, mutation of transcription factor STAT4 cause an increase in IFN pathway sensitivity while being associated with low levels of IFN-α (8,9).

Family linkage studies show that genetic predisposition is a necessary but not sufficient condition for SLE development. Gene expression and serological features were not different between the affected and the unaffected twin, pointing to the fact that there could be epigenetic and environmental dissimilarities (11).

1.2.2 – Environmental factors

The strongest evidence that supports a significant role of the environment in the development of SLE and its symptoms severity can be found in current cigarettes smoking, crystalline silica exposure, and exogenous estrogen intake. Other environmental aspects have been studied such as UV radiation, pesticides and air pollution exposure, vitamin D levels, previous infections, and dietary habits; however, despite positive correlation with SLE occurrence, all of them only reach a mild level of evidence because of discordant studies results or unclear cause-effect relationships (12,13).

Toxic components from cigarette smoke (i.e. nicotine, carbon monoxide, polycyclic aromatic hydrocarbons, and free radicals) are responsible of direct DNA and proteins damage and oxidative stress. Both processes contribute to genetic mutations, gene activation and pro-inflammatory cytokines spreading, leading to an immune system dysregulation state that could start the development of SLE. A meta-analysis of studies analyzing the connection between smoking and SLE risk revealed that only current smoking had a modestly elevated SLE risk (OR 1.5; 95% CI 1.09, 2.08) compared to non-smoking. Past smokers had the same risk of the non-smokers in that meta-analysis. The same risk dissimilarities were confirmed in other analyses within the Nurses' Health Study prospective cohorts, where current, but not past, smokers were strongly associated to the risk of having anti-double stranded DNA-positive subtype of SLE (1.86; 95% CI 1.14, 3.04]). All this data

supports the evidence that smoking is involved in the pathogenesis of some specific subtype of SLE (7,13).

Respirable crystalline silica (<10 μm), also known as silica dust produced in quartz quarry, is strongly related to the development of many autoimmune disease including SLE. Crystalline silica has the capability to induce apoptosis and intracellular antigens release, leading to an increase in pro-inflammatory cytokines, oxidative stress, and T-cell responses. There is a dose-dependent risk association between respirable crystalline silica and SLE development based on both occupational and residential exposure, even though the minimum required dose and the exposure timing are aspects that have yet to be elucidated (7,12,13).

Another major environmental factor implicated in SLE etiology is exogenous estrogen intake, not only in the form of oral contraceptives but also with hormonal replacement therapy. Studies conducted on women taking HRT for menopausal symptoms and on patients who underwent male to female gender transition showed a higher number of SLE diagnosis in these types of patients compared to general population. These results on the exogenous estrogen effects support the theory of endogenous estrogens being involved in the pathogenesis of SLE, providing an explanation on why women of childbearing age are affected at an higher rate than man (4,7).

1.3 – Pathogenesis

The current model for SLE pathogenesis considers both genetic background and environmental factors. In genetically predisposed healthy individuals, exposure of some environmental agents (smoking, silica dust, UV light, infections, dietary habits and hormonal changes) act as triggers that set the stage to immune system dysregulation. At this point, patients are still asymptomatic, but at cellular level the loss of tolerance has already begun. As a matter of fact, autoantibodies precede the clinical manifestations of SLE by years and can be found in asymptomatic patients' plasma (14). This provides an explanation on the relatively low penetrance of SLE in monozygotic and dizygotic twins (11).

For the disease to be clinically evident, other events that are yet to be identified must happen to trigger the spread of autoimmunity. Once this phase is reached, adaptive and innate immunity dysfunction together with pro-inflammatory cytokines lead to tissue damage, which in turn provides new autoantigens that amplify the autoimmune process in a self-sustaining feed-forward loop. This

positive feedback pattern causes irreversible organ damage and chronic inflammation (14).

Several subsets of immune cells and signaling pathways are involved in SLE pathogenesis. Although a comprehensive explanation to SLE development still represents a demanding challenge, knowing the key actors involved could offer new therapeutical strategies.

1.3.1 – Type 1 IFN

One of the fundamental aspects of pathogenesis is the imbalance between the production of apoptotic cells and the removal of apoptotic material. Nuclear antigens are usually not accessible to the immune system, but during apoptosis, the cell membrane forms vesicles that detach, containing fragments of cellular material, including nuclear antigens. These apoptotic debris are usually cleared quickly, remaining inaccessible to the immune system.

Exposure to UV light, infections, and toxins, which are associated with SLE, can increase the apoptotic cell load. Persistent apoptotic debris containing nucleic acids can trigger an inflammatory response through the activation of nucleic acid recognition receptors, such as Toll-like receptors (TLR). Cytosolic nucleic acid sensors identify viral infections and initiate defenses based on type I IFN production. Type I and type II IFNs have been identified as key cytokines in the pathogenesis of SLE (and other autoimmune diseases), with their increased levels preceding the development of autoantibodies. Type I IFNs and other cytokines also facilitate B-cell differentiation and loss of tolerance (14).

1.3.2 – T cells

There are several pathways through which T-cell tolerance may be defective in SLE. One of the earliest described phenomena is abnormal signalling through the T-cell receptor. This issue is not intrinsic to the cell and can be induced in normal T-cells by serum IgG from SLE patients. Despite this hyperactivated state, T-cell production of IL-2 is impaired (14).

Patients with SLE may also exhibit an imbalanced T-cell cytokine profile, characterized by reduced IL-2 and increased IL-17 levels (10). IL-2 is crucial not only for the development and function of Treg cells but also for limiting IL-17 expression. In SLE, IL-17 can cause local tissue damage by inducing inflammatory cytokines and chemokines, and by recruiting other immune cells. In fact, the

production of IL-17 by T-cells contributes to organ infiltration by neutrophils, and activated T-cells also enhance IFN production by plasmacytoid DCs (pDCs).

T-cells play a role beyond providing signals for class switching; they are a critical checkpoint for autoreactive B-cells in SLE. T cell–B cell interactions are a major focus of current SLE research, as these interactions occur outside their typical locations in secondary lymphoid organs (14).

1.3.3 – B cells

Abnormalities in T-cells and B-cells have been well-documented in SLE and play a central role in the disease process.

In SLE, B-cell activation and autoantibody production are driven by BAFF (B-Cell Activating Factor). Serum levels of BAFF are elevated in SLE patients and show a positive correlation with autoantibody titers. BAFF is essential for B cell homeostasis, and high levels of BAFF may decrease the stringency of B-cell selection, allowing autoreactive clones to survive in the periphery.

B-cells can respond to nucleic acids through direct antigen recognition and via surface IgM receptors for proteins complexed with nucleic acids. Once autoantibodies are formed, B-cells can also internalize nucleic acids through Fc receptors and B-cell receptors recognizing rheumatoid factor. Once activated, these B-cells mature, proliferate, and start secreting more antibodies, thereby enhancing the adaptive immune response (14).

1.3.4 – Autoantibodies

The autoantibodies found in SLE are typically high-affinity, somatically mutated IgG, indicating their origin in germinal centers where T cells assist with class switching. Autoantibodies contribute to SLE by forming immune complexes, acting as direct agonists or antagonists, and interfering with intracellular functions. Immune complexes activate complement and bind to Fc receptors, thereby driving inflammation (14).

Autoantibodies against double-stranded DNA and small nuclear RNA-binding proteins such as Ro, La, Sm, and nRNP are characteristic of SLE. Along with other cellular and soluble mediators of inflammation, they contribute to end-organ damage (8,10,14).

1.3.5 – Innate immune cells

As previously discussed, profound defects in innate immunity are related with the onset and progression of SLE, as well as tissue damage. Dysfunctional phenotypes and impaired functions have been identified in neutrophils, monocytes, macrophages, and dendritic cells in SLE patients. These abnormalities play crucial roles in the pathogenesis of SLE, including ineffective clearance of apoptotic debris, presentation of self-antigens, and production of inflammatory cytokines (10).

Neutrophils and apoptotic cells are central to the cascade of pathogenetic mechanisms in systemic lupus erythematosus, providing critical ligands that induce the expression of type I (IFNs) (8). Neutrophils are key participants in inflammation-mediated organ damage, and they release neutrophil extracellular traps (NETs), a source of citrullinated peptide and nucleic acid antigens. SLE patients exhibit an aberrant subset of neutrophils prone to NETosis, contributing to the type I IFN signature of SLE by stimulating IFN production by plasmacytoid DCs (pDCs). Apoptotic debris can also activate the expression of inflammatory cytokines, which contribute in the recruitment of cells into tissues (14).

While various cells produce type I IFNs, plasmacytoid dendritic cells produce these cytokines at the highest levels. SLE patients display multiple abnormalities in DCs, including a decrease in circulating conventional DCs but an increase in pDC numbers. The pDC subset primarily secretes type I IFNs in response to nucleic acids via TLR7 and TLR9. Furthermore, conventional DCs in SLE tend to promote autoreactivity rather than tolerance (14).

Moreover, monocytes and macrophages are potent phagocytes crucial for clearing apoptotic debris. Defects in this process can disrupt immune tolerance by presenting autoantigens that trigger adaptive immunity against self (8,10).

1.4 – Clinical manifestations

As previously mentioned, immune activation in systemic lupus erythematosus is marked by a breakdown in immune tolerance to self-antigens, leading to the production and impaired clearance of antibodies. The presence of circulating immune complexes, and their deposition in tissues, as well as the activation of complement and cytokines, contribute to the diverse clinical manifestations observed in SLE.

The onset of lupus often resembles a viral infection. Common symptoms include weight loss, fatigue, and low-grade fever, which are often accompanied by joint pain or arthritis. However, the clinical presentation of SLE is highly variable, with the potential to affect any organ system, sometimes leading to severe and life-threatening organ damage (1).

1.4.1. – Skin involvement

Cutaneous involvement is a common feature of SLE, and, in some cases, the skin may be the only organ affected. Approximately 90% of SLE patients experience skin manifestations, which include lupus-specific conditions such as acute cutaneous lupus (characterized by indurated or flat erythematous lesions), subacute cutaneous lupus (featuring annular lesions in photosensitive areas that do not scar), and chronic cutaneous lupus, with discoid lupus being the most prevalent form. Non-lupus-specific manifestations encompass alopecia, vasculitis, livedo reticularis, periungual telangiectasias, and Raynaud's phenomenon.

Acute cutaneous lupus is almost invariably associated with systemic lupus, whereas discoid lupus, marked by indurated plaques with scarring and hypopigmentation, is infrequently linked to systemic disease (14,15).

1.4.2 - Musculoskeletal involvement

Joint pain and arthritis are very common in SLE, occurring in almost 90% of patients. They typically present as symmetric polyarthritis, primarily affecting the metacarpophalangeal, proximal interphalangeal, and knee joints (15).

Lupus arthritis manifests with prolonged morning stiffness and mild to moderate joint swelling. In contrast to rheumatoid arthritis, significant effusions are less prevalent in lupus, and the synovial fluid generally exhibits lower levels of inflammation. Furthermore, joint deformities and erosions are less frequently noted in lupus cases (1).

1.4.3 – Hematologic manifestations

Cytopenias frequently occur in individuals with lupus, with moderate to severe lymphopenia being linked to heightened disease activity and organ damage. Following lymphopenia, anemia represents one of the most prevalent hematologic abnormalities and is typically associated with disease onset, alongside thrombocytopenia (1,16).

1.4.4. Renal involvement

Kidney involvement is a frequent manifestation in lupus, significantly impacting prognosis due to its propensity for organ failure. Around half of lupus patients experience renal involvement, with a higher incidence among certain ethnic groups, notably African Americans (70%). A renal biopsy is crucial for confirming the diagnosis, ruling out other potential causes, assessing the presence of active inflammation versus irreversible damage, determining prognosis, and guiding treatment (15,17).

The Renal Pathology Society/International Society of Nephrology (or RPS/ISN) classification include 6 classes:

- Minimal mesangial lupus nephritis.
- Mesangial proliferative lupus nephritis.
- Focal lupus nephritis
- Diffuse lupus nephritis
- Membranous nephropathy.
- Advanced sclerosing lupus nephritis (15,17).

End-stage kidney disease due to lupus is linked to poorer survival outcomes among patients undergoing dialysis or transplantation compared to other causes of end-stage kidney disease (1,18).

1.4.5 – CNS involvement

Neuropsychiatric manifestations in systemic lupus erythematosus (SLE) can result from vasculopathy, autoantibodies, and inflammatory mediators. Impairment of the blood-brain barrier allows immunoglobulins, cytokines, and immune cells to penetrate brain tissue, serving as a central mechanism in neuropsychiatric lupus. The complement system plays a pivotal role in disrupting the integrity of the blood-brain barrier (1,14).

Only a handful of neurological features exhibit a certain degree of specificity for SLE and are useful in diagnosis. These include seizures, psychosis, mononeuritis multiplex, myelitis, peripheral or cranial neuropathy, and acute confusional state. Magnetic resonance imaging (MRI) and analysis of cerebrospinal fluid (IgG and oligoclonal bands) play essential roles as diagnostic tools in these contexts (15).

1.4.6 – Respiratory involvement

Pulmonary complications are a significant concern in SLE and contribute significantly to morbidity and mortality. Pleuritis represents the most prevalent respiratory manifestation of SLE, affecting 30-50% of patients. Vascular involvement can lead to conditions such as diffuse alveolar hemorrhage, pulmonary hypertension, or thromboembolic disease. Parenchymal damage, however, is less frequently observed (1).

1.5 – SLE and thrombosis

Thrombosis is a significant contributor to morbidity and mortality among patients with SLE, occurring more frequently and at a younger age compared to the general population. Studies have shown that 7.2-12% of SLE patients experience thrombosis, which accounts for 26% of mortality rates, akin to active SLE and infections. Factors such as ethnicity, duration of disease, and the type of thrombotic event play a role in the incidence of thrombosis in these patients (19).

Thrombosis in SLE is mediated by both disease-related factors and extrinsic factors. Traditional risk factors (male sex, diabetes, arterial hypertension, smoking, hyperhomocysteinemia), inflammation and endothelial damage are all involved in SLE thrombosis pathogenesis. Well-known intrinsic risk factors for thrombosis in SLE are disease activity, prednisone dosage, nephrotic syndrome, cutaneous vasculitis and the presence of antiphospholipid antibodies (aPL) (19).

The presence of aPL is associated with an increased risk of thrombosis. This heightened risk is primarily due to resistance to natural anticoagulants like protein C, impaired fibrinolysis, the activation of endothelial cells to a pro-coagulant state, and platelet activation. The strongest correlation between these antibodies and thrombosis is observed with persistent positivity, moderate to high titers, and triple marker positivity for aPL (20,21).

However, not all SLE patients who are aPL positive develop thrombosis, and conversely, not all thrombotic events in these patients can be solely attributed to the presence of these antibodies. Thus, thrombosis arises from the involvement of multiple factors that may synergistically contribute to the risk of thrombosis (19). Furthermore, a SLE patients without aPL still has a two-fold higher risk of thrombosis than general population (22).

A less known actor in SLE thrombotic events are antibodies against U1-ribonuclear protein (U1-RNP or RNP/Sm). Though typically linked with mixed connective tissue disease (MCTD), these antibodies are also prevalent in SLE and systemic sclerosis, exhibiting varied clinical features (23). Studies have shown that anti-U1-RNP antibodies function as anti-endothelial cell antibodies (AECA), interacting with mononuclear and endothelial cells to facilitate tissue damage and vasculopathy in connective tissue diseases by enhancing the production of IL-1 and IL-6 (24).

Chapter 2 – Antiphospholipid syndrome (APS)

2.1 – Definition and epidemiology

Antiphospholipid syndrome (APS) is at the same time an autoimmune disease and an acquired thrombophilia owing to presence of some antibodies against cell-membrane proteins anchored to membrane antiphospholipids or directly against antiphospholipids (25). APS exists in two forms: primary APS (PAPS) where the syndrome is not associated with other defined disease, and secondary APS (SAPS) in which another systemic autoimmune condition is present, most of the time systemic lupus erythematosus (26).

Prevalence and incidence of this syndrome is yet to be elucidated cause well-designed populations-based studies are scarce. In a recent review, annual prevalence for APS ranged between 40 to 50 cases per 100.000 adults, whereas incidence was between 1 to 2 cases per 100.000 persons/years (27). Female to male ratio is 5:1, but it is higher in SAPS (7:1) and lower in PAPS (3:1) (28).

Historically, APS was divided into thrombotic APS and obstetric APS, depending on patient's symptoms. However, not only this dichotomy it's often nonexistent since there are patients with combined symptoms, but some experts claim that APS should not be approached as a single entity but rather as a set of different disorders united by the presence of aPL (25).

2.2 – Antiphospholipid antibodies (aPL)

Antiphospholipid antibodies are a heterogeneous group of antibodies that target phospholipid binding proteins and heterotypic phospholipid complexes or that can directly bind to phospholipids. They are secreted by long lived plasma cells and since they exhibit high degree of maturation Th-cells play an important role in their production. Immunomodulating medications are generally ineffective on APS symptoms and prognosis (29).

Different aPLs carry different prognostic value, thus are associated to a different weight of clinical severity. Some are used in clinical settings for APS diagnosis, while others such as antiphosphatidylserine/prothrombin antibodies despite demonstrating a certain degree of involvement in the pathogenesis of the disease are not well characterized and its value is not standardized in clinical practice (29,30).

It is important to note that up to 5% of the population could temporarily be positive to aPL without having APS, also known as aPL carriers. In fact, aPL antibodies without clinical significance could be present in plasma patients due to different scenarios such as infections (27). Even within SLE patients 25 to 40% of patients are aPL positive, but only 50 to 70% of this group will develop SAPS after a 20 year interval (26,31).

2.2.1 – Lupus anticoagulant (LAC)

Lupus anticoagulant is the type of aPL that is mostly related to thrombosis, hence indicating an high-thrombotic risk profile (20). Despite its well-known importance since the 1990s, its structure and epitope has not been identified yet. In fact, LAC is estimated not with an ELISA test, but through a functional essay test such as dilute Russel's viper venom time (dRVVT) or aPTT (29).

Strangely, LAC in vitro behaves in the opposite way than in vivo, meaning that it prolongs coagulation time. The test to be labeled as positive must ascertain prolonged coagulation time of patient's plasma even when it has been mixed with normal plasma; this effect is quenched with the addition of an excess of phospholipids. Since it is a functional essay, its result can be unconclusive if patients are on warfarin anticoagulation therapy (29).

2.2.2 – Anticardiolipin antibodies (aCL)

Anticardiolipin antibodies are a heterogenous group of antibodies that recognize cardiolipins, a type of phospholipids that are widely present in mitochondrial membrane. However, their specific epitope is yet to be determined. Elevated titers of aCL IgG are closely related to susceptibility to thrombotic events; IgM role remains unclear. ELISA solid-phase assay and automated testing are the most used method to measure aCL titers (29,32).

2.2.3 – Anti-β_2-glycoprotein I antibodies (aβ2GPI)

Anti-β_2-glycoprotein I antibody is the only clinically relevant antibody in which its epitope and pathogenetic role has been discovered, that is a plasma protein β2GPI, a protein involved in vWF activity inhibition. Despite its well-defined activity, its positivity does not have the same weight as LAC nor as aCL in terms of thrombotic risk, although high IgG titers are included in high-risk aPL profile (20,29,32).

2.3 – Pathogenesis

To this day, APS pathogenesis remains puzzling and a comprehensive vision that can put together every single mechanism involved is lacking. However, the most accredited theory of the two hits tries to explain the diversity in clinical manifestations in aPL positive patients. This hypothesis claims that aPL create a prothrombotic environment easily susceptible to external stimuli (first hit), and then a second event, which could be subclinical (vascular injury) or evident (infections), triggers an already prone to thrombosis setting to activate in an dysfunctional manner (29).

2.3.1- Dysregulated activation of hemostatic factors

Plasma protein β2GPI can bind to anionic antiphospholipid preventing that its GPI-α subunit could bind von Willebrand factor. However, the complex of aβ2GPI-β2GPI exposes that particular subunit so that the binding with vWF could be established. This leads to the release of proinflammatory cytokines and chemokines through NF-kB pathway leading to intima hyperplasia and vascular cells inflammation (29,32).

In addition, aβ2GPI-β2GPI complex could include apolipoprotein E receptor 2 (apoER2) and the annexin A2-TLR4 complex, both of which in this state cannot prevent coagulation systems activation and platelets aggregation. Furthermore, antiprothrombin antibodies can activate normal thrombocytes in sole presence of calcium and prothrombin. This could explain why DOACs failed to be as effective as warfarin in reducing thrombotic events (29,32).

2.3.2- Immunothrombosis

Antiphospholipid antibodies are able to activate complement system and trigger IL-6 and TNF-α secretion in monocytes. This innate immune system recruitment triggers endothelial proliferation and enhances platelets adhesion to vascular wall, constituting a link between autoimmunity and prothrombotic state. It is important to underline that augmented levels of C5b-C9 deposition, responsible for complement mediated cell-death, correlate with triple aPL positive status and thrombosis recurrence (29,32).

2.3.3 – Neutrophil extracellular traps (NETs)

Antiphospholipid antibodies induce neutrophils activation kicking of the formation of neutrophils extracellular traps (NETs) through ROS production and p38 MAPK mediated pathways. Neutrophils elastase present within NETs milieu is responsible for creating a prothrombotic environment through cleavage of tissue factor pathway inhibitor. Furthermore, NETs structure provides an optimal scaffold for platelets and red blood cells adhesion and aggregation. The synergistic effect of NETs and aPL induces endothelial proliferation and complement activation (29,32).

2.4 – Clinical Manifestations

Because of the copresence of a prothrombotic state and an autoimmune environment, the changing conjunct impact of these two fundamental aspects of APS are responsible for a plethora of clinical manifestations (25). Below, the most characteristic clinical features of APS are described, from the most frequent to the rarest.

2.4.1- Venous thromboembolism

Venous thrombosis is the most common thrombotic event in APS affecting up to 60% of patients. Lower limbs deep vein thrombosis is the most usual manifestation (30-40% of patients), but other sites that are rarely interested in general population could be involved such as jugular, subclavian vein or inferior vena cava. Pulmonary embolism is another common clinical feature, occurring both isolated and as a consequence of deep vein thrombosis. Recurrent pulmonary embolism can lead to a very rare condition named chronic thromboembolic pulmonary hypertension caused by irresolution of thrombi in pulmonary small vascular bed (27).

Other venous thrombotic events manifest in approximately 1% of patients with APS: cerebral sinus or veins thrombosis, retinal vein thrombosis or splanchnic vein thrombosis. More accurately, retinal vein thrombosis is more frequent in PAPS, whereas splanchnic thrombosis such as renal thrombosis is more frequent in SAPS (27).

2.4.2 – Arterial thrombosis

Although less common than venous thromboembolism, arterial thrombosis is the first cause of death and disability in APS (22.5% of patients). Ischemic stroke

and transient ischemic attack are the most common arterial thrombotic events, with the former being more frequent than the latter (30% and 10%, respectively). Peripheral arterial thrombosis is much less common than DVT, and so are cardioembolic events due to Liebmann-Sacks endocarditis or intracardiac thrombi compared to venous thromboembolism (27).

On the other hand, splanchnic arterial thrombosis is more common than the venous counterpart. Mesenteric, spleen and renal artery thrombosis are usual spot of splanchnic thrombosis. In addition, myocardial infarction with or without coronary atherosclerosis is a frequent first clinical presentation of APS (27).

2.4.3 – Microvascular thrombosis

The exact epidemiology of microvascular thrombosis is still unknown, since it is a rare clinical presentation and studies addressing this topic are not uniform in the way they collect data. It is possible to know the prevalence of some of its presentations, such as aPL nephropathy and pulmonary microvascular thrombosis (27).

Nine to 30% of PAPS patients and over 30% of SAPS patients are affected by acute or chronic aPL nephropathy. This renal disease is determined by the presence of lesions to glomerula and/or arterioles with microthrombi. Diagnosis is challenging given that several differential diagnoses must be excluded (TTP, HELLP syndrome, or atypical HUS) and biopsy is required to exclude immune complexes depositions, especially in SAPS patients. On the other hand, pulmonary microvascular thrombosis is less prevalent (1% of patients) and consists of small capillary arteries and alveoli capillary lumens obstruction with or without capillaritis. Symptoms vary from dyspnea and hemoptysis to alveolar hemorrhage and ARDS (27).

2.4.4 – Dermatological disease

Livedo racemose, livedo reticularis, livedoid vasculopathy lesions and skin ulcerations are all possible skin manifestations of APS.

Livedo racemose is a dermatological disorder characterized by ischemic skin lesions that appears widespread, violaceous, symmetric and in a net-like pattern. It is distinguished from livedo reticularis by its peculiar irregular 'broken' appearance. While livedo reticularis is present in 20% of APS patients, it is not

specific for this syndrome, given that occurs in other diseases such as TTP, connective tissue disorders and cryoglobulinemia (27,33).

Livedoid vasculopathy lesions are painful chronic ischemic skin lesions due to microvascular thrombosis of small dermal vessels. Skin ulcerations instead could be the result of venous insufficiency, peripheral arterial thrombosis or microvascular thrombosis (27).

2.4.5 – Obstetric morbidity

Pregnancy morbidity is one of the hallmarks of APS. Typical presentations of patients with obstetric morbidity are three or more consecutive early spontaneous abortion before the 10^{th} week of gestation or fetal death without fetal malformations or chromosomal anomalies after the 10^{th} week of gestation. Other typical clinical features are preterm birth due to pre-eclampsia, eclampsia or placental insufficiency. The reason behind all these symptoms lays in the fact that placenta has an enriched expressed level of β2GPI, a key ligand of aPL. Clinically and histologically, this translates in reduced trophoblast development and endometrial decidualization all the way to placental infarction, decidual inflammation and defective spiral artery remodeling (34,35). Patients with only obstetric involvement are not at the same level of risk of thrombosis as other APS patients (20).

2.4.6 – Cardiac valve disease

Another clinical manifestation of APS is cardiac valve disease, especially involving mitral valve. Thickening or vegetation depositions on the valve due to Liebmann Sacks endocarditis. This autoimmune endocarditis pathogenesis involves fibrin thrombi that cause valvular dysfunction. aPL take part in this process of valvular damage through promoting formation of thrombin on endothelial cells (36).

2.4.7 – Hematologic involvement

Thrombocytopenia is the most common hematologic manifestation in APS with higher prevalence in SAPS, although in this case thrombocytopenia could be determined by SLE. Other rarer forms of hematologic disorders in APS include neutropenia or autoimmune hemolytic anemia (AIHA) (26).

2.4.8 – CNS involvement

Most of neurological presentations are determined by arterial thrombosis. Large cerebral infarcts or repeated small lacunar strokes could result in cognitive deficits. In fact, MRI scans of APS patients may present typical multifocal white matter lesions associated with cognitive impairment (27).

Other neurological presentations are associated with APS, but their pathogenesis is yet to be elucidated. Migraine, seizures, transvers myelitis and chorea are all rare manifestations where the role of thrombosis is not demonstrated yet. Seizures are more common in SAPS and chorea occurs more frequently in young adult females with a level of severity that increases along with aPL titers (37).

2.4.9 – Catastrophic APS (CAPS)

Catastrophic APS (CAPS) is a rare life-threatening condition of APS that occurs in approximately 1% of patients. CAPS is characterized by thrombosis in three or more different organs or systems within 1 week because of microvascular involvement (25).

Mortality rate is 50%, so a tempestive diagnosis and subsequent treatment is crucial to increase chances of survival. Unfortunately, CAPS is also known for not responding to usual APS anticoagulation therapy, so immunomodulators that act on antibody secretion and complement activity are used such as rituximab and eculizumab (20).

2.5 – Therapy

Antithrombotic therapy in APS changes based on the type of thrombotic event and the type of thrombotic and cardiovascular risk factors (20).

One important risk factor of thrombotic propensity is aPL profile:

- High-risk aPL profile:
    - LAC positivity on two or more separate occasions, spaced at least 12 weeks apart.
    - Double positivity: any combination of LAC, aCL or aβ2GPI
    - Triple positivity: LAC, aCL and aβ2GPI.
- Low-risk aPL profile:
    - Isolated low to medium titers of either aCL or aβ2GPI (20).

While aPL profiles plays a crucial role in thrombotic risk prediction, it does not consider other thrombotic and cardiovascular factors that could influence the thrombotic profile of a patient. Thus, adjusted Global APS Score (aGAPSS) was developed. aGAPSS score assigned to each risk factor a determine number of points:

- Hyperlipidemia: 3 points.
- Arterial Hypertension: 1 points.
- aCL IgG/IgM: 5 points.
- aβ2GPI IgG/IgM: 4 points.
- LAC: 4 points (38).

Studies are not concordant on the performance of aGAPSS score. While some studies have shown that this score might help to stratify patients at risk of developing recurrent thrombosis, others found that aGAPSS performance was suboptimal and further research is needed to stratify correctly patients at risk of re-thrombosis (39,40)

2.5.1 – Primary thromboprophylaxis

Primary thromboprophylaxis in aPL positive patients consists of daily low dose aspirin (75-100 mg) therapy. This treatment is recommended in asymptomatic aPL carriers or in SLE aPL carriers with a high-risk aPL profile without any history of thrombotic events or pregnancy morbidity. The same therapy may be prescribed to SLE aPL carriers with a low risk aPL profile (20).

2.5.2 – Secondary thromboprophylaxis

In patients that had a venous thrombotic event, warfarin therapy with a target INR of 2-3 is recommended. Long term anticoagulation therapy is recommended in patients that had a first unprovoked thrombosis, whereas anticoagulation therapy could be carefully discontinued in patients with provoked thrombosis, even if a longer anticoagulation period should be considered for high-risk aPL profiles. In addition, rivaroxaban is not indicated in patients with high-risk aPL profiles since there are limited data supporting their efficacy and safety (20,41).

On the other hand, in patients that had an arterial thrombotic event a warfarin therapy with a target INR of either 2-3 or 3-4 is recommended. Choice should be made pondering thrombotic and bleeding risk factors. DOACs are not

indicated since large trials were interrupted because of not acceptable rates of recurrent thrombosis in patients on rivaroxaban (20,41).

2.5.3 - High intensity secondary thromboprophylaxis

High intensity secondary thromboprophylaxis is recommended in patients with recurrent venous or arterial thrombosis despite their target INR is reached. It consists of either warfarin with a target INR of 3-4 or double antithrombotic therapy with LDA plus warfarin with a target INR of 2-3. While the first one is more effective in reducing re-thrombosis rates it is also characterized by more frequent bleeding complications. Furthermore, only for recurrent arterial thrombosis one may consider triple therapy with warfarin with a target INR of 2-3, LDA and clopidogrel (20,42,43).

2.5.4 – Antithrombotic therapy withdrawal

ACR/EULAR recommendations for management of APS in adults do not identify a period after which LDA can be stopped in primary or secondary thromboprophylaxis, leaving to clinicians the decision of ever interrupting the therapy (20). Since thrombotic recurrence occurs in 16.6% of patients within the first 5 years of disease and in 14.4% within the second 5 year period, well-established predictive factors of re-thrombosis are essential to overcome subjective judgement based on individual clinical and serological profile (44).

Although several studies have addressed this topic, results are discordant. One prospective study conducted in a single aPL patients cohort found that thrombotic recurrence was significantly higher in patients that had their warfarin therapy stopped after six months of treatment compared to patients that continued the same therapy for other six months. Similar results were obtained in retrospective studies with either warfarin or aspirin based therapy (42). On the other hand, a prospective study conducted on a SAPS cohort found that oral anticoagulation withdrawal in patients who became persistently seronegative to all aPL do not cause any thrombotic relapse. Seronegativity occur only in patients where APS diagnosis was made after SLE diagnosis, while patients that developed APS prior or with SLE remained persistently positive. Immunosuppressive therapy was a predictor of aPL negativization , while triple aPL positivy prior to SLE diagnosis was a predictor of persistent positivity (44).

Chapter 3 – APS classification criteria

3.1 – Definition of classification criteria

Antiphospholipid syndrome involves multiple systems, and its causes are unclear. As could be deduced from the previous chapter, APS presentation, progression and outcomes is extremely variable, lacking a definitive clinical, laboratory or pathological feature that serves as a "gold standard" for diagnosis. In fact, diagnostic criteria, intended as sets of signs, symptoms, and tests used in routine clinical care to guide patient management, are yet to be developed and validated. At the moment, clinician have to establish a diagnosis upon a subjective combination of clinical manifestations and laboratory tests. For this reason, clinical expertise plays a crucial role in identifying all the heterogeneous presentations of APS (45).

Conversely, classification criteria are standardized definitions designed to create well-defined, relatively uniform groups of patients for clinical research. They aim to capture the majority of patients with key features of APS, not the entire spectrum of possible patients. Validated classification criteria are pivotal for interpreting study results and comparing outcomes between studies. However, even if classification criteria can aid in diagnosis, they typically have high specificity, meaning few false positives, but lower sensitivity, resulting in some false negatives. This specificity makes them less suitable for routine clinical care, as they might miss some individuals with the disease (45,46).

Since the first description of this rheumatic disease in 1983 (47), three sets of APS classification criteria were designed: Sapporo criteria in 1999 (48), revised Sapporo criteria also known as Sydney criteria in 2006 (49) and lastly 2023 ACR/EULAR classification criteria (50).

3.2 – Sapporo APS classification criteria

The first APS classification ever created consider clinical criteria and laboratory criteria. A patient is eligible to be classified as APS if he presents at least one feature considered by clinical criteria and one within laboratory criteria. Clinical criteria consider two clinical presentations of APS:

- Vascular thrombosis:

- One or more episodes of venous, arterial or small vessels thrombosis confirmed by imaging, doppler studies or histopathology and not otherwise explained by other diseases.

– Pregnancy morbidity:
  - Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation without maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal anomalies.
  - One or more unexplained fetal deaths without morphologically anomalies (confirmed by ultrasound or direct examination) at or beyond the 10th week of gestation.
  - One or more premature births of a morphologically normal newborn at or before the 34th week of gestation because of severe preeclampsia or eclampsia, or severe placental insufficiency.

Laboratory criteria consider:

– LAC positivity in plasma on 2 or more separate occasions at least 6 weeks apart using functional assays such as activated partial thromboplastin time, kaolin clotting time, dilute Russell's viper venom time, dilute prothrombin time or Textarin time.
– Medium to high titer of aCL IgM and/or IgG isotypes tested with an ELISA essay (48).

3.3 – Sydney APS classification criteria

Sydney classification criteria make amendments to Sapporo criteria. While the overall structure of the previous classification method was maintained (at least one clinical criterion and at least one laboratory criterion), some key changes were introduced, specifically in terms of laboratory criteria.

According to these criteria in order to fulfill laboratory criteria must test positive to aPL in two or more separate times at least 12 weeks apart, a period of time doubled compared to Sapporo criteria with the aim of reducing the inclusion of transient positive patients. Furthermore, besides LAC and aCL IgG/IgM, also aβ2GPI IgG/IgM antibodies are considered a sign of definite APS if they reach moderate or high titer in plasma or serum. Minimum titer cut-off value accepted for

fulfilling laboratory criteria is >40 GPL or MPL, or rather >the 99th percentile depending on the measurement method. Titer estimation should be measured by a standardized ELISA.

Even if no updates were made to clinical criteria, Sydney criteria suggest to carry out a further stratification of patients that meet the criteria based on a list of thrombotic risk factors: age (>55 in men and >65 in women), hypertension, diabetes mellitus, elevated LDL or low HDL cholesterol, cigarette smoking, family history of premature cardiovascular disease, BMI>30 kg/m^2, microalbuminuria, estimated GFR <60 mL/min, inherited thrombophilia, oral contraceptives, nephrotic syndrome, malignancy, immobilization, and surgery. However, these classification criteria do not provide a scoring system through which these differences between classified patients could be easily spotted (49).

3.4 – 2023 ACR/EULAR APS classification criteria

With the publication of the 2023 ACR/EULAR APS classification criteria a considerable upgrade to Sydney criteria was made. For the first time, entry criteria were created, and a weighting system was attributed to every single clinical and laboratory feature involved in definite APS. In order to be classified as APS, patients must reach 3 points within clinical domains and 3 points within laboratory domains.

Clinical criteria are now divided into six domains (macrovascular venous and macrovascular arterial, microvascular, obstetric, cardiac valve and hematologic) all of which incorporate a list of clinical manifestations with their respective scores (*Figure 1*). Certain APS presentations that were considered "extra criteria" are now part of the clinical features included in these criteria; these are livedo racemosa, aPL nephropathy, pulmonary hemorrhage, adrenal hemorrhage, cardiac valve disease and thrombocytopenia. These adjustments not only can reach different subsets of patients that were once excluded from the classification, but also allows researchers to shed light on rarer forms of APS giving them the opportunity to better understand their pathogenesis and therapeutical targets. In addition, different magnitude is assigned to macrovascular thrombotic events depending on patient's VTE and CVD profile risk (*Figure 2*).

Clinical criteria are not the only aspect that was changed since two different laboratory domains were developed: one dedicated to LAC positivity, the other to aCL and aβ2GPI titer and isotypes. Within the latter, different weight is assigned to

IgM and IgG positivity in a way that a single moderate to high titer aCL or aβ2GPI IgG positivity is enough to meet laboratory criteria, while patients that tested positive only to aPL IgM do not reach a sufficient laboratory score, even if at high titer. Another important aspect that differentiates the new criteria from the previous ones was that titre thresholds for moderate and high titer are respectively 40-70 units and >80 units, GPL or MPL. The only type of essay allowed for aPL titer estimation is a solid-phase ELISA.

These criteria were tested by the steering committee in two independent validation cohorts reaching a sensitivity of 84% and a specificity of 99%. Sydney criteria were also applied to the same cohorts and reached a sensitivity of 99% and a specificity of 86%. This reflects the purpose of the authors, since the main goal was to create more homogenous subsets of definite APS patients. Thus, the steering committee deliberately prioritized the specificity at the cost of sensitivity (50).

Despite its unequivocable qualities, some critical issues were noted. More in details, some types of patients once classified as APS according to Sydney criteria now would be excluded by the ACR/EULAR criteria:

- Patients with high-risk VTE or high-risk CVD profile that only had either venous thromboembolic events or arterial thrombotic events.
- Patients with obstetric APS with three or more consecutive miscarriages before the 10^{th} week of gestation and/or fetal losses after the 16^{th} week of gestation without pre-eclampsia, eclampsia or placental insufficiency.
- Patients with exclusively moderate to high aCL and/or aβ2GPI IgM titer.

This aspect was highlighted even by the steering committee itself. Nonetheless, the panel of expert found it in some degree acceptable since the goal was to maximize specificity (50).

However, several experts criticized some of these issues, focusing on either obstetric manifestations (51,52) or IgM positive patients (53,54). Others contested that choosing a fixed 40 U cutoff for moderate aPL titer estimated with an ELISA assay was problematic since different results are obtained with the same sample depending on the different ELISA systems available on the market; a problem that could easily be resolved considering the >99^{th} percentile as a valid method to confirm aPL positivity (54–56).

At the same time, the ACR/EULAR criteria were tested in different types of cohorts in terms of ethnicity, age and secondary autoimmune disease presence; all studies confirmed specificity and sensitivity estimated in the validation cohorts, sometimes with minimal but insignificant differences(57–59).

Chapter 4 – Objective of the study

This study aimed analyzed the performance of 2023 ACR/EULAR APS classification criteria in a cohort of primary APS and in cohort of secondary APS. Since in clinical practice the Sydney classification criteria have been often used as diagnostic criteria, our goal was also to evaluate the effect of using the new 2023 classification criteria in the diagnosis of PAPS and SAPS. Another object of the study was to compare specificity and sensitivity between ACR/EULAR criteria and Sydney criteria both estimated in the SAPS cohort.

Besides identifying any significant difference between classified and not classified patients, another goal was to estimate the rate of thrombosis recurrence between classified and unclassified subjects in order to see if the new classification criteria are able to correctly sort patients in high and low risk of thrombotic relapse.

Finally, one more intent was to compare the characteristics of patients with PAPS versus those of patients with SAPS.

Chapter 5 - Methods

5.1 - Sample populations

For this study, we considered two different sample populations: one with patients classified as having primary APS (PAPS) and the other with patients diagnosed with SLE classified as having secondary APS (SAPS). PAPS sample population was formed exclusively by patients diagnosed with thrombotic APS.

The target SLE population of this retrospective study corresponded to the U.O.C. Reumatologia of Azienda Ospedale Università degli Studi di Padova monocentric cohort which consists of 570 patients diagnosed with SLE between 1980 and 2023 and followed-up in the same period. The ethic committee Territoriale Area Centro-Est Veneto approved this study.

A first selection was made with the aim of considering patients who tested positive for at least one aPL (LAC, aCL IgG and/or IgM and aB2GPI IgG and/or IgM) for at least two consecutive times at least 12 weeks apart.

This aPL-positive population was previously divided into two groups based on their clinical manifestations. Patients were classified as having APS or not having APS according to the 2006 revised Sapporo criteria also known as Sydney criteria (49). Those who were not classified with APS were defined as aPL carriers. The group of patients classified as having APS was the SAPS sample population of this study.

As for the formation of the PAPS population, a monocentric U.O.C. Reumatologia of Azienda Ospedale Università degli Studi di Padova cohort made up of patients diagnosed of PAPS between 1980 and 2023 and followed-up in the same period was the sample population of the study. This population did not include any aPL carrier.

5.2 - Data collection

Clinical characteristics and laboratory tests of patients included in this study were re-evaluated to apply a new categorization in line with the 2023 ACR/EULAR criteria (50). The following data were retrospectively collected from patients' medical records and listed anonymously in a designated database:

- Demographics:
  - Sex.

- Date of SLE diagnosis.
- Age at SLE diagnosis.
- Date of APS diagnosis.
- Age at APS diagnosis.
- Previous pregnancies.

– Clinical characteristics:
  - SLE-related manifestations:
    - Rash.
    - Alopecia.
    - Arthritis.
    - Serositis.
    - Proteinuria.
    - Hematuria.
    - Thrombocytopenia.
    - Leukopenia.
    - Neurological involvement.
    - Vasculitis.
  - 2023 ACR/EULAR APS clinical domains events:
    - Venous thromboembolism.
    - Arterial thrombosis.
    - Microvascular thrombosis.
    - Obstetric morbidity.
    - Cardiac valve involvement.
    - Hematology involvement.
  - Other APS-related events.
  - Thrombotic risk factors.
  - CVD risk factors.
  - APS-related relapses.
  - Active SLE at the time of the APS-related thrombotic event.
  - Nephrotic syndrome at the time of the APS-related thrombotic event.

– Laboratory characteristics:
  - C3 and C4 serum levels.
  - ANA titer > 1:80.

- Anti-SSA and/or anti-SSB positivity.
- Anti-dsDNA positivity.
- aPL positivity:
  - LAC positivity.
  - aCL IgM titer.
  - aCL IgG titer.
  - aB2GPI IgM titer.
  - aB2GPI IgG titer.

– Therapeutical characteristics:

- Prednisone >25 mg/die at the time of the APS-related thrombotic event.
- First antithrombotic treatment.
- Last antithrombotic treatment.
- Antithrombotic treatment at the time of the APS-related thrombotic relapse (if any).

5.3 - Score estimation

The 2023 ACR/EULAR APS classification criteria total score was computed utilizing clinical domain events data and aPL antibodies titer of each patient weighted as indicated by *Table I*.

For the assessment of clinical domain score, when addressing the weight of a venous or arterial thrombotic event, venous thromboembolic risk profile and cardiovascular disease risk profile were evaluated based on major and minor risk factors as indicated by *Table II*.

The RheumCalc online tool (https://rheumcalc.com/APS) was used to calculate the total score of each patient.

5.4 - Statistical analysis

A retrospective analysis of the prospectively collected data was carried out. Continuous variables were analyzed by t-test if normally distributed, Mann-Whitney test if not. Categorical variables were analyzed with Chi-square test, with Fisher's correction for samples lower than 5 units.

ROC curves were generated to evaluate the performance of Sydney and EULAR/ACR 2023 classification criteria, using the physician diagnosis as the

reference standard. Sensibility and specificity were also calculated. Statistical analyses were performed using SPSS Statistics version 29.0 (Chicago, Illinois, USA).

Table I: 2023 ACR/EULAR APS Classification Criteria score system

ENTRY CRITERIA **At least one** documented clinical criterion listed below (domain 1-6) **AND** **A positive antiphospholipid antibody test** (aPL) (a lupus anticoagulant test or moderate to high titers of anti-cardiolipin or anti-??-glicoprotein- I antibodies [IgM or IgG] **within 3 years** of the clinical criterion If absent, do not attempt to classify as APS - If present, apply additive criteria			
ADDITIVE CLINICAL AND LABORATORY CRITERIA Do not count a clinical criterion if there is an equally or more likely explanation than APS. Within each domain, only count the highest weighted criterion towards the total score			
CLINICAL DOMAINS			
	Weight		Weight
D1. Macrovascular (Venous Thromboembolism [VTE])		**D2. Macrovascular (Arterial Thrombosis [AT])**	
VTE with a high-risk VTE profile	1	AT with a high-risk CVD profile	2
VTE without a high-risk VTE profile	3	AT without a high-risk CVD profile	4
D3. Microvascular		**D4. Obstetric**	
Suspected	2	?3 Consecutive pre-fetal (<10w) and/or early fetal (10 w 0 d - 15 w 6 d) deaths	1
Livedo racemosa (exam) Livedoid vasculopathy lesions (exam) Acute/chronic aPL nephropathy (exam or lab) Pulmonary hemorrage (symptoms and imaging)		Fetal death (16 w 0 d - 33 w 6 d) in the absence of pre-eclampsia (PEC) with severe features or placental insufficiency (PI) with severe features	1
Established	5		
Livedoid vasculopathy (pathology) aPL nephropathy (pathology) Pulmonary hemorrage (BAL or pathology)		PEC with severe features (<34 w 0 d) or PI with severe features (<34 w 0 d) with/without fetal death	3
Myocardial disease (imaging or pathology) Adrenal hemorrage (imaging or pathology)		PEC with severe features (<34 w 0 d) and PI with severe features (<34 w 0 d) with/without fetal death	4
D5. Cardiac valve		**D6. Hematology**	
Thickening	2	Thrombocytopenia (lowest 20-130×10 ?/L)	2
Vegetation	4		
LABORATORY DOMAINS			
	Weight		Weight
D7. aPL test by coagulation-based functional assay (lupus anticoagulant test [LAC])		**D8. aPL test by solid phase assay (anti-cardiolipin antibody [aCL] ELISA and/or anti- ??-glicoprotein-I antibody [a??GPI] ELISA [persistent])**	
Positive LAC (single - one time)	1	Moderate or high positive (IgM) (aCL and/or a??GPI)	1
Positive LAC (persistent)	5	Moderate positive (IgG) (aCL and/or a??GPI)	4
		High positive (IgG) (aCL or a??GPI)	5
		High positive (IgG) (aCL and a??GPI)	7
TOTAL SCORE Classify as Antiphospholipids Syndrome for research purposes if there are **At least 3 points from clinical domains AND at least 3 points from laboratory domains**			

Table II: 2023 ACR/EULAR APS Classification Criteria definitions to determine high-risk VTE profile and high-risk CVD profile.

Definitions of high-risk venous thromboembolism (VTE) and cardiovascular disease (CVD)
1. To determine if a thrombotic event occurred in a patient with a high-risk VTE or high-risk CVD profile, investigators should make every effort to collect and review risk factor data based on patient report or medical record review. if clinically relevant VTE or CVD risk factors at the time of an historical thrombotic event are unknown in the data source, then the lowest possible non-zero weight should be assigned to the macrovascular event to avoid overestimation of antiphospholipid antibody (aPL) contribution to thrombosis.
2. High-risk VTE profile is defined based on 1 or more major OR 2 or more minor VTE risk factors, if timeline/severity is associated with the event based on investigator's judgement (timelines based on general population guidelines are provided when available).
Major VTE risk factors (any of the following at the time of the event): - Active malignancy with no or noncurative treatment received, ongoing curative treatment including hormonal therapy, or recurrence/progression despite curative treatment at the time of the event. - Hospital admission confined to bed (only bathroom privileges) with an acute illness for at least 3 days within 3 months prior to the event. - Major trauma with fractures or spinal cord injury within 1 month prior to the event. - Surgery with general/spinal/epidural anaesthesia for >30 min within 3 months prior to the event. **Minor VTE risk factors** (2 or more of the following at the time of the event): - Active systemic autoimmune disease or Active inflammatory bowel disease using disease activity measures guided by current recommendations. - Acute/active severe infection according to guidelines, for example, sepsis, pneumonia, SARS-CoV-2. - Central venous catheter in the same vascular bed. - Hormone replacement therapy, estrogen containing oral contraceptives, or ongoing in vitro fertilization treatment. - Long distance travel (≥8 hours). - Obesity (body mass index (BMI) ≥30 kg/m2) - Pregnancy or postpartum period within 6 weeks after delivery. - Prolonged immobilisation not counted above, for example, leg injury associated with reduced mobility, or confined to bed out of hospital for at least 3 days. - Surgery with general/spinal/epidural anesthesia for<30 min within 3 months prior to the event.
3. High-risk CVD profile is defined based on 1 or more high CVD risk factors OR 3 or more moderate CVD risk factors, if timeline/severity is associated with the even based on investigator's judgement (timelines based on general population guidelines are provided when available). **High CVD risk factors** (any of the following at the time of the event): - Arterial hypertension with systolic blood pressure (BP) ≥180mm Hg or diastolic BP ≥110mm Hg. - Chronic kidney disease with estimated glomerular filtration rates60mL/minute for more than 3 months. - Diabetes mellitus with organ damage or long disease duration (type 1 for ≥20 years; type 2 for10 years). - Hyperlipidemia (severe) with total cholesterol ≥310 mg/dL (eight mmoles/litre) or low-density lipoprotein (LDL)-cholesterol>190mg/dL (4.9 mmoles/litre). **Moderate CVD risk factors** (3 or more of the following at the time of the event): - Arterial hypertension on treatment, or with persistent systolic BP≥140mm Hg or diastolic BP≥90mm Hg. - Current tobacco smoking. - Diabetes mellitus with no organ damage and short disease duration (type 1<20 years; type 2<10 years). - Hyperlipidemia (moderate) on treatment, or with total cholesterol above normal range and <310mg/dL (eight mmoles/litre), or LDL-cholesterol above normal range and<190 mg/dL (4.9 mmoles/litre). - Obesity (BMI≥30 kg/m2).

Chapter 6 – Results

6.1 – PAPS

6.1.1 – Population characteristics

205 patients (145 females and 60 males, *Figure 1*) represented the PAPS sample population. All patients were classified as having APS according to Sydney classification criteria. The mean age at diagnosis was 43.9 years with a mean disease duration of 13.6 years (*Table III*).

Figure 1

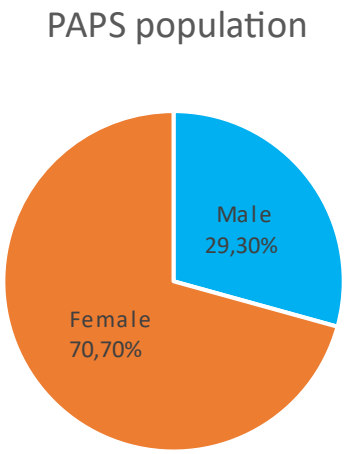


Table III

PAPS sex, age and disease duration			
	Total (N=205)		
	Mean	Standard deviation	Range
Age at diagnosis	43.9	14.2	6 - 76
Disease duration (years)	13.6	9.0	1 - 33
Sex	Number of Patients	Percentages	
Female	145	70.7%	
Male	60	29.3%	

76.9% of patients took only warfarin as their first antithrombotic therapy, but the same medication was prescribed alone in 60% of patients at the end of follow-up; at diagnosis, the remaining 14.4% of the population on both warfarin and LDA. During follow-up, 23 patients (11.2%) had a thrombotic relapse, being 57.1% of them on warfarin at the time of the thrombotic event (*Table IV*).

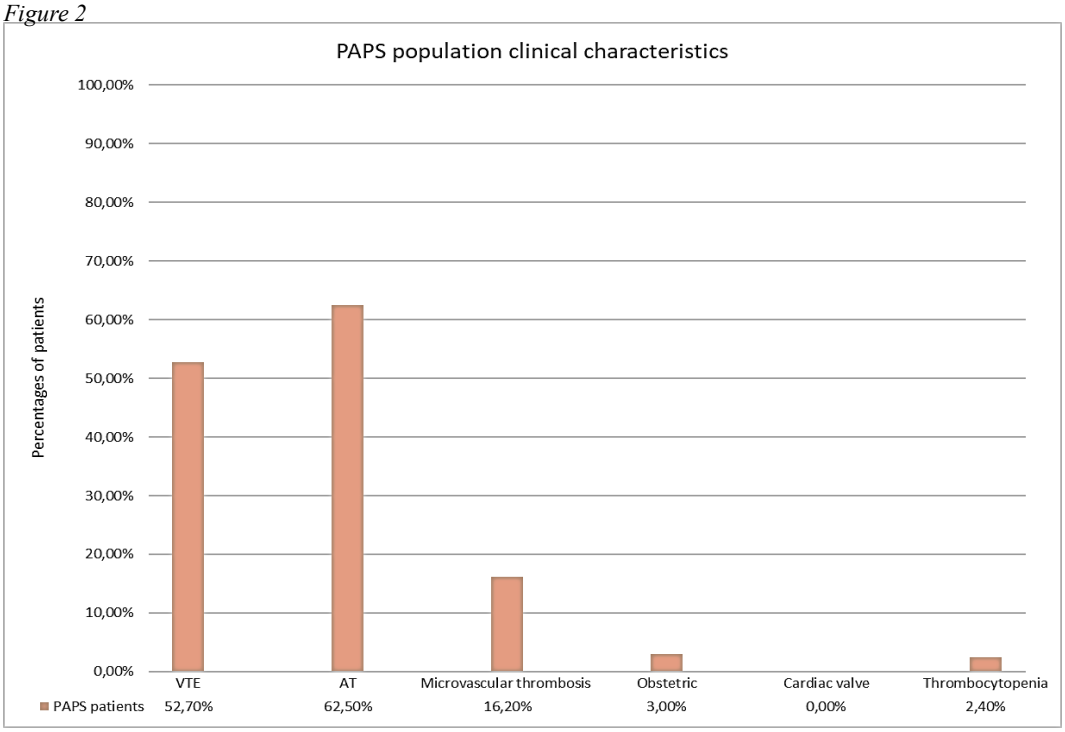
Table IV

PAPS therapeutical and relapse characteristics		
	Total (N=205)	
	Number of Patients	Percentages
First antithrombotic therapy		
Warfarin	150	76.9%
ASA	41	21%

DOAC	2	1%
Clopidogrel	2	1%
Last antithrombotic therapy		
Warfarin	117	60%
LDA	35	17.9%
Warfarin +LDA	28	14.4%
LDA + Clopidogrel	2	1%
DOAC	1	0.5%
Clopidogrel	2	1%
Warfarin + HCQ	2	1%
Warfarin +LDA + HCQ	3	1.5%
LDA + HCQ	4	2.1%
DOAC + HCQ	1	0.5%
Thrombotic relapse	23	11.2%
Antithrombotic therapy at thrombotic relapse		
Warfarin	12	57.1%
LDA	5	23.8%
DOAC	1	4.8%

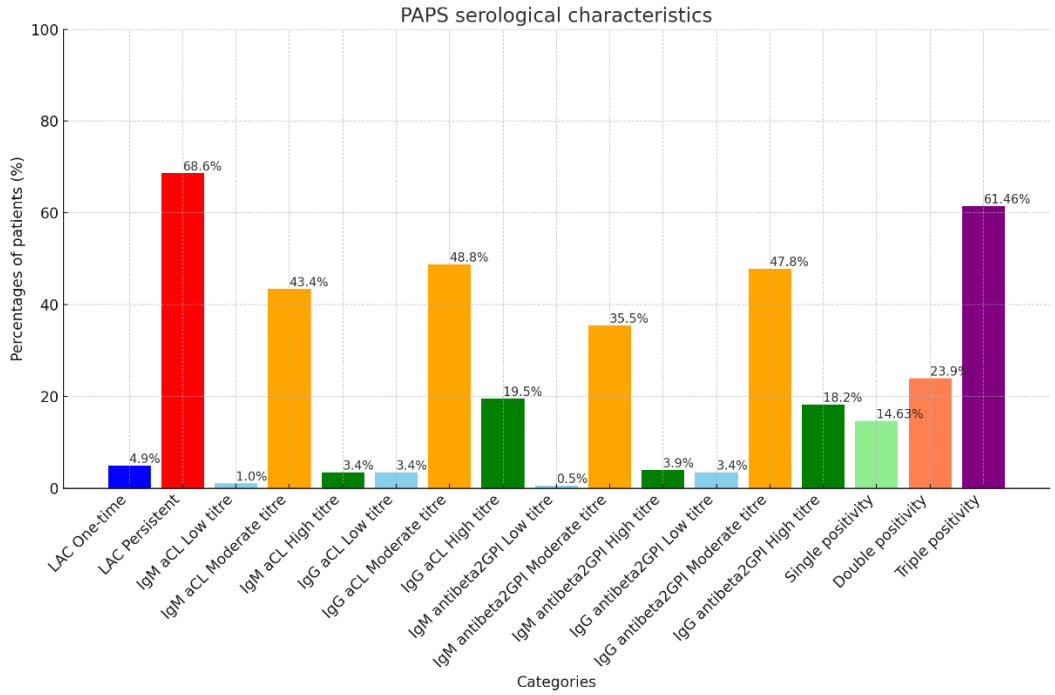
The most frequent clinical manifestation was arterial thrombosis: 61.5% of patients had an arterial thrombosis event without having a high-risk CVD profile risk, whereas 1% had the same event although with a high-risk CVD profile. Venous thromboembolism was the second most common thrombotic event happening in 52.7% of patients. Other APS-related clinical features were less common: microvascular thrombosis 16.2%, obstetric morbidity 3%, thrombocytopenia 2.4% and cardiac valve involvement 1% (*Figure 2*).

Figure 2



Considering laboratory biomarkers, LAC positivity was the most common laboratory item in the population with 68.6% of patients with persistent LAC positivity. Regarding titers of aCL and aβ2GPI, the majority of patients had moderate titer of both aPL, whether IgG or IgM (aCL IgM: 43.8%; aCL IgG: 48.8%; aβ2GPI IgM: 35.5%; aβ2GPI IgG: 47.8%). Furthermore, 61.5% of patients had a triple positive antibody profile (LAC, aCL and aβ2GPI), 23.9% were double positive and 14.6% tested positive for a single type of aPL (*Figure 3*).

Figure 3

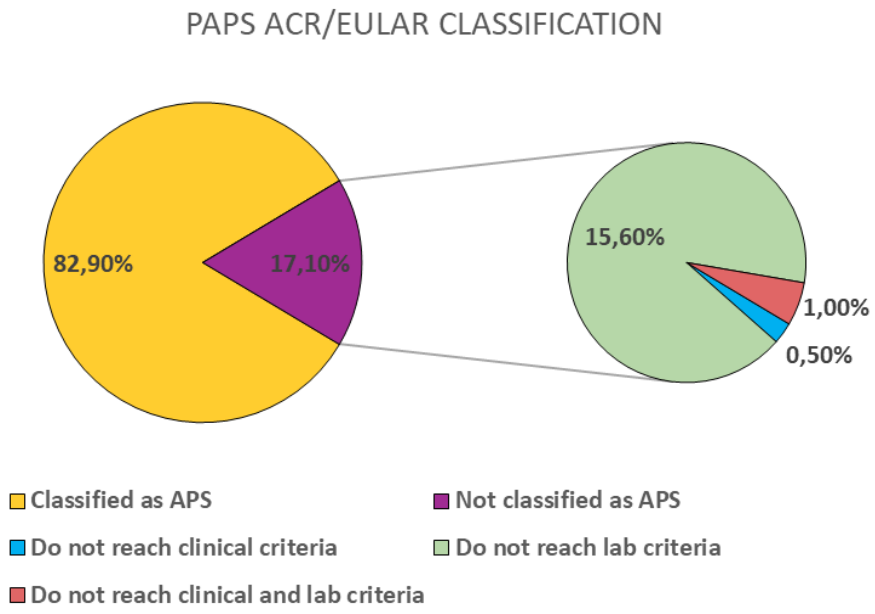


6.1.2 – ACR/EULAR APS classification criteria performance

According with our aims, we applied the new classification criteria to our cohort. Among 205 patients classified according to Sydney criteria, 170 patients (82.9%) were classified as having APS while 35 patients (17.1%) were excluded. Reason for not being classified were:

- Clinical criteria score not reached (1 patient, 0.5%).
- Laboratory criteria score not reached (32 patients, 15.6%).
- Clinical and laboratory criteria score both not achieved (2 patients, 1.0%) (*Figure 4*).

Figure 4



A statistically significant difference for age at diagnosis between classified and unclassified as APS patients was noted (p=0.002): mean age at diagnosis was 50.3 years in unclassified patients, while it was 42.6 years in the classified as APS group (*Table V*). Women and men were respectively 65.7% and 34.3% in the unclassified group, 71.8% and 28.2% in classified group (*Table VI*).

Table V

Age and disease duration in patients fulfilling or not the EULAR/ACR classification.				
	Mean	Standard deviation	Range	*p-value*
Age at APS diagnosis				
NOT classified as APS (N=35)	50.3	12.2	22-66	***0.002**
Classified as APS (N=170)	42.6	14.3	6-76	
APS disease duration				
NOT classified as APS (N=35)	13.5	9.5	1-32	0.814
Classified as APS (N=170)	13.7	8.9	1-33	

Table VI

Sex in patients fulfilling or not the EULAR/ACR classification.					
	Not classified as APS (N=32)		Classified as APS (N=170)		*p-value*
Sex					

Female	23	65.7%	122	71.8%	0.474
Male	12	34.3%	48	28.2%	

Considering other follow-up aspects, a statistically significant difference was noted between the two groups regarding thrombotic relapses (p=0.003): 8.2% of patients classified as APS developed a relapse vs. 25.7% of not classified patients. Notably, 3 patients in the not classified group had a thrombotic relapse after suspending antithrombotic therapy. In order to avoid the bias of this phenomenon, the same statistical analysis was performed excluding these 3 patients. After this, the proportion of unclassified patients who had a thrombotic relapse was 18.8%: even though the difference remained between the two groups, it failed to reach statistical significance (p=0.068).

To further analyze the population of relapsing patients, we evaluated the antithrombotic therapy at the time of thrombotic relapse. We found that a different therapeutic approach was undertaken in patients classified vs. non classified: among the unclassified patients 16.7% were on warfarin and 66.7% were on LDA; on the other hand, 91.7% of classified patients were on warfarin upon thrombotic relapse, with only 8.3% on LDA (p<0.001) (*Table VII*).

Table VII

Therapeutical and relapse characteristics in patients fulfilling or not the EULAR/ACR classification. Exclusion of patients with provoked thrombosis, i.e. those with relapse after anticoagulation withdrawal (selection bias).					
	Not classified as APS (N=32)		Classified as APS (N=170)		*p-value*
First antithrombotic therapy					
Warfarin	25	78.1%	123	76.9%	0.378
LDA	6	18.8%	35	21.9%	
DOAC	0	0%	2	1.3%	
Clopidogrel	1	3.1%	0	0%	
Last antithrombotic Therapy					
Warfarin	22	68.8%	93	58.1%	0.372
LDA	5	15.6%	30	18.8%	
Warfarin +LDA	3	9.4%	25	15.6%	
LDA + Clopidogrel	1	3.1%	1	0.6%	
DOAC	0	0%	1	0.6%	
Clopidogrel	1	3.1%	0	0%	
Warfarin + HCQ	0	0%	2	1.3%	
Warfarin +LDA + HCQ	0	0%	3	1.9%	
LDA + HCQ	0	0%	4	2.5%	
DOAC + HCQ	0	0%	1	0.6%	

Thrombotic relapse	6	18.8%	14	8.2%	0.068
Type of thrombotic relapse					
DVT	1	16.7%	3	21.4%	0.296
PE	1	16.7%	0	0%	
MI	2	33.3%	3	21.4%	
Stroke	1	16.7%	0	0%	
TIA	1	16.7%	0	0%	
Microvascular thrombosis	0	0%	3	21.4%	
DVT + MI	0	0%	1	7.1%	
DVT + Stroke	0	0%	1	7.1%	
DVT + Microvascular thrombosis	0	0%	2	14.3%	
MI + Microvascular thrombosis	0	0%	1	7.1%	
Antithrombotic therapy at relapse					
Warfarin	1	16.7%	11	91.7%	***<0.001**
LDA	4	66.7%	1	8.3%	
DOAC	1	16.7%	0	0%	

Table VIII shows the clinical characteristics of the two groups. What emerged was that venous thromboembolism, type of arterial thrombosis and suspected microvascular thrombosis all were statistically significant different between classified and unclassified patients (p=0.004, p <0.001 and p=0.025, respectively). Notably, among patients within the unclassified group there were more patients with high-risk VTE profile (9.4% versus 0.6%), less patients who had a stroke (15.6% versus 29%) and none that had microvascular thrombosis, neither suspected (0% versus 14.7%) or established (0% versus 4.7%).

Table VIII

Clinical characteristics in patients fulfilling or not the EULAR/ACR classification. Exclusion of patients with provoked thrombosis, i.e. those with relapse after anticoagulation withdrawal (selection bias).					
	Not classified as APS (N=32)		Classified as APS (N=170)		*p-value*
Venous thromboembolism					
with high-risk VTE profile	3	9.4%	1	0.6%	***0.004**
without high-risk VTE profile	14	43.8%	88	51.8%	
Type of VTE					
DVT	15	46.9%	68	40.2%	0.925
PE	1	3.1%	11	6.5%	
Retinal thrombosis	0	0%	1	0.6%	
DVT + PE	1	3.1%	6	3.6%	
DVT + splanchnic thrombosis	0	0%	2	1.2%	
Arterial thrombosis					
with high-risk CVD profile	0	0%	2	1.2%	0.782

without high-risk CVD profile	19	59.4%	105	61.8%	
Type of AT					
MI	10	31.3%	52	30.8%	
Retinal thrombosis	1	3.1%	0	0%	
Stroke	5	15.6%	49	29%	***<0.001**
TIA	3	9.4%	0	0%	
MI + Stroke	0	0%	5	3%	
Microvascular suspected					
Livedoid vasculopathy lesions	0	0%	24	14.1%	***0.025**
Pulmonary hemorrage	0	0%	1	0.6%	
Microvascular established					
Livedoid vasculopathy	0	0%	5	2.9%	1.000
aPL nephropathy	0	0%	3	1.8%	
Obstetric					
Preeclampsia with severe features	0	0%	1	0.6%	1.000
Fetal death	0	0%	5	3%	
Cardiac valve	0	0%	2	1.2%	1.000
Hematologic					
Thrombocytopenia	0	0%	5	2.9%	1.000

Significant differences between classified and unclassified patients were also found regarding serological profile, both in terms of aPL titers and number of positive tests. 73.5% of patients classified as APS had triple antibody positivity against 3.1% of not classified patients (*Table IX*).

Table IX

Serological characteristics in patients fulfilling or not the EULAR/ACR classification. Exclusion of patients with provoked thrombosis, i.e. those with relapse after anticoagulation withdrawal (selection bias).					
	Not classified as APS (N=32)		Classified as APS (N=170)		P-value
LAC					
One-time	0	0%	10	5.9%	***<0.001**
Persistent	1	3.1%	139	82.2%	
aCL IgG					
Low titer	7	21.9%	0	0%	
Moderate titer	0	0%	100	58.8%	***<0.001**
High titer	1	3.1%	39	22.9%	
aCL IgM					
Low titer	2	6.3%	3	2%	
Moderate titer	14	43.8%	62	36.5%	***0.028**
High titer	7	21.9%	7	4%	
antiB2 IgG					
Low titer	6	18.8%	1	0.6%	***<0.001**

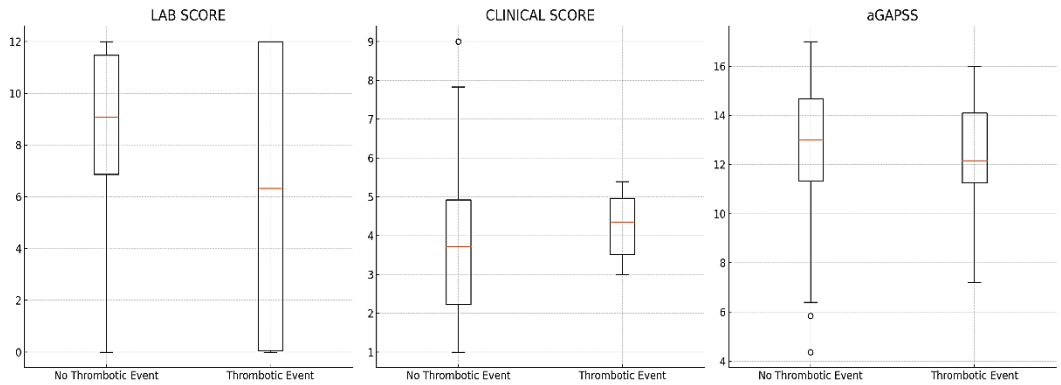
Moderate titer	0	0%	97	57.7%	
High titer	1	3.1%	36	21.4%	
antiB2 IgM					
Low titer	1	3.1%	8	5%	
Moderate titer	11	34.4%	48	28.2%	***0.001**
High titer	7	21.9%	3	2%	
Antibody profile					
Single positivity	12	37.5%	18	10.6%	
Double positivity	19	59.4%	27	15.9%	***<0.001**
Triple positivity	1	3.1%	125	73.5%	

6.1.3 – Thrombotic relapses

As shown in *Table VII*, there is not significant difference in thrombotic relapses between unclassified and classified patients according to the ACR/EULAR criteria (18.8% vs 8.2% respectively, p=0.068) with not classified patients that have a slightly higher rate of thrombotic recurrence. For this reason, we studied the characteristics of patients that had a new thrombotic event (N=20, 10%) and patients that did not experienced other thrombotic event (N=182, 90%) in order to find out whether a parameter could predict which patients are more at risk of a new thrombotic episode.

There were no significant differences between patients who had a thrombotic relapse and those who had no further thrombotic events even for the lab score and clinical score as shown in the box plots below (*Figure 5*). Interestingly, even applying a score that considers antibody profile positivity and CVD profile risk with the aim of predicting which patient is more likely to develop a recurrent thrombosis like aGAPSS score, no significant difference came to light between patients with and without a thrombotic relapse (*Figure 5*).

Figure 5: lab score, clinical score and aGAPSS score box plots in patient with no thrombotic event and with thrombotic event



Sex proportion between patients with and without thrombotic relapses is approximately the same (Females 70.9% in no thrombotic relapse group vs 65% in thrombotic relapse group). Therapeutical characteristics were similar at diagnosis with slightly less patients on warfarin in patients without re-thrombotic event (75.3% vs 94.4%), but a significant difference was found for the last antithrombotic therapy between the two groups (p=0.037): 61.5% of patients without thrombotic relapses were on warfarin against 44.4% of their counterparts, but in the latter group 44.4% of patients were on high-intensity anticoagulation therapy with warfarin plus LDA, a therapy that only 11.5% of patients without thrombotic recurrence had (*Table X*).

Table X

Sex and therapeutical characteristics in patients with or without thrombotic relapses. Exclusion of patients with provoked thrombosis, i.e. those with relapse after anticoagulation withdrawal (selection bias).					
	No thrombotic relapse (N=182)		Thrombotic relapse (N=20)		*p*-value
Sex					
Female	129	70.9%	13	65%	0.585
Male	53	29.1%	7	35%	
First antithrombotic therapy					
Warfarin	131	75.3%	17	94.4%	0.333
LDA	40	23.0%	1	5.6%	
DOAC	2	1.1%	0	0%	
Clopidogrel	1	0.6%	0	0%	
Last antithrombotic Therapy					
Warfarin	107	61.5%	8	44.4%	***0.037**
LDA	34	19.5%	1	5.6%	
Warfarin + LDA	20	11.5%	8	44.4%	
LDA + Clopidogrel	2	1.1%	0	0%	
DOAC	1	0.6%	0	0%	
Clopidogrel	1	0.6%	0	0%	
Warfarin + HCQ	2	1.1%	0	0%	
Warfarin + LDA + HCQ	2	1.1%	1	5.6%	
LDA + HCQ	4	2.3%	0	0%	
DOAC + HCQ	1	0.6%	0	0%	

The only significant difference within baseline clinical characteristics was noted for the type of arterial thrombosis (p=0.045). In particular, within patients that experienced a thrombotic relapse there was a higher percentage of myocardial infarction than in patients without thrombotic relapse (55% vs 28%, respectively). And patients that had myocardial infarction are more likely to have a thrombotic

relapse with RR of 1.14 (95% CI, 1.01-1.29). Interestingly, patients with thrombotic relapse that had a stroke were only 15% compared to 28% of patients without thrombotic recurrence (*Table XI*).

Table XI

Clinical characteristics in patients with or without thrombotic relapses. Exclusion of patients with provoked thrombosis, i.e. those with relapse after anticoagulation withdrawal (selection bias).					
	No thrombotic relapse (N=182)		Thrombotic relapse (N=20)		*p*-value
Venous thromboembolism					
with high-risk VTE profile	4	2.2%	0	0%	0.350
without high-risk VTE profile	89	48.9%	13	65%	
Type of VTE					
DVT	74	40.9%	9	45%	0.383
PE	10	5.5%	2	10%	
Retinal thrombosis	1	0.6%	0	0%	
DVT + PE	6	3.3%	1	5%	
DVT + splanchnic thrombosis	1	0.6%	1	5%	
Arterial thrombosis					
with high-risk CVD profile	2	1.1%	0	0%	0.192
without high-risk CVD profile	108	59.3%	16	80%	
Type of AT					
MI	51	28%	11	55%	***0.045**
Retinal thrombosis	1	0.6%	0	0%	
Stroke	51	28%	3	15%	
TIA	3	1.7%	0	0%	
MI + Stroke	3	1.7%	2	10%	
Microvascular suspected					
Livedoid vasculopathy lesions	21	11.5%	3	15.0%	0.742
Pulmonary hemorrage	1	0.5%	0	0%	
Microvascular established					
Livedoid vasculopathy	5	2.7%	0	0%	1.000
aPL nephropathy	3	1.6%	0	0%	
Obstetric					
Preeclampsia with severe features	1	0.6%	0	0%	0.711
Fetal death	5	2.8%	0	0%	
Cardiac valve	1	0.6%	1	5%	1.000
Hematologic					
Thrombocytopenia	5	2.7%	0	0%	0.453

There was no significant difference between the two groups of patients with or without thrombotic relapses in terms of aPL positivity, except for aCL IgG

(p=0.024). Regarding aCL IgG titers, patients with a thrombotic relapse were more frequently aCL IgG positive (40%) compared with patients without thrombotic relapse (17.6%). Finally, no significant difference was found in antibody profile, with similar proportion of single, double and triple positivity between the two groups (*Table XII*).

Table XII

Serological characteristics in patients with or without thrombotic relapses. Exclusion of patients with provoked thrombosis, i.e. those with relapse after anticoagulation withdrawal (selection bias).

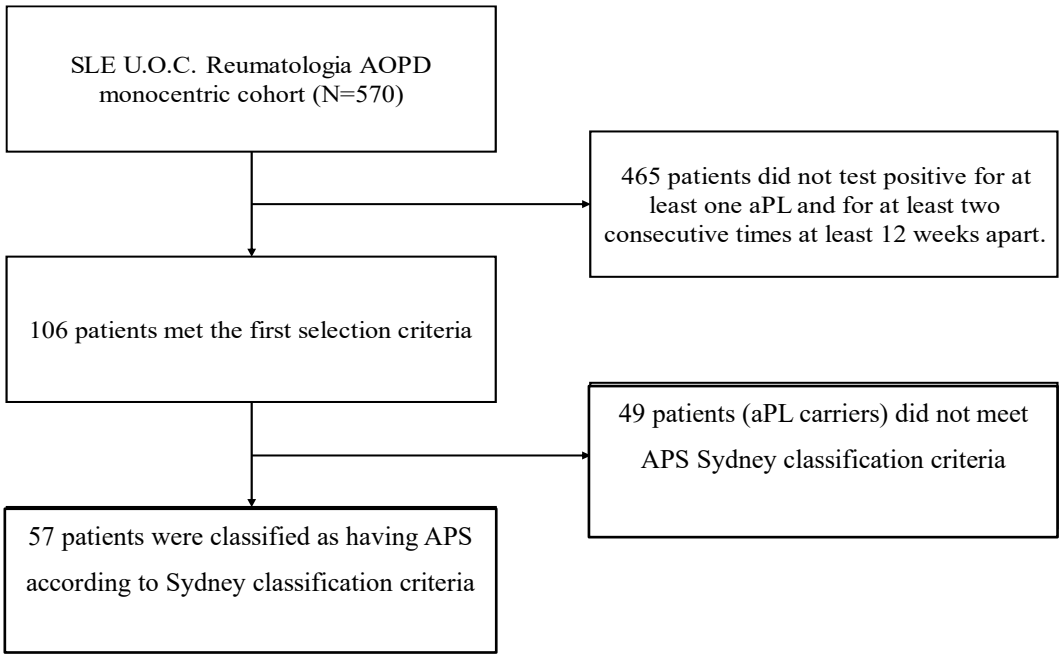
	No thrombotic relapse (N=182)		Thrombotic relapse (N=20)		*p*-value
LAC					
One-time	9	5%	1	5%	0.880
Persistent	127	70.2%	13	65%	
aCL IgG					
Low titer	5	2.7%	2	10%	***0.024**
Moderate titer	94	51.6%	6	30%	
High titer	32	17.6%	8	40%	
aCL IgM					
Low titer	2	1.1%	0	0%	0.388
Moderate titer	78	42.9%	8	40%	
High titer	5	2.7%	2	10%	
antiB2IgG					
Low titer	7	3.8%	0	0%	0.120
Moderate titer	91	50.6%	6	30%	
High titer	29	16.1%	8	40%	
antiB2 IgM					
Low titer	1	0.6%	0	0%	0.647
Moderate titer	61	33.9%	9	45%	
High titer	7	3.9%	0	0%	
Antibody profile					
Single positivity	27	14.8%	3	15%	1.000
Double positivity	43	23.6%	4	20%	
Triple positivity	112	61.5%	13	65%	

Five patients had a thrombotic relapse when their anticoagulation therapy was suspended; consequently, their new event was considered a provoked re-thrombosis. Three patients were single aPL positive and two were triple aPL positive; they were all classified because they either have LAC persistent positivity or moderate to high titre of aCL or aβ_2GPI IgG. Considering clinical aspect, all patients had a venous thromboembolic event but only two presented an arterial thrombosis event; thrombosis occurred without the presence of VTE risk factors,

but in one patient arterial thrombosis occurred in the context of a high-risk CVD profile. One patient also had an episode of microvascular thrombosis. First anticoagulation therapy and therapy after thrombosis recurrence remained the same for three out of five: previously all on warfarin, then 2 switched to warfarin plus LDA. This shift in therapy is due to the fact that those patients had an arterial re-thrombosis.

6.2 – SAPS

Figure 6 Flow chart used for SAPS sample population creation.[1]45 patients did not fulfill Sydney criteria because they had no thrombotic events.



6.2.1 – Population characteristics

Among 570 patients with SLE, 57 patients (10%) were classified as having APS according to Sydney classification criteria (*Figure 6*) with 37 women and 20 men (*Figure 7* and *Table XIII*).

The mean age at APS diagnosis was 31.9 years with a mean disease duration period of 21.2 years, whereas the corresponding characteristics for SLE were 28.6 years and 24.6 years, respectively (*Table XIV*). In fact, most of the sample population (43.1%) was diagnosed with APS after being diagnosed with SLE, while a smaller portion received a diagnosis of APS that of SLE (*Table XV*).

Figure 7

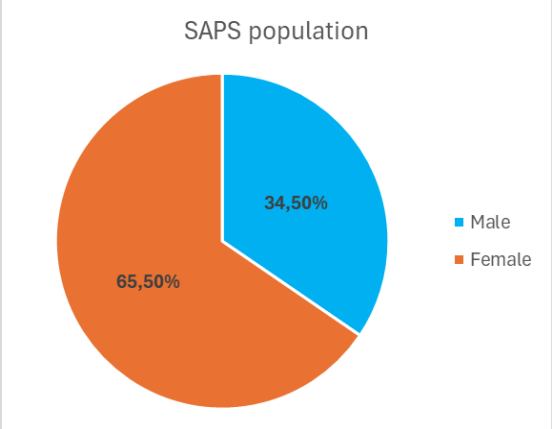


Table XIII

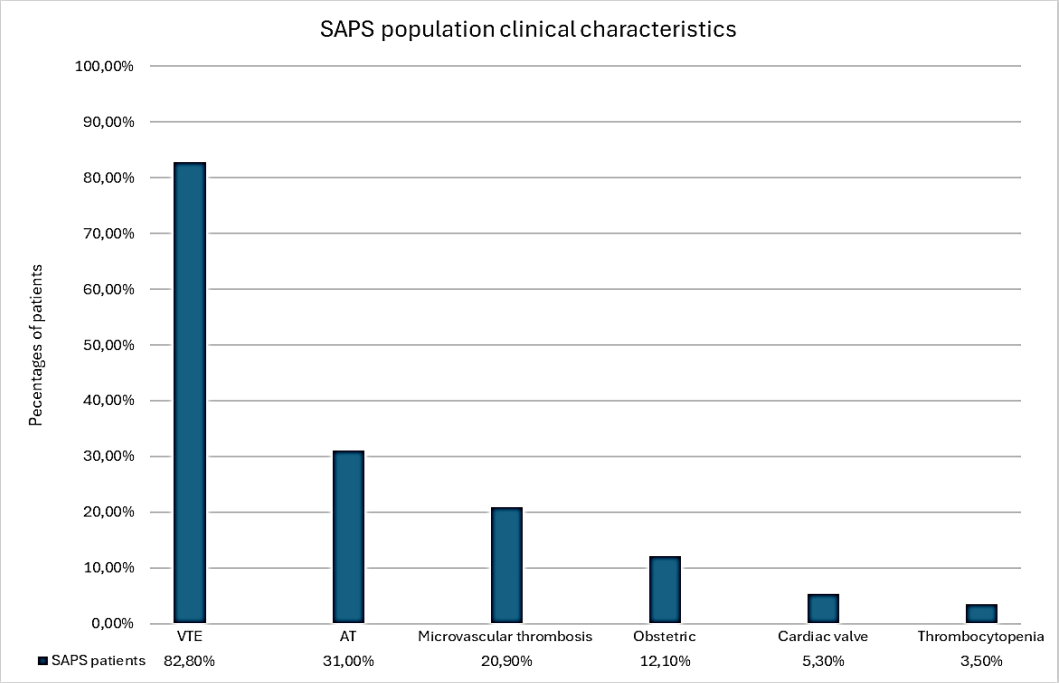
SAPS sex characteristics		
	Total (N=57)	
	Number of Patients	Percentages
Sex		
Male	20	34.5%
Female	37	65.5%

Table XIV

SAPS age and disease duration characteristics			
	Total (N=57)		
	Mean	Standard deviation	Range
Age at SLE diagnosis, years	28.6	12.3	6 - 64
SLE disease duration, years	24.6	9.8	4 - 45
Age at APS diagnosis, years	31.9	12.9	6 - 63
APS disease duration, years	21.2	9.5	4 - 43

Considering clinical features of this sample population, venous thromboembolism was the most common clinical manifestation affecting 82.8% of patients, having only 8 patients (13.8%) a high VTE profile risk. Arterial thrombosis occurred in 31% of patients, while microvascular thrombosis, obstetric morbidity, cardiac valve involvement and thrombocytopenia occurred in 20.9%, 12.1%, 5.3% and 3.5%, respectively (*Figure 8*).

Figure 8



Regarding serological characteristics, 79.3% of patients tested persistently positive to LAC; aCL and aβ2GPI IgG antibodies not only were more prevalent than their respective IgM isotypes, but their titer was higher compared to IgM: 32.8% of patients had high titer of aCL IgG vs 6.9% with high titer of aCL IgM; similarly, 29.3% of patients had high titer of aβ2GPI IgG with only 5.2% who had high titer of aβ2GPI IgM. In addition, 24.1% of patients tested positive for a single aPL antibod, 17.2% had a double positivity and 58.2% were simultaneously positive to LAC, aCL and aβ2GPI (IgG and/or IgM) (*Figure 9*).

Warfarin was prescribed as first antithrombotic therapy in 77.2% of patients, but only 43.1% of them remained on a single warfarin therapy until the end of follow-up. Despite being treated with antithrombotic therapy, 48.3% patients had at least one thrombotic relapse with 44.4% of them that developed a new event while taking warfarin. Venous thromboembolism was the most frequent type of thrombotic relapse. In particular, DVT occurred in 17.2% of the population (*Table XV*). Median remission period since APS diagnosis was 9 years, with a minimum of less than a year (1 month) and a maximum of 27 years.

Figure 9

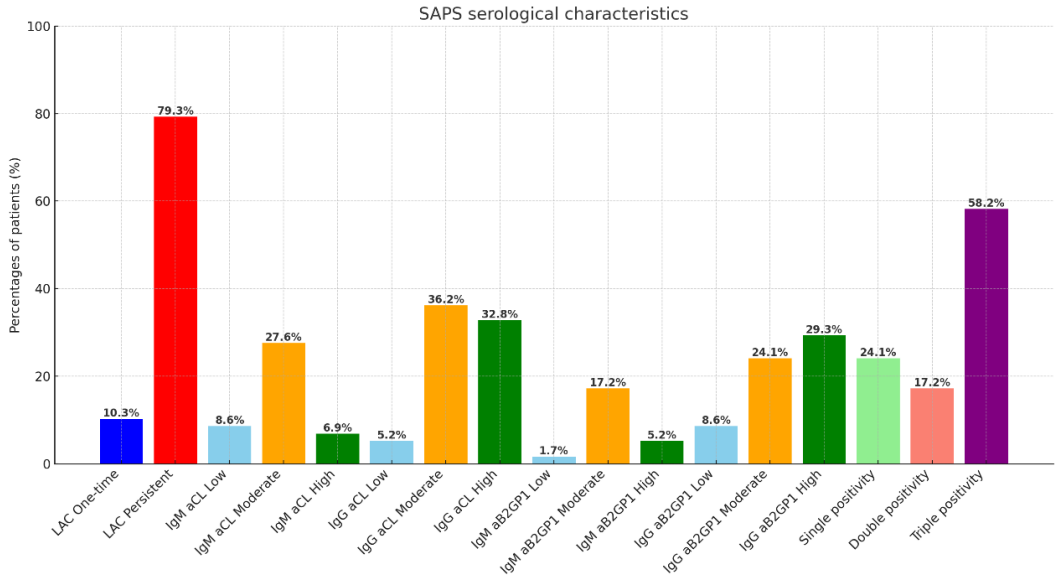


Table XV

SAPS Therapeutical and relapse characteristics		
	Total (N=57)	
	Number of Patients	Percentages
Moment of APS diagnosis		
Before SLE	13	22.4%
With SLE	19	34.5%
After SLE	25	43.1%
First antithrombotic therapy		
Warfarin	43	77.2%
LDA	9	15.8%
Warfarin +LDA	1	1.8%
LDA + Clopidogrel	1	1.8%
DOAC	2	3.5%
Last antithrombotic therapy		
Warfarin	25	43.1%
LDA	16	29.3%
Warfarin +LDA	8	13.8%
DOAC	3	5.2%
Clopidogrel	2	3.4%
Fondaparinux	1	1.7%
Warfarin +LDA + Clopidogrel	1	1.7%
Thrombotic relapse	28	48.3%
Antithrombotic therapy at thrombotic relapse		
Warfarin	12	44.4%
LDA	7	25.9%

LMWH	1	3.7%
Warfarin +LDA + Clopidogrel	2	7.4%

Data collected about SLE clinical and serological characteristics showed that arthritis, rash, proteinuria and leukopenia were the most common clinical manifestations (63.8%, 55.2%, 51.7%, 50%, respectively), whereas hypocomplementemia and anti-dsDNA were the most prevalent serological features (77.6% and 65.5%, respectively). Almost the entirety of the population had an ANA titer > 1:80 (98.6%). Notably, 60.3% of patients were in a state of active SLE at the time of the APS-related event (*Table XVI*).

Table XVI

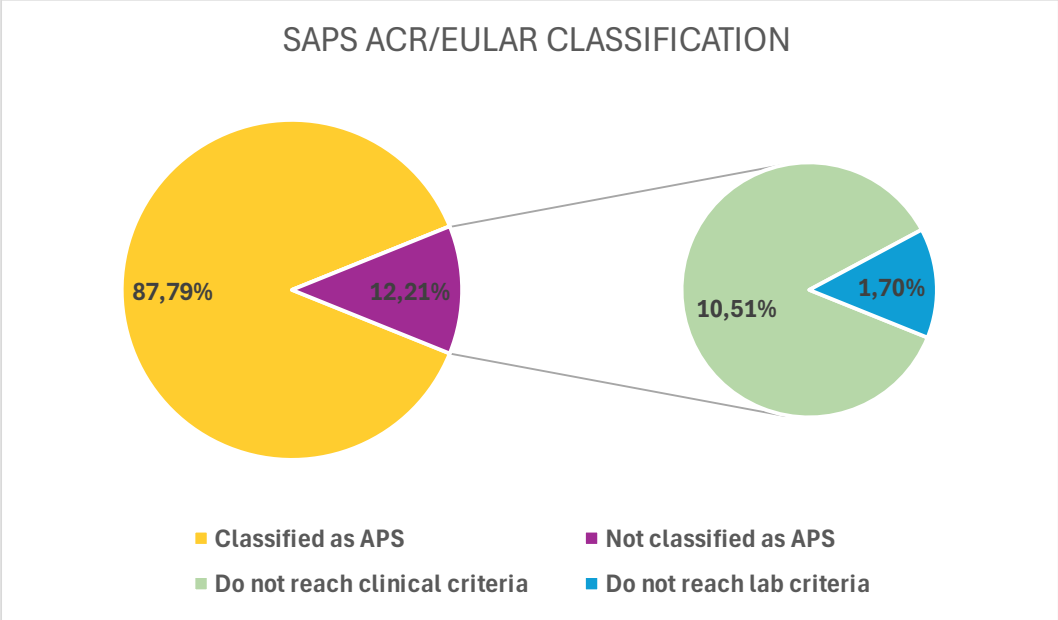
SLE clinical and serological characteristics		
	Total (N=57)	
	Number of Patients	Percentages
Rash	32	55.2%
Alopecia	3	5.2%
Arthritis	37	63.8%
Serositis	11	19%
Proteinuria	30	51.7%
Hematuria	24	41.4%
Thrombocytopenia	13	22.4%
Leukopenia	29	50%
Neurologic involvement	16	27.6%
Vasculitis	3	5.2%
Hypocomplementemia	45	77.6%
Anti-dsDNA	38	65.5%
ANA titer > 1:80	56	98.6%
Anti-SSA and/or anti-SSB	16	27.6%
Anti-U1RNP	12	20.7%
Active SLE[1]	35	60.3%
Nephrotic syndrome[1]	10	17.2%
Prednisone >25 mg[1]	8	13.8%
[1]At the time of the APS-related event		

6.2.2 – ACR/EULAR APS classification criteria performance

According with our aims, we applied the new classification criteria to our cohort of SAPS patients. Among 57 patients, 50 were classified as having APS (87.2%) while 7 patients failed to reach 2023 ACR/EULAR APS classification criteria (*Figure 10*). Of this latter group:

- 6 patients (10.5%) did not reach clinical criteria score.
- 1 patient (1.7%) did not reach laboratory criteria score.

Figure 10



The two subsets of patients had similar mean age at APS diagnosis and mean disease duration period as shown in *Table XVII*. Women and men were respectively 87.8% and 12.2% in the unclassified group, 62% and 38% in classified group (*Table XVIII*).

Table XVII

Age and disease duration in patients fulfilling or not the EULAR/ACR classification.

	Mean	Standard deviation	Range	*p*-value
Age at SLE diagnosis				
NOT classified as APS (N=8)	28.2	13.3	7 - 49	0.937
Classified as APS (N=50)	28.6	12.3	6 - 64	
SLE disease duration				
NOT classified as APS (N=8)	22.1	11.2	11 - 43	0.448
Classified as APS (N=50)	25.0	9.6	4 - 45	
Age at APS diagnosis				
NOT classified as APS (N=8)	29.5	12.1	13 - 49	0.559

Classified as APS (N=50)	32.3	13.0	6 - 63	
APS disease duration				
NOT classified as APS (N=8)	20.9	12.1	7 - 37	0.927
Classified as APS (N=50)	21.2	9.2	4 - 43	

Table XVIII

Sex in patients fulfilling or not the EULAR/ACR classification.					
	NOT classified as APS (N=7)		Classified as APS (N=50)		*p*-value
Sex					
Male	1	14.3%	19	38%	0.241
Female	6	85.7%	31	62%	

Table XIX shows the impact of clinical manifestations on being classified as APS. There was a significant impact of thrombotic risk factors between unclassified and classified patients for venous thromboembolism (p=0.002). More in details, 57.2% of patients not classified as APS who developed a VTE accident had a high-risk VTE profile, while only 8% of classified patients had the same risk profile. In fact, 78% of classified patients had a VTE episode despite not having a high-risk VTE profile.

Table XIX

Clinical characteristics in patients fulfilling or not the EULAR/ACR classification.					
	NOT classified as APS (N=7)		Classified as APS (N=50)		*p*-value
Venous thromboembolism					
with high-risk VTE profile	4	57.2%	4	8%	***0.002**
Without high-risk VTE profile	1	14.3%	39	78%	
Type of VTE					
DVT	3	42.9%	27	54%	0.795
PE	0	0%	3	6%	
Splanchnic thrombosis	0	0%	1	2%	
Renal thrombosis	0	0%	1	2%	
Retinal thrombosis	0	0%	1	2%	
DVT + PE	2	28.6%	9	18%	
DVT + PE + Splanchnic thrombosis	0	0%	1	2%	
Arterial thrombosis					
High risk CVD profile	1	14.3%	7	14%	0.355
Without high-risk CVD profile	0	0%	10	20%	

Type of AT					
MI	0	0%	6	12%	0.151
Peripheral thrombosis	0	0%	3	6%	
Stroke	0	0%	5	10%	
TIA	0	0%	2	4%	
Intestinal thrombosis	0	0%	1	2%	
MI + peripheral thrombosis	1	12.5%	0	0%	
Microvascular suspected					
Livedo racemosa	0	0%	1	2%	0.750
Livedoid vasculopathy lesions	0	0%	3	6.1%	
Microvascular established					
aPL nephropaty	0	0%	3	6%	0.529
Microthrombosis	0	0%	4	8%	
Obstetric					
Preeclampsia with severe features	0	0%	3	6%	0.607
Fetal death	1	12.5%	2	4%	
Cardiac valve					
Thickening only	0	0%	2	4.1%	0.772
Vegetation with or without thickening	0	0%	1	2%	
Hematologic					
Thrombocytopenia	0	0%	2	4.1%	0.561

Table XX clarifies the role of each laboratory item on classifying a patient as having APS. Only LAC positivity showed a significant impact on classification (p=0.030), with a greater prevalence of persistent LAC positivity in patients classified as having APS (84% against 57.2%).

Table XX

Serological characteristics in patients fulfilling or not the EULAR/ACR classification.					
	NOT classified as APS (N=7)		Classified as APS (N=50)		*p*-value
LAC					
One-time	1	14.3%	5	10%	**0.030**
Persistent	4	57.2%	42	84%	
IgM aCL titer					
Low titer	0	0%	5	10%	0.110
Moderate titer	1	14.3%	15	30%	
High titer	2	28.6%	2	4%	
IgG aCL titer					
Low titer	0	0%	3	6%	0.767
Moderate titer	3	42.9%	18	36%	
High titer	2	28.6%	17	34%	
IgM antibeta2GPI titer					

Low titer	0	0%	1	2%	0.742
Moderate titer	1	14.3%	9	18%	
High titer	1	14.3%	2	4%	
IgG antibeta2GPI titer					
Low titer	1	14.3%	4	8%	0.778
Moderate titer	1	14.3%	13	26%	
High titer	2	28.6%	15	30%	
Antibody profile					
Single positivity	3	42.9%	10	20%	0.178
Double positivity	1	14.3%	9	18%	
Triple positivity	3	42.9%	31	62%	

There were not significant differences between the two groups regarding the impact of sex and first and last antithrombotic therapy. Thrombotic relapses were more common in patients classified as having APS (54% of patients versus 14.3% of those not classified). Notably, 5 patients in the classified group had a thrombotic relapse after suspending antithrombotic therapy. In order to avoid the bias of this phenomenon, the same statistical analysis was performed excluding these 5 patients.

After this, the proportion of classified patients who had a thrombotic relapse was 48.9%: even though the difference remained between the two groups, it failed to reach statistical significance (p=0.117). Furthermore, the classified group experienced a broader range of thrombotic relapses typologies, although not reaching a statistical significance. Interestingly, antithrombotic therapy at the time of thrombotic relapse were extremely different between the two groups but it failed to reach a statistical significance as it is shown in *Table XXI*.

Table XXI

Therapeutical and thrombotic relapse characteristics in patients fulfilling or not the EULAR/ACR classification. Exclusion of patients with provoked thrombosis, i.e. those with relapse after anticoagulation withdrawal (selection bias).					
	NOT classified as APS (N=7)		Classified as APS (N=45)		*p*-value
Moment of APS diagnosis					
Before SLE	1	14.3 %	11	24.4 %	0.501
With SLE	4	57.1 %	13	28.9 %	
After SLE	2	28.6 %	21	46.7 %	
First antithrombotic therapy					
Warfarin	5	71.4 %	33	73.3 %	0.885
LDA	2	28.6 %	7	15.6 %	
Warfarin +LDA	0	0,0%	1	2.2 %	
LDA + Clopidogrel	0	3,3%	1	2.2 %	

DOAC	0	6,7%	2	4.4 %	
Last antithrombotic therapy					
Warfarin	2	28.6 %	19	42.2 %	0.526
LDA	5	71.4 %	12	26.7%	
Warfarin +LDA	0	0%	6	13.3%	
DOAC	0	0%	3	6.7 %	
Clopidogrel	0	0%	2	4.4 %	
Fondaparinux	0	0%	1	2.2 %	
Warfarin +LDA + Clopidogrel	0	0%	1	2.2 %	
Thrombotic relapse	1	14.3%	22	48.9%	0.117
Type of thrombotic relapse					
Venous Thromboembolism	1	100%	10	45.5 %	1.000
Arterial Thrombosis	0	0%	5	22.7 %	
Microvascular Thrombosis	0	0%	1	4.5 %	
Venous Thromboembolism + Arterial Thrombosis	0	0%	4	18.2 %	
Arterial Thrombosis + Microvascular Thrombosis	0	0%	2	9.1 %	
Antithrombotic therapy at thrombotic relapse					
Warfarin	0	0%	13	59%	0.075
LDA	0	0%	7	32%	
LMWH	1	100%	0	0%	
Warfarin +LDA + Clopidogrel	0	0%	2	1%	

SLE disease activity characteristics were studied to evaluate any statistical relationship with APS classification criteria. Several dissimilarities were noted and reported in *Table XXII*, but the only feature which resulted significant was the presence of anti-U1RNP antibody (p=0.049). Patients that were not classified as having APS tested more frequently positive to anti-U1RNP than patients classified as having APS (57.2% versus 16%).

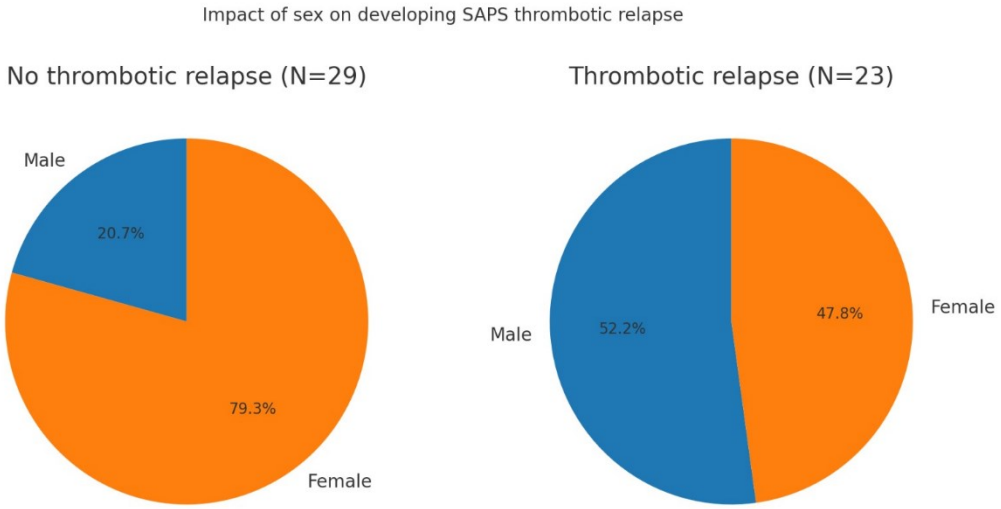
Table XXII

SLE characteristics in patients fulfilling or not the EULAR/ACR classification.					
	NOT classified as APS (N=7)		Classified as APS (N=50)		p-value
Rash	5	71.5%	27	54%	0.654
Alopecia	0	0%	3	6%	0.477
Arthritis	6	85.8%	31	62%	0.477
Serositis	0	0%	11	22%	0.141
Proteinuria	5	71.5%	25	50%	0.511
Hematuria	4	57.2%	20	40%	0.594
Thrombocytopenia	2	28.6%	11	22%	0.850

Leukopenia	2	28.6%	27	54%	0.128
Neurologic involvement	2	28.6%	14	28%	0.860
Vasculitis	0	0%	3	6%	0.477
Hypocomplementemia	6	85.8%	39	78%	0.850
Anti-dsDNA	4	57.2%	34	68%	0.320
ANA titer > 1:80	7	100%	49	98%	0.565
Anti-SSA and/or anti-SSB	4	57.2%	12	24%	0.127
Anti-U1RNP	4	57.2%	8	16%	***0.049**
Active SLE[1]	6	75%	29	58%	0.361
Nephrotic syndrome[1]	1	14.3%	9	18%	0.702
Prednisone >25 mg[1]	1	14.3%	7	14%	0.909
[1]At the time of the APS-related event					

6.2.3 – Thrombotic relapses

Figure 11



As written in previous chapter, the proportion of patients that had a thrombotic relapse was higher in the classified as APS group although without statistical significance (48.9% vs 14.3%, *p*=0.117). We then proceeded to analyze the characteristics of patients with and without thrombotic relapses (N=23, 44% and N=29, 56%, respectively) with the aim of evaluating any significant differences between these two groups. As *Figure 11* shows, in the no thrombotic relapse group there is a higher percentage of female (79.3% vs 20.7%), while in the group of patients that had a thrombosis recurrence there is a higher percentage of male (47.8% vs 52.5%). This difference reaches statistical significance (*p*=0.014).

Figure 12

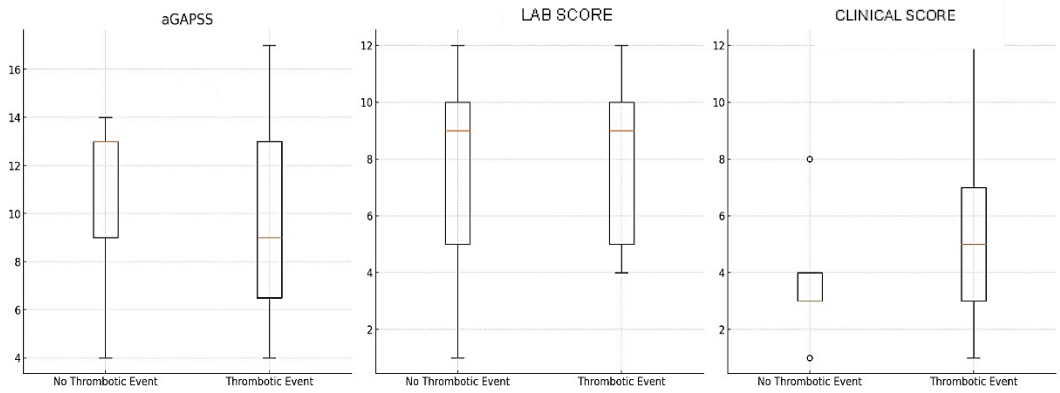


Figure 12 shows the distribution of aGAPSS score, ACR/EULAR laboratory score and ACR/EULAR clinical score. There are no significant differences between patients with or without thrombotic relapses for both aGAPSS score and laboratory score, even though median aGAPSS score is higher in patients that had no thrombotic relapse rather than in patients with thrombotic relapse (13 points vs 9 points, respectively). However, clinical score was significantly different between the two groups with a median of 3 points in no thrombotic relapse group vs 5 points in thrombotic relapse group (p=0.005).

Regarding therapeutical characteristics, no significant differences were found between the two groups. However, it is worth noting that in the no thrombotic relapse group there were higher percentages of patients on LDA both as first and last antithrombotic therapy (20.7% vs 13.6% and 41.4% vs 21.7%, respectively), while higher percentages of patients on warfarin and on warfarin plus LDA were found in the thrombotic relapse group (Table XXIII).

Table XXIII

Therapeutical characteristics in patients with or without thrombotic relapses. Exclusion of patients with provoked thrombosis, i.e. those with relapse after anticoagulation withdrawal (selection bias).					
	No thrombotic relapse (N=29)		Thrombotic relapse (N=23)		*p*-value
Moment of APS diagnosis					
Before SLE	7	24.1%	6	26.1%	0.347
With SLE	11	37.9%	5	21.7%	
After SLE	11	37.9%	12	52.2%	
First antithrombotic therapy					
Warfarin	20	69%	18	81.8%	0.393
LDA	6	20.7%	3	13.6%	
Warfarin + LDA	0	0%	1	4.5%	
LDA + Clopidogrel	1	3.4%	0	0%	
DOAC	2	6.9%	0	0%	

Last antithrombotic therapy					
Warfarin	11	37.9%	11	47.8%	0.147
LDA	12	41.4%	5	21.7%	
Warfarin + LDA	1	3.4%	5	21.7%	
DOAC	3	10.3%	0	0%	
Clopidogrel	1	3.4%	1	4.3%	
Fondaparinux	1	3.4%	0	0%	
Warfarin + LDA + Clopidogrel	0	0%	1	4.3%	

Table XXIV shows that the only clinical characteristics with a significant difference between the two groups were arterial thrombosis (p=0.049): percentages of patient with and without a high-risk CVD profile that had an AT event were both higher in the thrombotic relapse group than in patients with no thrombotic relapse (21.7% vs 6.9% and 26.1% vs 10.3%, respectively).

Table XXIV

Clinical characteristics in patients with or without thrombotic relapses.
Exclusion of patients with provoked thrombosis, i.e. those with relapse after anticoagulation withdrawal (selection bias).

	No thrombotic relapse (N=29)		Thrombotic relapse (N=23)		p-value
Venous thromboembolism					
with high-risk VTE profile	4	13.8%	4	17.4%	0.253
Without high-risk VTE profile	17	58.6%	17	73.9%	
Type of VTE					
DVT	13	44.8%	15	65.2%	0.383
PE	1	3.4%	2	8.7%	
Splanchnic thrombosis	1	3.4%	0	0%	
Renal thrombosis	1	3.4%	0	0%	
Retinal thrombosis	1	3.4%	0	0%	
DVT + PE	5	17.2%	4	17.4%	
DVT + PE + Splanchnic thrombosis	0	0%	0	0%	
Arterial thrombosis					
High-risk CVD profile	2	6.9%	5	21.7%	***0.049**
Without high-risk CVD profile	3	10.3%	6	26.1%	
Type of AT					
MI	1	3.4%	3	13%	0.124
Peripheral thrombosis	0	0%	3	13%	
Stroke	2	6.9%	3	13%	
TIA	1	3.4%	1	4.3%	
Intestinal thrombosis	0	0%	1	4.3%	
MI + peripheral thrombosis	1	3.4%	0	0%	
Microvascular suspected					
Livedo racemosa	1	3.4%	0	0%	0.657

Livedoid vasculopathy lesions	1	3.4%	2	8.7%	
Microvascular established					
aPL nephropaty	3	10.3%	2	8.7%	0.666
Microthrombosis	0	0%	2	8.7%	
Obstetric					
Preeclampsia with severe features	1	3.4%	2	8.7%	0.431
Fetal death	2	6.9%	2	8.7%	
Cardiac valve					
Thickening only	0	0.%	2	9.1%	0.114
Vegetation with or without thickening	0	0.%	1	4.5%	
Hematologic					
Thrombocytopenia	0	0%	1	4.5%	0.238

Interestingly, no significant difference was found between the two groups in neither of serological biomarkers (*Table XXV*). Even the antibody profile positivity was similar between the two groups, with akin percentages of single, double and triple aPL positive patients, although triple positive patients were slightly more in the no thrombotic relapse group (69% vs 52.2%).

Table XXV

Serological characteristics in patients with or without thrombotic relapses. Exclusion of patients with provoked thrombosis, i.e. those with relapse after anticoagulation withdrawal (selection bias).					
	No thrombotic relapse (N=29)		Thrombotic relapse (N=23)		*p*-value
LAC					
One-time	4	13.3%	2	7.1%	0.741
Persistent	23	76.7%	19	82.1%	
IgM aCL titre					
Low titre	2	6.7%	2	10.7%	0.098
Moderate titre	13	43.3%	3	10.7%	
High titre	2	6.7%	1	7.1%	
IgG aCL titre					
Low titre	1	3.3%	2	7.1%	0.573
Moderate titre	13	43.3%	8	28.6%	
High titre	8	26.7%	8	39.3%	
IgM antibeta2GPI titre					
Low titre	0	0%	1	3.6%	0.175
Moderate titre	8	26.7%	2	7.1%	
High titre	1	3.3%	2	7.1%	
IgG antibeta2GPI titre					
Low titre	4	13.3%	1	3.6%	0.422
Moderate titre	8	26.7%	6	21.4%	
High titre	9	30%	6	28.6%	
Antibody profile					

Single positivity	4	13.8%	6	26.1%	0.452
Double positivity	5	17.2%	5	21.7%	
Triple positivity	20	69%	12	52.2%	

Table XXVI shows SLE characteristics between the patients with and without thrombotic relapse. The only significant difference that was noted was a higher presence of alopecia in patients that had a thrombotic recurrence (13% vs 0%, p=0.042). SLE disease characteristics at the time of APS-related events were approximately the same between the two groups, with a prevalence of active SLE and nephrotic syndrome slightly higher in patients with thrombotic relapse (65.2% vs 55.2% and 21.7% vs 17.2%, respectively).

Table XXVI

SLE characteristics in patients with or without thrombotic relapses. Exclusion of patients with provoked thrombosis, i.e. those with relapse after anticoagulation withdrawal (selection bias).					
	No thrombotic relapse (N=29)		Thrombotic relapse (N=23)		*p*-value
Rash	19	65.5%	12	52.2%	0.196
Alopecia	0	0%	3	13%	***0.042**
Arthritis	19	65.5%	13	56.5%	0.940
Serositis	3	10.3%	5	21.7%	0.071
Proteinuria	15	51.7%	12	52.2%	0.786
Hematuria	10	34.5%	11	47.8%	0.198
Thrombocytopenia	9	31%	3	13%	0.152
Leukopenia	15	51.7%	12	52.2%	1.000
Neurologic involvement	10	34.5%	4	17.4%	0.311
Vasculitis	2	6.9%	1	4.3%	0.595
Hypocomplementemia	26	89.7%	16	69.6%	0.086
Anti-dsDNA	17	58.6%	18	78.3%	0.142
ANA titre > 1:80	28	97%	23	100%	0.207
Anti-SSA and/or anti-SSB	9	31%	3	13%	0.670
Anti-U1RNP	8	27.6%	3	13%	0.245
Active SLE[1]	16	55.2%	15	65.2%	0.259
Nephrotic syndrome[1]	5	17.2%	5	21.7%	0.905
Prednisone >25 mg[1]	5	17.2%	2	8.7%	0.511
[1]At the time of the APS-related event					

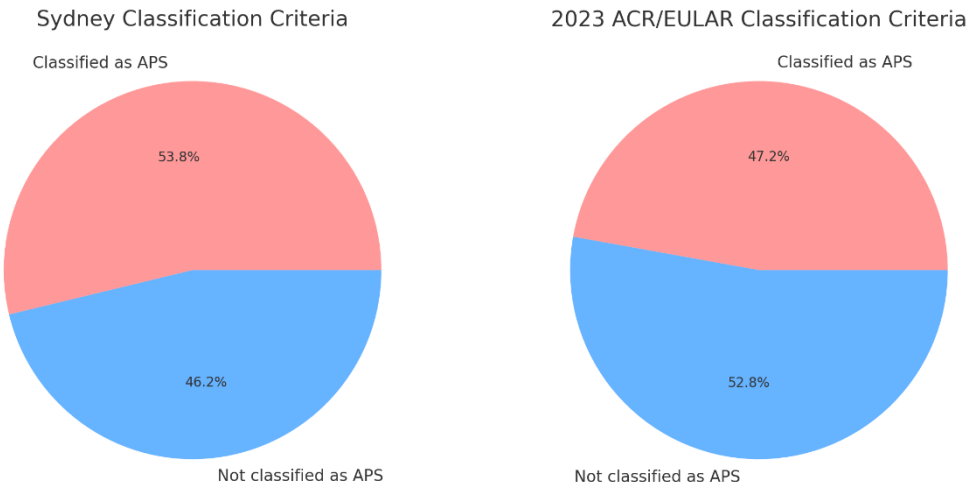
Five patients had a thrombotic relapse when their anticoagulation therapy was suspended; consequently, their new event was considered a provoked re-thrombosis. Three patients were single aPL positive and two were triple aPL positive; they were all classified because they either have LAC persistent positivity

or moderate to high titre of aCL or aβ$_2$GPI IgG. Considering clinical aspect, all patients had a venous thromboembolic event but only two presented an arterial thrombosis event; thrombosis occurred without the presence of VTE risk factors, but in one patient arterial thrombosis occurred in the context of a high-risk CVD profile. One patient also had an episode of microvascular thrombosis. First anticoagulation therapy and therapy after thrombosis recurrence remained the same for three out of five: previously all on warfarin, then 2 switched to warfarin plus LDA. This shift in therapy is due to the fact that those patients had an arterial re-thrombosis.

6.2.4 - *ACR/EULAR APS classification criteria sensitivity and specificity*

Both Sydney criteria and 2023 ACR/EULAR criteria were tested as diagnostic criteria by putting them in comparison with gold standard, that was clinical judgment.

Figure 13



We found that Sydney criteria have greater sensitivity than 2023 ACR/EULAR criteria (93% versus 82%), with both reaching a specificity rate of 100% (*Table XXVII* and

Table XXVIII). *Figure 13* shows how patients who tested positive to at least one aPL for two consecutive times at least 12 weeks apart were classified based on Sydney criteria and 2023 ACR/EULAR classification criteria. *Figure 14* and *Figure 15* show ROC respectively Sydney criteria and ACR/EULAR criteria ROC curves.

Table XXVII

Sydney classification criteria performance used as diagnostic criteria.			
	Diagnosed with APS	Not diagnosed with APS	Total
Classified as APS	57	0	57
Not classified as APS	4	45	49
Total	61	45	106
Sensibility	57/61	93%	
Specificity	45/45	100%	

Table XXVIII

2023 ACR/EULAR classification criteria performance used as diagnostic criteria.			
	Diagnosed with APS	Not diagnosed with APS	Total
Classified as APS	50	0	50
Not classified as APS	11	45	56
Total	61	45	106
Sensibility	50/61	82%	
Specificity	45/45	100%	

Figure 14: Sydney criteria ROC curve

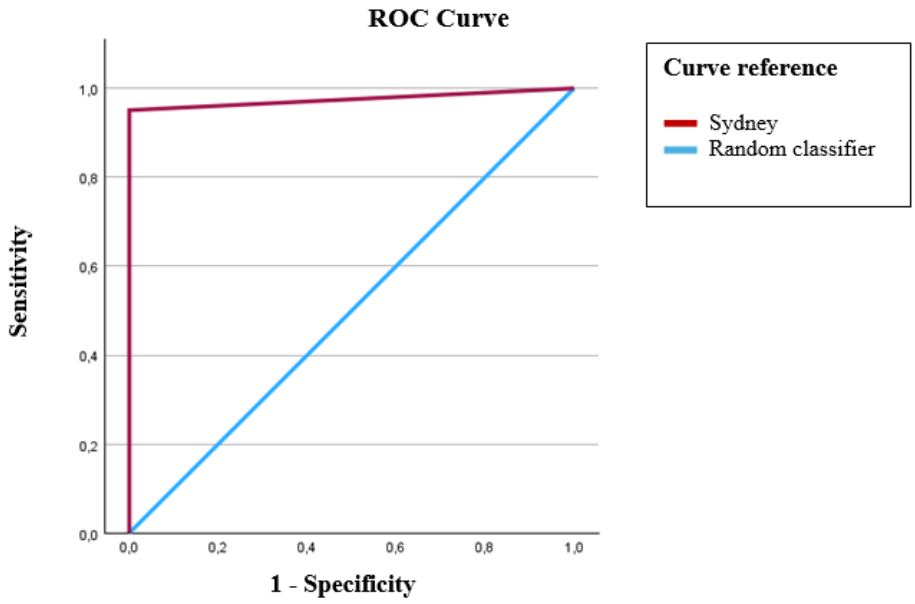
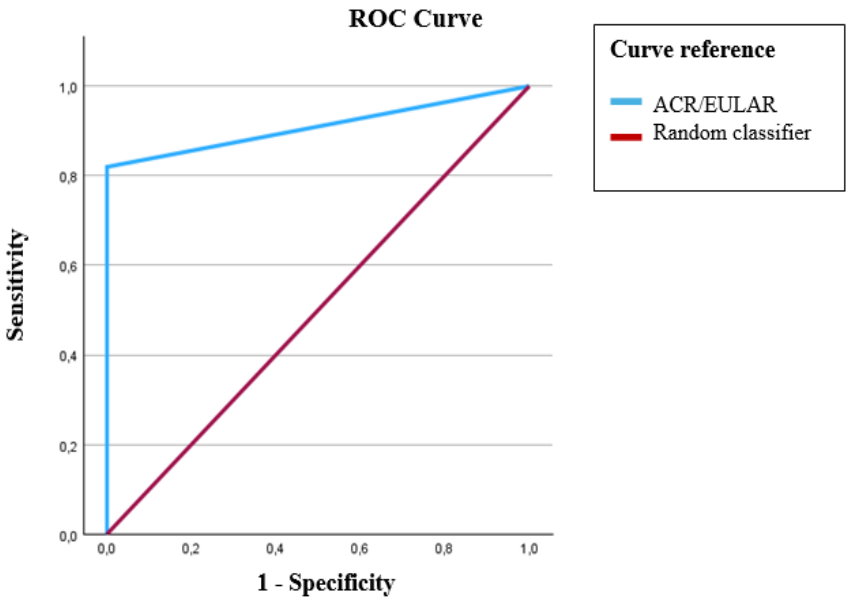


Figure 15: ACR/EULAR criteria ROC curve



In addition, we also tested the performance of both Sydney and 2023 ACR/EULAR classification criteria on accurately predicting which patients are more likely to have a thrombotic relapse (*Table XXIX* and *Table XXX*). While Sydney criteria have a higher sensibility compared to ACR/EULAR criteria (100% vs 96%, respectively), the latter have better specificity than the 2006 criteria (63% vs 72%).

Table XXIX

Sydney classification criteria performance performance on predicting thrombotic relapses in SAPS population			
	Thrombotic relapse	No thrombotic relapse	Total
Classified as APS	28	29	57
Not classified as APS	0	49	49
Total	28	78	106
Sensibility	28/28	100%	
Specificity	49/78	63%	

Table XXX

2023 ACR/EULAR classification criteria performance on predicting thrombotic relapses in SAPS population			
	Thrombotic relapse	No thrombotic relapse	Total
Classified as APS	27	22	50
Not classified as APS	1	56	56
Total	28	78	106
Sensibility	27/28	96%	
Specificity	56/78	72%	

Chapter 7 – Discussion

7.1 – ACR/EULAR APS classification criteria performance

We assessed the ACR/EULAR APS classification criteria performance in a PAPS cohort of 205 patients and a SAPS cohort of 57 patients. Data about sex, age, clinical manifestations, laboratory tests, therapeutical and thrombotic relapses were collected in other to find out potential significant differences between classified and not classified patients.

7.1.1 - PAPS

Among 205 patients classified according to Sydney criteria, 170 patients (82.9%) were classified as having APS while 35 patients (17.1%) were excluded. Reasons for exclusion were not sufficient clinical score (1 patient, 0.5%), not sufficient laboratory score (32 patients, 15.6%) and not sufficient clinical and laboratory score (2 patients, 1%).

The only patient not classified solely for not reaching 3 points in clinical domains was excluded despite having an episode of DVT because of his/her high-risk VTE profile. In fact, deep vein thrombosis occurred within 3 months from major surgery. Conversely, among 32 patients who did not reach 3 points in laboratory domains 22 had moderate to high titers of aCL and/or aβ2GPI IgM, thus reaching a laboratory score of 1 point, while the remaining 10 had low titre of aCL and/or aβ2GPI. In addition, the 2 patients that failed to meet the minimum of both clinical and laboratory score to be classified as APS were characterized by moderate to high titers of aCL and aβ2GPI IgM together with a DVT episode which happened within 3 months from major surgery.

Unclassified patients were significantly older at the time of diagnosis compared to classified patients (50.3 years vs 43.6 years, p=0,002). Interestingly, this difference in age is not described by other studies that tested the new classification criteria (57–59). One possible explanation is that aging increases the probability of having cardiovascular risk factors other than aPL, thus older patients resulting to be included among patients with high-risk CVD profile and for this reason excluded by the classification criteria. This occurrence can lead to two different considerations: on one side, we can conclude that the new classification criteria are more accurate and clinically meaningful than the previous one, since they allow the exclusion of patients in whom the thrombotic event was possibly

related to non-aPL risk factors (older patients with comorbidities); on the other side, one can conclude that by giving a strong impact to other cardiovascular risk factors other than aPL in the classification process, the new classification criteria reduce the diagnostic impact of aPL, thus reducing the sensibility in the classification.

Three patients in the PAPS, who were all part of the not classified group, had a thrombotic recurrence after discontinuation of anticoagulation therapy. The rationale behind the therapy suspension was that these patients had low risk aPL profile (and, in fact, were not able to fulfill the laboratory score of the new classification criteria). However, it is worth noting that all APS patients have a risk of thrombotic recurrence that ranges of 50-73% per year after antithrombotic medications withdrawal and that to this date there are no particular risk factors which have the capability to correctly distinguish patients who are more likely to have a thrombotic relapse (42). In order to eliminate the bias generated by provoked thrombosis after stopping anticoagulants, these three patients were not considered in our analysis of re-thrombosis. Our findings that unclassified patients were at high risk of relapse (as high as patients classified) suggest that these patients should be carefully evaluated and followed-up despite the low risk antibody profile: in fact, rheumatologists should keep into consideration all risk factors for thrombosis before withdrawing anticoagulant/anti-platelets therapy in these patients.

Percentage of unclassified patients who developed VTE was the same as the percentage of classified patients, but significant differences in their VTE profile risk was noted with unclassified patients that have more frequently high-risk VTE profile rather than their classified counterparts (9.4% vs 0.6%, p=0.004). Undergoing a venous thrombotic event with a high-risk VTE profile is weighted only 1 point by the new classification criteria. So, it is not a surprise that more patients with high-risk VTE profile are present in the not classified as APS group.

Another significant clinical divergence worth of notice was the type of arterial thrombosis (p<0.001), with higher stroke prevalence in classified patients (29% vs 15.6%). As stated before, stroke is the most common arterial thrombotic event in APS patients (42) and the fact that occurred more frequently in definite APS patients confirms this epidemiological data. One could speculate that not classified APS patients could have a less aggressive disease activity which explains why in some of them cerebral thrombotic events happened in the form of TIA rather than ischemic stroke.

Another significant difference in clinical features was that suspected microvascular thrombosis events occurred only in the classified group (14.7% vs 0%, p=0.020) with 24 cases of livedoid vasculopathy lesions and 1 with pulmonary hemorrhage. The association between microvascular disease and more severe APS disease course has been previously described (27).

Since publication of the 2023 ACR/EULAR classification criteria, some studies have been made with the intent of further assessing the new criteria in cohorts different from the validation one used by the steering committee (50). The serological data we obtained were in line with other cohorts tested for the new classification criteria (51,58,59).

7.1.2 - SAPS

Among 57 patients classified as APS according to Sydney criteria, 50 (88%) were confirmed as APS according to ACR/EULAR classification criteria, while 7 (12%) did not fulfill the classification criteria. In the not classified group, 6 patients (86%) did not reach a sufficient clinical domain score and one did not reach a sufficient laboratory score. More in details, this patient had a single high titre aCL IgM positivity. Conversely, within the 6 patients not classified for not reaching clinical criteria 5 had a clinical score of 1 point: 4 had a VTE event having high-risk VTE profile (1 for active malignancy, 2 for hospital admission confined to bed and 1 for long distance travel and concomitant active SLE) and 1 for fetal loss >10 weeks of gestation. The last patient of this group had an AT event but reached a clinical score of only 2 points because of his chronic kidney disease (high-risk CVD profile).

Contrary to PAPS population, the majority of unclassified patients was excluded because of low clinical score, meaning that for SLE patients is easier to reach the minimum laboratory score indicated by the new classification criteria. In fact, SLE is notoriously known for his epitope spreading phenomena (14) and aβ2GPI is one of the earliest antibodies to appear in patients sera. Both mice and human studies have shown that aβ2GPI are able to trigger a T cell response that is associated with epitope spread of SLE-related autoantibodies (60). This could be the explanation of our results.

Regarding SLE-associated autoantibodies (aPL excluded), we found that anti-U1RNP positivity were significantly higher in not classified patients (p=0.049). This characteristic was not studied nor noted by other works assessing

the performance of the new criteria (51,57,59), but since the sample size was little this result could have low clinical meaning. One possible explanation is that anti-U1RNP antibodies are associated with a more inflammatory disease phenotype (23), and inflammation could act as a second hit in inducing thrombosis. This means that in this subset of patients, even a low risk profile of aPL could be sufficient to induce thrombosis. Moreover, an in vitro study suggested that the anti-U1RNP antibody could bind with human pulmonary arterial endothelial cell and directly recognize antigens on its surface, being a possible trigger of endothelial cell inflammation (24).

Considering clinical characteristics, there was a significant difference among unclassified and classified patients which was VTE profile: the percentage of VTE occurred in high-risk VTE profile patients was considerably higher in unclassified patients compared to classified as APS group (57.2% vs 8%, p=0.002). SLE disease activity was part of the minor criteria for defining high-risk VTE profile indicated by the ACR/EULAR classification criteria, therefore is logical that this aspect is present in a SAPS cohort. However, since there were no significative difference between unclassified and classified subsets regarding active SLE at the time of the APS-related event, it is not possible to state that SLE disease activity was the cause of this significant difference.

Finally, serological characteristics were similar between the two groups, with the only exception being LAC persistent positivity (p=0.030) which was present in 84% of classified patients compared to 57.2% of unclassified patients. Despite this difference, we highlight the fact that only one patient did not fulfill laboratory criteria, meaning that this data, although statistically significant, was not the reason of exclusion of the majority of unclassified patients. In fact, the almost entirety of unclassified patients had moderate to high titer of aCL and/or aβ2GPI IgG (6 out of 7).

7.2 – ACR/EULAR APS classification criteria and thrombosis relapse

7.2.1 – PAPS

Considering the percentage of patients that had a thrombotic relapse, no significant difference was found between not classified and classified as APS patients (18.8% vs 8.2%, respectively). Given that the new classification criteria

addressed little weight to moderate to high titer of aCL and/or aβ2GPI IgM, an antibody profile shared by the majority of unclassified patients (22 out of 32 patients), this data suggests that aCL and aβ2GPI IgM could have an impact on thrombotic APS. In fact, some experts rose a doubt about the decision of the ACR/EULAR steering committee of assigning only one point to moderate to high levels of aPL IgM (54,61).

Since there was no significant difference in rate of recurrent thrombosis between unclassified and classified groups, we split the entire PAPS cohort into two other groups: patients without a thrombosis recurrence and patients with a thrombosis recurrence.

What we found was that classical risk factors which should predict high-risk thrombotic APS such as antibody profile positivity and aGAPSS score (20,40) failed to identify patients that later in their follow-up developed a thrombotic relapse. In fact, percentages of single, double and triple positive aPL patients were roughly the same between patient without thrombotic relapse and with thrombotic relapse. Even aGAPSS score, which takes into account not only aPL antibodies positivity but also two CVD risk factors as arterial hypertension and dyslipidemia, had a similar distribution within the two groups. These results line up with another study that found that aGAPSS score had a suboptimal performance in identifying patients at risk of thrombotic recurrence (21).

A possible explanation to these results is that patients tested positive to only aPL IgM have a certain risk of thrombotic recurrence, but this risk is amplified by the fact that their anticoagulation therapy is not as intense as it should be. Indeed, in our cohort, physicians taking care of these patients did perceive this aPL profile as benign and, as a consequence, in some cases discontinued the anticoagulation. Although a study found that aPL IgM are independently responsible for obstetric APS but not for thrombotic APS (62), this last aspect still remains controversial (63). What is highly accepted by experts is that IgG contribute more to APS pathogenesis than IgM (63), meaning that a therapy with low-dose aspirin is more appropriate in patients positive to only IgM (20). This concept is reflected in the significant difference between unclassified and classified patients regarding antithrombotic therapy at the time of thrombotic relapse ($p<0.001$); 66.7% patients not classified as APS were on LDA at the time of thrombosis recurrence, versus only 8.3% in the classified group.

7.2.2 - SAPS

Contrary to what happened with PAPS cohort, within patients classified as APS happened more thrombotic relapses than in unclassified patients (48.9% vs 14.3%) although lacking statistical significance. As stated before, unclassified patients were often excluded because of their high-risk VTE profile. In other words, one could argue that this subset of patient had a thrombotic event not for APS activity per se, but for the compound effect of APS and their thrombotic and cardiovascular risk factors. EULAR recommendations for management of APS state that patients will benefit if they can change their habits with the objective of reducing the weight of their VTE and CVD risk factors (20,27). However almost all patients had a thrombotic event because of a major surgery, long distance travel or active SLE, situations that not always are avoidable nor predictable.

We then proceeded to compare the characteristics of patients that had a thrombotic relapse with patients that had no other thrombotic events aiming to find out significant differences that could be useful in further stratifying patients more at risk.

Interestingly, men were more prone to develop thrombotic recurrence than women. While it is true that APS men are more at risk of myocardial infarction and arterial thrombosis in lower leg and feet (28), no studies have tried to study the effect of sex in thrombosis recurrence.

Considering the widely accepted APS thrombotic risk factors, aGAPSS score not only showed not significant difference between the two groups, but median aGAPSS score was higher in patients that had no new thrombotic events rather than in the thrombotic relapse group (13 vs 9 points, respectively).

Considering clinical features, thrombotic relapse was significantly more prevalent in patients that had an arterial thrombotic event (p=0.049), regardless of the type of CVD profile risk. This difference could be due to the higher percentage of males in the group that had thrombotic recurrence, since a recent Italian study has shown that myocardial infarction and arterial thrombosis is more frequent in male patients (64).

Another surprising result was that no significant difference in serological characteristics emerged between the two subsets. Neither LAC nor antibody positivity were different between the two groups, even though LAC persistent

positivity and triple positive patients are widely considered more at risk of thrombotic events (20,21,64,65).

In addition, the only significant difference in SLE characteristics was alopecia, while contrary to other studies, there were no significant differences between the two subsets in terms of active SLE, nephrotic syndrome, high-dose prednisone therapy and anti-U1RNP. All this SLE aspects have been associated with greater rates of thrombotic recurrence in previous studies (19). No other studies found that a link between alopecia and thrombotic recurrence, and we do not have an explanation for this association.

7.3 – ACR/EULAR APS classification criteria sensitivity and specificity

We also compared the sensitivity and specificity of the ACR/EULAR APS classification criteria and of the Sydney classification criteria in diagnosing secondary APS, using the clinical judgement as the gold standard. The new criteria had a lower sensitivity compared to the Sydney criteria (82% vs 93%, respectively), but specificity reached 100% in both cases. While sensibility and specificity rates of ACR/EULAR criteria were in line with those observed in the validation cohort used by the ACR/EULAR steering committee (50) and in various different tested in other studies, the specificity of Sydney criteria resulted strangely higher than usual (85-90%) (57–59).

This high level of specificity is linked to the characteristic of the control group, which in our case was represented by aPL carriers affected with SLE. Within carriers, no patient had clinical manifestations previously known as “extra-criteria” (49) nor had thrombotic events.

One patient with livedoid vasculopathy lesions diagnosed with APS was not classified as APS either by the Sydney criteria or by the new criteria because of not sufficient clinical score: livedoid vasculopathy lesions are weighted 2 points, so a patient with only this clinical manifestation could not be classified as APS. Furthermore, 3 patients diagnosed with APS were not classified by both classification criteria because their clinical features related to APS (29) but not included in definite APS: 2 patients had neurological involvement (1 suffered from migraine, the other presented with chorea), and one had pulmonary hypertension.

Sydney criteria and ACR/EULAR criteria performance in predicting patients at risk of thrombotic recurrence were also tested. In this analysisis, specificity of Sydney criteria and ACR/EULAR criteria was 62% and 73%

respectively, while sensitivity was 100% and 96% respectively. In other words, all patients that had a thrombotic relapse were classified as APS according to Sydney criteria, but not all patients classified as APS had recurrent thrombosis. The same scenario is present for the new criteria, although with less false positives.

Both performance results of the two sets of criteria prove that classification criteria should not be considered and used as diagnostic criteria, especially the 2023 ACR/EULAR classification criteria. The purpose of classification criteria is to form homogenous cohort of definite APS for research purposes and clinical trials (46). Accordingly, the steering committee deliberately created a new set of criteria with higher specificity at the cost of sensitivity.

7.4 – Similarities and discrepancies between PAPS and SAPS

We found that patients having primary APS and patients having secondary APS have some common traits and other distinguishing characteristics.

Firstly, while female to male proportion is roughly the same (70.7% females and 29.3% males in PAPS; 65.5% females and 34.5% males in SAPS), patients with primary APS are on average 10-years-older at APS diagnosis than patients with secondary APS (mean age at diagnosis 41.9 years vs 31.9 years). This difference could be explained by the fact that 77.6% of SAPS patients were diagnosed with APS at the same time or after receiving a SLE diagnosis. Patient with SLE undergo diagnostic and follow-up laboratory exams that include testing for aPL (1). As a result, APS diagnosis could be made earlier since patients are already followed up for SLE.

Comparing clinical manifestations, venous thromboembolism was the main cause of thrombosis in SAPS cohort (52.7% in PAPS vs 82.8% in SAPS), whereas arterial thrombosis (AT) was the most frequent thrombotic event in PAPS cohort (62.5% in PAPS vs 31% in SAPS). While SAPS population prevalence of venous thromboembolism was greatly higher than what is commonly reported by the majority of studies (26), this is not the case for PAPS cohort, where AT occurred more frequently than in other APS cohorts (58,59). In both populations, deep vein thrombosis was the most frequent VTE clinical presentation (78.7% in PAPS, 51.7% in SAPS), but the second VTE event in frequency was pulmonary embolism alone in PAPS (11.1%), DVT and PE altogether in SAPS (19%). Considering arterial thrombotic events, myocardial infarction was the first most common cause of AT both in PAPS and SAPS (49.2% and 10.3%, respectively) with stroke being

at second place (43% in PAPS and 8.6% in SAPS). Microvascular thrombosis was more common in SAPS population than in PAPS population (20.90% vs 16.2%, respectively), as for cardiac valve disease (5.3% vs 1%, respectively). Thrombocytopenia was approximately the same between the two cohorts (3.5% in SAPS vs 2.4% in PAPS). The prevalence of VTE in PAPS and SAPS populations was in line with literature (28), whereas in both our cohorts myocardial infarction was slightly more common than stroke, which should be, according to literature, the most prevalent arterial thrombotic event (26,66).

Other dissimilarities were found within serological characteristics. While the proportion of triple positive aPL patients were almost the same between the two cohorts (61.5% in PAPS vs 58.2% in SAPS), there were more double positive patients in PAPS population (23.9% vs 17.2%) and more single positive patients in SAPS population (14.6% vs 24.1%). Based only on antibody positivity, these two cohorts share the same thrombotic risk given that roughly 60% of both groups are positive for LAC, aCL and aβ2GPI at the same time (20). When we compared single aPL positivity and titer, we found that persistent LAC positivity was more frequent in SAPS population (68.6% vs 79.3%), but moderate titres of aCL and aβ2GPI for both IgM and IgG isotypes were much more frequent in PAPS population (aCL IgM: 43.4% vs 27.6%; aCL IgG: 48.8% vs 36.2%; aβ2GPI IgM: 35.5% vs 17.2%; aβ2GPI: IgG 47.8% vs 24.1%).

7.5 – Limitations

This study presented some limitations. Firstly, we could not obtain data regarding the aPL profile at the time of thrombotic relapse; this is a monocentric study and ethnic differences were not taken into consideration. Lastly, we could not assess the performance of the new criteria in the PAPS cohort since our dataset did not include asymptomatic aPL carriers.

Chapter 8 - Conclusions

The new ACR/EULAR classification criteria for APS represent a step forward in APS clinical research. The weighting system and domain divisions give to researchers the possibility of forming homogenous subsets of definite APS patients stratified for laboratory and clinical traits of the syndrome in order to conduct clinical trials.

However, given the characteristics of certain unclassified patients, assigning little weight to aPL IgM positivity remains controversial. Patients that do not fulfill these classification criteria not necessarily have a milder form of APS. As a proof of this, in our study we found no significant differences in rate of thrombotic relapse between classified and unclassified patients.

In our study, some unclassified patients in our study who had a re-thrombosis were considered at low risk of thrombosis due to their aPL profile, meaning that their anticoagulation therapy was not as intense as the one of classified patients. This behavior may have left patients with a not enough intense anticoagulation, exposing them to further thrombotic events.

LAC persistent positivity, triple aPL positivity and aGAPSS score were similar between patients with or without thrombosis recurrence, so other biomarkers should be studied with the aim of better stratifying patients at high risk of thrombotic relapse (65).

Nevertheless, it is important to highlight that classification criteria must not be used as diagnostic criteria, since they do not consider all the numerous clinical manifestations associated with APS.

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