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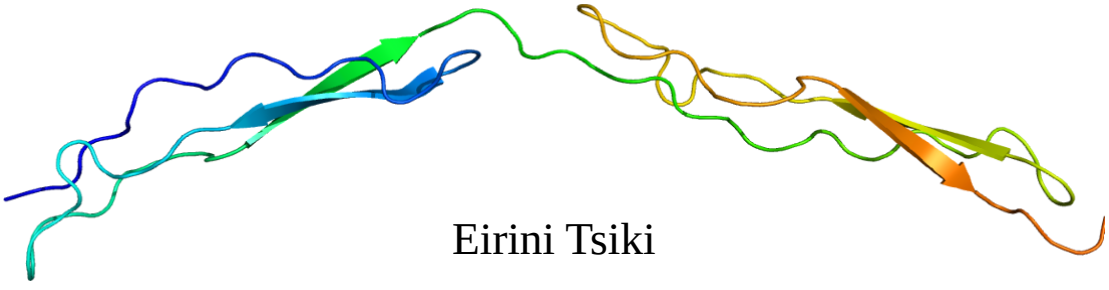
'The role of Thrombospondin 1 in Antiphospholipid Syndrome'

Master Thesis

Supervisor: Prof. Christos Tsatsanis

Committee: Prof. Panayiotis G. Vlachoyiannopoulos

Dr. Panagiotis Verginis



Eirini Tsiki

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‘Ο ρόλος της Θρομβοσπονδίνης-1 στο Αντιφωσφολιπιδικό Σύνδρομο’

Διπλωματική Εργασία Μεταπτυχιακού

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Abstract

Antiphospholipid syndrome (APS) is an acquired thrombophilia characterized by recurrent thrombosis and/or pregnancy morbidity, in the presence of antibodies to β2 glycoprotein-I (β2GPI) or prothrombin. Anti-β2GPI antibodies recognize complexes of β2GPI dimers with CXCL4 chemokine and activate platelets. Thrombospondin 1 (TSP-1) is secreted by platelets and exhibits prothrombotic and proinflammatory properties. Therefore, in this study we investigated the role of TSP-1 in APS. Human Umbilical Vein Endothelial Cells (HUVECs) treated with plasma, total IgG or anti-β2GPI from APS patients and healthy donors were found to secrete TSP-1 as quantified by ELISA and Real-Time PCR. Immunofluorescent stainings for markers of endothelial cell dysfunction and APS were conducted in HUVECs and in placenta from APS patients and Healthy donors. Titration experiments with various concentrations of TSP-1 in HUVECs were also conducted in order to confirm the effect of TSP-1 in the cells. In a parallel clinical and laboratory study, higher plasma levels of TSP-1 were observed in APS but not HDs. Patients with arterial thrombotic events or those undergoing a clinical event had the highest TSP-1 levels. These findings support a possible implication of TSP-1 in APS pathophysiology. *In vitro* cell treatments along with patient plasma measurements in APS patients suggest that high TSP-1 levels could mark a prothrombotic state and an underlying inflammatory process.

Περίληψη

Το Αντιφωσφολιπιδικό σύνδρομο είναι μια αυτοάνοσοση θρομβοφιλία που χαρακτηρίζεται από επαναλαμβανόμενες θρομβώσεις και νοσηρότητα στην εγκυμοσύνη, παρουσία αντισωμάτων εναντι β2 γλυκοπρωτείνης (β2GPI) ή προθρομβίνης. Anti-β2GPI αντισώματα αναγνωρίζουν διμερή β2GPI αφού προσδεθούν στη CXCL4χυμοκίνη και ενεργοποιήσουν τα αιμοπετάλια. Η θρομβοσπονδίνη -1 1 (TSP-1) εκκρίνεται από ενεργοποιημένα αιμοπετάλια παρουσιάζοντας προ-θρομβοτικές και προ-φλεγμονώδη ιδιότητες. Σε αυτή την έρευνα, μελετήθηκε ο ρόλος της TSP-1 στο αντιφωσφολιπιδικό σύνδρομο. Ενδοθηλιακά κύτταρα από ανθρώπινες ομφαλικές φλέβες από υγιείς δότες υπό την επίδραση πλάσματος, total IgG ή anti-β2GPI από τους ασθενείς καθώς και από υγιείς δότες βρέθηκε ότι εκκρίνουν TSP-1 όπως μετρήθηκε με ELISA και Real-Time PCR. Πραγματοποιήθηκαν χρώσεις ανοσοφθορισμού για μόρια μάρτυρες ενδοθηλιακής καταστροφής και αντιφωσφολιπιδικού συνδρόμου σε ενδοθηλιακά κύτταρα από ομφαλικές φλέβες και πλακούντες από ασθενείς και υγιείς. Πειράματα με επίδραση διάφορων συγκεντρώσεων TSP-1 στα ενδοθηλιακά κύτταρα ομφαλικής φλέβας έγιναν έτσι ώστε να επιβεβαιωθεί η επίδραση της TSP-1 στα ενδοθηλιακά κύτταρα. Τα αποτελέσματα αυτά υποστηρίζουν μια πιθανή εμπλοκή της TSP-1 στην παθοφυσιολογία του συνδρόμου.

Introduction

Materials and Methods

Results

Discussion

Conclusions-Future Perspectives

References

Introduction

Antiphospholipid syndrome (APS)

Antiphospholipid syndrome (APS) is an autoimmune prothrombotic disorder characterized by arterial or venous thrombosis and/or pregnancy morbidity in the presence of antiphospholipid antibodies (aPL). Antiphospholipid antibodies comprise the anti-β2glycoprotein I (anti-β2GPI) and the anti-prothrombin antibodies or lupus anticoagulant (LA) [3]. This disorder was first described by the physician Graham R.V. Hughes in 1983, thus APS is also known as Hughes' syndrome.

APS's more frequent clinical manifestations are recurrent of arterial and venous thrombosis and pregnancy complications, while other severe clinical features can also be present [4]. The presence of aPL are associated with multiple conditions such as asymptomatic ''aPL carrier'' patients; ''classical'' APS with recurrent vascular events (venous and/or arterial thrombosis); APS limited to pregnancy morbidity (obstetric APS); or aPL-positive patients presenting with non- thrombotic or obstetric clinical manifestations. A small portion of APS patients can develop a life-threatening disease, called catastrophic APS (CAPS), illustrated by the rapid occurrence of multiple occlusive events ending up to multiorgan failure [5]. Additionally, the definition ''seronegative APS'' was proposed for patients with clinical manifestations of APS and persistently negative aPL, in those no other explanations for thrombosis are evident. Positive aPL may also be related to infections, drugs, and malignancies, but in those conditions aPL antibody levels are transient and low, and patients have not presented thrombosis or pregnancy problems [5].

APS is characterized as ''primary'' APS when there is no association with other underlying disorders and as ''secondary'' if it occurs in association with other diseases mainly, but not exclusively SLE. Clinical manifestations of primary or secondary APS can be identical; however, the clinical picture of secondary forms could be complicated by the expression of the underlying disease.

Endothelial deregulation compatible with innate immunity activation has been shown to be a characteristic of the syndrome [6,7]. Experimental models indicate that

inhibition of angiogenesis as well as endothelial apoptosis play a significant role in the fetal loss in APS [8,9]. Patients with APS display increased circulating levels of proinflammatory cytokines [10,11]. Toll Like Receptor (TLR) 2, 4 and 6 activation lead to monocyte and endothelial cell activation and subsequent tissue factor (TF) and proinflammatory cytokine expression [12-14]. Platelet derived chemokines are elevated in the plasma of APS patients [15]. Platelets are activated by complexes of anti-β2GPI autoantibodies and β2GPI, especially when it is dimerized by binding to CXCL4 chemokine [16].

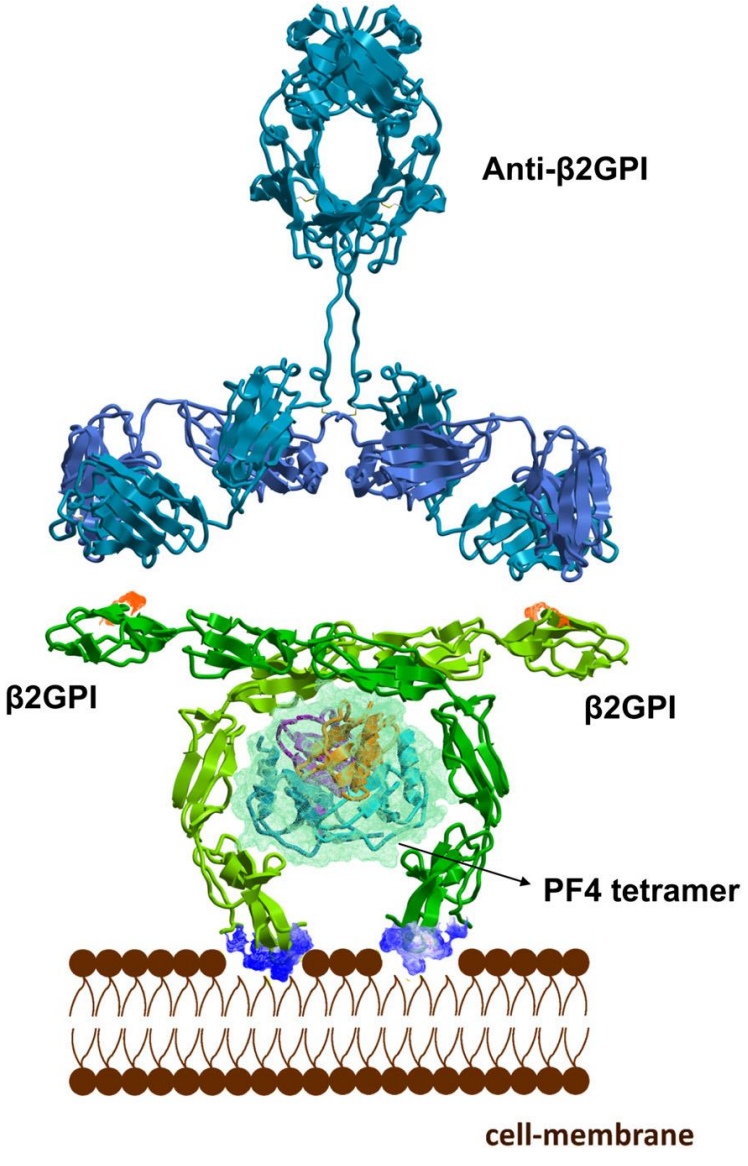

Figure 1 Model of recognition of b2GPI antigen by autoantibodies. *Platelet factor 4 tetramer binds to b2GPI, promoting its dimerization. Epitopes that thrombosis-associated antibodies recognize the 2 antigen-binding sites of an antibody, allowing their bivalent recognition by low-affinity autoantibodies.*[16]

Epidemiology

APS patients are aPL-positive individuals. Prevalence of aPL ranges from 1 to 5 % but the percentage is higher in older patients. APS generally affects young and middle-aged adults and about 85 % of patients are between 15 and 50 years old. Moreover, APS is more common in women than men, and the male-to-female ratio differentiated and the form of APS ranging from 1:3.5 for primary APS to 1:7 for secondary mainly with SLE as underlying disease [17]. The incidence of APS is approximately 5 new cases per 100,000 people per year and the prevalence is about 40–50 cases per 100,000 people. The prevalence of catastrophic APS is estimated to be <1% of all cases of APS. [18,19]

aPL are found in higher frequency in individuals with blood hypercoagulability (i.e. venous or arterial thrombosis) and in women with recurrent miscarriages or pregnancy morbidity. aPL positivity is present in about 13 % in stroke, 11 % in myocardial infarction, and 9.5 % in deep vein thrombosis [20]. Approximately 1 % of women deal with recurrent miscarriages, and in 10–15 % of these a diagnosis of APS. Additionally, positivity to aPL has been observed in 11–29 % of women with preeclampsia and in 25 % with growth-restricted fetuses.

Finally, aPL are present in up to 50 % of SLE patients, whereas aPL prevalence in various connective tissue disorders (i.e. rheumatoid arthritis, dermatomyositis, Sjogren's syndrome, and systemic sclerosis) is about 5 to 20 % [5,21]. Approximately 33 % of these patients will develop thrombotic events[22].

Clinical manifestations

Vascular thrombosis and pregnancy morbidity are the two clinical hallmarks of APS, but disease includes additional common features that cannot be explained only by a prothrombotic state. Low specificity of these manifestations hinders the classification of APS patients [23].

i. Thrombosis

Single or multiple thrombi in veins, arteries and the microvasculature and the time interval between these manifestations may vary from days to years. This variability in location of the thrombi results in the wide spectrum of clinical presentations that may involve many organ systems[17].

The most common symptom of APS patients is venous thromboembolism, specifically deep vein thrombosis of the lower limbs (prevalence ~39%, Euro-Phospholipid Project)[17], differentiated from thrombosis in congenital thrombophilias.. Pulmonary embolism and chronic thromboembolic pulmonary hypertension are secondary manifestations[24]. Arterial thrombosis is rare but life threatening as it takes place in the cerebral circulation leading to stroke and transient ischemic attacks. Indeed, 20% of APS patients developed a stroke and 11% developed a transient ischemic attack. Moreover, thrombotic APS manifestations occurring in atypical sites can provoke clinical syndromes such as acute coronary syndromes, hepatic arterial or venous (Budd–Chiari syndrome) thrombosis, portal, mesenteric, or splenic ischemia, pancreatic and adrenal insufficiency secondary to acute vascular infarction[5].

Recurrence of thrombotic events is very common in APS and data from cohort studies found the recurrence thrombosis's rates in patients that don't take drugs ranging from 19 to 29 % per year. Positivity for lupus anticoagulant, triple positivity and isolated, persistent positivity for anticardiolipin antibodies are related to higher risk of developing thrombosis. Thromboprophylaxis is the treatment that lowers the possibility of thrombotic episodes and also, recurrences[25].

ii. Obstetrical morbidity.

Pregnancy morbidities are the second major feature of syndrome. Recurrent miscarriage, including more than three pregnancy losses, normally occurs before the 10th

week of gestation. Early spontaneous abortion and aPL positivity is not related to APS, as early miscarriages are common in general population (fetal chromosomal anomalies) [26]. Secondary obstetric adverse events related to APS are fetal deaths after the 10th week of gestation, intrauterine growth restriction (fetal weight that is below the 10th percentile for its gestational age).

The maternal pregnancy morbidity of APS consists of pre-eclampsia, eclampsia and placental abruptions. Furthermore, women with APS often deal with gestation-related maternal thrombosis and premature births before the 34th week of gestation because of placental insufficiency. Despite the transplacental transfer of maternal antiphospholipid antibodies, babies born to mothers with APS not necessarily have thrombosis or SLE. [27]

iii. Thrombocytopenia.

Thrombocytopenia occurs in at least 30% of individuals with APS and is most marked at times of thrombosis formation[2] . However, thrombocytopenia might also be associated with other systemic manifestations of APS, such as obstetrical morbidity, venous and/or arterial thrombosis, myocardial infarction and valve vegetations [27].

aPL are found in patients diagnosed with idiopathic thrombocytopenic purpura (ITP), and ITP may be the first symptom in patients with APS or SLE. Various mechanisms may synergize in the pathogenesis of platelet destruction[28]. APS-associated thrombocytopenia is mild ([50,000/mm3) without clinical manifestation. If the number of platelets is markedly decreased and thrombocytopenia is symptomatic, ITP treatments are used[5].

iv. Cardiac manifestations.

Cardiac features associated with APS include valve lesions, accelerated atherosclerosis, myocardial infarction, intracardiac thrombi, pulmonary hypertension, cardiomyopathy and diastolic dysfunction[29,30].

Cardiac valve abnormalities are being observed in 30–50% of individuals with APS together with other systemic autoimmune diseases[2]. Valvular abnormalities are present in up to one-third of patients with APS. The most frequent heart problem is heart valve thickening mainly in the mitral valve, followed by the aortic valve results in impaired function characterized by regurgitation. Valve vegetations (Libman–Sacks endocarditis) are also common. These are sterile fibrinous vegetations on the endocardial surface of the valve that usually increase the risk of superadded bacterial endocarditis.[5]

Myocardial ischemic events can be the consequence of coronary thrombosis without underlying atherosclerosis, accelerated atherosclerosis of the coronary arteries or microvascular injury[31].

v. Pulmonary manifestations.

Pulmonary embolism and infarction is present in ~14% of individuals with APS. Additionally, pulmonary hypertension, acute respiratory distress syndrome, fibrosing alveolitis and intra-alveolar hemorrhage has been described in APS patients[2,32].

vi. Dermatological manifestations.

Livedo reticularis occurs in 16–25% of patients and can be a marker of severe disease linked with the arterial and microangiopathic subtypes of APS. Pathological livedo is defined as ‘‘the persistent, non-reversible with rewarming, violaceous, red and blue, reticular or mottled pattern of the skin of trunk, arms or legs, consisting of regular unbroken circles (livedo reticularis) or irregular broken pattern (livedo racemosa)’’.Livedo reticularis is an independent risk factor for arterial thrombosis. Furthermore, other dermatological problems such as digital gangrene, skin ulcerations, acrocyanosis, anetoderma, superficial phlebitis, superficial skin necrosis, pseudovasculitis lesions and pyoderma gangrenosum-like lesions, characterized by deep, necrotic ulcers can possibly be related to aPL[2,33].

vii. Neurological manifestations.

Neurological disorders are often associated with APS, since the initial description of APS made by Hughes and colleagues[5]. Thrombo-occlusive events in the cerebral circulation are referred and associated with stroke, which is the most common and severe neurological manifestation of APS. In addition to thrombotic events, aPL binding nervous tissues and interfering with neuronal function has been suggested[34]. Cognitive dysfunction (owing to several cerebral small vessel thromboses), untreatable headaches and migraine, visual disturbances, myelopathy, multiple sclerosis-like syndrome, epilepsy and chorea have all being associated to aPL antibodies. Additionally, epilepsy has also been observed in patients with APS as well as strokes and transient ischemic attack, SLE, valvulopathy and livedo reticularis[2].

viii. Renal manifestations.

Thrombotic microangiopathy due to antiphospholipid antibodies may cause hematuria, proteinuria and renal insufficiency, or it may even cause acute renal failure and hypertension. The main lesions of APS-related nephropathy are renal artery stenosis, venous renal thrombosis, and small-vessel vaso-occlusive nephropathy. APS nephropathy may be acute (thrombotic microangiopathy involving glomerular capillaries) or chronic (fibrous intimal hyperplasia, tubular atrophy, arteriolar occlusions, and focal cortical atrophy). Furthermore, in patients with APS and SLE, APS renal disease may coexist with immune complex-mediated lupus nephritis[35].

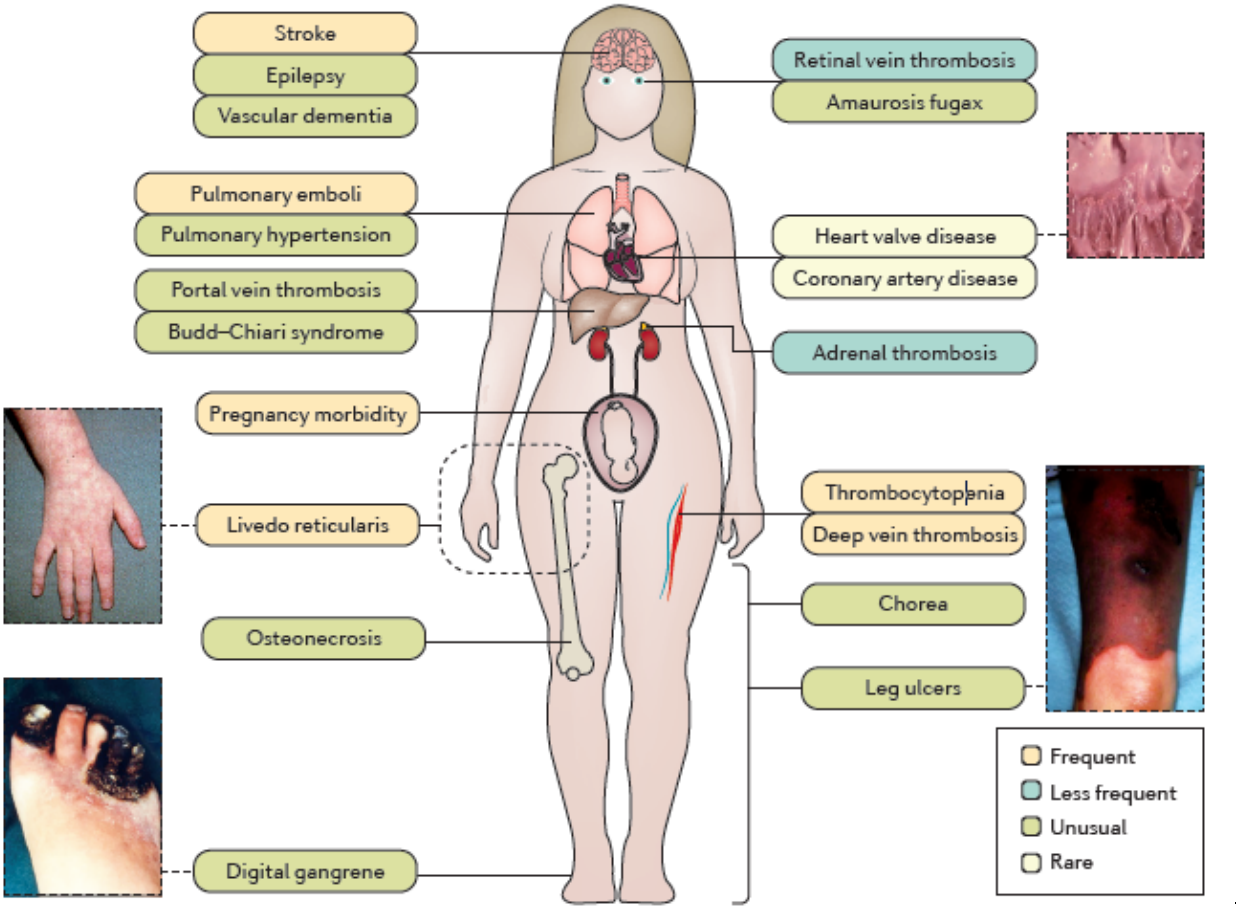

Figure 2 ***Clinical manifestation of antiphospholipid syndrome.*** *aPL antibodies are associated with a variety of symptoms.*[2]

Etiopathogenesis

The mechanism underlying the production of aPL in APS patients is not fully understood yet, but there is a commonly acceptable hypothesis about comorbid infections and exposure to various environmental agents[5]. The pathogenic mechanism underlying thrombotic events and pregnancy difficulties in APS has also not been elucidated and maybe a multitude of mechanisms are involved. A lot of studies mention infections and probably other environmental stimuli trigger the initial events related to APS such as the production of aPL antibodies and thrombosis. There is evidence that activation of monocytes, endothelial cells, platelets, complement and coagulation cascade proteins leading to a prothrombotic condition.

Antiphospholipid antibody family are a heterogeneous group of autoantibodies that interact with many different phospholipid (PL)-binding plasma proteins. The most known PL-binding plasma proteins are b2-glycoprotein I (b2GPI), prothrombin, thrombomodulin, kininogens, antithrombin III, protein C, protein S and annexin I, II, and V [5].These proteins bind to phosphatidylserine, a phospholipid which is located in the inner surface of the cell membrane. Phosphatidylserine is exteriorized on activated or apoptotic cell membranes, including those of trophoblasts, monocytes, endothelial cells, and platelets, causing cell activation, clearance of apoptotic cells, and coagulation.

Implications of PF4-b2GPI complex in APS

PF4 found to implicated in pro-coagulant conditions. Firstly, PF4 seems to inhibit the upregulation of thrombin inactivation by antithrombin, through heparin inhibition. Secondary, found to be involved in platelet aggregation, as in PF4 knockout mice treated with thrombin lead to platelets defection. Strong evidences have described about the PF4 release from platelets to compensate the impaired platelet thrombus formation. In addition, PF4 involved in protein C activation in vitro and in vivo expressing anti-coagulant action[16].

PF4-heparin complex induces anti-PF4/heparin antibodies in heparin-treated patients. These autoantibodies make the complexes to activate platelets via FcgRIIa receptors. Although, blocking FcgRIIa receptor, platelets activation in presence of anti-b2GPI/PF4/ b2GPI complex implying that antib2GPI in APS are responsible for the binding in F(ab)2 fragments.

The mechanism has been described as followed:

Platelets are activated and secrete a high amount of PF4, which is complexed with b2GPI dimmers and attached on platelets and monocytes cell surface and are antigenic targets for anti-b2GPI on platelets, monocytes and endothelium. Anti-b2GPI bind and activate platelets, monocytes and ECs, regulating the production of pro-coagulant molecules[36].

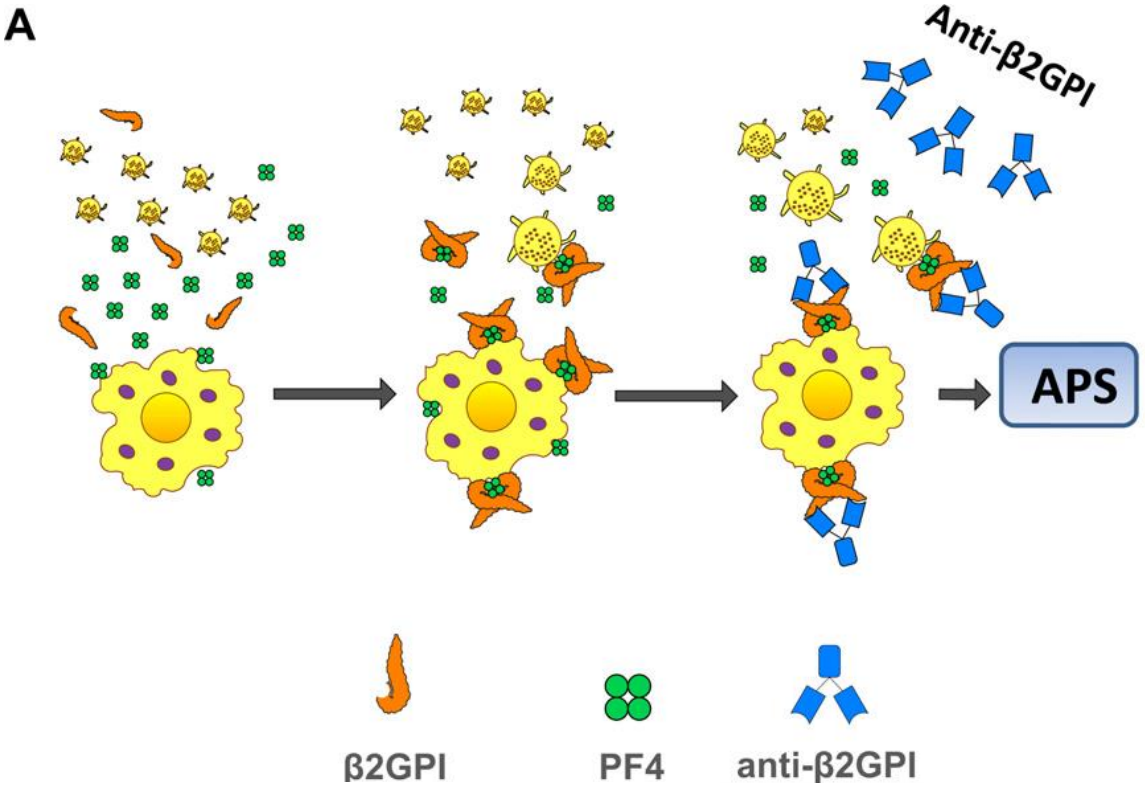

Figure 3 ***Mechanistic basis of APS:*** *Binding of anti-b2-GPI autoantibodies to PF4/β2-GPI e containing complexes generate pathologic immune complexes, , one via the F(ab)2 part of the β2-GPI-IgG*[36].

Anti-b2GPI antibodies are responsible for upregulation of tissue factor, leading to an extrinsic coagulation cascade activation in monocytes and endothelial cells[5]. Furthermore, aPL act on endothelial cells resulting in higher expression of adhesion molecules i.e. intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin (SEL-E), and proinflammatory cytokines, leading to proinflammatory and procoagulant phenotype of ECs[5]. Also, aPL may activate platelets, increasing expression of glycoprotein IIb–IIIa, synthesis of thromboxane A2, and secretion of platelet factor-4, a chemokine with procoagulant and prothrombotic effects[16]. Annexin V, a physiological anticoagulant bind to phosphatidylserine surfaces and the complex regulates the formation of procoagulant complexes. Anti-β2GPI autoantibodies in complex with β2GPI impede the formation of this anticoagulant shield, exposing procoagulant phosphatidylserine promoting thrombosis.

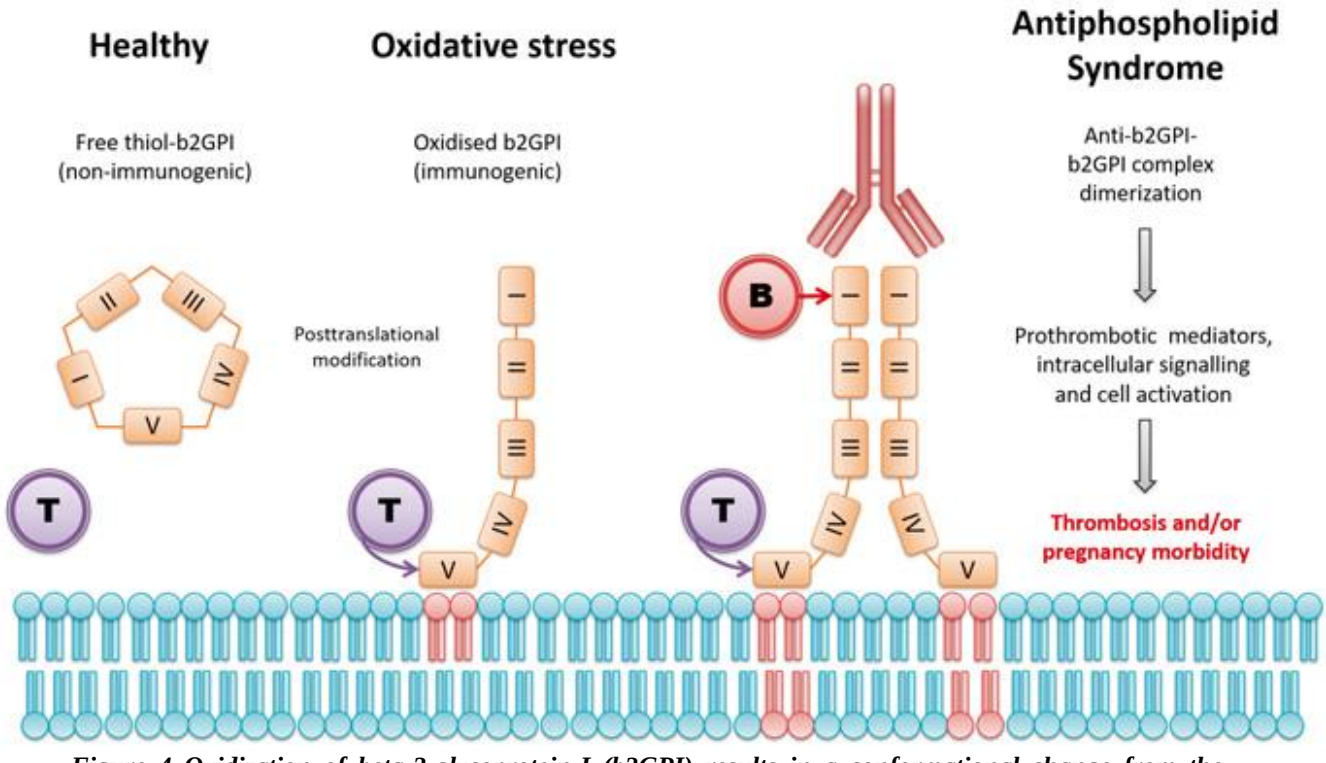

Figure 4 Oxidization of beta-2-glycoprotein-I (b2GPI) results in a conformational change from the circular form to the J-form[37]

B2GPI protein is a natural anticoagulant and plays a role in innate immunity. Increased oxidative stress changes β2GPI configuration from the circular non-immunogenic, free thiol form to the J-shaped immunogenic, oxidized form which

increases immunogenicity by exposing hidden epitopes to T cells and B cells and enables anti-β2GPI antibody binding. Increased levels of oxβ2GPI probably raise T cell immunogenicity and stimulate the production of pathogenic antibodies[37].

Studies in mice reveal that obstetric complications caused by aPL-induced complement activation affects fetal loss and induce thrombosis[38]. Antibody- b2GPI complexes stimulate complement activation and C5a, which induce inflammation and placental insufficiency. C5a can bind and activate neutrophils upregulating the expression of TF. TF interacts with factor VIIa and activating factor X, generating thrombin, and clot formation. aPL were found to interact with proteins in the coagulation cascade, mostly with prothrombin, factor X, protein C, protein S, and plasmin, which seems to alter the balance of procoagulant and anticoagulant factor levels and induce fibrinolysis[5].

Studies suggesting that the direct activity of aPL are implicated in non-thrombotic cerebral manifestations and pregnancy morbidity. Indeed, aPL can bind to trophoblast cells resulting to cellular injury and the lowering secretion of human chorionic gonadotropin can cause placental abnormalities[39].

Recently, it was found that microvesicles take part in APS related thrombosis, vascular reactivity, and inflammation representing a novel biomarker and a therapeutic target[40]. APS patients present high levels of circulating platelet, monocyte, and endothelial microparticles[41]. Microvesicles, are implicated in procoagulant activities, and have been related to membrane expression of phosphatidylserine and TF, increase the expression adhesion molecules, interfere with nitric oxide- prostacyclin pathways, and secret more cytokines and chemokines[42]. Assuming that, microRNA that released from exosomes provoke the secretion of proinflammatory cytokines from trophoblast (i.e. IL-8) by activating TLR-8[43].

‘‘Antigenic specificity’’ of aPL promote different APS phenotypes depending the specific epitope. The pathogenetic aPL autoantibodies bind to phospholipid-binding proteins (i.e. b2GPI), which are linked to phospholipids (b2GPI-dependent aPL). Infectious diseases, drugs, or malignancies trigger the production of aPL that bind directly to phospholipids (b2GPI-independent aPL), but those autoantibodies are not related to thrombosis[5]. Studies from animal models and human suggest that anti-b2GPI antibodies with specificity against the first domain of the molecule are more strongly associated with thrombosis and obstetric complications than those directed against other

epitopes of the b2GPI, indicating that this specific epitope is of primary importance of APS pathogenesis.

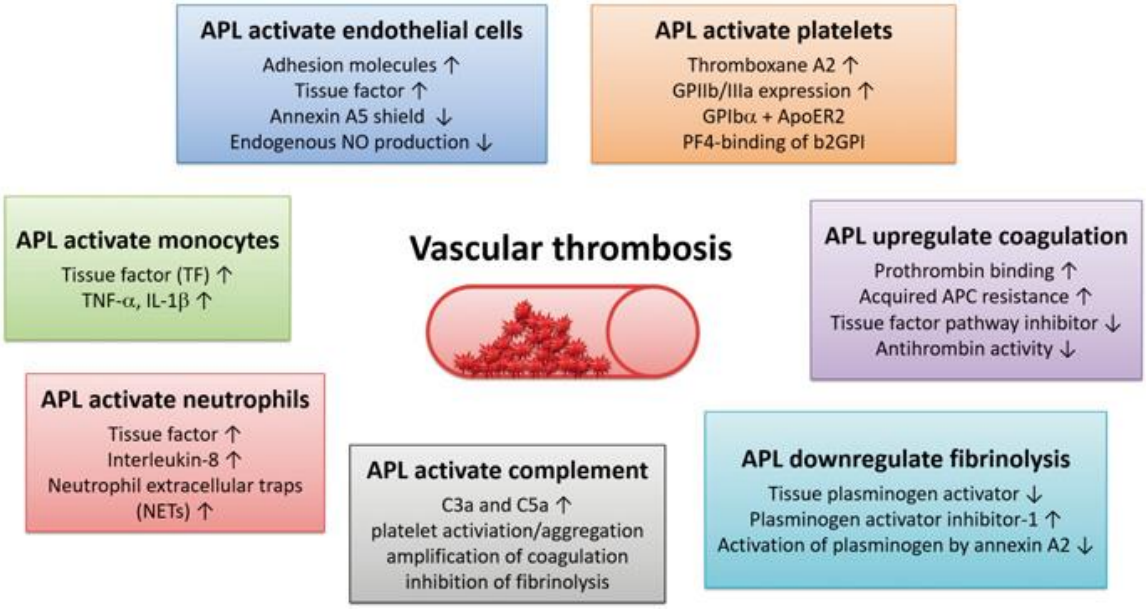

Figure 5 Factors leading to vascular thrombosis in APS[37].

Antiphospholipid antibody formation

Infectious agents are culpable for antiphospholipid antibodies generation, mainly anti-β2-glycoprotein 1 antibodies[2]. Studies have implicated molecular mimicry mechanisms between bacterial and viral structures and β2-glycoprotein-1-derived amino acid sequences. Another mechanism can be the misfolding of β2-glycoprotein 1. Binding of β2-glycoprotein 1 to the surface protein H of *Streptococcus pyogenes* induces a conformational change in β2-glycoprotein 1, causing the exposure of a cryptic epitope in domain 1 of β2-glycoprotein 1. Mice injected with the mouse protein H–β2-glycoprotein 1 complex developed antibodies against this epitope[44]. In this way, healthy individuals may produce antibodies against β2-glycoprotein 1, even though only in the appropriate genetic background or following secondary triggers these antibodies can be pathogenetic.

There is a highly acceptable hypothesis of a ‘‘two-hit model’’; this supposes that aPL augment the risk of thrombosis (‘‘first hit’’) but thrombus formation only occurs following another procoagulant condition (‘‘second hit’’) (estrogens, surgical procedures, trauma, or infections)[2,5].

Two-hit model

In APS patients, antiphospholipid antibodies are continuously present, while thrombotic events take place only periodically. Probably, the production of antiphospholipid antibodies is a necessary but insufficient condition for APS pathology and other factors participate. 'Second hits' push the hemostatic balance leading to thrombotic events and include environmental factors (infections), inflammatory factors (concomitant connective tissue diseases) or other non-immunological procoagulant factors (oestrogen-containing contraceptives, surgery and immobility)[45]. Patients' genetic background, i.e genes encoding inflammatory mediators, might also be a pivotal variable in the development of clinical APS manifestations. Familial studies found genetic predisposition to APS, in part accounted for by the human leukocyte antigen (HLA) system, with the most consistent associations being those with *HLA-DR4* and *HLA-DRw53*[2].

The presence of both lupus anticoagulant and anticardiolipin antibodies are also related to these HLA haplotypes. Other genes related to APS, are *IRF5* (encoding interferon regulatory factor 5) and *STAT4* (encoding signal transducer and activator of transcription 4)[46].

As the two-hit model postulates, apL trigger the thrombus formation and the thrombotic response is much harsher after a second hit (vascular injury) owing to the priming of immune cells, platelets and endothelial cells by anti-β2-glycoprotein 1 antibodies (the first hit). (figure 6).

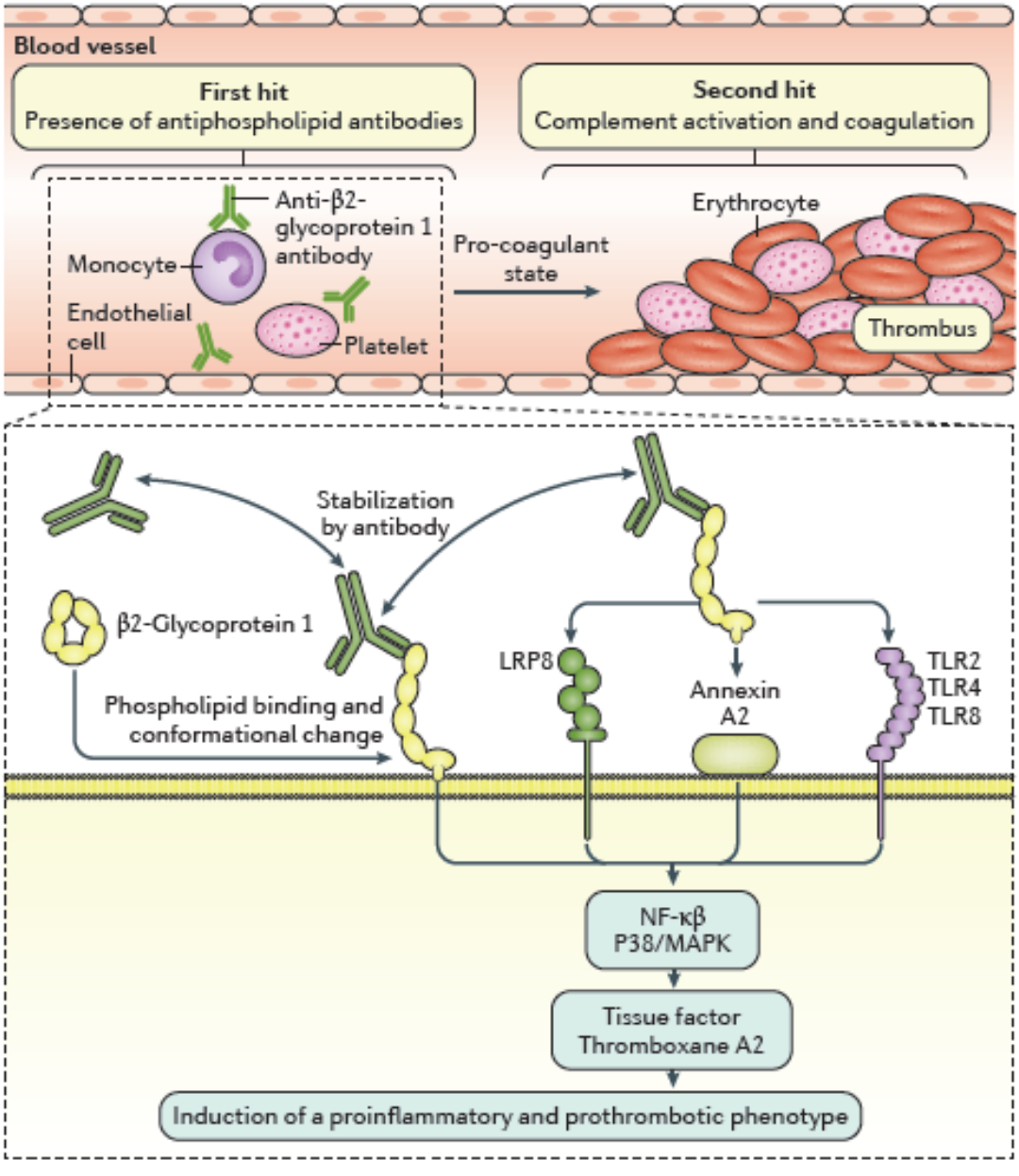

Figure 6 ***Pathophysiology of antiphospholipid antibody-associated thrombosis.*** *LRP8, low-density lipoprotein receptor-related protein 8; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; TLR, Toll-like receptor*[2].

Various risk factors exist for thrombotic complications related to arterial thrombosis and venous thromboembolism (including deep vein thrombosis and pulmonary embolism)[2].

Several mechanisms explaining the prothrombotic effects of antiphospholipid antibodies have been proposed. One of the most acceptable hypotheses is that the anti-β2GPI antibodies bind to monocytes, platelets and endothelial cells and trigger a

conformational change of β2GP1 membrane protein. The changed protein structure stabilized by anti-b2gpi and the complex seems to bind in Annexin A2 or LRP-8 or in TLRs (TLR-2/TLR-4/TLR8) depending on each cell receptor. This binding results in activation of cells towards proinflammatory and prothrombotic pathways. Transcription factors NFkB and P38 MAPK, have pivotal role to inflammation, indeed, these transcription factors upregulate the expression of TF and Thromboxane A2 (TA2). A large proportion of β2GPI in the sera of patients with APS is oxidized and the oxidized form [47] is associated with APS AND and not with other reasons for thrombosis. Furthermore, β2GPI changes conformation of C3 complement component, so the regulator factor it attaches and induces subsequent degradation by factor I[48]. (figure 2)

Antiphospholipid antibodies injections in mice, rats or hamsters do not cause severe thrombotic events. However, squeezing a clip of cremaster veins, induces thrombus formation in animals pre-treated with antiphospholipid antibodies than after infusion of a control antibody in mice[49]. This evidence strongly support the idea that antiphospholipid antibodies are risk factors for thrombosis in humans[2].

Studies in animal models have proven that antibodies against β2-glycoprotein 1, especially those against domain 1, can induce a strong prothrombotic phenotype[50]. Administration of human anti-β2-glycoprotein 1 antibodies in mice create a good model for the human disease. A prothrombotic phenotype was also induced by anti-prothrombin antibodies, but it is not yet known which is the key epitope on this protein that these autoantibodies are directed in mice[2].

Anti-β2-glycoprotein 1 antibodies in complex with β2-glycoprotein 1 at the cell surface results in the activation of cultured endothelial cells, platelets, monocytes, neutrophils, fibroblasts and trophoblasts as well as expression and release of cell type-dependent activation markers. Important target cells of this complex are platelets, endothelial cells and monocytes which are involved directly or indirectly via the shedding of prothrombotic microparticles [51].

Complexes of anti-β2GPI bound to Toll-like receptor 2 (TLR2), TLR4, annexin A2 or low-density lipoprotein receptor-related protein 8 (LRP8; also known as apolipoprotein E receptor 2) and activate intracellular signal transduction pathways, changing the phenotype of cells to a prothrombotic cellular phenotype [2](figure 4).

Evidence from knockout mice studies reveals that cell activation via LRP8, annexin A2 and TLR4 by anti-β2GPI antibodies results in a prothrombotic or thrombotic phenotype of cells[52].

Antiphospholipid antibodies seem to strongly interfere with complement activation. Mice deficient in complement factors C3, C5 and C6 exhibit a reduced thrombotic response in the presence of antiphospholipid antibody combined with a vascular challenge compared with wild type mice[2].

aPL antibodies induce activated protein C resistance *in vitro* suggesting that autoantibodies compete with activated protein C for the binding to the catalytic phospholipids, limiting the access of protein C to its substrates[2]. In vitro experiments have shown that activated protein C resistance predisposes to venous thromboembolism and antiphospholipid antibodies augment the size and the persistence of thrombus[2].

Antiphospholipid antibodies have been associated with recurrent first-trimester pregnancy loss and is different from pathogenesis of morbidity occurring in late pregnancy. First-trimester pregnancy loss has been attributed to a direct inhibition of trophoblast cell proliferation[2]. Late obstetrical problems in APS patients, such as pre-eclampsia, intrauterine growth restriction and stillbirths, are attributed to placenta dysfunction. Failure of extravillous trophoblasts to adequately remodel the spiral arteries, causing low maternal blood flow to the placenta and hypoxic injury has been proposed as a major factor. Another mechanism that seems to cooperate in fetal loss is the inadequate delivery of nutrients to the fetus while concurrently high-velocity and high-pressure blood flow can damage the placenta. Antiphospholipid antibodies decrease proliferation and invasion of extravillous trophoblasts and increase the inflammation at the maternal–fetal interface, both of them leading to placental failure (figures 7,8,9).

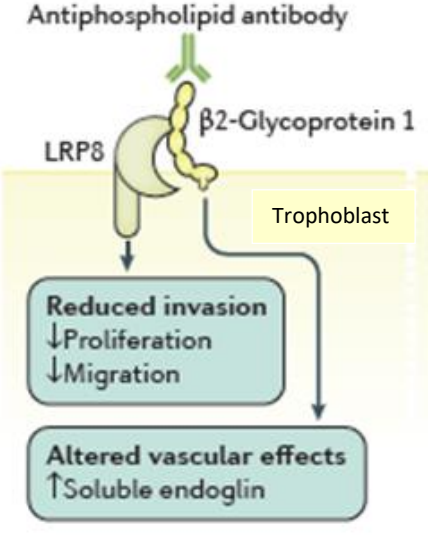

Figure 7 aPL antibodies on trophoblasts[1]

β2GPI is expressed in the cell surface of endothelial cells in trophoblasts. Anti-β2-GPI bind to trophoblasts and endothelium through the phospholipid binding site in domain 5 of β2-glycoprotein 1 and in

different cell surface receptors[2]. I*n vitro* studies proved inhibition of trophoblast migration, extravagant trophoblast antiangiogenic soluble endoglin secretion and impaired trophoblast–endothelial signal transduction in a model of spiral artery transformation[53]. LRP8 activated by β2-glycoprotein 1 - anti-β2-glycoprotein 1 complex, reducing IL-6 levels and STAT3 activity impede cell migration[53]. Indeed, LRP8 implication in antiphospholipid antibody-mediated fetal loss and intrauterine growth restriction was shown *in vivo*[54] (figure 7).

Polyclonal IgG antibodies with high titers of antiphospholipid antibodies or monoclonal human antiphospholipid antibodies injected to pregnant mice cause fetal resorption and growth restriction. Antiphospholipid antibodies localized to the placenta and associated with complement activation or recruitment and stimulation of neutrophils, are the basic components of placental insufficiency, fetal loss and growth restriction. Other *in vitro* studies, with human first-trimester extravillous trophoblasts proposed that anti-β2-GP1 antibodies trigger production of pro-inflammatory cytokines and chemokines (mostly IL-1, IL-7 and IL-8) through TLR4[2]. (figure 8).

Complement activation regulates tumor necrosis factor (TNF) secretion and the antiangiogenic factor soluble vascular endothelial growth factor receptor 1 (sVEGFR1) by infiltrating leukocytes, facts related to placenta loss and pre-eclampsia[55].

Binding of C5a to C5aR located in the cell membranes of neutrophils and monocytes promotes placental injury, through TF expression, reactive oxygen species release, and TNF and antiangiogenic factors secretion (sVEGFR1)[56] (figure 9).

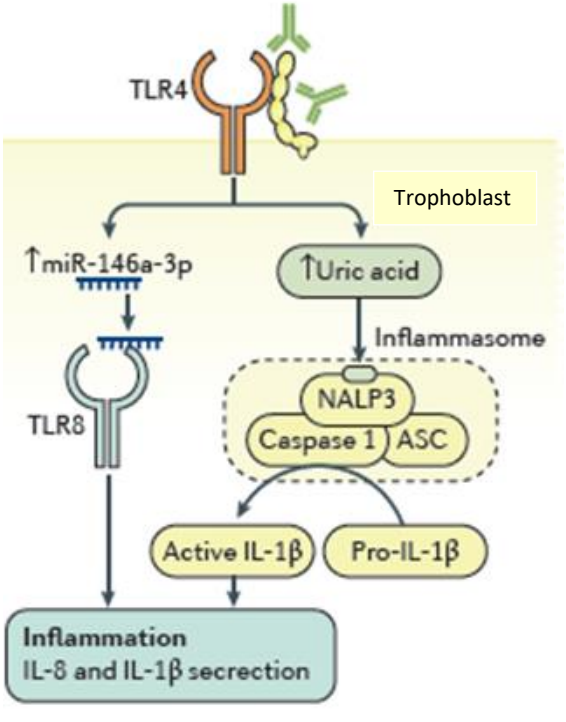

Figure 8 Secretion of inflammatory cytokines and chemokines by activating Toll-like receptor (TLR) and inflammasome pathways[2]

TNF contribution to adverse pregnancy outcomes in humans is supported by the fact that increased TNF levels are being found in the maternal blood and amniotic fluid of women with preeclampsia and at the fetal–maternal interface in intrauterine growth restriction[2]. In addition, mice with TNF deficiency or treated with TNF blockers have lower possibilities for fetal loss caused by antiphospholipid antibodies.

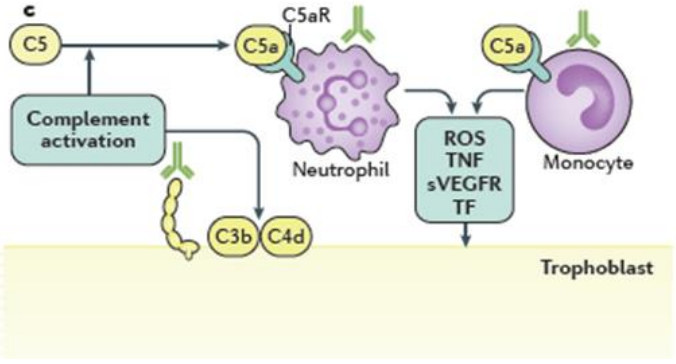

Figure 9 activate complement on the cell surface, leading to neutrophil and monocyte activation with release of reactive oxygen species (ROS), tumour necrosis factor (TNF), antiangiogenic factors (soluble vascular growth factor receptor (sVEGFR)) and tissue factor (TF)[2]

Activated complement recruits and activates neutrophils (figure 9)[2]. In mouse models of pre-eclampsia, neutrophils infiltrate the placenta and their depletion improves placental morphology, recovers spiral artery remodeling and improves pregnancy outcomes. In pregnant mice injected with aPL antibodies, neutrophil infiltration in placenta affected fetal survival and growth. When in those mice the neutrophils were depleted, there was no pathology[2]. In both models, recruitment of neutrophils attributed to complement activation at the maternal–fetal interface was followed by higher TNF levels, lower VEGF levels resulting in abnormal placentation and fetal death[57].

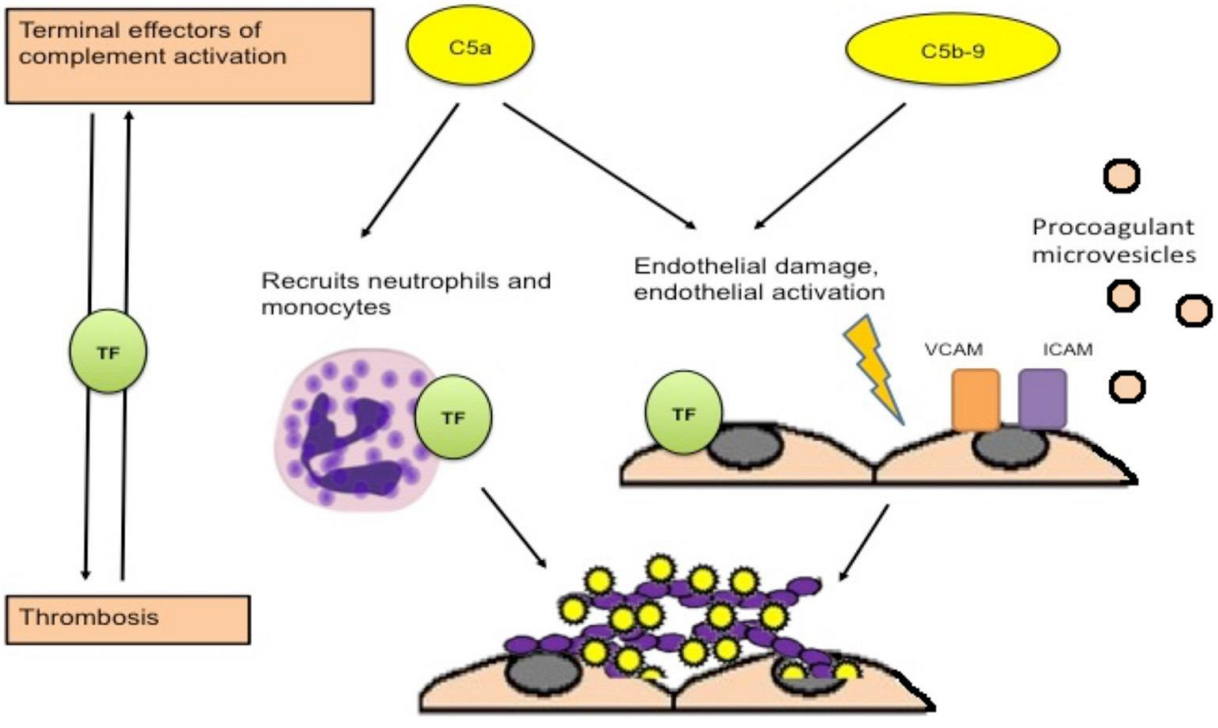

Figure 10 Procoagulant effects of complement activation.[57]

Complement activation includes the expression of C5a and C5b, which complex with other complement components to form the membrane attack complex. C5a is an anaphylatoxin that recruits neutrophils and triggers TF expression on neutrophils, monocytes and endothelial cells. Accumulation of membrane attack complex in endothelium drives endothelial injury and procoagulant phenotype apart from upregulation of adhesion molecules, release of von Willebrand factor and procoagulant microvesicles. (figure 10)

A study demonstrated that anti-β2GP1 antibodies in complex with β2-glycoprotein can stimulate neutrophil extracellular trap (NET) formation through ROS and TLR4 pathways[58]. In APS patients increased NET formation, impaired NET clearance and higher numbers of circulating low-density granulocytes have been found resulting in the production of cytokines and type 1 interferons. NETs are also found in placental intervillous spaces in SLE patients and women with pre-eclampsia[59].

Heparin, a complement activation inhibitor, can be administrated in order to diminish the possibility of pregnancy loss. In contrast, anticoagulation therapy with hirudin or fondaparinux, that don't affect complement activation, when administrated to APS model mice was found not to prevent pregnancy loss[60]. Complement fragment C4d

is found in the placentae of women with SLE and APS and preeclampsia, but not in healthy individuals. Inherited hypofunctional variants of complement regulators are also associated with the risk of pre-eclampsia in women with SLE. Moreover, studies have found mild hypocomplementaemia in APS patients, demonstrating continuous activation and consumption of complement components[2].

Catastrophic antiphospholipid syndrome

A rare, life-threatening form of APS occurring in less than 1% of patients, is defined as catastrophic APS (CAPS)[5]. CAPS include intravascular thrombosis affecting three or more organs, systems and tissues either simultaneously or within 1 week with histological confirmation of small vessel occlusion. CAPS is characterized by the rapid onset of small vessel thrombosis in multiple organs, commonly associated with microangiopathy, resulting in acute multiorgan dysfunction and failure, with more than 50 % mortality. Moreover, large vessels may be affected[61].

Hypotheses that different factors (infection, surgery, therapy discontinuation) can be precipitant factor(s) of endothelial injury driving cytokine overproduction and development of a systemic inflammatory response resulting in CAPS have been described. Nearly 50% of catastrophic APS patients have had a previous infection[61].

Organs involved in catastrophic APS are the kidneys (73% of CAPS patients), pulmonary system (60%), brain (56%), cardiac system (50%) and skin (47%). In addition, thrombocytopenia is commonly observed in CAPS patients (67%), followed by schistocytes (22%). Of all individuals who develop CAPS, 60% only have isolated APS, while 40% have APS associated with another systemic autoimmune disease[19].

Endothelial Cell Activation

The concept of EC activation is based on ultrastructural microscopic alterations of the vascular endothelium found in the tuberculin reaction in guinea pigs treated with azobenzenearsonate-insulin. This concept has been expanded to investigate the

expression of specific gene products and specific elements of inflammation, coagulation factors, and immunity [62-64]

Endothelial cell activation comprises ‘‘EC stimulation’’ (‘‘Type I EC activation’’) and ‘‘EC activation’’ (‘‘Type II EC activation’’). Type I EC activation takes place after stimulation and does not require *de novo* protein synthesis or gene transcription. The surface of the type I-activated ECs is capable of shedding endothelial adhesion and antithrombotic molecules, i.e P-selectin, thrombin, heparin, antithrombin III, and thrombomodulin. In Type I, the endothelium in the venules and small veins rapidly retracts causing hemorrhage, edema, and higher vascular permeability [65].

Type II EC activation is a latter response that is dependent on the activation of gene transcription and the *de novo* synthesis of proteins [63,64]. The genes involved in Type II produce proteins such as adhesion molecules, cytokines, chemokines, and procoagulant factors [66]. The endothelium expresses E-selectin on its surface and secretes von Willebrand factor (vWF), chemokines (IL-8), nitric oxide (NO), prostacyclin and platelet-activating factor (PAF) [67]. Type II EC activation is manifested by protrusion of ECs into the lumen of blood vessels, hypertrophy of ECs (plump cuboidal appearance), increased numbers of biosynthetic organelles (Golgi complex, rough endoplasmic reticulum, and ribosomes), and higher permeability (pinocytotic vesicles).

Monocytes and lymphocytes circulating around activated ECs, harboring Fc receptors (FcR), can be exposed to the basement membranes, extravasated through the interaction of selectins and integrins, leading to immune activation [68].

Endothelial cell activation is a state of reversible morphological rearrangement (increased size and cytoplasmic organelles) and altered expression of various molecules. When EC activation is uncontrolled, progress to EC apoptosis is evident. Electron microscopy has shown that morphological alternations are reversible, lowering the levels of cytokines, tumor necrosis factor, and interferons.[69]

Endothelial cell activation may progress to EC dysfunction described as a disruption in its vasoactive role in regulating tissue perfusion and blood pressure homeostasis [70]. EC activation becoming EC dysfunction and EC injury is through a series of immediate and delayed events. Endothelial cell dysfunction with irreversible EC injury are induced by uncontrolled chronic and persistent EC activation. Endothelial cell activation and EC apoptosis are regulated by protective gene expression (IkB-a, A20,

Bcl-2) that down-regulate the expression of NF-kB. Activation of NF-kB triggers EC activation leading to apoptosis. Activated ECs express specific proteins that play significant role in inflammation, coagulation, and immunity. Persistent EC activation produces increased levels of endothelial adhesion molecules, procoagulant molecules, vasodilators, cytokines, chemokines, and lead to vasculitis thrombosis EC necrosis, and other mural cell injury [69]. The expression of CAMs are major markers of EC dysfunction that occurs at the early stages of inflammation and atherosclerosis development.

Endothelial cell activation can progress to vascular EC injury and vasculitis development, thrombosis, and other vascular diseases that are mediated by leukocyte adhesion molecules, vasoactive mediators, pro-inflammatory cytokines, chemokines, and procoagulant molecules released by activated ECs. Type I EC activation and Type II EC activation are reversible when EC activators are withdrawn, whereas chronic EC activation leads to EC injury with EC detachment from the underlying basement membrane and denudation of the vessel wall, resulting in circulating endothelial cells (CECs) and the release of EC microparticles (EMPs) and EC caveolin-1 (Cav-1) from the plasmalemmal membranes[69] (figure 11).

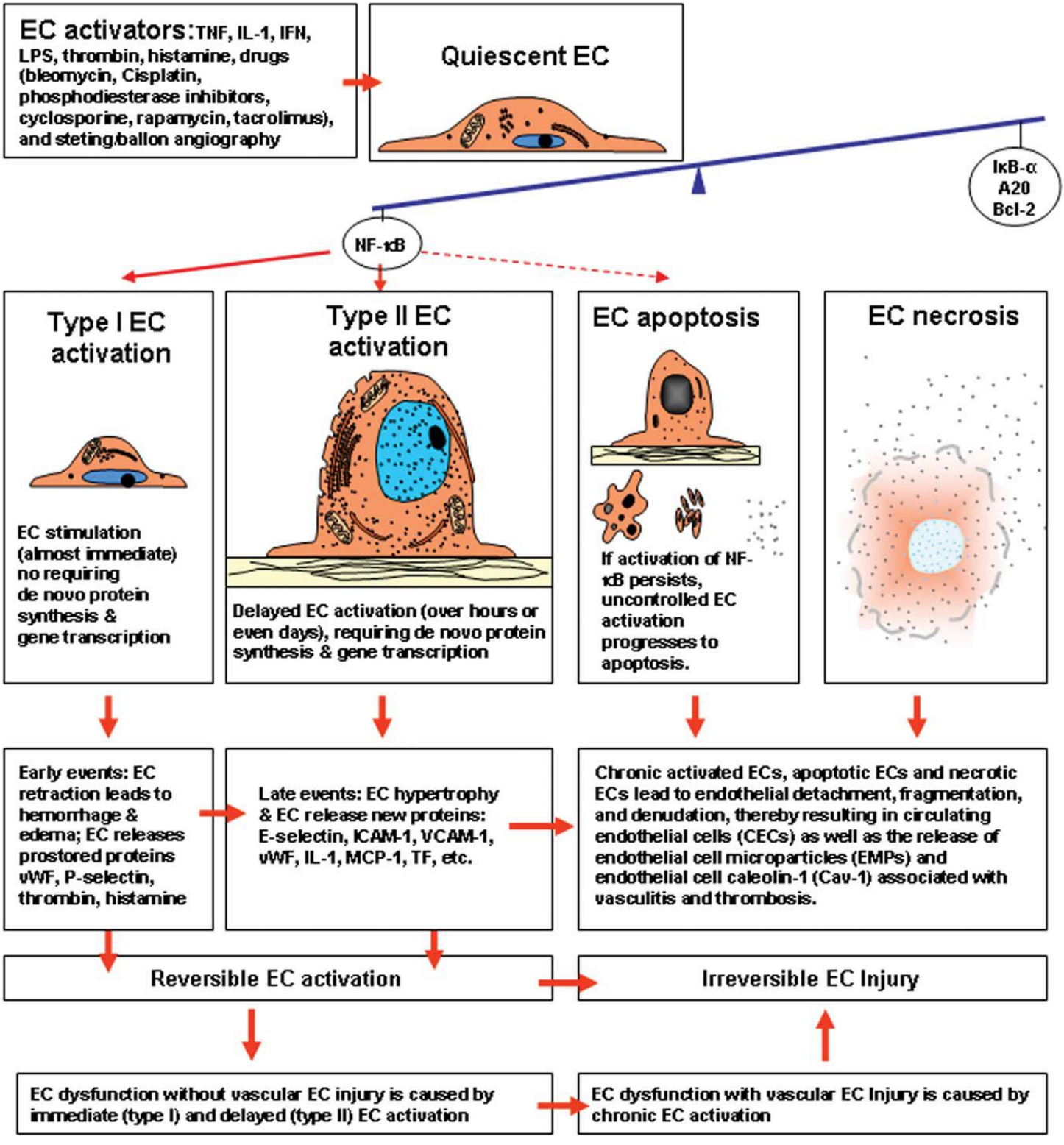

Figure 11 Endothelial cell activation process *TNF, tumor necrosis factor; IL-1, interleukin-1; IFN, interferon; LPS, lipopolysaccharide* [69]

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Thrombospondin 1 (TSP-1)

Extracellular matrix (ECM) is a group of proteins forming a 3D scaffold that is remodeled depending on synthesis, deposition and degradation processes. Thrombospondins (TSPs) are a family of ECM proteins expressed in embryonic and adult tissues, and constitute a family of five members: TSP1, TSP2, TSP3, TSP4, and TSP5[71].

Thrombospondin-1 is mainly expressed and secreted by cell types including ECs, fibroblasts, muscle cells, immune cells, platelets (and megakaryocytes) and transformed parenchymal cells[72] (Figure 12). Secretion rates of TSP-1 protein from the stromal components (eg fibroblasts, ECs) are one to two fold greater than the rates from tumor cells. Between different types of stromal cells, ECs produce and secrete TSP-1 at higher levels. ECs comprise approximately 1%-2% of total cells in a tissue, but this percentage is higher in highly vascularized tissues (tumors, lungs and heart)[72].

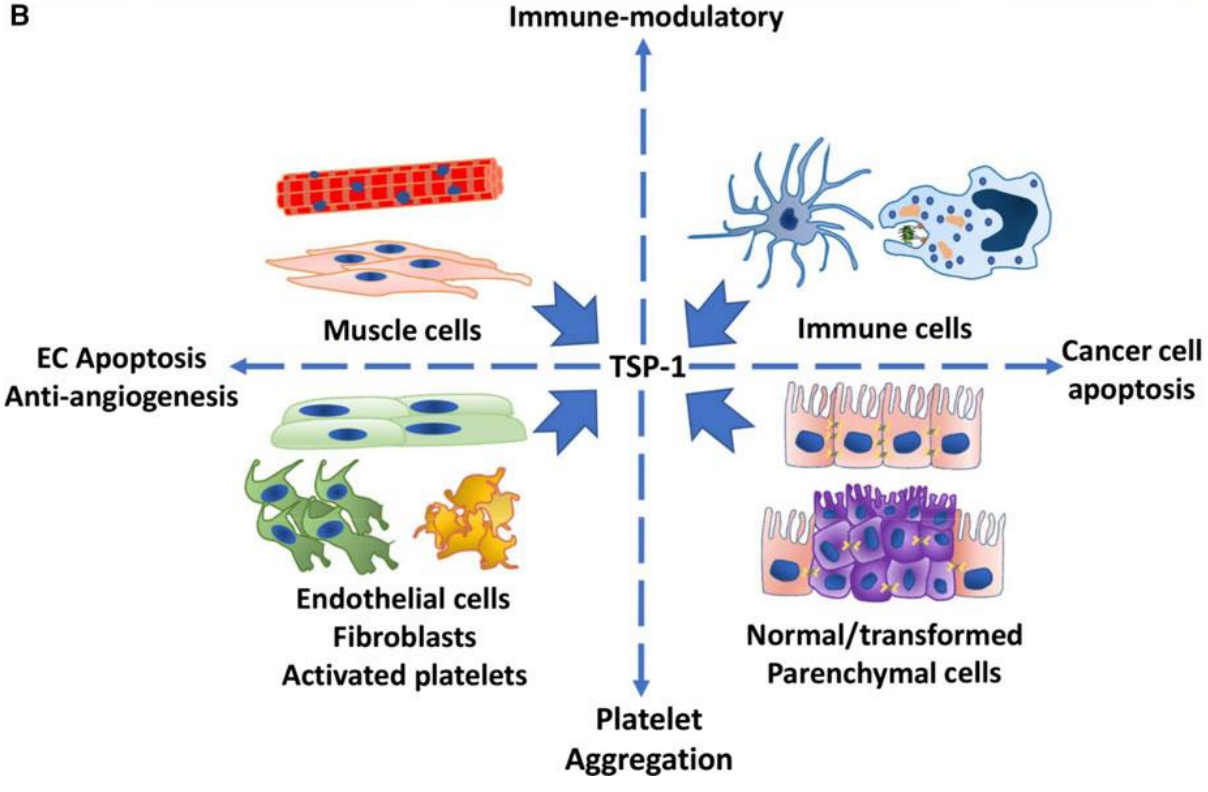


Figure 12 Various types of cells in humans produce and secrete TSP-1, which can potently regulate many important cellular processes.[72]

TSP1 is mainly an angiogenesis inhibitor, hence is a target of many drugs against angiogenesis-driven diseases. TSP1 involved in tumor progression, playing multiple roles, depending on the molecular and cellular composition of the area

microenvironment. Indeed, TSP1 regulates diverse processes such as adhesion, invasion, migration, proliferation, apoptosis, immunity response and treatment response by interacting with multiple ligands.

TSP1 is a 450-kDa homotrimeric glycoprotein, and its mature polypeptide single chain contain 1152 amino acids (180 kDa), connected with disulfide bonds in cysteine 252 and 256 . Each TSP1 subunit has N- and C-terminal globular domains and a thin connecting strand. The N domains are three polypeptidic segments with a heparin-binding domain (HBD), whose affinity helps to purify TSP1 from platelets. Next to the N-terminal, is a region homologous to procollagen involved in the assembly of the protein into a trimer, that could inhibit antigenicity. Three type I repeats (TSR1s) placed around six cysteinyl residues and type II repeats (TSR2s) are three motifs of 60 amino acids containing six cysteinyl residues as TSR1. Seven type III repeats (TSR3s) are aspartic residues related with calcium binding interacting with αvβ3 and αIIβ3 integrins in different cells. A peptide in G domain (cell-binding domain (CBD)), seems to stimulate platelet aggregation through binding with integrin-associated protein (IAP/CD47)[71].(figure 13)

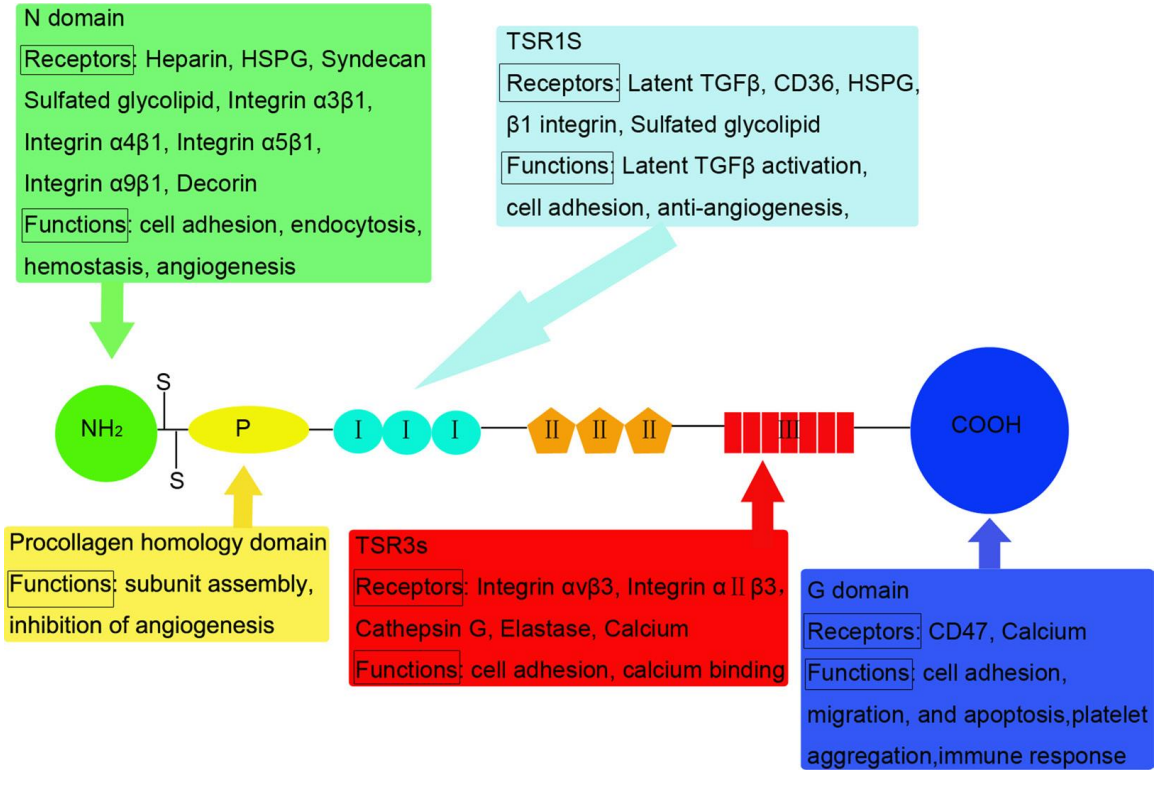


Figure 13 Structural and functional domains of TSP1: Mature polypeptide chains are linked by disulfide bonds. *TSP1 monomer comprises of N and G domains and a thin connecting strand. Interactions between structural domains and multiple cell surface molecules determine the diverse functions of TSP1.*[71]

The secreted TSP-1 trimeric protein is heavily modified by glycosylation and weighs, as mentioned above, over 450 kD 7. Interactions between TSP1 structural domains and multiple cell surface molecules determine its role. Well-studied receptors include low density lipoprotein receptor-related protein (LRP), proteoglycans and sulfatides, CD36, integrins, integrin-associated protein (IAP). Receptors with G domains are calcium and CD47.

TSP-1 gene transcription is regulated by multiple transcription factors and gene methylation status. Some already known factors that positively regulating TSP-1 expression include HIF, p53, NFAT, FOXO1 and SMAD2, whereas TSP-1 is downregulated by c-Myc, Id-1 and YY-1. In addition, TSP-1 mRNA can be targeted by microRNAs, such as miR-18a, miR-194 Let-7g.[72](figure 14).

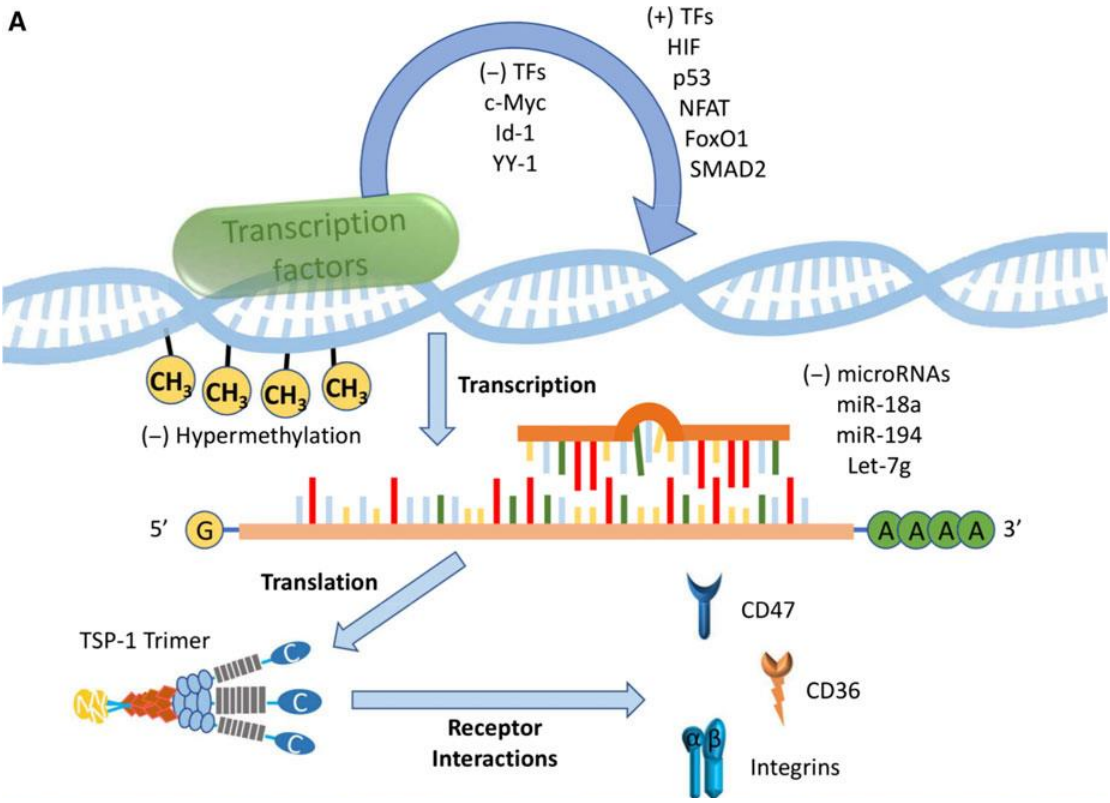


Figure 14 Regulation of thrombospondin-1 (TSP-1) at multiple levels and TSP-1 secretion by different cells. [72]

TSP-1 is a major contributor in disease pathophysiology evidenced by its expression in different cells and body compartments. Systematic characterization and quantification of TSP-1 expression in cancer shows variability to the point of forestalling prediction as to its role in many of these cases. In the same study, it was

found that TSP-1 expression levels remained continuously elevated in inflammatory and cardiovascular diseases[72].

Secreted TSP-1 directly transduces signals as it binds to cell-surface receptors. Alternatively, TSP-1 can regulate cell signaling through binding to enzymes and growth factors. Regulation of cell function from TSP-1 is concentration- and cell type-specific. Studies found that in the presence of high levels of TSP-1 i self-renewal and proliferation are inhibited in primary cells, and at certain concentrations, cell death was induced[72]. TSP-1 deteriorates pro-growth signals of metabolism and its expression controls tissue repair, injury and stress signals, an ability that is augmented by upregulating transforming growth factor beta (TGF-beta). In CNS, TSP-1 is secreted by astrocytes playing a role in synapse formation, neuronal proliferation and differentiation. In the immune system, TSP-1 seems to have a supportive role as it can activate monocytes, dendritic cells, macrophages and T cells[72]. TSP-1 may also act to suppress inflammation and take part in the resolution of inflammation[72].

Angiogenesis

Regulation of angiogenesis depends upon the balance between pro-angiogenic and anti-angiogenic factors. TSP1 promotes neovascularisation and is recognized as an endogenous angiogenesis inhibitor[71]. TSP-1 downregulates angiogenesis, mediated by endothelial cells, by blocking vascular endothelial growth factor (VEGF) and gasotransmitter nitric oxide (NO) activity. Therefore, TSP-1 secretion, owing to decrease of NO-mediated vasorelaxation, can control angiogenesis and blood flow and regulate blood pressure homeostasis by changing cardiovascular responses. TSP-1 suppresses tumor growth, hence is down-regulated in the tumor microenvironment. This role is supported by the finding that higher TSP-1 levels are found in patients with good tumor prognosis. However, TSP-1 role in cancer is not clear as other studies proposed that TSP-1 supports tumor growth and spread.[71,72]

The role of TSP1 in angiogenesis rely on the environment, different receptors and binding domain. CD36-TSP1 binding is considered to be a negative regulator of angiogenesis proved by the fact that HUVECs transfected with a CD36 expression vector had a slower growth rate. The specific domains reflecting angiogenic activity are located in the procollagen homology domain and TSR1s. Short peptidic fragments from these domains inhibit angiogenic processes both *in vivo* and *in vitro*. Substituted

peptides prevent migration of capillary endothelial cells, showing antiangiogenic activity[71]. The second and third TSR1s deteriorate chorioallantoic membrane angiogenesis and endothelial cell proliferation. The expression of 4N1K peptide derived from the G domain inhibits tumor angiogenesis[73]. In addition, the 25 kDa heparin binding domain has been linked with angiogenic activity. However, the 140kDa heparin fragment seem to be an inhibitor of FGF2-induced angiogenesis[71].

Endothelial cell apoptosis

Peptides from TSR1s stimulate endothelial cell apoptosis , by dephosphorylation, since tyrosine and serine-threonine phosphatase inhibitors blocked bovine aortic endothelial cell apoptosis[71].

Antibodies that neutralized TSP1 or blocked access of TSP1 to CD36, inhibited TSP1-induced apoptosis. HUVECS treated with a CD36 activating antibody induced apoptosis, confirming this observation. Higher endothelial cells apoptosis was found in TSP1-treated mice with cancer compared to saline-treated, in the area of active neovascularization around the tumor. The CD36-TSP1-dependent signaling pathways include the activation of CD36-p59fyn-caspase 3-p38MAPK cascade, c-Jun N-terminal kinases and Fas/Fas ligand[71].

Another mechanism seems to be the lack of hemodynamic forces or irregular flow conditions that are important factors for survival of vascular endothelial cells. The disturbance of laminar flow drive apoptosis[71]. Autocrine loop of TSP1 and the αvβ3 integrin/IAP complex is involved in these processes. Despite this, administration of αvβ3 antagonists found to result to regression of angiogenic blood vessels[71].

Inhibition of endothelial cell migration and proliferation

CD36 and β1 integrins could regulate TSP1-mediated migration inhibition depending on the microenvironment and the migration stimulus. CD36 is important in endothelial cell migration and tube formation by TSP1 hence, anti-CD36 /TSP-1 treatment expand cell migration. CD36 transfection in large vessel endothelial cells impedes cell migration. TSRs were pan-specific ligands for β1 integrins[71]. β1 integrins are essential for TSP1-mediated inhibition of migration, confirmed by the observation that TSR inhibit VEGF-induced migration after β1 integrins-activating

antibody treatment or by suppressing β1 integrins. β1 integrins may be implicated in cell migration through a PI3K-dependent, Akt-independent pathway[71].

TSP1 induces cell-cycle arrest as it upregulates p21 expression mediated by p53[74]. The human microvascular endothelial cells (HMVECs) proliferation is inhibited by interaction of TSP1 with the very low-density lipoprotein receptor (VLDLR)[75].

Acute Inflammation

Acute inflammation is followed by resolution, progress to the formation of fibrotic capsule, or tissue destruction, fibrin and collagen deposition, while in some cases, is followed by chronic inflammation[76]. The vascular system is actively involved in acute inflammation. Vasodilation widens capillary permeability and promotes the formation of exudate. Slow blood flow controls the release of cytokines and enhances local edema.

The acute inflammatory process starts from injury, followed by cytokines release. Local macrophages express toll-like receptors (TLR) that recognize specific types and epitopes of antigens. TLR activation in turn is causing more cytokine release leading to inflammation and white blood cell activation. Cytokines induce factors that provoke the rapid release of neutrophils from red bone marrow. Circulating neutrophils enter the blood stream and emigrate outside the blood vessels. Other attracted leukocytes are monocytes and macrophages, engulfing any on-site cell debris or pathogens. Furthermore, mast cells (producing histamine), injured tissue cells, phagocytes, lymphocytes, basophils, and blood proteins are inflammatory mediators.

TSP-1 is transiently secreted by various cell types at the beginning of acute inflammation, and multiple factors control TSP-1 release during this process. Accelerated production of TSP-1 presumably controls immune responses and protects tissues from excessive damage. TSP-1 mediates macrophage phagocytosis of apoptotic cells through binding to CD36[76].

Binding CD36 to TSP-1 regulates blood flow and leukocyte infiltration via NO pathway activation. The inducible isoform (iNOS) is expressed upon inflammation and produces NO gas. NO is expressed in basal levels and is anti-inflammatory and antiangiogenic whereas, after injury, high levels of NO are released promoting angiogenesis and leukocyte adhesion to the endothelium[77]. TSP-1 binding

to CD36 and CD47, impedes the soluble guanylyl cyclase system in ECs. In this way, TSP-1 inhibits NO pathway resulting in inflammation by limiting adhesion and activating leukocytes to endothelium and deteriorating angiogenesis[76].

During early inflammation, the nuclear hormone receptor transcription factor called peroxisome proliferator-activated receptor (PPAR) seem to bind to TSP-1. Ablation of this transcription factor in leukocytes results in high levels of TSP-1 release. PPARγ enhances the proapoptotic effects of the TSP-1-derived peptide ABT510, which binds to TSR and induces vascular apoptosis through CD36[76].Treatment with PPARγ agonist increases CD36 expression in ECs, enhancing peptide's antiangiogenic properties.

CD47 is mainly regulating leukocytes migration through endothelial and epithelial barriers. CD47 is expressed in polymorphonuclear cells and augments TSP-1 expression of TSP-1 in leukocytes. Leukocytic apoptosis activated via CD47 pathway, through mitochondrial mechanisms, or activation of the Fas/CD95 pathway. CD47 activation in apoptotic granulocytes modulates phagocytic functions of macrophages in inflammatory regions, which might control resolution[76].(figure 15)

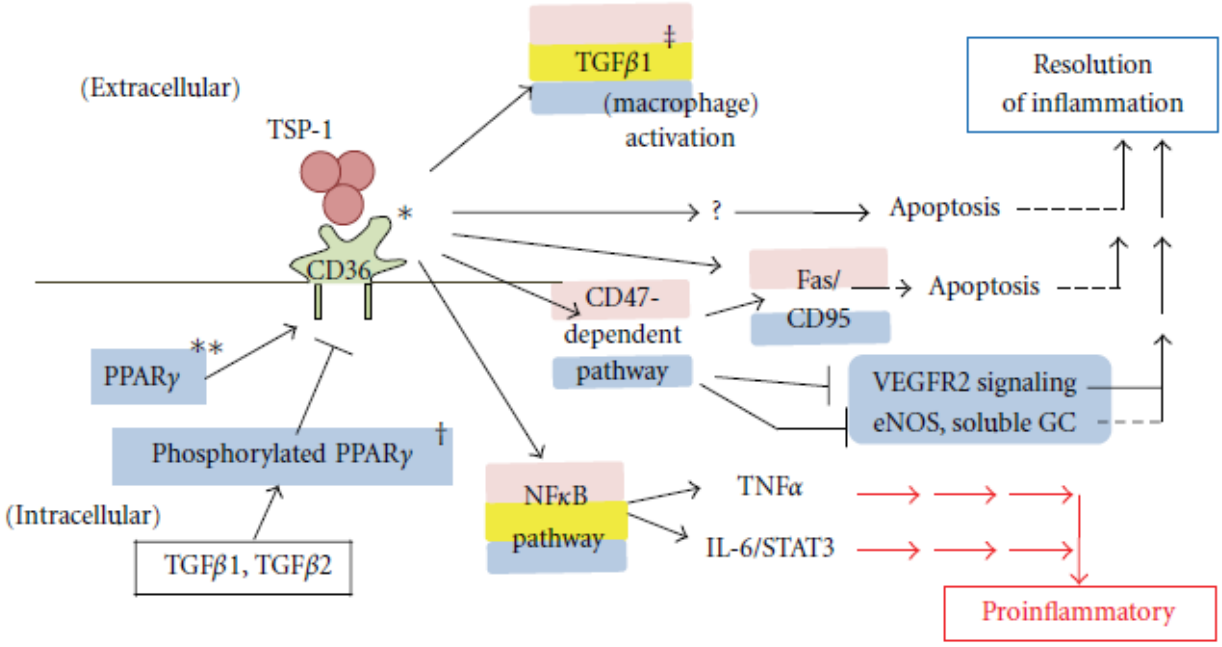


Figure 15 CD36-TSP-1 interaction in inflammation.[76]

Chronic Inflammation and Adaptive Immunity

Chronic inflammation is characterized by the infiltration of mononuclear cells, macrophages, lymphocytes, and plasma cells. Chronically inflamed tissues have fibroblast proliferation, angiogenesis, tissue destruction, and fibrosis.

Persistent chemokine and cytokine secretion trigger monocyte production and their transformation to macrophages. Monocytes and macrophages invade injured regions during the acute process and when the injury persists, infiltration by macrophages is enhanced. Also, lymphocyte infiltration, fibroblast proliferation, tissue destruction, and fibrosis are promoted. Lymphocytes are produced by bone marrow hemoblasts and promote immunocompetence and self-tolerance. T lymphocytes mature in thymus and are a major part in cell-mediated immunity. Plasma cells maturing from B lymphocytes produce antibodies providing humoral immunity. Dendritic cells and macrophages are called antigen-presenting cells (APCs) as antigen-specific receptors appear on their surface. Dendritic cells (DCs) internalize antigens and present antigenic determinants on their surface for T lymphocytes recognition in the lymph nodes.

High levels of TSP-1 increase DC tolerance to antigens, resulting in inflammatory response. TSP-1 regulates interleukin 10 (IL10) expression in DCs. On the other hand, treating DCs with IL-6, IL-10, or TGFβ1, developed immune tolerance and elevated levels of intracellular TSP-1 levels[78]. TSP-1 suppresses APC capacity to sensitize T-cells in host, established when corneal TSP-1 null allografts are rejected[79].

TSP-1 can also bind to CD47 and activate thymus-derived CD4+ CD25+ T regulatory cells (Tregs) in order to retain self-tolerance. Repressing CD47 activity results in non-maturing DCs. Deletion of CD47 or TSP-1 genes in DC protects newborn mice against bacterial meningitis [80]. In addition, TSP-1/CD36 binding can activate macrophages and phagocytosis. CD36 deficiency worsens symptoms of bacterial and malaria infection, while CD36 null mice presented proinflammatory response to infection, higher cytokine levels and mortality[76].

Antibodies secreted by B lymphocytes, bind to infectious agents and mark them for elimination. Inside a cell, a pathogen is protected from these antibodies therefore cytotoxic T cells have to destroy them by inducing host cell apoptosis. TSP-1 decrease immune responses as well as inhibit T-cell effectors actions, or induce T

cell apoptosis[76]. Also, TSP-1 binding to $\alpha 4\beta 1$ integrin T-cell adhesion improves chemotaxis. During the immune response, leukocytes produce reactive oxygen species (ROS), that are important molecules for pathogens elimination, but they can produce cell damage too. TGFβ1 pathway catalyzes ROS formation, and ROS can also activate TGFβ1 inducing apoptosis and fibrosis[76].(figure 16)

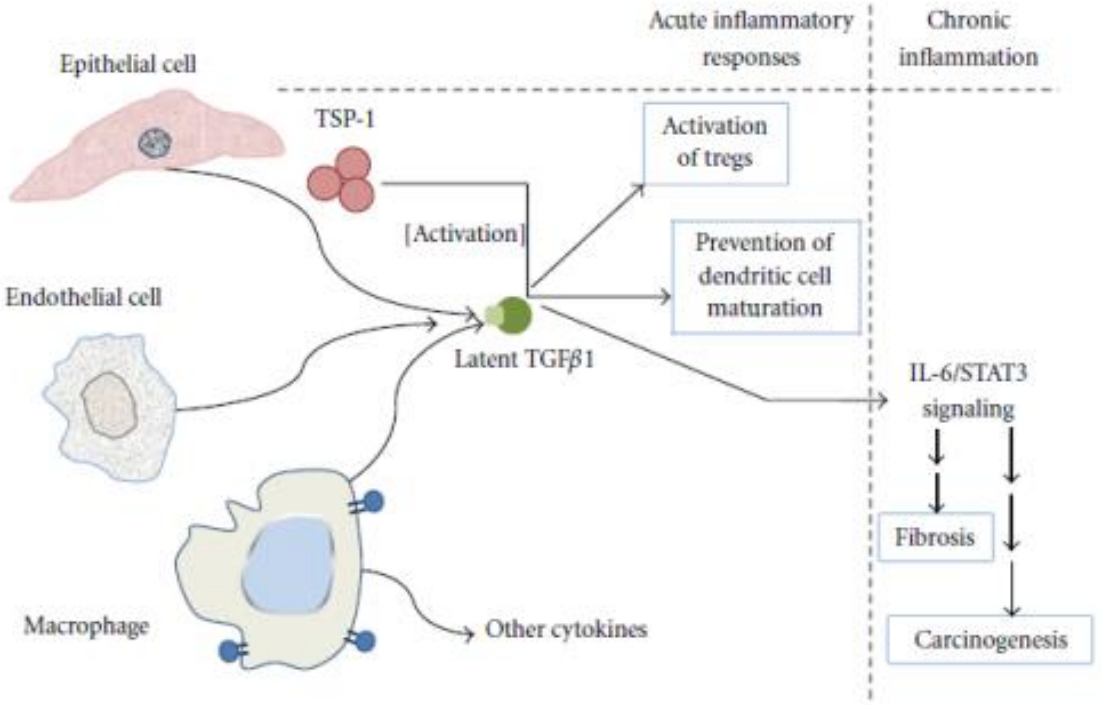


Figure 16 Activation of TGFb1 through TSP-1.[76]

Inflammatory Diseases and Animal Models to elucidate TSP-1 pathophysiology

TSP-1 -/- mice present extensive acute pneumonia, leukocytosis, pancreatitis, and inflammatory infiltrates in the lacrimal glands. This animal model shows that TSP-1 is involved in anti-inflammatory and proinflammatory responses owing to interactions with various receptors or to specific matricellular proteins in injured tissue as observed in animal models of disease[81]. TSP-1 might act by a biphasic or dose-dependent mechanism.

Work in a rat kidney disease model, found that TSP-1 binds to TGFβ1 and lead to fibrosis and renal damage, whereas a TSP-1 antagonist, LSKL inhibits the TSP-1/ TGFβ1 binding, and decreases renal interstitial fibrosis. TSP-1 is supposed to be an early marker of inflammation and fibrosis as it overexpressed in

glomerulopathies. TSP-1-deficient mice develop diabetic nephropathy followed by lower rates of glomerulosclerosis, glomerular matrix accumulation, podocyte injury, renal infiltration, and changes of renal functional parameters.

In addition, TSP-1 regulate TGFβ1 effects in cardiovascular diseases, atherosclerosis, obesity and moreover, monocytes and macrophages secrete TSP-1 in acute phase of the healing in myocardial infarction. Infarcted hearts exhibited upregulation of TGFβ1 in mice, and TSP-1 is expressed in the infarcted border consider that the binding inhibits expansion of inflammation. TSP-1 deficiency accelerates inflammation as it improves plaque formation paralleled by higher levels of circulated metalloproteinases and extensive necrosis in an atherosclerosis mouse model (ApoE−/−TSP-1−/−)[82].

The mRNA levels of TSP-1 are linked with obesity and insulin resistance in nondiabetic patients. TGFβ1 activation upregulating plasminogen activator inhibitor 1 (PAI-1) and higher PAI-1 levels are detected in insulin resistance and metabolic syndrome[83]. TSP-1−/− mice and integrin β6 subunit (Itgb6) −/− mice have an inflammatory phenotype not so severe as the one found in Tgfb1−/− mice[84].

TSP-1 knockout mice have acutely induced colitis, as well as increased bleeding and colonic inflammation. Furthermore, they display megacolon and peritonitis due to deeper infiltration of leukocytes into the muscularis and intestinal perforation. TSP-1 deficiency enhances angiogenesis and dysplasia. TSP-mimetic peptide ABT510 drastically reduces intestinal inflammation and angiogenesis and CD36 is upregulated, providing evidence that CD36 controls inflammation[85].

TSP-1 is overexpressed in monocytes and tissues in rheumatoid arthritis (RA) patients and high levels in plasma are associated to higher levels of proinflammatory cytokines[86]. A synthetic peptide from type 3 repeats, lowers inflammation in a model of erosive arthritis, as well as reducing angiogenesis, leukocyte infiltration, and thickening of the synovial lining of the joint. Also, in the spleen and liver, diminishes granuloma formation[76].

TSP-1 is also associated with proinflammatory effects in myositis. TSP-1 binding to CD47 exerts chemotactic agents in leukocytes leading to muscle inflammation in response to high levels of TNF-alpha[76].

TSP-1 in endothelium activation

TSP-1 is up-regulated at vascular injury areas and seems to control CAM expression on endothelium. More specifically, TSP-1 upregulates ICAM-1, VCAM-1, and E-selectin expression in a concentration-dependent manner, while expression levels of CD36 and integrin α5β1 are at basal levels. CAM is stimulated by IAP-specific peptide derived from TSP-1. Inhibition of TSP-1-IAP interaction (antibodies or siRNA) decreased CAM expression. TSP-1, through IAP, targets endothelium and stimulates CAM expression followed by monocytes attachment[76].

EC activation by TNF-α depends on TSP-1 expression and its binding to IAP. TNF-α stimulates the expression of TSP-1 and IAP on EC in a concentration-dependent manner. Knockdown of IAP or blockade of TSP-1-IAP interaction diminishes TNF-α-induced CAM expression[76]. The above evidence confirm that TNF-α stimulated through TSP/IAP complex results in higher CAM expression on ECs.

TSP-1 has an important role in inflammatory processes and in atherosclerosis development as described above. TSP-1 regulates expression of CAM and monocyte adhesion to endothelium suggesting that TSP-1 which is abundant in sites of vascular injury and platelet activation, may trigger the initial stages of inflammation and atherogenesis. As shown in another study, activated platelets trigger atherosclerosis in mice. CAM stimulation from TSP-1 does not affect the expression of other adhesion molecules such as CD-36 and β1 integrins[87,88].

The effects of TSP-1 on vascular cells are mediated by interactions with cell-surface receptors. CD36, binds TSP-1 and mediates signal transduction in monocytes confirmed by SVTCG peptide, derived from TSP-1, which binds to CD36 with high affinity[88].

The RFYVVMWK peptide, which is conserved in thrombospondin family, has been identified as an IAP agonist[88]. RFYVVMWK sequence seems to be buried and not available for the direct interaction with IAP, while, studies with IAP deficient mice, supported that RFYVVMWK peptide is essential for TSP-1-IAP binding, so conformational changes can occur in TSP-1 protein in order to interact Moreover, platelets from IAP-null mice, treated with this peptide, cannot aggregate on fibrinogen-coated surface. A study shows that RFYVVMWK peptide, triggers CAM

expression following by monocyte adhesion, confirm IAP involvement[88]. Transfection with siIAP, lead to complete ablation of the effect of TSP-1 and TNF-α on CAM production in ECs[88]. ICAM-1 follows the TSP-1 expression pattern. Also, TNF-α up-regulates TSP-1 expression in ECs in a dose-response manner, found to be regulated through TNFRII but not TNFRI. In addition, TSP-1 treatment of EC activates the NF-κB transcriptional factor, major regulator of CAM expression.

IAP overexpressed on the endothelium upon TNF-α treatment and evidences by blockade of TSP-1-IAP interaction or by knockdown of IAP lead to lower response to TNF-α, showing that TSP-1-IAP complex is essential for CAM expression[88]. Finally, recently found that the TSP-1-IAP interaction enhances vascular cells proliferation induced by insulin-like growth factor-1[89].

Materials and Methods

Patients and specimens

Patients:

APS (n=8)

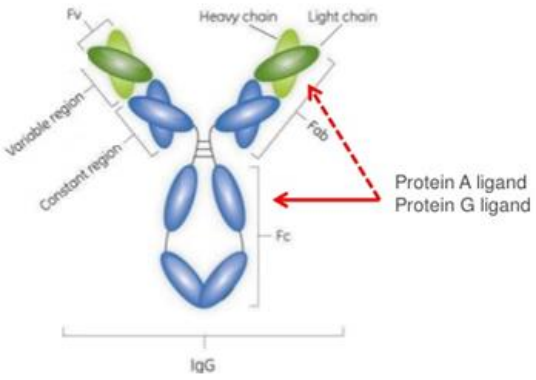
I. Plasma, collected in CTAD (Citrate, Theophylline, Adenosine and Dipyridamole) tubes.
II. Serum isolated from all participants and kept frozen at -80 ^{0}C.
III. Human umbilical vein endothelial cells (HUVECs) were isolated from 2 APS patients and 3 HDs after uncomplicated pregnancy and cultured in Endothelial Basal Medium-2.

Clinical events compatible with APS were extracted from patient medical records.

IgG isolation with Affinity Chromatography

Affinity chromatography is a liquid chromatography technique which separates biomolecules based on highly specific biological interactions. In order to isolate IgG immunoglobins from plasma or serum protein A or protein G used as the affinity ligand.

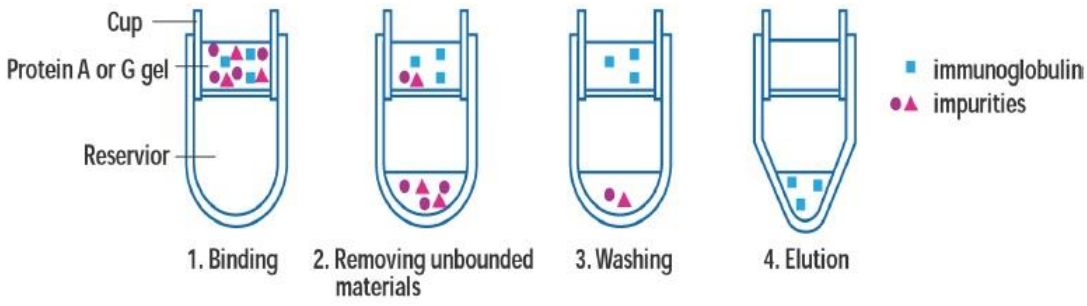
Total IgG was isolated from 8 patients with APS and 8 HDs. Serum from patients and control individuals was incubated with using Protein G Sepharose beads according to manufacturer' s instructions (GE Healthcare Life Sciences)



Total IgG was eluted according to the manufacturer's protocol using spin columns as follow:

1. Centrifuge column at 500g for 1 min to remove buffer
2. Pour buffer from falcon and repeat step 1

3. Add 5ml Glycine-HCl 0,1 M pH=2.5 and invert column several times until all beads are in the slurry
4. Centrifuge column at 500g for 1 min to remove buffer. Pour buffer from falcon and repeat step 1
5. Add Tris-HCl 0.1M pH=8.0 and invert column several times until all beads are in the slurry
6. Centrifuge column at 500g for 1 min to remove buffer. Pour buffer from falcon and repeat step 1
7. Add 5ml PBS to wash the column and invert column several times until all beads are in the slurry. Centrifuge column at 500g for 1 min to remove buffer. Pour buffer from falcon and repeat this step 3 times
8. Add 4 ml PBS and 1 ml patients' serum, screw the cap and place it on the rotator for 1h at room temperature.
9. Centrifuge column at 500g for 1 min to remove buffer
10. Keep the flow through at a falcon and repeat the step 9
11. Wash the column with PBS. Repeat 3 times the wash
12. Add Glycine- HCl Glycine-HCl 0,1 M pH=2.5 and invert column several times until all beads are in the slurry
13. Centrifuge column at 500g for 1 min to remove buffer.
14. Keep the flow through
15. Repeat the step 12-14
16. Centrifuge column at 500g for 1 min and keep the flow through
17. Put the flow through from steps 14-15-16 in a falcon and add 1 ml Tris-HCl.
18. Add Tris-HCl 0.1M pH=8.0 and invert column several times until all beads are in the slurry
19. Centrifuge column at 500g for 1 min to remove buffer. Pour buffer from falcon and repeat step 1
20. Add 5ml PBS to wash the column and invert column several times until all beads are in the slurry. Centrifuge column at 500g for 1 min to remove buffer. Pour buffer from falcon and repeat this step 3 times
21. Screw the bottom cap and add 5ml of 70% EtOH in the column, screw upper cap and store the column in 4 ^{o}C .



Sample concentration for proteins > 50 MWCO

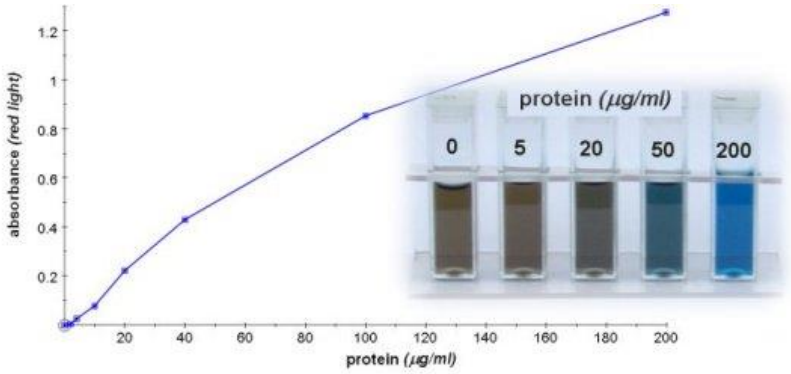
The Protein Concentrators are disposable ultrafiltration centrifugal devices that provide reliable and consistent results for concentrating, diafiltrating and buffer exchanging of biological samples such as proteins and nucleic acids.

1. Samples were added to 50K MWCO concentrators.
2. Centrifuge the samples at 3000g for 30 min.
3. Repeat the centrifugation until all the liquid has passed in the lower place.
4. Discard the flow through and add 15 ml of PBS and repeat the step 2, until the sample reaches the 500 λ.
5. Collect the sample which remained in the upper place of concentrator and measure the protein concentration with Bradford assay and check the purification of IgG with Coomasie staining.

Bradford assay

Bradford assay is a sensitive and accurate method for protein quantitation. The method based on the binding of Coomasie Brilliant Blue G-250 to Proteins that lead to the change of colour dye from red (465nm) to blue (596nm) under acidic conditions. To quantify the concentration of protein samples, first, must measure the protein absorbance of already known protein concentration samples.

1. In a 96 well plate put 295 λ Bradford reagent
2. Add 5 λ protein sample
3. Pipette up and down the sample
4. Measure the change of color with spectrophotometer at 595 nm



Coomasie Blue staining

1. Fix gel in Fixing solution (50% methanol and 10% glacial acetic acid) for 1 hr to overnight with gentle agitation. Change solution once at first 1 hr.
2. Stain gel in Staining solution (0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid) for 20 min with gentle agitation.
3. Destain gel in Destaining solution (40% methanol and 10% glacial acetic acid). Replenish the solution several times until background of the gel is fully destained.
4. Store the destained gel in Storage solution (5% glacial acetic acid).

Anti-β2GPI IgG isolation with Affinity Chromatography

Based on B2GPI-resin beads binding of anti-B2GPI IgG antibodies.

1. Centrifuge column at 500g for 1 min to remove buffer
2. Pour buffer from falcon and repeat step 1
3. Add 5ml Glycine-HCl 0,1 M pH=2.5 and invert column several times until all beads are in the slurry
4. Centrifuge column at 500g for 1 min to remove buffer. Pour buffer from falcon and repeat step 1
5. Add Tris-HCl 0.1M pH=8.0 and invert column several times until all beads are in the slurry
6. Centrifuge column at 500g for 1 min to remove buffer. Pour buffer from falcon and repeat step 1

7. Add 5ml PBS to wash the column and invert column several times until all beads are in the slurry. Centrifuge column at 500g for 1 min to remove buffer. Pour buffer from falcon and repeat this step 3 times
8. Add 4 ml PBS and 1 ml Total IgG preparations from the APS patients were incubated with the β2GPI conjugated beads overnight at 4^{O}C on the rotator (or for 1h at room temperature).
9. Centrifuge column at 500g for 1 min to remove buffer
10. Keep the flow through at a falcon and repeat the step 9
11. Wash the column with PBS. Repeat 3 times the wash
12. Add Glycine- HCl Glycine-HCl 0,1 M pH=2.5 and invert column several times until all beads are in the slurry
13. Centrifuge column at 500g for 1 min to remove buffer.
14. Keep the flow through
15. Repeat the step 12-14
16. Centrifuge column at 500g for 1 min and keep the flow-through
17. Put the flow through from steps 14-15-16 in a falcon and add 1 ml Tris-HCl.
18. Add Tris-HCl 0.1M pH=8.0 and invert column several times until all beads are in the slurry
19. Centrifuge column at 500g for 1 min to remove buffer. Pour buffer from falcon and repeat step 1
20. Add 5ml PBS to wash the column and invert column several times until all beads are in the slurry. Centrifuge column at 500g for 1 min to remove buffer. Pour buffer from falcon and repeat this step 3 times
21. Screw the bottom cap and add 5ml of PBS in the column, screw upper cap and store the column in 4 ^{o}C.

Optimization step:

Instead of pool IgGs, platelet extraction used in order to check the binding affinity of B2GPI column. From previous studies it is known that B2GPI binds to PF4 which secreted by activated platelets. The binding was checked by western blot.

Protein precipitation (Sample concentration for proteins < 50 MWCO)

1. In one volume of protein sample add 4 volumes of ice-cold acetone

2. Vortex and place at -80ºC for 1h
3. Centrifuge at 3500g at 4 ºC for 3 h
4. Carefully discard supernatant
5. Remove the pellet with acetone 100 % and place it in a 1,5 ml Eppendorf
6. Centrifuge at 20000 g for 15 min
7. Discard the supernatant and let the pellet dry for 30 min at room temperature
8. Resuspend the pellet in SDS-sample buffer 1x and place the samples in heat block for 10 min at 95ºC.

HUVECs culture cells

I. Culturing HUVEC

A. PREPARING CELL CULTURE FLASKS FOR CULTURING HUVEC

1. Pipette 3 ml of 2% sterile gelatin to a T-75 flask or culturing plates and place them in 37ºC for 1 h or 4ºC overnight.
2. Remove gelatin from the flasks and let them fry in sterilized hood for 20 min.
3. Take the Endothelial Cell Growth Medium from the refrigerator
4. Pipette 20 ml of Endothelial Cell Growth Medium* to a T-75 flask.

B. THAWING AND PLATING HUVEC

1. Use cryopreserved vial of HUVEC and remove the vial cap carefully. Resuspend the cells in the vial by gently pipetting the cells 5 times with a 2 ml pipette.
2. Pipette the cell suspension (~1ml) from the vial into the T-75 flask containing 20 ml of Endothelial Cell Growth Medium.
3. Place the T-75 flask in a 37oC, 5% CO_2 humidified incubator. Loosen the cap to allow gas exchange.
4. Change to fresh Endothelial Cell Growth Medium after 24 hours or overnight to remove all traces of DMSO.
5. Change Endothelial Cell Growth Medium every other day until the cells reach 60% confluent.
6. Double the Endothelial Cell Growth Medium volume when the culture is >60% confluent or for weekend feedings.

III. Subculturing HUVEC

Subculture the cells when the HUVEC reach 80% confluent

1. Remove the medium from culture flasks by aspiration.
2. Wash the monolayer of cells with PBS and remove the solution by aspiration.
3. Pipette 5 ml of Trypsin/EDTA Solution into the T-75 flask. Rock the flask gently to ensure the solution covers all the cells. Let the flask in 37oc in order to activate the thrypsin solution.
4. Release the rounded cells from the culture surface by hitting the side of the flask against your palm until most of the cells are detached.
5. Pipette 5 ml of Medium to the flask to inhibit further tryptic activity.
6. Transfer the cell suspension from the flask to a 15 ml sterile conical tube.
7. Centrifuge the conical tube at 300xg for 10 minutes to pellet the cells.
8. Aspirate the supernatant from the tube without disturbing the cell pellet.
9. Resuspend the cells in 2 ml of Endothelial Cell Growth Medium by gently pipetting the cells to break up the clumps.
10. Count the cells with a hemocytometer or cell counter.
1. Inoculate at 10,000 cells per cm2 for rapid growth, or at 5,000 cells per cm2 for regular subculturing.

HUVEC stimulation

HUVECs were stimulated in 2 separate settings:

a) by human plasma, using HD plasma as control and

b) by anti- β2GPI, either with or without β2GPI

c) by TSP-1 native protein

Two separate protocols simulating the APS syndrome were used for the activation of HUVECs.

➔ 1st protocol

1. The first protocol involved the stimulation of HUVECs with human plasma. HUVECs isolated from 2 HDs were cultured to 70% confluence in 75 cm2 flasks. Subsequently cells were trypsinized, seeded in 24-well plates and cultured in EBM-2 complete medium containing FGF-b, VEGF, IGF-1, EGF and 2.5% FBS.
2. Upon confluence, the medium was removed and replaced with starvation medium containing EBM-2 and 1% FBS for 24h. EBM-2 containing 20%

recalcified plasma from either APS patients or HDs was added to the wells for 20 h.

3. After this period the supernatants were removed, cells were washed twice with PBS in order to remove any residual plasma and fresh medium without FBS was added to the wells for 2 h.
4. Supernatants were collected and stored until further analysis.

➔ 2nd protocol

1. The second protocol concerned the stimulation of HUVECs with purified IgG from APS patients and EBM without serum and growth factors. The cells were cultured for 4h and 6h.
2. Supernatants were collected and stored until further analysis.

➔ 3rd protocol

1. Cells were cultured as previous described and treated with TSP-1 native and recombinant proteins.
2. Cells were cultured in starvation conditions (without growth factors and serum)
3. Titration experiments with different concentrations of TSP-1 (0,5 μg/ml, 1 μg/ml, 2 μg/ml, 4 μg/ml, 8 μg/ml) protein in starvation medium and in different time points (4h, 6h, 12 h).

Human TSP-1 immunoassay (ELIZA)

In order to quantitate thrombospondin 1 protein in supernatant from HUVECs treated with plasma of APS patients compared to supernatant from HUVECs treated Healthy Donors, ELIZA kit (RnD systems) used.

1. Prepare all reagents, working standards, and samples.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack and reseal.
3. Add 100 μL of Assay Diluent RD1-56 to each well.
4. Add 50 μL of standard, control, or sample per well. Cover with the adhesive strip.
5. Incubate for 2 hours at room temperature on a microplate shaker.
6. Aspirate each well and wash with Wash Buffer (400 μL). 3X repeats.

7. Add 200 μL of Human Thrombospondin-1 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
8. Repeat wash as in step 5.
9. Add 200 μL of Substrate Solution to each well. Incubate for 30 minutes at room temperature on the benchtop. Protect from light.
10. Add 50 μL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

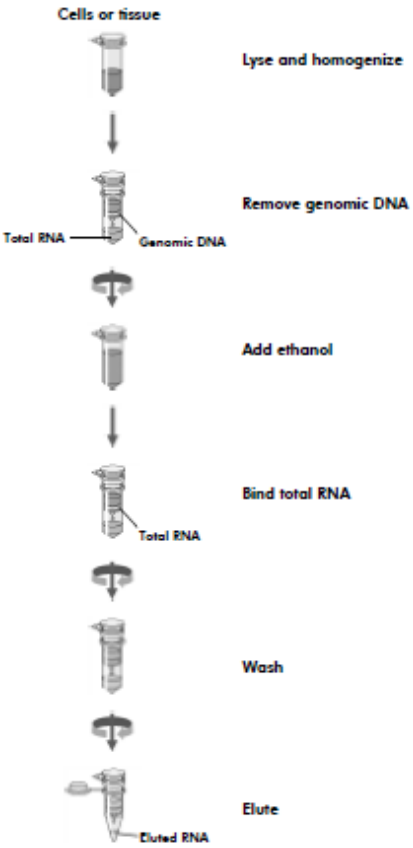
Immunofluorescence after HUVECs stimulation

1. Discard the supernatant from the well (or store them for eliza measurements).
2. Wash every well with 500 μλ PBS 1x.
3. Add PFA 4% 500 μλ for 10 min at RT
4. 2 washes με PBS 1x
5. Add 500 μλ PBS 1x Triton x 0.1% for 10 min at RT.
6. Add 500 μλ PBS 1x goat serum 10% 1h at RT (blocking buffer)
7. Discard the blocking buffer and add 250 μλ primary antibodies (1:100 dilution σε PBS 1x goat serum 10%) overnight at 4^{o}C.
8. 3 washes με PBS 1x
9. Add 250 μλ secondary antibodies (1:250 dilution σε PBS 1x goat serum 10%) for 1h at RT.
10. 3 washes με PBS 1x
11. Counterstain with DAPI for 5 min at RT (1:1000 dilution in water)
12. 3 washes with water
13. Mounting with mounting medium (1-2 drops in the ibidi plate after discard the plastic wells)
14. Cover the plate with cover slips.

15. Let the plate in 4 oC until further use (fluorescent microscope)

RNA extraction

In order to extract RNAs from culture cells two different RNA isolation kits were used depending on cells concentration. Protocols from quiagen RNA plus mini kit and quiagen RNA micro kit.



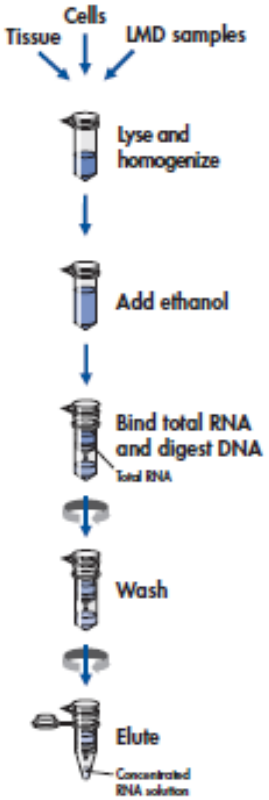
➔ RNA mini kit

1. Cells grown in a monolayer in cell-culture vessels can be either lysed directly in the vessel (up to 10 cm diameter) or trypsinized and collected as a cell pellet prior to lysis. Cells grown in a monolayer in cell-culture flasks should always be trypsinized.
2. Disrupt the cells by adding 350 λ Buffer RLT Plus. Vortex or pipet to mix, and proceed to step 3.
3. Pass the lysate at least 5 times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. Proceed to step 4.
4. Transfer the homogenized lysate to a gDNA Eliminator spin column placed in a 2 ml collection tube (supplied). Centrifuge for 30 s at ≥8000 x g (≥10,000 rpm). Discard the column and save the flow-through.
5. Add 1 volume (usually 350 µl or 600 µl) of 70% ethanol to the flow-through, and mix well by pipetting. Do not centrifuge.
6. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.*
7. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.*
8. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.

9. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane.
10. Optional: Place the RNeasy spin column in a new 2 ml collection tube and discard the old collection tube with the flow-through. Centrifuge at full speed for 1 min.
11. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.

➔ RNA micro kit

1. Cells grown in a monolayer (do not use more than 1 x 10^6 cells): Cells grown in a monolayer in cell-culture vessels can be either lysed directly in the vessel (up to 10 cm diameter) or trypsinized and collected as a cell pellet prior to lysis.



2. Disrupt the cells by adding Buffer RLT.
3. Pass the lysate at least 5 times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. Proceed to step 4.
4. Add 1 volume of 70% ethanol to the lysate and mix well by pipetting. Do not centrifuge. Proceed immediately to step 5.
5. Transfer the sample, including any precipitate that may have formed, to a RNeasy MinElute spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flowthrough.
6. Add 350 µl Buffer RW1 to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.
7. Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube.
8. Add the DNase I incubation mix (80 µl) directly to the RNeasy MinElute spin column membrane, and place on the benchtop (20–30°C) for 15 min.
9. Add 350 µl Buffer RW1 to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through and collection tube.

10. Place the RNeasy MinElute spin column in a new 2 ml collection tube. Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.
11. Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through and collection tube.
12. Place the RNeasy MinElute spin column in a new 2 ml collection tube. Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the flow-through and collection tube.
13. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube. Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

After RNA extraction measurements of RNA samples are conducted in nanodrop.

Reverse Transcription – PCR (c-DNA generation)

In order to make c-DNAs we use TAKARA primescript mix.

1. Prepare the following reaction mixture on ice.

<Per reaction>

Reagent	Amount	Final conc.
5X PrimeScript Buffer (for Real Time)	2 µl	1X
PrimeScript RT Enzyme Mix I	0.5 µl	
Oligo dT Primer (50 µM)*1	0.5 µl	25 pmol
Random 6 mers (100 µM)*1	0.5 µl	50 pmol
total RNA		
RNase Free dH_2O		
Total	10 µl*2	

2. Place 3.5 µλ from master mix on every pcr tube
3. Add 6.5 µλ from every RNA
4. Place the reaction mixture in a cycler incubate them in the following program:

37℃	15 min*3	(Reverse transcription)
85℃	5 sec	(Inactivation of reverse transcriptase with heat treatment)
4℃		

5. Store the tubes at 4°C.

Quantitative -PCR (q-PCR)

1. Prepare a master mix containing the appropriate volume of all reaction components common to all or a subset of reactions to be performed.

2. Calculate the required volume of each component based on the following table:

Component	Volume	Final conc.
PCR-grade water	Up to 20 µL	N/A
KAPA SYBR FAST qPCR Master Mix (2X)[2]	10 µL	1X
10 µM forward primer	0.4 µL	200 nM
10 µM reverse primer	0.4 µL	200 nM
Template DNA[3]	As required	<20 ng

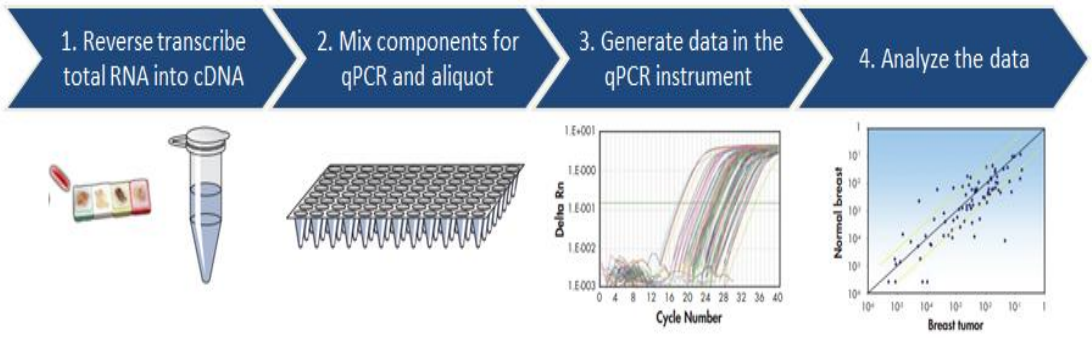
Transfer the appropriate volumes of qPCR master mix, template, and primers to each well of a PCR plate/tube(s). Cap or seal the reaction plate/tube(s) and centrifuge briefly.

3. Place the plate/tubes in the pcr machine and the qPCR protocol to be used conforms to the following parameters:

Step	Temp.	Duration	Cycles
Enzyme activation	95°C	3 min[1]	Hold
Denaturation	95°C	1 – 3 sec	40
Annealing/ extension/ data acquisition[2]	60°C	≥20 sec[3]	
Dissociation	According to instrument guidelines		

4. Data Analysis:

For Data analysis the 2 $^{-\Delta ct}$ mathematical relative expression model used. (Livak and Schmittgen, 2001). Relative expression gene is b2m and the control condition of each experiment.



Placenta sections in cryotome

1.Specimens from freshly taken tissue (and aldehyde fixed or not) are placed on a piece of aluminium foil covered by a drop of embedding medium

2. frozen tissue blocks are transferred to the cryo- chamber for sectioning or are stored in firmly closed vials at -70oC until use.

3. Tissue that stored before cutting sections should equilibrate to the temperature of the cryostat.

4. Sections of 4-8 μm thickness are cut with the cryo-microtome (C- profile steel knife) at -25^{o}C to -30^{o}C) using an anti-roll plate

5. Collected on glass slides, preferably coated for improved adherence of tissue sections.

6. Slides with tissue sections are immediately used or stored frozen until needed at -70^{o}C in a sealed slide box.

Hematoxylin- Eosin staining of placenta sections

1. Stain the samples in hematoxylin solution for 3 min.
2. Place the slides under running tap water at room temperature for at least 5 min.
3. Stain the samples in working eosin Y solution for 2 min.
4. Place the slides under running tap water at room temperature for at least 5 min
5. Dehydrate the samples as follows.
   i. Dip the slides in 95% ethanol about 20 times.
   ii. Transfer to 95% ethanol for 2 min.
   iii. Transfer through two changes of 100% ethanol for 2 min per change.
6. Clear the samples in three changes of xylene for 2 min per change.
7. Place a drop of mounting over the tissue on each slide and add a coverslip.
5. View the slides using a microscope.

Immunofluorescence of placenta sections

1. Sections are warmed at room temperature and air-drye for 30 min
2. Frozen sections from unfixed tissue blocks are fixed on the slide by organic solvents.
3. Incubate the slides in Precooled 80% ethanol for 5 min at -20^{o}C

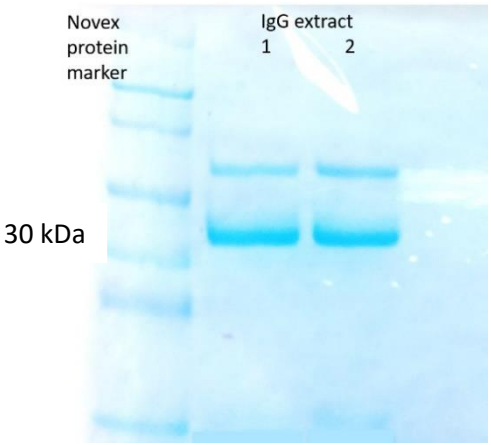
4. The sections were placed in Precooled acetone (-20^{o}C) and methanol (1:1) for 10 min in – 20^{o}C.
5. Air dry 30 min
6. Carefully trace around the tissue with a PAP pen.
7. 3 washes pbs 1x
8. blocking with pbs 10% goat serum for 1h at rt
9. primary antibody (1: 100 dilution) overnight at 4 ^{o}C
10. 3 washes με PBS 1x
11. Add 250 μλ secondary antibodies (1:250 dilution σε PBS 1x goat serum 10%) for 1h at RT.
12. 3 washes με PBS 1x
13. Counterstain with DAPI for 5 min at RT (1:1000 dilution in water)
14. 3 washes with water
15. Mounting with mounting medium (1-2 drops)
16. Cover the plate with cover slips.
17. Let the plate in 4 oC until further use (fluorescent microscope)

Results

i. IgG Isolation

Serum and plasma from APS patients were pooled and passed through a sepharose bead column that isolates IgGs. Patient sera had already been checked regarding the anti-b2GP1 IgG concentration, while clinical events were extracted from patient medical records.

The pooled plasma and serum were passed through the affinity chromatography column at 1 ml fractions and pooled isolated IgGs were concentrated in concentrators. Then, the protein concentration of the pooled IgGs was quantified by Bradford assay and checked for purity by gel electrophoresis and staining with Coomassie blue (figure 17 extract 1).



A small portion of concentrated IgG was eluted from the anti-b2GP1 sepharose column and concentrated. Again, the protein concentration measured and checked for purify (figure 17, extract 2)

Figure 17 Commasie blue staining, IgG extract 1 is extract from IgG sepharose column and extract 2 is the extract from anti-b2GP1 sepharose column.

ii. Treatment with IgG and b2gp1 stimulated Endothelial cell Activation

HUVECs derived from Healthy Donors were incubated (HD HUVECs) in culture with a mix of pooled IgGs and native b2GP1 for 6 hours in order to stimulate them. Then, markers for endothelial cell activation and inflammation were investigated by immunofluorescent assays in order to detect the differences between treated and untreated cells.

In order to test for cell activation, we measured the expression of cell adhesion molecules, which are major endothelial cell activation markers using antibodies.

i. I-CAM-1 (Intracellular Adhesion Molecule 1) was tested using a monoclonal antibody. As it is shown in Figure 18 in treated HUVECs the molecule is overexpressed in the cell membranes.

➢ICAM

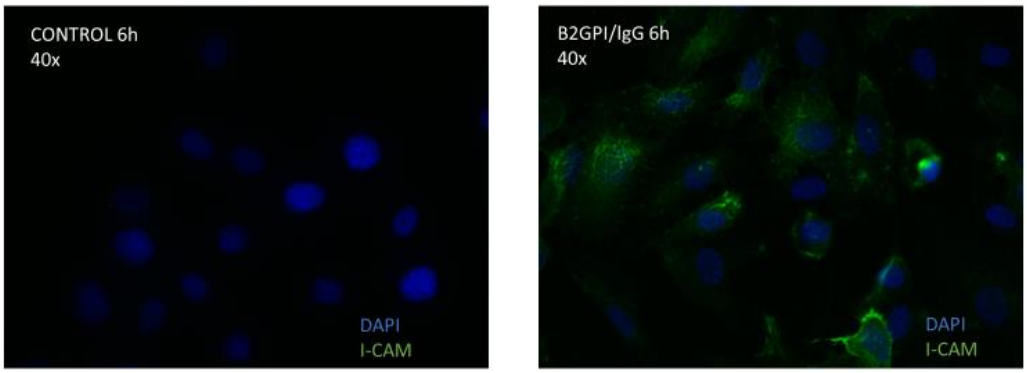


Figure 18 left: HUVECs in stavation, right : HUVECs after stimulation with antiB2GP1 and B2GP1.In these figures ICAM1 expression depicted in the different cases showng icreased levels of ICAM1 protein in treated cells

ii. V-CAM 1 molecule was found to be upregulated in treated HUVECs although its expression levels in the membrane are not so increased compared to I-CAMs. (figure 19)

➢V-CAM 1

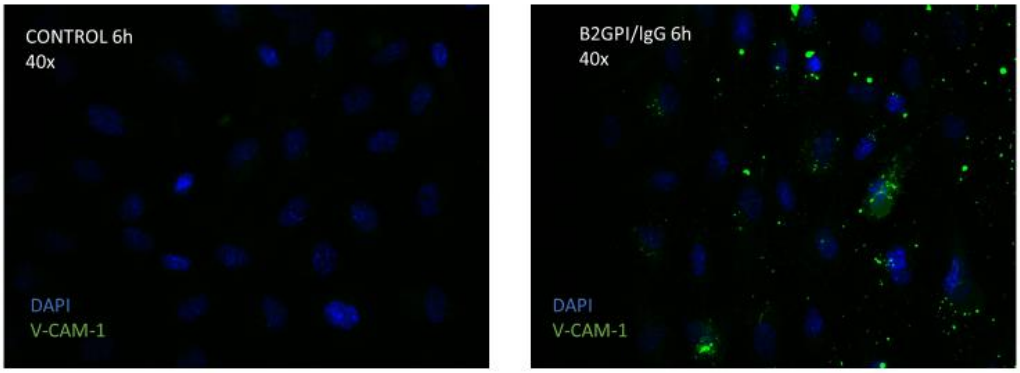


Figure 19 left: HUVECs in stavation, right : HUVECs after stimulation with antiB2GP1 and B2GP1.In these figures VCAM-1 expression depicted in the different cases showng slight icreased levels of VCAM1 protein in treated cells

iii. E- selectin is another adhesion molecule that was found overexpressed in treated cells compared to controls. (figure 20)

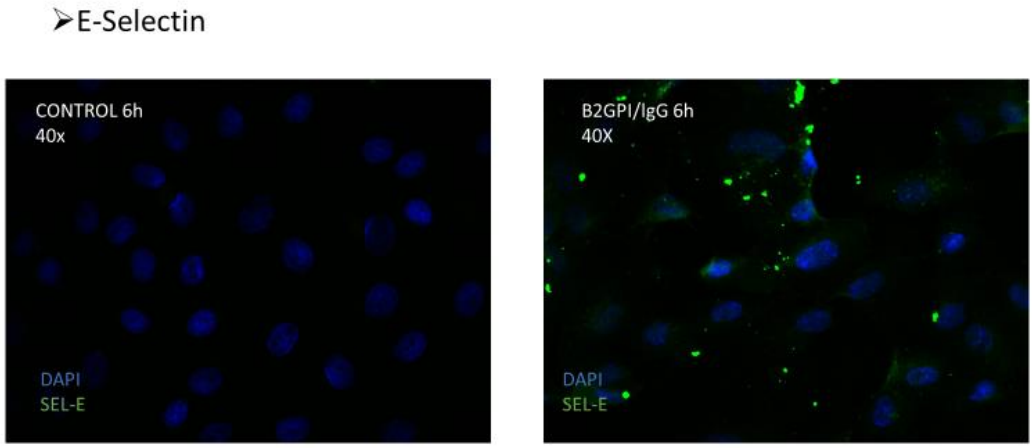


***Figure 20** left: HUVECs in stavation, right : HUVECs after stimulation with antiB2GP1 and B2GP1. E-Selectin expression depicted in the different cases found higher protein levels in treated cells, in the cell membrane areas.*

iv. The P-selectin protein was also expressed at relatively higher levels in treated cells, but the difference in expression levels, as quantified visually, were not significant. (figure 21)

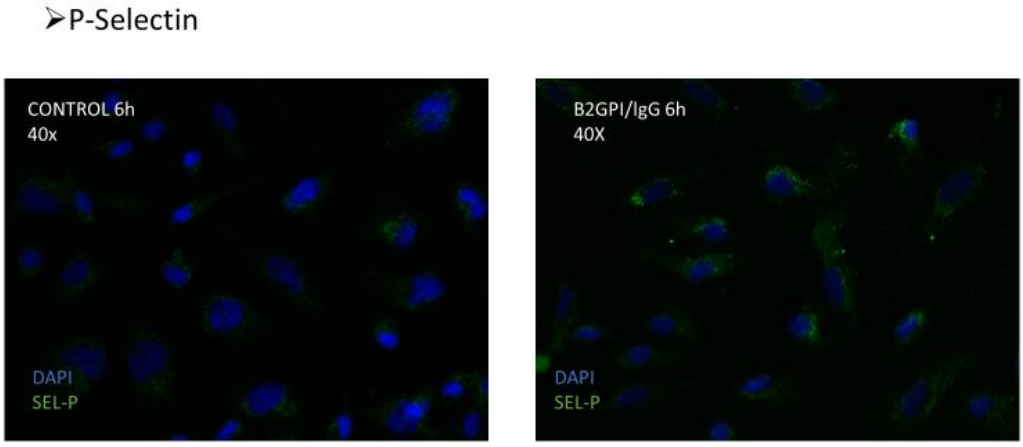


***Figure 21** left: HUVECs in stavation, right : HUVECs after stimulation with antiB2GP1 and B2GP1.-P-Selectin expressed either in treated or untreated, where depicted invreased.*

v. Tissue factor expression in untreated and treated HUVECs is showed in figure 21. The expression of tissue factor was higher in treated cells and its expression was located in cell membranes, as expected. (figure 22)

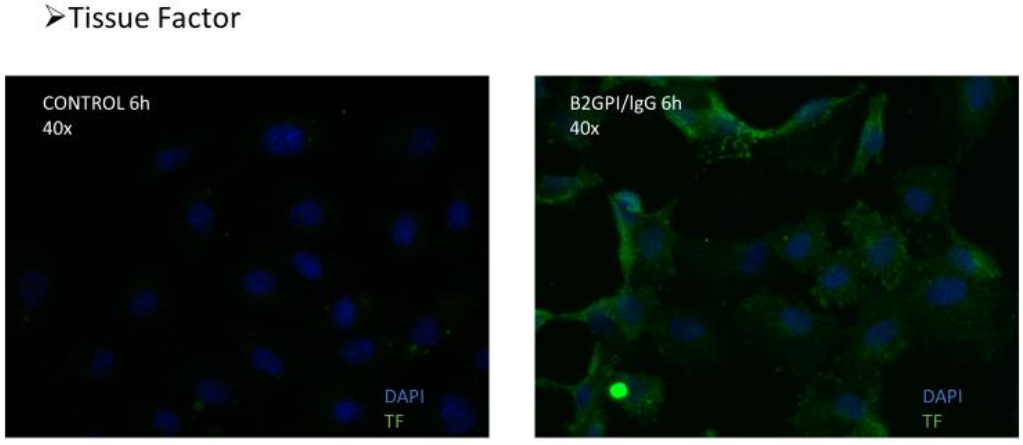


Figure 22 left: HUVECs in stavation, right : HUVECs after stimulation with antiB2GP1 and B2GP1. TF expression illustrated aberant to tretaed HUVECS compared to untreated.

vi. TM protein is expressed in low levels in the cell's cytoplasm in controls conditions as depicted in the figure, but it was found slightly translocated in the extracellular space in treated cells. (figure 23)

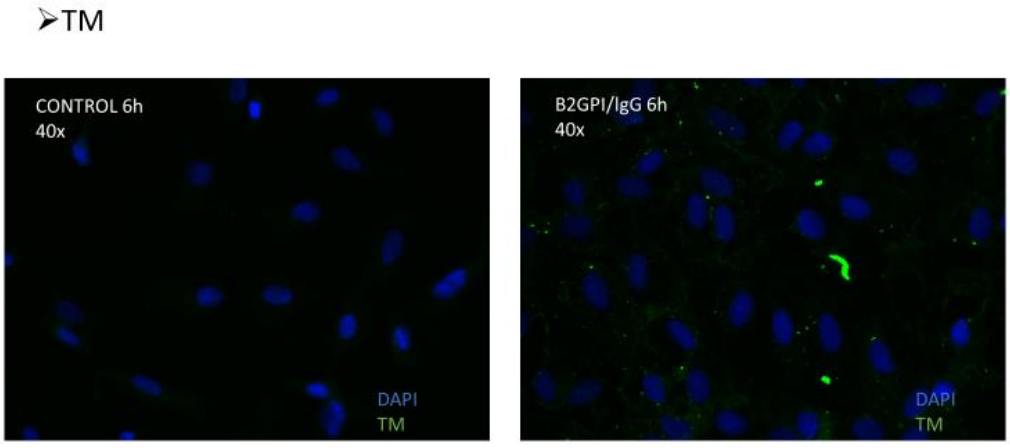


Figure 23 left: HUVECs in stavation, right : HUVECs after stimulation with antiB2GP1 and B2GP1. TM protein found in minorquantities in untreated. Althought, TM protein detected in extracellular areas in treated HUVECs.

vii. In the same experiment, I found significantly higher amounts of the cytokine interleukin-6 in the treated cells compared to control as depicted in figure 24.

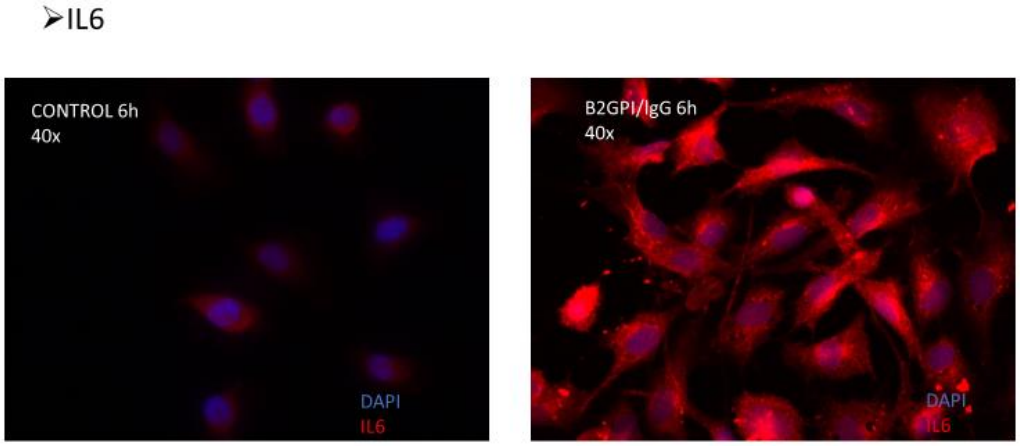


Figure 24 left: HUVECs in stavation, right : HUVECs after stimulation with antiB2GP1 and B2GP1. IL-6 observed in cytoplasmic areas in both conditions, expressed in low levels in control , and in very high levels in treated cells.

viii. Regarding another cytokine, interleukin 8, a minute expression of the protein was observed in controls, localized around the nucleus of each cell. In contrast in treated cells IL-8 was expressed in much higher levels as assessed visually. (figure 25)

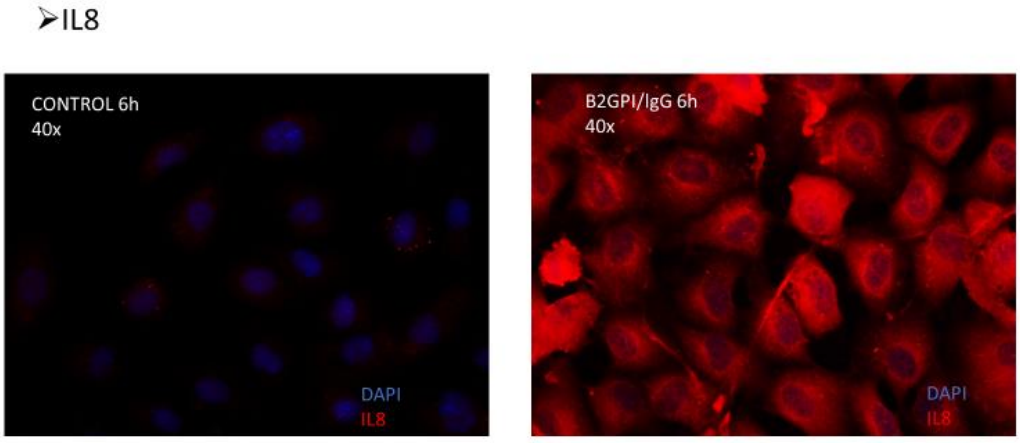


Figure 25 left: HUVECs in stavation, right : HUVECs after stimulation with antiB2GP1 and B2GP1. IL-8 found only in treated cells and not in untreated.

ix. The same trend au par with IL-6 and IL-8 was found when we tested NFk-B 1 protein as depicted. NFkB 1 is a subunit of the Nuclear factor NF-kappa-B and in humans is encoded by the *NFKB1* gene. This gene encodes a 105 kD protein apart which is a protein-specific transcription inhibitor and a DNA binding subunit of the NF-kappaB (NF-κB) protein complex. (figure 26)

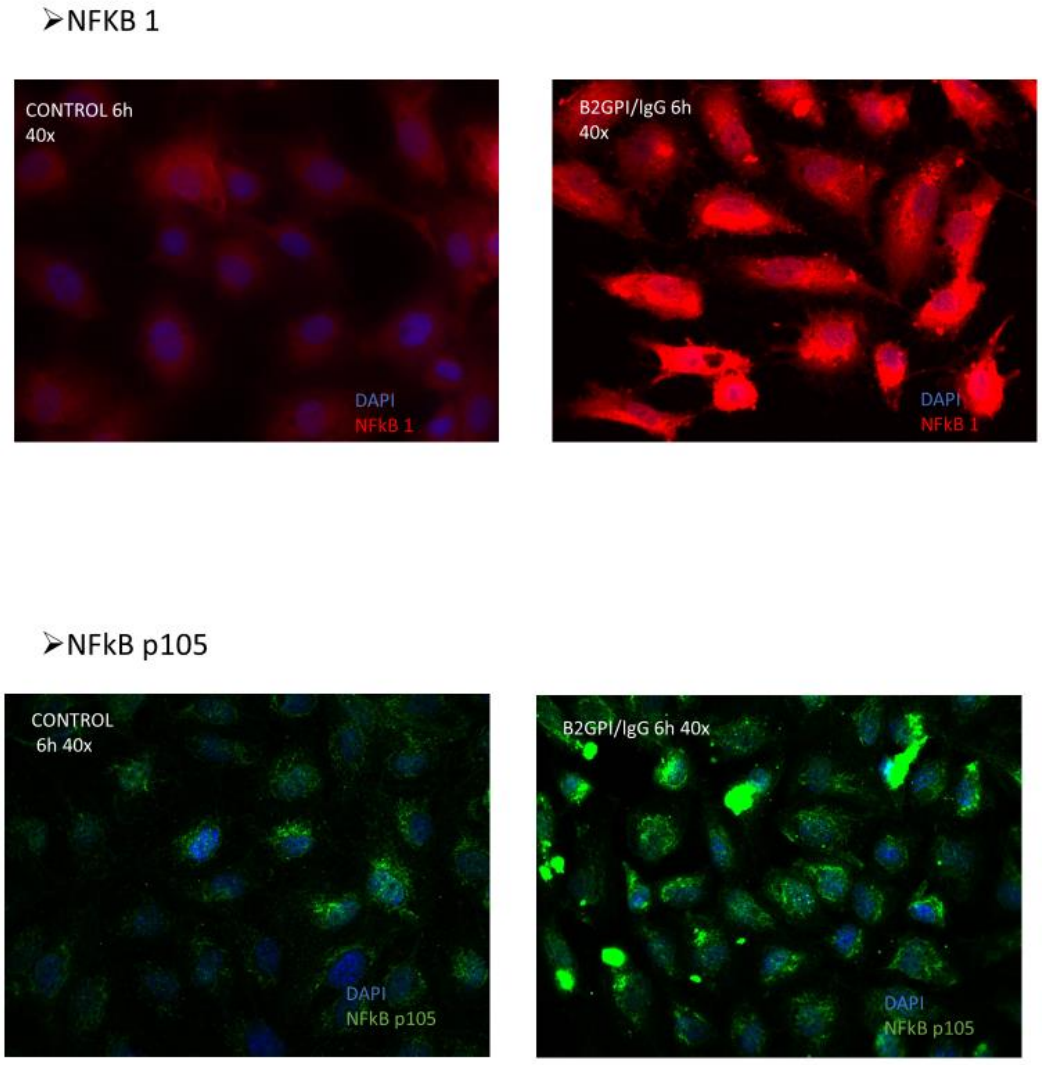


Figure 26 left: HUVECs in stavation, right : HUVECs after stimulation with antiB2GP1 and B2GP1. In these figures NFkB expression depicted in the different cases showng icreased levels of protein in treated cells compare to untreated. The difference in upper pictures detected have to do with different antibody from the lower pictures. (up:cusabio down:santacruz)

x. TGFβ-2 expression was similar between the two conditions, even though the expression in treated cells was more distinctive compared to the untreated cells. (Figure 27)

➢TGFβ-2

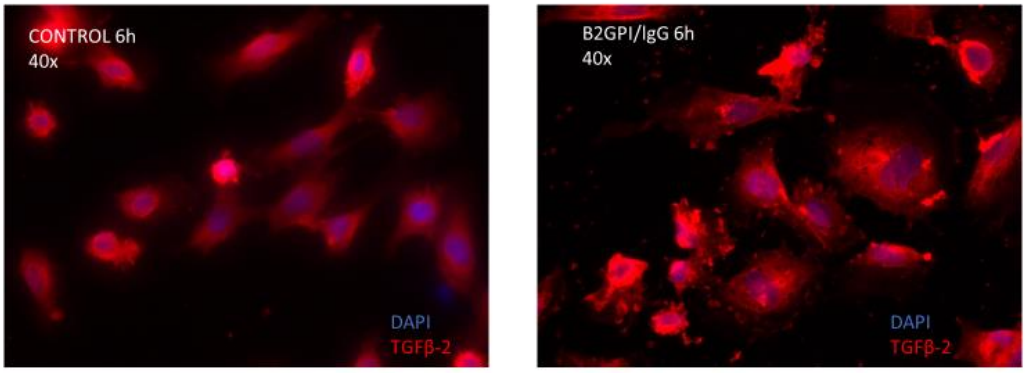


Figure 27 left: HUVECs in stavation, right : HUVECs after stimulation with antiB2GP1 and B2GP1.In these figures TGFβ-2 expression depicted in the different cases showing same levels of TGFβ-2 protein in treated cells.

xi. TGFR-1 protein was expressed in low levels in the cultured endothelial cells under control conditions as can be seen in the figure. Its protein expression was not upregulated by the anti-B2GPI/ B2GPI complex (figure 28).

➢TGFR-1

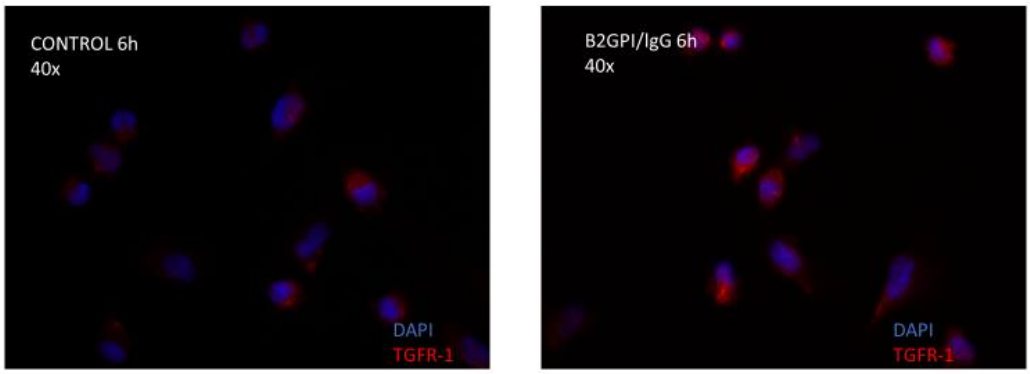


Figure 28 left: HUVECs in stavation, right : HUVECs after stimulation with antiB2GP1 and B2GP1.In these figures TGFR-1 expression depicted in the different conditions found to be unaffected by treatment and expressed in aproximately same levels.

xii. The TSP-1 protein is expressed in endothelial cells either in untreated or in cells treated with β2GP1/IgG complexes, but in treated cells the expression and secretion of protein is slightly higher. This phenomenon is showed in figure 29.

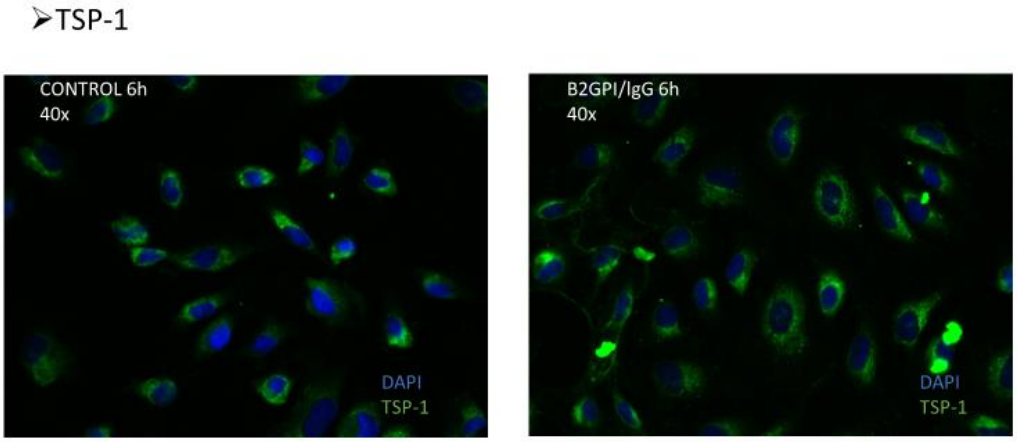


Figure 29 left: HUVECs in stavation, right : HUVECs after stimulation with antiB2GP1 and B2GP1.In these figures TSP-1 expression depicted to be expressed in both conditions, but in higher levels in treated cells.

Summarizing the above presented results, ICAM-1, E-selectin, Tissue Factor, Il-6, Il-8 and NFkB p 105 protein levels were substantially increased in treated HUVECs. This increase was visually assessed, and representative figures are being shown throughout. Indeed, used statistical analysis tools, as depicted in Table 1, those differences found to be statistically significant (*). There was also a small to moderate increase in proteins level in VCAM-1, P-Selectin, TGFb2, TSP-1 even though these observations, due to the smaller effect size, need to be confirmed. Furthermore, a decrease in protein levels was showed for TM while there was no observable difference between treated and control cells in TGFR-1 protein levels. These results confirmed as measured in Image J. (figure 30, Table 1)

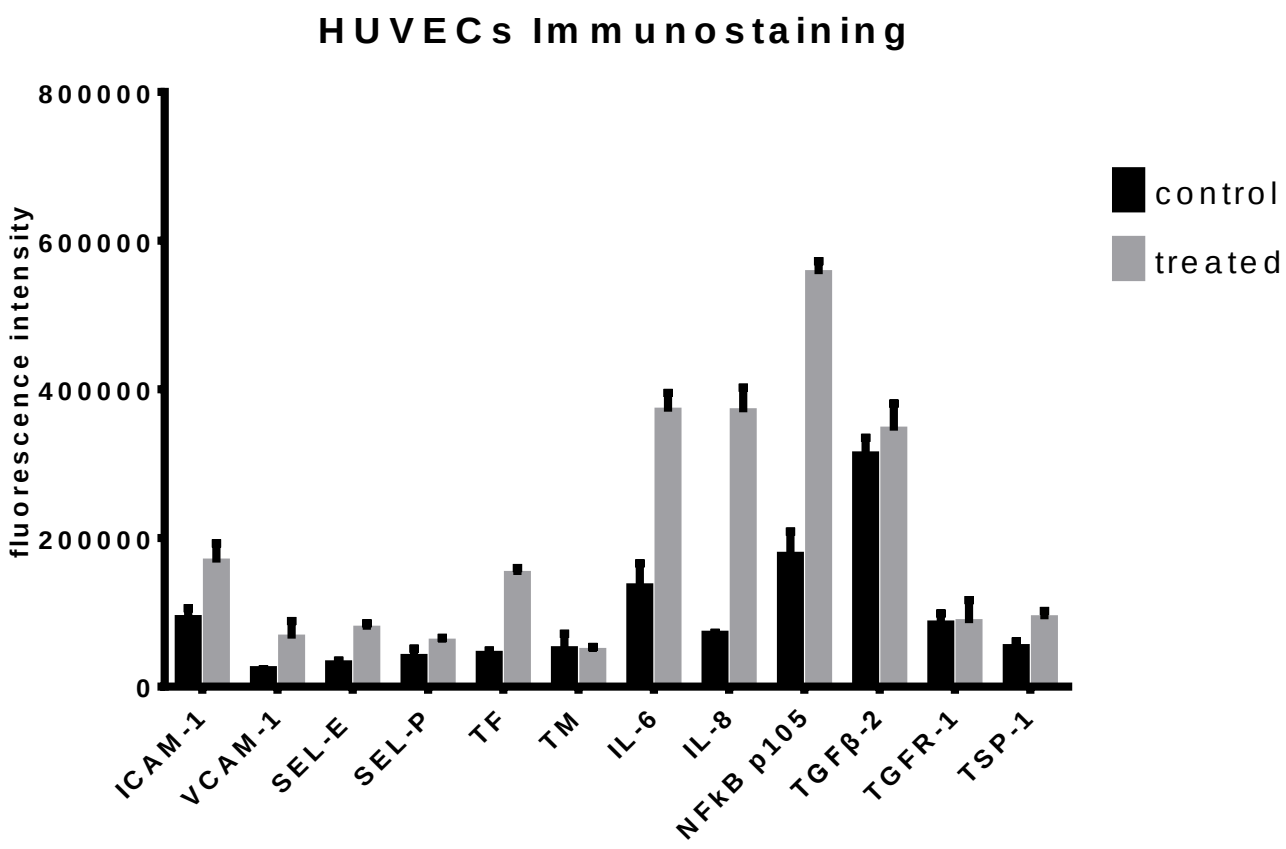


Figure 30 Quantitation of fluorescence intensity with image-J in immunofluorescent experiments depicted above.

Multiple t tests t tests		Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
1	ICAM-1	*	< 0.0001	90930.1	166950.0	-76019.6	16503.7	4.60621	48.0
2	VCAM-1		0.0132573	22167.0	64617.0	-42450.0	16503.7	2.57215	48.0
3	SEL-E	*	0.00688162	30083.0	76694.0	-46611.0	16503.7	2.82427	48.0
4	SEL-P		0.222502	38768.7	59166.0	-20397.3	16503.7	1.23592	48.0
5	TF	*	< 0.0001	42813.3	150177.0	-107364.0	16503.7	6.50541	48.0
6	TM		0.891131	49084.3	46813.4	2270.94	16503.7	0.137601	48.0
7	IL-6	*	< 0.0001	133578.0	369874.0	-236296.0	16503.7	14.3177	48.0
8	IL-8	*	< 0.0001	69941.3	369193.0	-299252.0	16503.7	18.1324	48.0
9	NFkB p105	*	< 0.0001	175986.0	554981.0	-378995.0	16503.7	22.9642	48.0
10	TGFβ-2		0.0477415	311028.0	344558.0	-33530.3	16503.7	2.03168	48.0
11	TGFR-1		0.913684	83575.0	85373.3	-1798.33	16503.7	0.108965	48.0
12	TSP-1		0.0228691	51876.0	90678.9	-38802.9	16503.7	2.35116	48.0

Table 1: Statistical analysis data from GraphPad Prism about HUVECs immunostaining differences. Statistical significance determined using the Holm-Sidak method, with alpha=5.000%.

iii. HUVECs treated with plasma from APS patients were found to secrete TSP-1.

TSP-1 expression *in vitro* was assessed by stimulating HUVECs derived from HD in cell culture. It was shown that HD HUVECs incubated with APS plasma secreted significantly higher levels of TSP-1 compared to control cells incubated with HD plasma. This was determined by Figure 31.

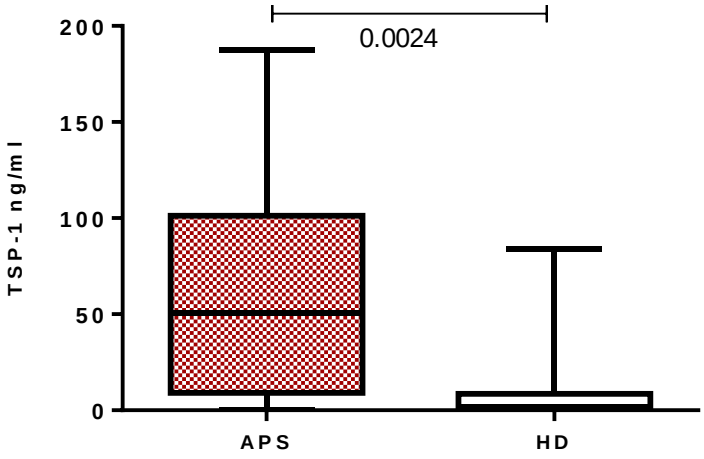


Figure 31 HD HUVECs incubated with APS plasma secreted significantly higher levels of TSP-1 compared to the ones incubated with HC plasma (p=0.0024)

iv. Treatment with IgG and b2gp1 in HUVECs demonstrated higher TSP-1 secretion.

HD HUVECs were also stimulated with anti-β2GPI and anti-β2GPI -β2GPI complex which gave the higher TSP-1 levels, while healthy IgG alone did not induce TSP-1 secretion (Figure 32).

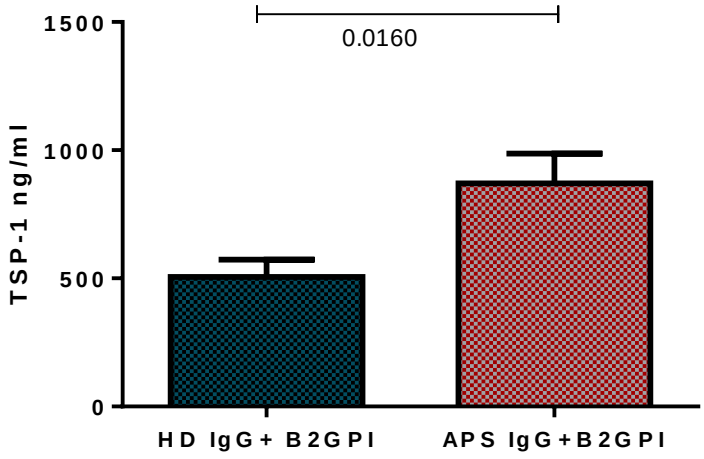


Figure 32 Treatment with anti-β2GPI IgG combined with β2GPI leads to secretion of significantly higher levels of TSP-1 compared to treatment with IgG derived from HDs and the untreated control in HD HUVECs (n=3, p<0.05)

v. Placenta sections and H/E staining

Placentas were derived from patients with APS who were under treatment with heparin and/or aspirin during pregnancy and delivered healthy infants. Additionally, placentas of healthy women were used as controls. In those placentas, hematoxylin-eosin staining was performed in order to demonstrate tissue architecture and integrity. In the figures below, we found distinct sides of big venous, (cotyledons), villous and intervillous spaces, where in physiological conditions there is maternal blood.

APS patients

Two APS patients were used for H/E staining. Tissues were sectioned in a cryotome placed in adherent microscope slides and the placenta sections were imaged in a light microscope. In the first APS patient, #94, it was difficult to distinguish much structure in the tissues except from the main venouses. The villi were very small and the distance between them is long. Moreover, in the second APS patient, #82, we observed less necrotic areas compared to the 94 APS patient whose epithelium was more dissected. In addition, in the first photograph of patient #82 patient and the last from patient #94, an accumulation of erythrocytes can be observed in the venous. (figures 33,34)

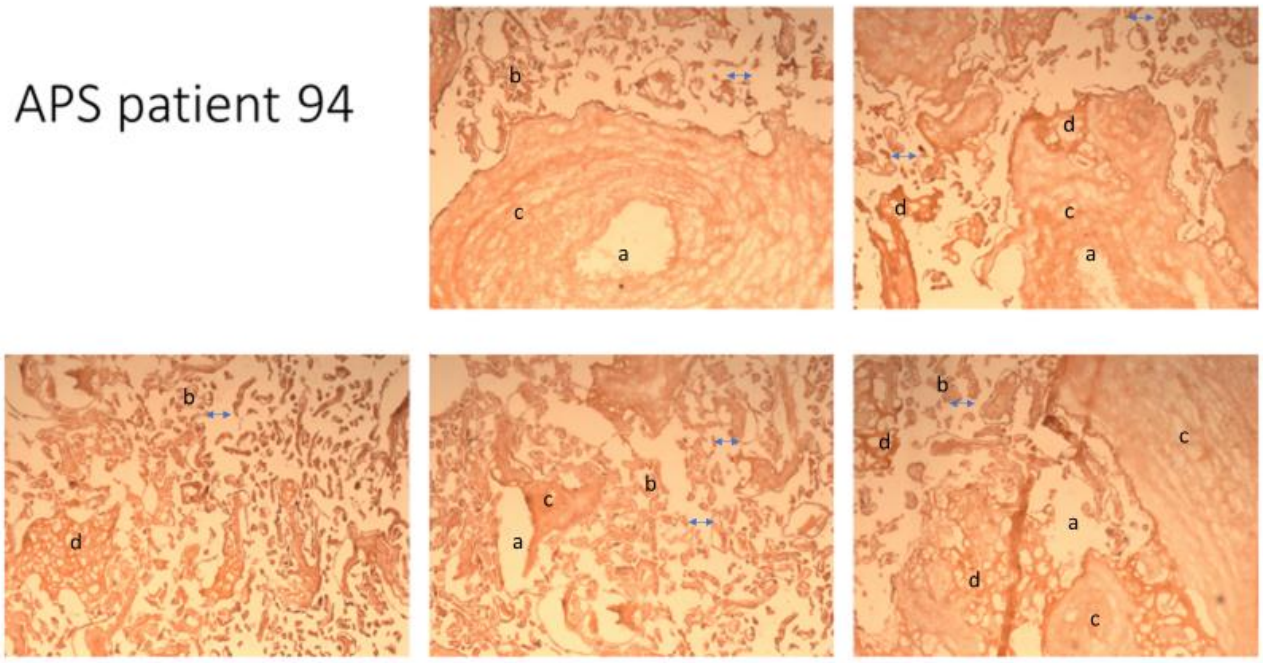


Figure 33 Placenta sections from patient #94, stained with H/E. a. venous b. villous c. epithelioum d. necrotic areas, ↔ peri-villous areas (distance between villous).

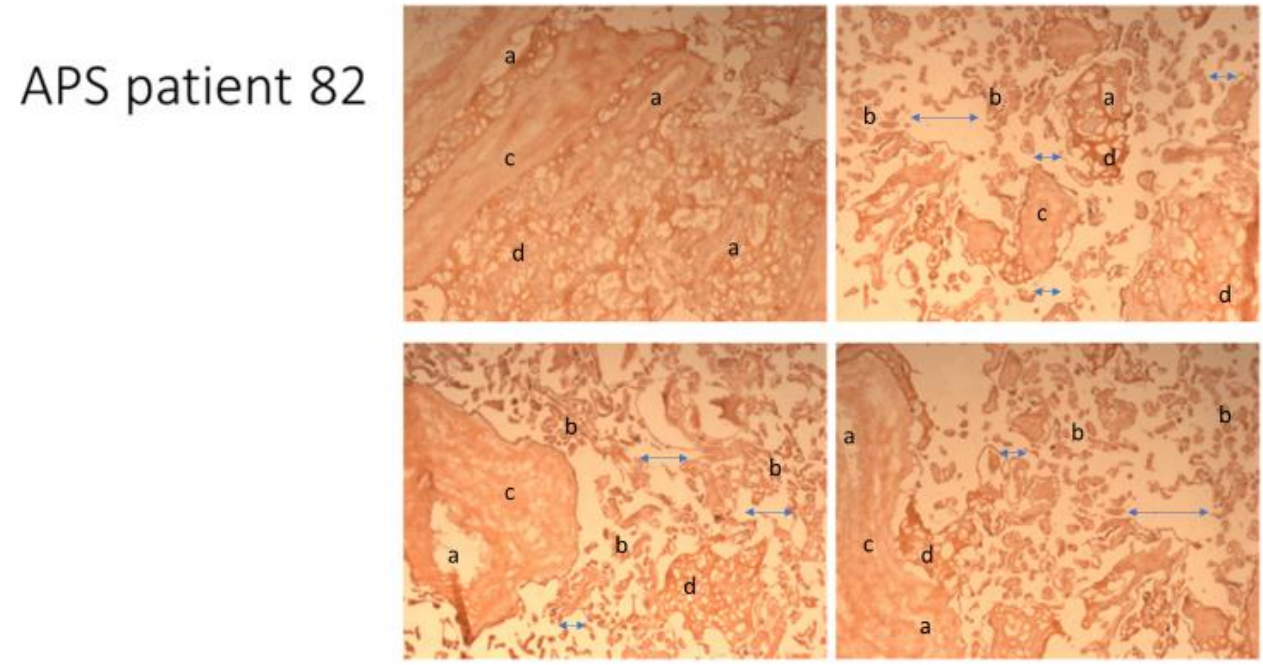


Figure 34 Placenta sections from patient #82, stained with H/E. a. venous b. villous c. epithelioum d. necrotic areas, ↔ peri-villous areas (distance between villous).

Healthy Donors

Two healthy donors were used for H/E staining HD 9 and HD 11. Tissues were sectioned in a cryotome placed in adherent microscope slides and the placenta sections were imaged in a light microscope. The various anatomical structures in APS patients were to be observed more atrophic than those ın healthy donors and there was no difference between the HDs. The intravillus distance was equal in each photographed section and there were no aggregations of erythrocytes in the venous. The epithelial tissue was found to be more compact in comparison to the APS patients' epithelial tissue. (figure 35)

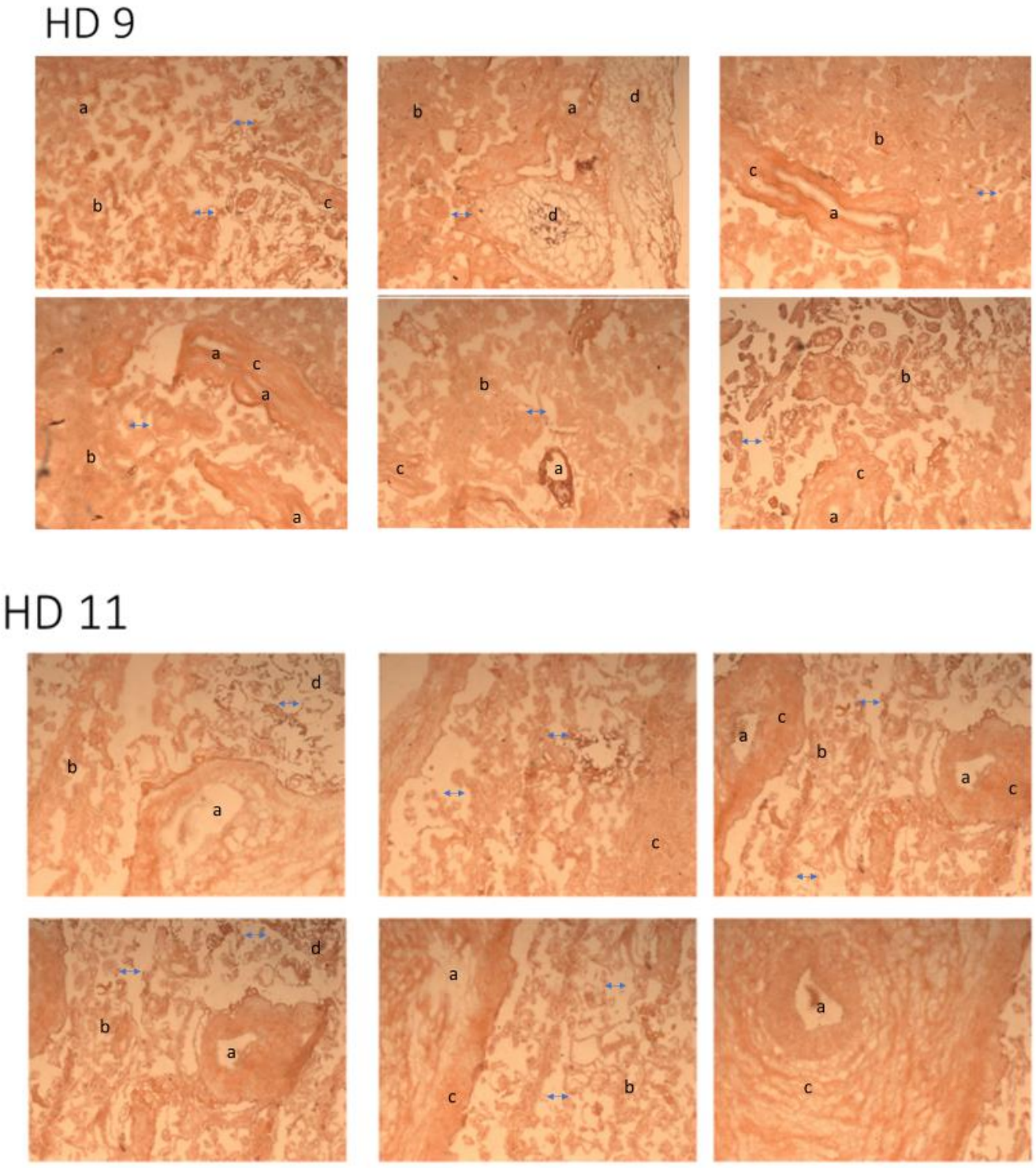


Figure 35 Placenta sections from Healthy Donors (HDs), stained with H/E. a. venous b. villous c. epithelioum d. necrotic areas, ↔ peri-villous areas (distance between villous).

vi. Immunochemistry placenta sections confirm the results from treated HUVECs.

In order to examine the expression patterns of the molecules that were found upregulated in treatments with anti-b2GP1/b2GP1 complexes in HUVECs, immunochemistry was performed in placentas from APS patients and HDs.

Staining with the ICAM-1 antibody showed a very high increase in protein expression in APS patients compare to HDs. Between the APS patients in #94 there was a wider expression compared to that in #82. ICAM-1 molecule is physiologically expressed in endothelial cells, found around the venous regions, as it can be observed in HDs placentas. In APS placentas, protein expression was apparent in the vilous and intarvillous areas in addition to the endothelial regions. (figure 36).

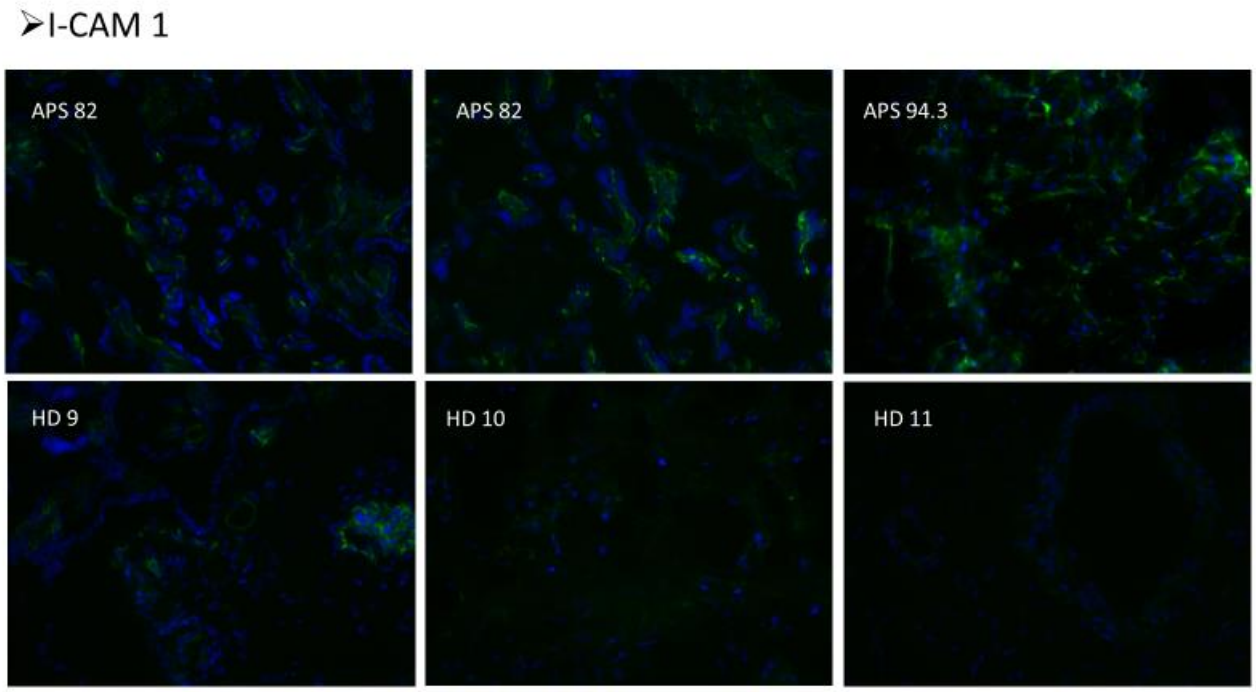


Figure 36 I-CAM 1 expression in placenta sections is aberrant in APS patients' placentas andin HDs the expression deteriorated in endothelium areas. Upper photographs: placenta from APS patients lower photographs: placenta from HDs.

Staining with interleukin 6 (IL-6) antibody illustrated that in APS patients' protein levels were increased relative to HDs. There was no difference in expression levels between the two APS patients. (figure 37)

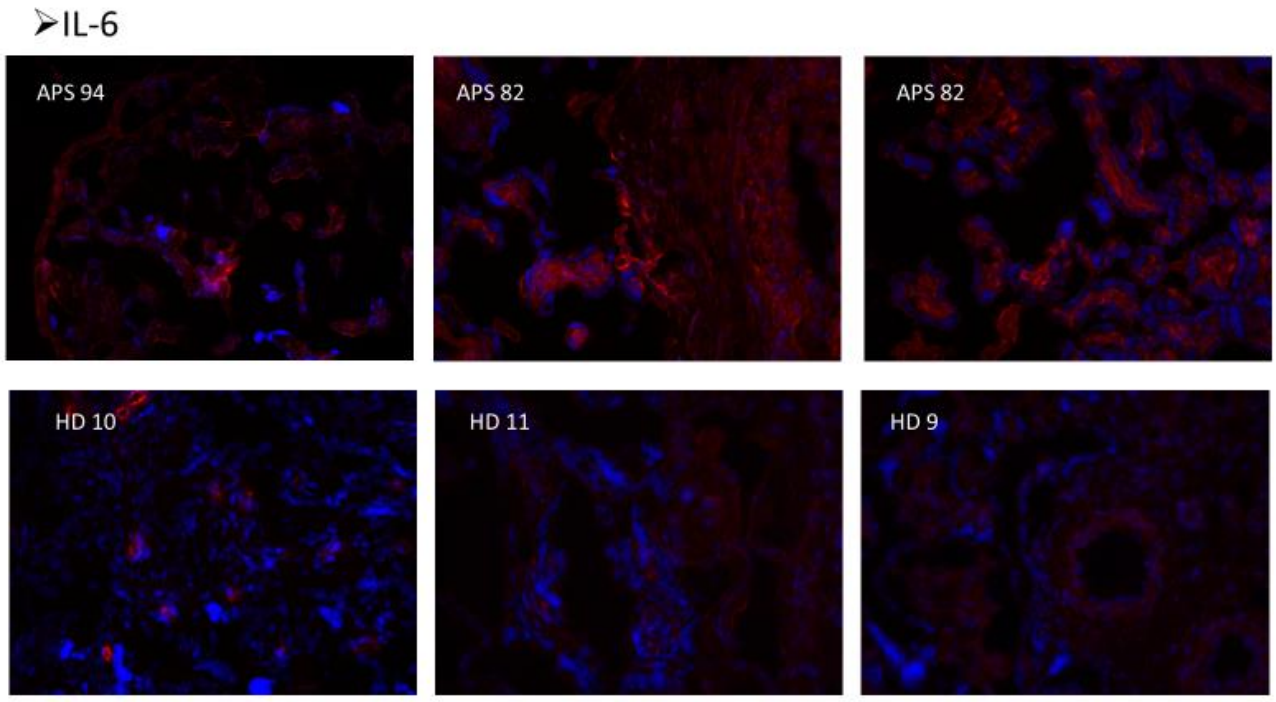


Figure 37 IL-6 expression in placenta sections is higher in APS patients' placentas compared to HDs. Upper photographs: placenta from APS patients lower photographs: placenta from HDs

Staining with TGFb-2 was found to be different between patients and healthy donors, as it was expressed less in the latter. In addition, there was a broader and higher expression of TGFb-2 in necrotic areas of the same patient. (figure 38)

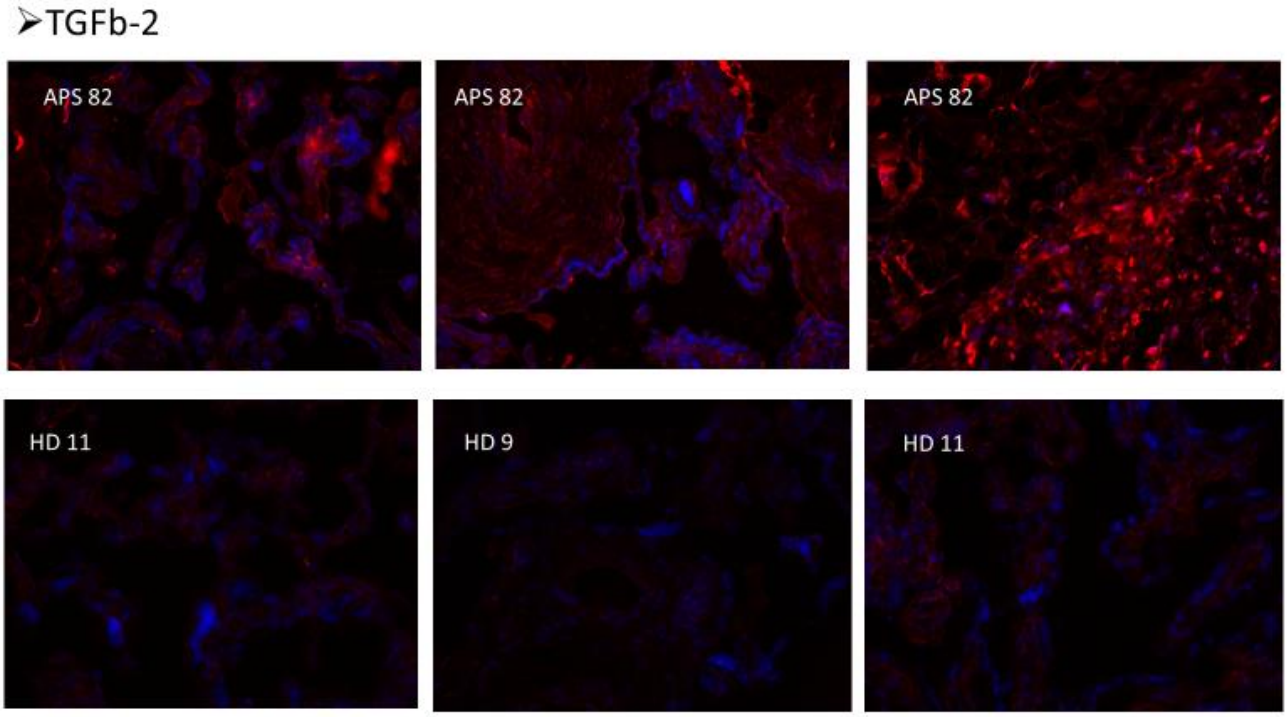


Figure 38 TGFb-2 expression in placenta sections is increased in APS patients' placentas and aberrant in necrotic areas of placentas, as depicted in the 3rd upper photograph. HDs placentas found to have low TGFb-2 expression. Upper photographs: placenta from APS patients lower photographs: placenta from HDs.

TNF-a protein levels in APS patients was higher than in HDs. In APS patients, TNF-a expression was detected in the villous, with even higher expression been found in endothelial cells of venouses and in necrotic areas. (Figure 39)

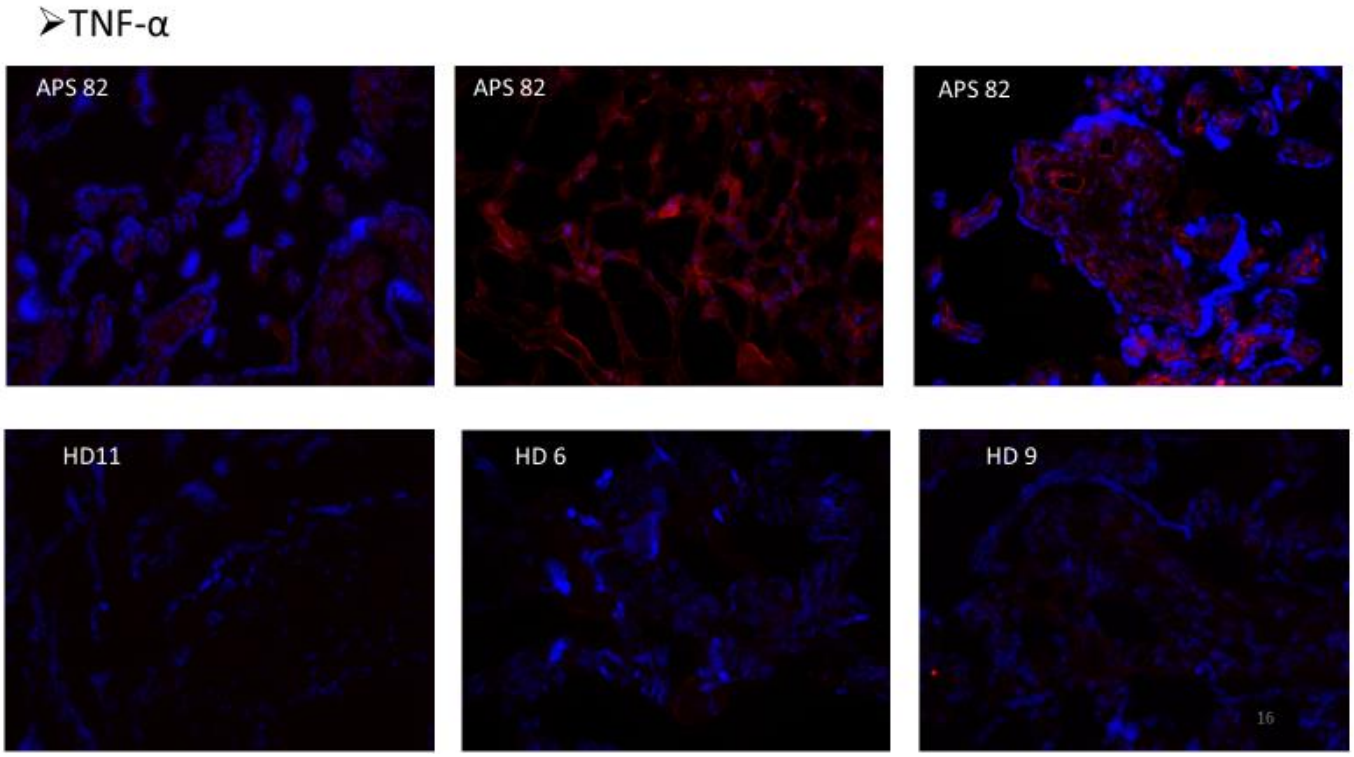


Figure 39 TNF-α expression in placenta sections is higher in APS patients' placentas and more especially in necrotic areas, found in the 2nd upper photograph. TNF-α also, found expressed in HDs, in basal levels. Upper photographs: placenta from APS patients lower photographs: placenta from HDs

Tissue factor protein expression was found to be the same between patients and healthy donors, while in patients the expression was more distinct compared to patients. (figure 40)

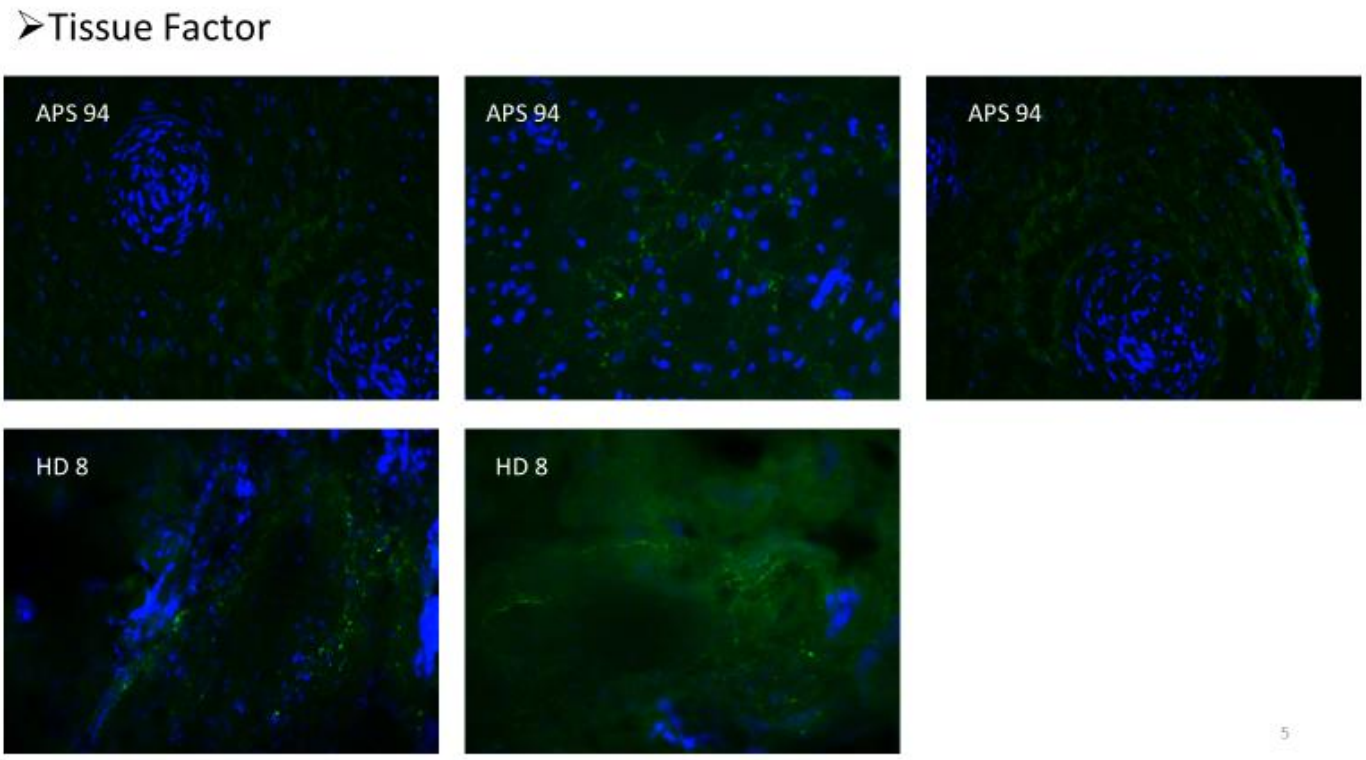


Figure 40 TF expression in placenta sections illustrated same in both conditions. TF expression detected in cells around the venous. Upper photographs: placenta from APS patients lower photographs: placenta from HDs

Interleukin-8 (Il-8) was found in higher levels in the placenta from APS patients compared to the placenta from healthy donors. Especially, Il-8 demonstrated increased expression in the necrotic areas of #94 patient compared to villous expression in patient 82. (figure 41)

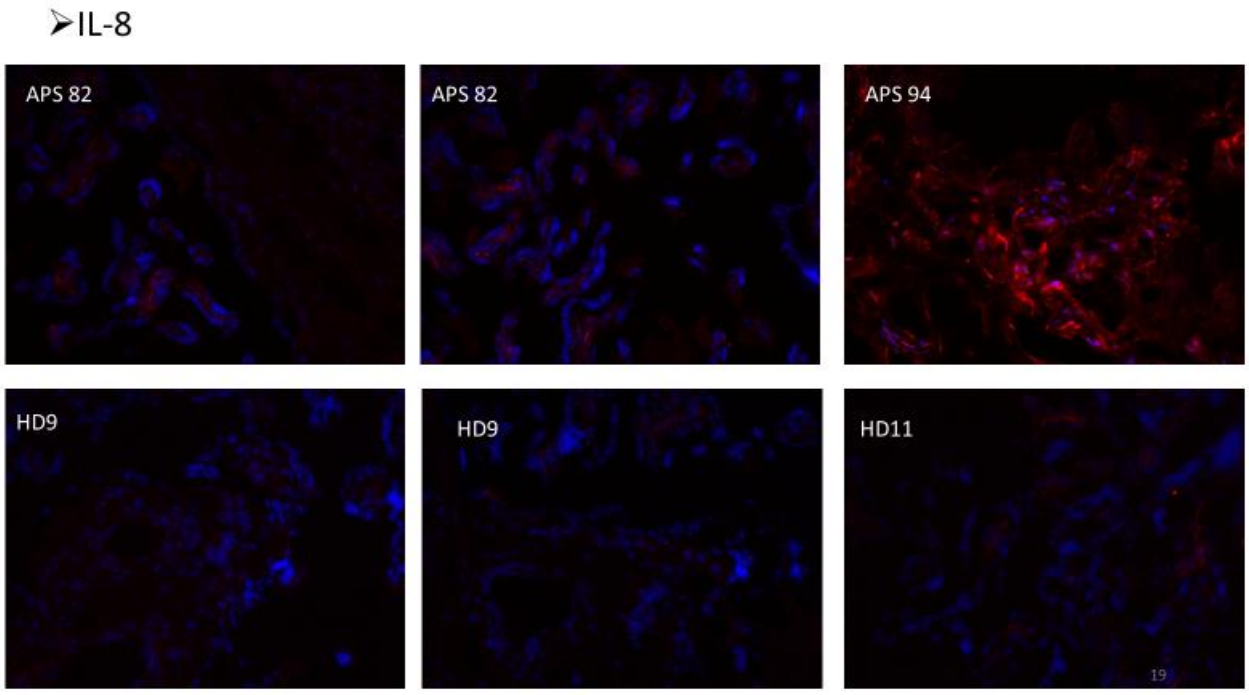


Figure 41 IL-8 found only a little increased in APS patients' placentas compare to HDs' placentas. In the 3rd photograph section from patient #94 depicts a necrotic area, where IL-8 expression showed very increased. Upper photographs: placenta from APS patients lower photographs: placenta from HDs

vii. Treatment with TSP-1 in HUVECs demonstrated ECs activation though increase of cell adhesion molecules expression.

HUVECs cells were cultured in medium enriched in serum and growth factors (EBM). Confluent cells were subjected in conditional starvation before any treatment in order to stop their proliferation. The appropriate titration experiments also took place. The treatment used was serum-free medium without and the addition of growth factors and different concentrations of TSP-1 in triplicates. Gene expression was assessed in different time points. RNA was isolated from cells and cDNA was generated. Then, the relative expression of two well-studied adhesion molecules that are overexpressed in APS patients' endothelium was measured.

The first molecule is E-Selectin (SEL-E), as shown in figure 42. The mRNA levels of treated cells in 4h were found increased in response to treatment and as the TSP-1 concentration augments SEL-E expression getting higher. In the 6h treatment we found the same trend in mRNA increase, but the expression levels were quite lower. In 12h the expression levels of SEL-E were in basal levels with the decreased trend among the different concentrations.

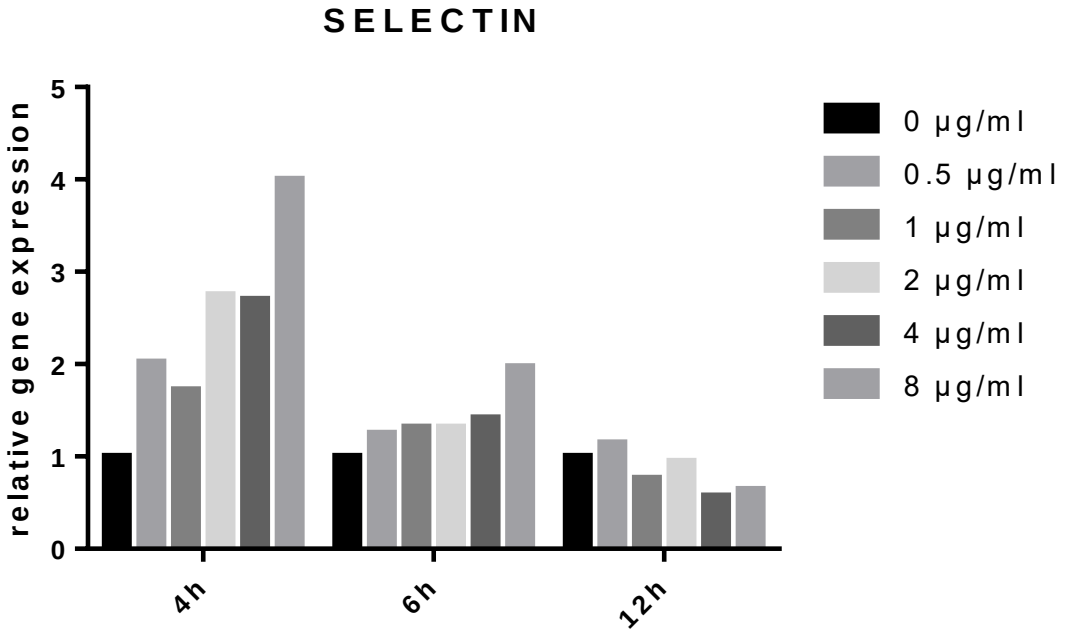


Figure 42 E-Selectin (Sel-e) relative gene expression in diferent time points and diferent TSP-1 concentrations.

Looking through the 4h treatment experiment the higher mRNA SEL-E gene expression was found in the 8μg/ml concentration, which aligns to the fact that this TSP-1 concentration is also found in the plasma of APS patients. The mRNA expression was associated to the increase of TSP-1 expression as shown in figure 43.

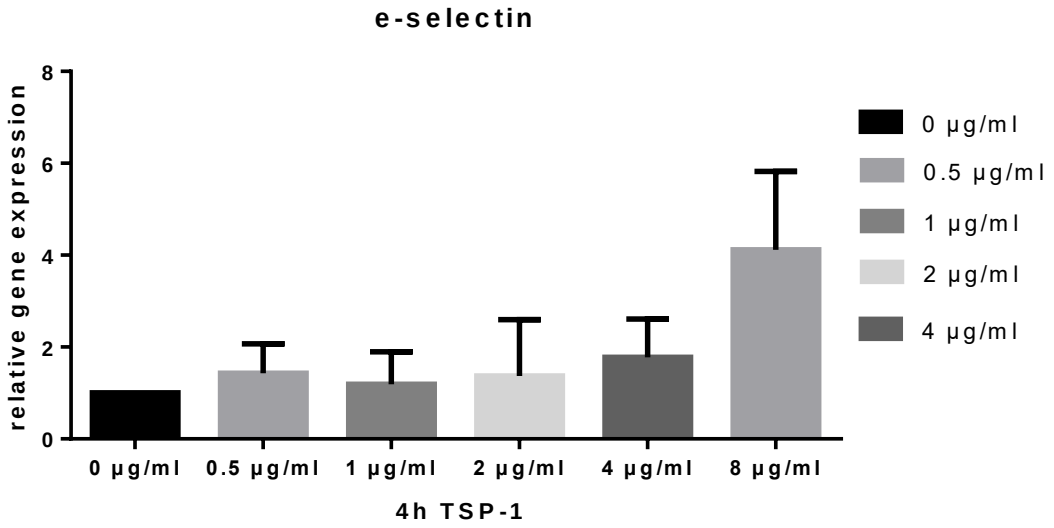


Figure 43 E-Selectin (Sel-e) relative gene expression in diferent TSP-1 concentrations.

The other mRNA that was studied was that of vascular cell adhesion molecule 1 (VCAM-1) as demonstrated in figure 43. The mRNA levels of treated cells in 4h were found to increase in response to treatment and as the TSP-1 concentration increased, VCAM-1 expression increased respectively. In the case of 6h treatment, the trend of mRNA increase changed. In TSP-1 0,5 μg/ml and 1μg/ml treatments the mRNA levels of VCAM-1 in HUVECs were lower than the untreated ones, while in the TSP-1 2 μg/ml treatment was quite lower than the untreated and as the TSP-1 concentration increased the mRNA expression didn't affected. In the 12h treatment, the expression levels of VCAM-1 mRNA were quite increased compared to basal levels with no distinguished trend among the different concentrations. This titration experiment showed that the 4h treatment is that the TSP-1 involved in ECs activation, same with the data occurred from SEL-E mRNA expression levels.

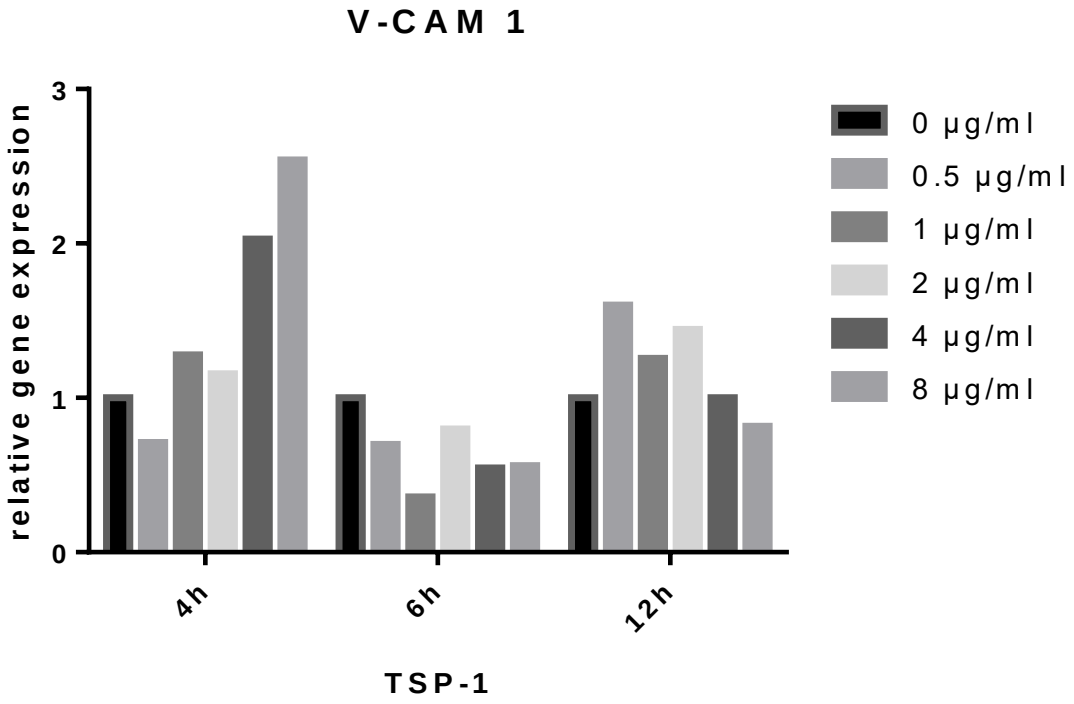


Figure 44 V-CAM-1 relative gene expression in diferent time points and diferent TSP-1 concentrations.

More precisely, when focused in the 4h time point, we found a slight increase in the mRNA levels in treatment with 0.5 µg/ml compared to the treatment with 1 µg/ml and 2 µg/ml. In 4 µg/ml treatment there was a significant increase in VCAM-1 transcription that augments in the treatment with 8 µg/ml. (figure 45)

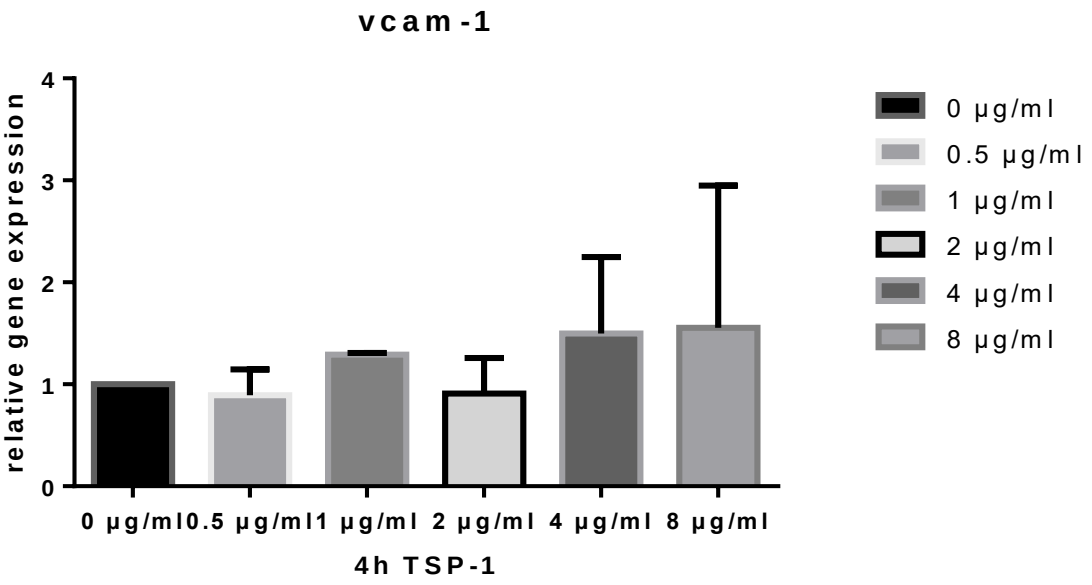


Figure 45 V-CAM-1 relative gene expression in diferent time points and diferent TSP-1 concentrations.

Discussion

Several studies have shown that ECs are activated in APS. This activation is characterized by the surface expression of adhesion molecules, chemokines such as IL/8, cytokines and TF. Although these observations were found in the plasma of patients as well as in cell cultures, the detection of these molecules was based on cell Elisa assays, while the cells have been cultured on the presence of whole patients' serum. Work from our laboratory has shown that platelets treated with a mixture of β2GPI/anti-β2GPI are activated as depicted by thromboxane β1 expression, this activation being more prominent when the chemokine CXCL4 (PF4) was added in the mixture of β2GPI/anti-β2GPI. It seems that CXCL4 dimerizes with β2GPI, the resulting complex being more accessible to anti-β2GPI binding, which eventually induces platelet activation. The present work investigates the role of β2GPI/anti-β2GPI complexes in activating HUVECs derived from normal human placenta. In addition, HUVECS were also treated with whole plasma from APS patients and they secreted high levels of TSP-1 protein, which stimulated as to evaluate its role in EC activation.

Unpublished data from our lab have shown that transcriptome analysis from HUVECs treated with β2GPI/anti-β2GPI or β2GPI normal human IgG plus β2GPI as control revealed 680 differentially expressed genes revealed 680 differentially expressed genes, among which 377 were upregulated and 303 downregulated. Characteristic examples of the upregulated genes are IL-6, IL-8, VCAM-1, SEL-E TGFB2 and TGFBR1. Further *in silico* analysis demonstrated that the upregulated genes belong mainly to the cytokine-cytokine receptor interaction, MAPK signaling, TNF signaling and NOD-like receptor pathways. Characteristic examples of the downregulated genes include the CBX4, CBX8, BCOR and HDAC7 genes. (unpublished results).

To corroborate further the previous findings, we performed immunochemistry experiments in treated HUVECs showing that at the protein level, similarly to the transcriptome level, ICAM-1, E-selectin, TF, IL-6, IL-8 and NFkB p 105 levels are increased compared to HUVECs treated with normal human IgG plus β2GPI. In the same experiment, a modest increase in protein level was found for

VCAM-1, P-Selectin and TGFb2, a finding not in agreement with the transcriptome analysis. Another interesting observation was that the TM protein, which normally has a protective role in inflammation, was found to be translocated from the cell cytoplasm in untreated cells to the extracellular space in treated cells. Possibly, TM was released from cells in order to control the inflammation induced cellular stress. Another rather unexpected observation was that TGFR-1 protein seemed to be expressed in the same levels between treated and untreated cells, something not anticipated from the transcriptome analysis. TGFR-1 protein is probably expressed in later stages. The immunohistochemical staining on placenta biopsies from APS patients and healthy controls for IL-6, IL-8, TNF-a, ICAM-1 and TGFB2 revealed increased signal intensity in the endothelial cells from APS tissues. Only in the case of TF, signal intensity was the same between patients and healthy donors.

TSP-1, a pluripotent molecule acting mainly as angiogenesis inhibitor, is elevated in patients with APS compared to HDs. This increase was observed both at the level of circulating TSP1 protein as well as in platelet-derived mRNA and mainly concerns those with a history of arterial thrombosis or those undergoing a clinical event at the time of assessment. (previous lab results/ unpublished). Titration experiments conducted in this study, validated the association between TSP-1 serum concentration and expression of proinflammatory molecules *in vitro*. As previously mentioned in this report, ECs dysfunction contributes to APS pathogenesis; finding that was further corroborated by our immunofluorescent experiments. It has been established that during thrombotic events in APS patients, high levels of circulating TSP-1 are found in their plasma. Significant changes in cell adhesion molecules were record at the mRNA level, in response to TSP-1 in HUVECs compared to untreated HUVECs.

The anti-angiogenic effects of TSP-1 are implicated in the pathophysiology of chronic inflammatory diseases pathophysiology (i.e. rheumatoid arthritis, atherosclerosis, airway inflammation). Activated EC cells secrete cytokines, chemokines, matrix metalloproteinases, and growth factors that can greatly influence the inflammatory reaction and promote carcinogenesis. This study's results suggest that TSP-1 affect cell adhesion molecules such as CAM motif molecules and lectins at the transcriptional level. In this report, mRNA levels of VCAM-1 and E-Selectin were found upregulated in response to TSP-1 treatment. More specifically, the

upregulation of SEL-E, that is a marker of EC activation[88] is proportionate to increasing concentrations of TSP-1. The changes in E-selectin's mRNA are controlled in a dose-response manner and the increase of mRNA expression is relative to TSP-1 concentration, a fact that supports the pathogenic role of TSP-1 in ECs.

This experiment supports the role of TSP-1 in the pathogenesis of antiphospholipid syndrome, supplementing previous work from our laboratory. It was shown that culture supernatant from HUVECs treated with plasma from APS patients contains about five-fold more TSP-1, compared to supernatant from HUVECs treated with plasma from healthy donors. As recorded, from clinical records TSP-1 concentration from APS patients was approximately 390 ng/ml while in plasma from healthy donors, the concentration was about 144 ng/ml. The above findings support the notion that TSP-1 plays a significant role in the pathogenesis of syndrome, in an autocrine or paracrine level.

Plasma TSP-1, also correlated with free active TGF-β1 and the pro-inflammatory cytokines IL-1β and IL-17A, implying that components of his proinflammatory axis could be examined as putative therapeutic targets.[91] Furthermore, this study suggests that TSP-1 promotes cell adhesion molecules expression, leading to adhesion of monocytes to endothelium through molecules of CAM and lectin families. IAP is involved in this process as a mediator of TSP-1-enodothelium interaction, which is also known from other studies. TSP-1-IAP complex is an important part of EC activation by TNF-α. In this pathway, NFkB is the mediator molecule between TSP-1 and TNF-a leading to the secretion of proinflammatory cytokines. This pathway is active in inflammatory conditions. This finding was further supported from immunofluorescence staining in placenta, where TNF-α was found to be overexpressed in APS patients compared to healthy donors. The data from both our immunofluorescent staining in HUVECs and transcriptome analysis showed that this pathway is probably activated as NFkB and IL-6 are found upregulated either in the mRNA or in the protein level. Therefore, these results propose a novel role for TSP-1 in the regulation of endothelial functions related to inflammation, vascular injury and atherosclerosis.

TSP-1 has been implicated in many pathological conditions. For instance, it has been proposed as a marker for brain infarct mortality, peripheral arterial disease complications [92,93], and sepsis-induced disseminated intravascular

coagulation [94]. Recent studies showed that TSP-1 expression is induced by CXCL4 in patients with Systemic Sclerosis and that it can be accurately used as a biomarker in Sjogren's syndrome [95]. Animal disease models also support a connection between TSP-1 and thrombosis and inflammation such as experimental renal ischemic injury [96] and cerebral ischemia [97].

TSP-1 has also been responsible for Th-17 responses in experimental autoimmune encephalomyelitis (EAE) and dry eye models [98,99]. Thus, the connection between TSP-1, TGF-β1, IL-1β and IL-17A (Th-17 response) could be possible in various inflammatory and thrombotic conditions [100-102]. However, a recent study showed that patients with pAPS had low frequency of circulating Th17 cells [103].

TSP-1 in association with TGF-β1, IL-1β and other cytokines, activate monocytes and secrete IL1-β [104]. Activated monocytes can drive naive T-cells toward the Th-17 phenotype through secretion of TGF-β1 along with IL-6. TSP-1 activates circulating TGF-β1 by dissociating the latent associated peptide and regulating its biological activity. IL-6 was found to be upregulated by TSP-1 through NFkB pathway [76]. In this report, NFkB and IL-6 were upregulated in treated cells with β2GP1/anti-β2GP1complex as described above, a finding that goes in parallel with preliminary evidence that in APS, Th-17 cell polarization occurred, and TSP-1 probably triggers this phenomenon.

In summary, the present report extends further the results of our laboratory by detecting significantly higher expression of the proteins ICAM-1, VCAM-1 TF, IL-6, IL-8, NFkB p105 in endothelial cells cultured in the presence of β2GPI/ antiβGPI complexes than in endothelial cells cultured in control conditions.

The above data strengthen further the idea that a local inflammatory response takes place in distinct areas of the vasculature and triggers thrombosis in APS. The higher levels of TSP-1 in plasma of APS patients as well as the overexpression of TSP-1 by HUVECs cultured in the presence of anti-β2GPI/β2GPI complexes supports the notion that TSP-1 may be a stimulator of endothelial cell activation. Furthermore, HUVECs cultured in presence of TSP-1 express VCAM-1, ICAM-1 and also TSP-1, implying that TSP-1 may act through a positive feedback loop as ECs activator.

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Conclusion – Future Perspectives

Our gene expression and immunofluorescent experiments in endothelial cells treated with anti-β2GPI and β2GPI revealed a robust pro-inflammatory and pro-coagulant phenotype. To validate those results, Western blot analysis could be performed in HUVECs, subjected to the same treatments, in order to quantify the differences in protein levels, between treated and untreated cells.

In addition, by Western blot, we could unravel the activation of pathways found in the RNAseq analysis (MAPK signaling, TNF signaling and NOD-like receptor pathways) in treated cells with the anti-β2GPI/β2GPI complex. More specifically, p38 MAPK was found to be transformed in its activated isoform by phosphorylation at Thr-180 and Tyr-182 (phospho-p38MAPK), and then translocated from the cytoplasm to the nucleus, acting as a transcription factor. We could perform a Western blot analysis in cytoplasmic and nuclear extracts to detect the activated protein and quantify its expression levels.

Regarding the experiments in the placental sections, more placenta from a greater number of APS patients could be checked for further validation. Also, more proteins studied in HUVECs such as VCAM-1, E-SELE, TM will be examined in immunofluorescent staining in the placenta.

In vitro experiments with anti-β2GPI/ β2GPI complex with the PF4 chemokine which is also involved in APS pathogenesis merit further investigation. Transcriptome analysis and immunofluorescence staining could be performed in order to explore whether PF4 chemokine in conjunction with anti-β2GPI/ β2GPI enhances the expression of proinflammatory factors and procoagulant proteins that are expressed in APS patients.

We are in a process of inactivating TSP-1 gene either by si- RNA in HUVECs or inactivating TSP-1 by a soluble TSP-1 inhibitor and reevaluate untreated and treated HUVECs with anti-β2GPI/ β2GPI plus or minus CXCL4 chemokines. Measuring adhesion molecules, TF or cytokines as described in the text will evaluate the pathogenic role of TSP-1 protein. Since TSP-1 seems to interact with CD36 and CD47, we will silence the respective genes in HUVECs, and the above-mentioned experiments will be repeated. Measurements of proinflammatory cytokines from

HUVECs treated with TSP-1 will be performed at time points taken from 0h to 8h, every hour.

Activated monocytes express a number of inflammatory mediators, including TGF-β1, IL-1β and IL-6. This generates an inflammatory milieu capable to drive Th-17 differentiation, which could affect the clinical outcome. There is evidence that APS patients present a Th-17 polarization and our study suggested that TSP-1 is implicated in this Th-17 response, however this issue is unresolved.

Preliminary experiments in our laboratory have shown that TSP-1 levels were associated with IL-17A in the placenta of APS patients and also supernatants of monocytes treated with anti-β2GPI/β2GPI/CXCL4 stimulate naïve CD4 T cells to produce IL-17A. Based on the above data, it is reasonable to design experiments by stimulating monocytes with TSP-1 in order to detect IL-17A, IL-1β, IL-6 and TGF-β – an armamentarium necessary for Th-17 differentiation.

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