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Review article
Pregledni članak

POTENTIAL MARKERS OF ARTERIAL AND/OR VENOUS THROMBOSES AND THEIR COMPLICATIONS IN PRIMARY ANTIPHOSPHOLIPID SYNDROME

POTENCIJALNI POKAZATELJI ARTERIJSKIH I/ILI VENSKIH TROMBOZA I NJIHOVIH KOMPLIKACIJA U PRIMARNOM ANTIFOSFOLIPIDNOM SINDROMU

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Summary: Antiphospholipid syndrome is characterized by venous or arterial thromboses and/or recurrent abortions accompanied by antiphospholipid antibodies and it can be primary (PAPS) or secondary (SAPS) to another disease. Arterial thromboses are less common than venous and most frequently they manifest as ischemia or infarction. Venous thromboses are usually multiple and bilateral and the most common complication of venous thromboses are pulmonary emboli. Considering that laboratory diagnosis of PAPS is currently based on persistently positive aCL, aβ2gpI and/or LA tests, and that neither one of those tests can discriminate between PAPS patients with arterial or venous thromboses or their complications, the aim of this study was to investigate the diagnostical significance of the determination of apo(a), oxLDL, anti-oxLDL antibodies, anti-anxA5 antibodies, hsCRP, C3 and C4 complement components and HPT for discrimination between PAPS patients with diverse clinical manifestations. Considering that elevated oxLDL and anti-oxLDL antibodies concentrations were found in PAPS patients, and also in subgroups of PAPS patients with MI or PE, it can be concluded that those parameters represent additional risk factors which together with other factors may lead to thromboses and their complications in PAPS. Regarding the fact that C3 and C4 concentrations were decreased in PAPS patients and that a positive correlation was found between hsCRP and C3 concentrations, this finding could indicate potential roles of these parameters as markers of atherosclerosis, which represents the leading cause of morbidity and mortality. HPT and apo(a) concentrations are not independent risk factors for MI in PAPS because lower levels were found in those

Kratak sadržaj: Antifosfolipidni sindrom karakterišu venske ili arterijske tromboze i/ili spontani pobačaji uz prisustvo antifosfolipidnih antitela, a može biti primarni (PAPS) ili sekundarni (SAPS), koji je povezan sa postojanjem drugog oboljenja. Arterijske tromboze su manje uobičajene nego venske i najčešće se ispoljavaju kao ishemija ili infarkt. Venske tromboze su obično multiple i bilateralne, a njihova najčešća komplikacija su plućne embolije. Kako se laboratorijska dijagnostika PAPS trenutno zasniva na perzistentno pozitivnim aCL, aβ2gpI i/ili LA testovima, od kojih nijedan ne može da napravi diskriminaciju između PAPS pacijenata sa arterijskim ili venskim trombozama i njihovim komplikacijama, cilj ovog istraživanja bio je da se ispita dijagnostička značajnost određivanja nivoa apo (a), oxLDL, anti-oxLDL antitela, anti-anxA5 antitela, hsCRP, C3 i C4 komponente komplementa i HPT za razlikovanje PAPS pacijenata sa različitim kliničkim manifestacijama. Na osnovu povišenih koncentracija oxLDL i anti-oxLDL antitela u pacijenata sa PAPS, kao i u podgrupama PAPS pacijenata čije su glavne kliničke manifestacije bili IM ili PE, može se zaključiti da ovi parametri predstavljaju dodatne faktore rizika koji zajedno sa drugim faktorima mogu da dovedu do tromboza i njihovih komplikacija u PAPS. Kako su koncentracije C3 i C4 komponente komplementa bile snižene u PAPS pacijenata i kako je pronađena pozitivna korelacija između koncentracija hsCRP i C3 komponente komplementa, moglo bi se ukazati na eventualnu ulogu ovih parametara kao pokazatelja ateroskleroze, koja predstavlja jedan od vodećih uzročnika morbiditeta i mortaliteta. Koncentracije HPT i apo(a) nisu nezavisan faktor rizika za infarkte miokarda u PAPS jer su dobijene niže vrednosti u

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Abbreviations: aCL – anticardiolipin antibodies; aß2gpI – anti-ß2glycoprotein I antibodies; anx – annexin; apo(a) – apolipoprotein (a); CRP – C-reactive protein; HPT – haptoglobin; LA – lupus anticoagulans; MI – myocardial infarction; oxLDL – oxidized LDL; PE – pulmonary emboli; PAPS – primary antiphospholipid syndrome.

patients in comparison to MI survivors without PAPS. No significant correlation of anti-anxA5 antibodies and the presence of arterial or venous thromboses or their complications was found, but increased concentrations of the IgG isotype of those antibodies could be a marker for recurrent abortions in PAPS, although this finding should be further investigated on a larger number of patients with this clinical finding. Determination of hsCRP in PAPS patients could not be an adequate parameter which would provide discrimination between patients with increased risk for development and/or recurrence of venous and/or arterial thromboses, nor for their complications, because no statistically significant difference in concentrations of this parameter was found among PAPS, IM, PE and SLE patients who were included in this study.

Keywords: anti-oxidized LDL antibodies and oxidized LDL, anti-annexin A5 antibodies, apolipoprotein (a), complement, C-reactive protein, haptoglobin, primary antiphospholipid syndrome

poređenju sa IM pacijentima bez PAPS. Za anti-anxA5 antitela nije pronađena nikakva korelacija sa prisustvom arterijskih ili venskih tromboza, niti njihovih komplikacija, ali bi zato povišene koncentracije ovih antitela IgG klase mogle da budu pokazatelj čestih pobačaja u PAPS pacijentkinja, iako bi se ovaj nalaz morao dodatno ispitati na većem broju pacijentkinja sa ovom odlikom PAPS. Određivanje hsCRP u PAPS pacijenata ne može da bude adekvatan parametar koji bi omogućio pravljenje diskriminacije između pacijenata sa povećanim rizikom od razvoja i/ili učestalosti venskih i/ili arterijskih tromboza, kao ni njihovih najčešćih komplikacija, jer nije pronađena statistički značajna razlika u koncentracijama ovog parametra između PAPS, IM, PE i SLE pacijenata koji su obuhvaćeni ovim istraživanjem.

Ključne reči: anti-oxLDL antitela i oksidovani LDL, anti-aneksin A5 antitela, apolipoprotein (a), komplement, C-reaktivni protein, haptoglobin, primarni antifosfolipidni sindrom

Introduction

Antiphospholipid syndrome is characterised by venous or arterial thromboses and/or recurrent abortions accompanied by antiphospholipid antibodies, and it can be primary (PAPS) or secondary (SAPS) to another disease (1–4).

Recurrent thromboses are the major clinical manifestation of APS and they can involve blood vessels of any organ. Depending on the size of the vessels affected, organ failure has two predominant causes, thrombotic microangiopathy and ischemia secondary to thrombotic events. One third of all APS patients experience only one thrombotic event; the other two thirds have recurrent thromboses. The time between the initial occurrence and recurrence may be several days to several years. Eighty percent of the patients have either recurrent venous thromboses or recurrent arterial thromboses, whereas only 20% have both arterial and venous thromboses. The reason why some patients develop venous thrombosis, whereas others develop arterial thrombosis, is still unexplained (4).

Arterial thromboses are less common than venous thromboses and most frequently manifest with features consistent with ischemia or infarction. The severity of presentation relates to the acuteness and the extent of occlusion. The brain is the most common site, with strokes and transitory ischemic attack (TIA) accounting for almost 50% of arterial occlusions. Coronary occlusions account for an additional 23%, the remaining 27% involve diverse beds, including subclavian, renal, retinal and pedal arteries. Not all arterial episodes of ischemia or infarction are thrombotic in origin. Emboli, especially from mitral valve or aortic valve vegetations, can also lead to cerebral events. The frequency of cardiac valvular abnormalities appears to be quite high, with echocardiography revealing at least one valvular abnormality in up to 63% of patients with APS. Although many of these abnormalities are of little clinical consequence, vegetations of the mitral or aortic valves are present in approximately 4% of patients with PAPS or SAPS (5, 6).

Cardiac manifestations of APS are valve abnormalities (valve thickening, vegetations, and dysfunction), thrombotic and atherosclerotic coronary occlusion, ventricular hypertrophy and dysfunction, intracardiac thrombus and pulmonary hypertension. The most common cardiac manifestation of APS are valvular pathologies. They include verrucous endocarditis, valvular thickening and insufficiency. Valvular stenosis is rarely seen. The mitral valve is the most commonly affected site, followed by the aortic valve, although the tricuspid or pulmonary valve can also be affected. APS patients suffer from increased rate of cardiovascular accidents: myocardial infarction appears at the same stage of the disease in up to 5.5% and it is the presenting manifestation in 2.8% of APS patients (7, 8).

Venous thromboses are more common than arterial thromboses and they are present in 65–75% of patients with APS. Antiphospholipid antibodies can be detected in 8–10% of all patients with venous thrombosis. Venous thromboses are usually localized in the lower extremities and are frequently multiple and bilateral. Around one third of the cases are complicated by pulmonary embolism which can lead to pulmonary hypertension. Antiphospholipid antibodies are detected in around 10% of all patients with pulmonary hypertension (4). André et al. (9) are the first who have reported the only two cases of asymptomatic thrombosis of ovarial vein.

The most common pulmonary complications of APS are pulmonary thromboembolism and pulmonary hypertension (3, 10, 11). Pulmonary embolism occurs in approximately 14% to 30% of the patients. It may be the initial feature in approximately 9% of the patients (3, 10). Recurrent pulmonary embolism may lead to thromboembolic pulmonary hypertension. Severe cases with pulmonary hypertension may be accompanied by isolated tricuspid valve insufficiency (11). Several cases of primary (non-thromboembolic) pulmonary hypertension complicating PAPS have been described. The outcome in patients with pulmonary hypertension and APS is usually fatal (11). Rare cases with diffuse alveolar hemorrhage, fibrosing alveolitis, adult respiratory distress syndrome and pulmonary artery thrombosis have all been reported (3, 10, 11).

Potential markers of arterial and/or venous thromboses and their complications in primary antiphospholipid syndrome

Considering that laboratory diagnosis of PAPS is currently based on persistently positive aCL, aβ2gpI and/or LA tests, and that neither one of those tests can discriminate between PAPS patients with arterial or venous thromboses or their complications, it is important to evaluate the diagnostic significance of the determination of apo(a), oxLDL, anti-oxLDL antibodies, anti-anxA5 antibodies, hsCRP, C3 and C4 complement components and HPT for discrimination between PAPS patients with diverse clinical manifestations.

Oxidized LDL and antibodies against oxLDL

Lipid peroxidation and especially oxidative modification of LDL has a key role in the pathogenesis of APS and atherosclerosis (12). LDL are susceptible to free radical oxidation leading to oxidized LDL (oxLDL). LDL oxidation affects both the lipid and protein components of LDL. Reactive aldehyde products result from the oxidation of polyunsaturated fatty acids and include MDA (malondialdehyde) and 4HNE (4-hydroxynoneal) (13), capable of attaching to the ε-amino groups of lysine residues of apoB (14), and also they react with the amino group of phosphatydilserine or phosphatydilethanolamine (15).

The first oxidative modification of native LDL occurs in blood where these modified LDLs, known as »minimally modified LDL«, are recognized by the receptor for native LDL. In endothelial cells, »minimally modified LDL« are oxidized from intracellular oxidative products and then recognized by the macrophage scavenger receptors, leading to the production of foam cells. In contrast, when anti-oxLDL antibodies-oxLDL complexes are generated in the vascular wall, the macrophages catch them by the Fc-receptor or via phagocytosis and destroy oxLDL in the lysosome system (16).

Oxidized LDL has numerous proatherogenic roles: it supports formation of foam cells, enhances scavenger receptor expression on macrophages, it is susceptible to aggregation which leads to enhanced uptake and it is a substrate for sphingomyelinases, which promote LDL aggregation; it changes the expression of inflammatory genes in vascular cells, induces tissue factor expression and platelet aggregation, and binds CRP, leading to complement activation (17).

It is suggested that anti-oxLDL antibodies could be a marker of oxidative stress and atherosclerosis such as LDL oxidation, endothelial dysfunction and inflammation of arterial wall (18, 19). It is not yet known whether *in vivo* immune response to oxLDL is proatherogenic or antiatherogenic. Elevated titers of anti-oxLDL antibodies were reported in patients with carotid atherosclerosis, coronary arterial disease, peripheral vascular disease, hypertension, preeclampsia (13), thrombosis (20–22), and this finding suggests that anti-oxLDL antibodies have an atherogenic role (13). It was shown that anti-oxLDL antibodies recognize antigens on the surfaces of atherosclerotic lesions but not on the normal arterial wall.

Lipoprotein oxidation is estimated by the measurement of the amount of autoantibodies against epitopes of oxidized LDL. It is supposed that anti-oxLDL antibodies do not have a direct influence on coagulation, but that they are important in the inflammation of blood vessels in atherosclerosis and vasculitis. Nevertheless, there are some studies which suggest that the anti-oxLDL antibodies titer is not a marker of atherosclerotic vascular disease in patients with insulin-independent diabetes (13). Controversy regarding the clinical significance of anti-oxLDL antibodies is a result of many different factors like methodological imprecision, interference with soluble immune complexes and lack of standardization criteria for tests that detect anti-oxLDL antibodies (14). It is suggested that IgM isotype of anti-oxLDL antibodies have a protective role, which is the basis for the »immunomodulatory« role which has recently been suggested for these antibodies, but this suggestion is a result of experiments on animal models, while for humans no confirmatory data are available. Dominant subclasses of affinity purified anti-oxLDL antibodies are proinflammatory IgG1 and IgG3 (23). IgM antibodies could predominate in LDL immune complexes. Pathogenic immune complexes are formed in atheromatous plaques, although it is unlikely that the IgM isotype is dominant in extravascular compartments because of the large molecular mass and predominant intravascular distribution. It is suggested that levels of circulating oxLDL could be a biochemical marker of risk for coronary heart disease

(24). Ehara et al. (25, 26), as well as Tsimikas et al. (27), reported that acute coronary syndrome is characterised by increased circulating oxLDL levels. Oxidized LDL concentrations are significantly increased in comparison to healthy controls, and oxLDL levels were not associated with age, gender and total cholesterol or apoB levels (24). Contradictory to the growing evidence for the atherogenic mechanism of LDL oxidation in arterial walls, clinical significance of circulating oxLDL has not been explained, mostly due to a lack of sensitive methods for specific detection of circulating oxLDL levels.

Antibodies against cardiolipin and oxLDL were independent risk factors for myocardial infarctions during a five-year follow-up of middle-aged dislipidemic men (28, 29). In 1993, it was reported that anticardiolipin antibodies in SLE patients cross-react with MDA-modified LDL (30). Some anticardiolipin antibodies obtained from APS patients cross-reacted with oxLDL, providing the conclusion that anticardiolipin antibodies are important for atherosclerosis (31), but another study (32) found no correlation between anticardiolipin antibodies of the IgG/IgM isotype and oxLDL concentrations, nor between these and anti-oxLDL antibodies concentrations, in PAPS patients.

Methodological differences in anti-oxLDL antibodies detection provide contradictory data regarding their significance. There are several antigenic forms of LDL which are used in assays, and the nature of their antigenic epitopes has not been clarified. Anti-oxLDL antibodies are present in different subclasses, like IgG, IgM, IgA. Karvonen et al. (33) have reported that only IgM anti-oxLDL antibodies have shown an inverse correlation with carotid atherosclerosis. These studies suggest the existence of functional differences in the epitope or isotype, and it is important to determine these differences in order to clarify the association between anti-oxLDL antibodies and atherosclerosis development. Independently of these problems, the immunological status in patients with autoimmune disorders is different in comparison to healthy subjects. Elevated anti-oxLDL antibodies titers were found in patients with autoimmune disorders, and anti-oxLDL antibodies titers in SLE patients were connected with disease severity. SLE patients had elevated anti-oxLDL antibodies concentrations in comparison to PAPS, IM and PE patients (32).

Studies which investigate the differences in isotypes, structure or avidity of anti-oxLDL antibodies will most probably clarify their protective or pathological role. PAPS patients and subgroups of PAPS patients whose main clinical manifestation of PAPS were MI, had increased anti-oxLDL antibodies concentrations in comparison to MI survivors without PAPS. In addition, the subgroup of PAPS patients with MI had elevated oxLDL concentrations in comparison to MI survivors without PAPS. Oxidized LDL and anti-oxLDL antibodies concentrations were elevated in PAPS patients in comparison to patients with PE without PAPS (34). This suggests that oxLDL and anti-oxLDL antibodies levels represent additional risk factors which together with other factors might lead to thromboses and their complications in PAPS. It was reported that healthy subjects (35) and patients with metabolic disorders (16) had an inverse correlation between oxLDL and anti-oxLDL antibodies concentrations, and an inverse correlation between oxLDL and anti-oxLDL antibodies concentrations in PAPS patients was also found (32).

Lipoprotein (a) and apolipoprotein (a)

Lipoprotein (a) (Lp(a)) particles contain apolipoprotein (a) (apo(a)) and apoB in 1:1 molar ratio, which are linked by a single disulphide bridge (36). Apo(a) is a glycoprotein that exhibits both intra- and interindividual variations in size. Uncomplexed apo (a), as well as apo(a) fragments, have been identified in arterial lesions. The fragments may correspond to cleavage products of Lp(a) generated by elastase-like enzymes present at the sites of local inflammation. This may indicate a link between Lp(a) function and the inflammatory state. The potential role of apo(a) /Lp(a) fragments in angiogenesis has been suggested owing to the similarity of these apo(a) fragments to proteolytic fragments of plasminogen that have been implicated in the inhibition of the process of angiogenesis. The size of apo(a) isoforms was inversely correlated with the Lp(a) plasma concentration. This inverse correlation may be attributable at least in part to less efficient secretion of large apo(a) isoforms from hepatocytes.

Components of Lp(a) resemble both LDL and plasminogen, suggesting that Lp(a) may represent a bridge between atherosclerosis and thrombosis. It is well-documented that Lp(a) is present in the arterial wall at the sites of atherosclerotic lesions and that Lp(a) accumulates at these sites to an extent that is proportional to plasma Lp(a) concentration. A number of studies have suggested that Lp(a) may inhibit fibrinolysis by competing with plasminogen for binding to fibrin and to cell surfaces, thereby interfering with plasminogen activation on these surfaces. Apo(a) may interfere with plasminogen activity by complexing with plasminogen in solution; this complex binds poorly to the fibrin surface, which may inhibit plasmin formation on this surface *in vivo*. Further studies are necessary for the evaluation of the prothrombotic/ antifibrinolytic role of Lp(a) in atherogenesis.

Some studies concluded that Lp(a) concentration is an independent risk factor for MI or coronary heart disease, while other studies reached opposite conclusions (37). Despite the fact that elevated Lp(a) concentrations have been found in patients with autoimmune disorders, the significance of Lp(a) and/ or apo(a) concentrations in PAPS patients is not being

studied enough. Furthermore, the role of Lp(a) and/or apo(a) concentrations in PAPS patients is not clear because most of the previous studies included a small number of PAPS patients and also SAPS and SLE patients. It was reported (38) that Lp(a) levels and cerebrovascular insults in PAPS patients were not correlated, although a statistically significant correlation was found between elevated apo(a) concentrations and cerebrovascular insults in PAPS patients. Therefore, it was suggested that measurement of apo(a) concentrations could be important for discrimination between PAPS patients with cerebrovascular insults and PAPS patients without this clinical finding. Furthermore, we suggested that this could help in the follow-up of PAPS patients with cerebrovascular insults and also for the identification of those PAPS patients who are at increased risk for recurrence of cerebrovascular insults (38).

The size of apo(a) could be significant for the development of coronary heart disease. It has been reported that small apo(a) isoform size is more frequent in patients with MI than in healthy subjects, and that it is a powerful predictor for the risk of advanced atherosclerosis. Considering that advanced atherosclerosis is associated with thrombotic events, this suggests an apo(a) isoform-dependent role in these events (39, 40). Atherosclerosis is an important concern in PAPS because it could contribute to cardiovascular and cerebrovascular burden and further morbidity. It was shown that Lp(a) and LDL could act additively in the development of coronary heart disease, that Lp(a) and cholesterol levels act synergistically, and that Lp(a) levels in men were not a predictor for the progression of coronary heart disease when LDL concentrations were decreased. This means that Lp(a) is not a primary causative agent in atherogenesis. Patients with MI without PAPS had significantly increased apo(a) concentrations in comparison to the subgroup of PAPS patients whose clinical manifestations were MI (32). This suggests that apo(a) concentrations were not an independent risk factor for MI in PAPS.

C3 and C4 complement components

The complement system is one of the major effector mechanisms of humoral immunity and an important effector mechanism of inborn immunity. It is composed of serum and cell surface proteins which react with each other, as well as other molecules of the immune system in a highly regulated manner. There are three pathways of complement activation:

1. *classical pathway* is activated by certain isotypes of antibodies bound to an antigen;
2. *alternative pathway* is activated on cell surfaces of microorganisms in the absence of antibodies;
3. *lectin pathway* which is activated by the binding of plasma mannose-binding lectin for terminal mannose-residues that are found in microbial proteins and polysaccharides, but not in mammalian molecules.

Although the pathways of complement activation differ in how they are initiated, all of them result in the generation of enzyme complexes that are able to cleave the complement protein C3. The central event in complement activation is proteolysis of the complement protein C3 to generate biologically active products, and the subsequent attachment of C3b component to microbial cell surfaces or to antibodies bound to antigen.

C3 complement component (185 kD) is composed of two subunits: alpha subunit has 110 kD and beta subunit has 75 kD. Serum concentrations of C3 protein are in range from 1000 to 1200 μg/mL. C3b binds to microbial surfaces (functioning as opsonin) and it is the component of C3 and C5 convertase, while C3a stimulates inflammation (anaphylatoxin). C3c is a stable C3 fragment which is formed as the result of effects of factor I on unstable C3b. C3c is an indicator of C3 turnover.

C4 protein (210 kD) is a trimer composed of three chains of 97, 75 and 33 kD. Serum concentrations range from 300 to 600 μg/mL. C4b covalently binds to the surface of a microbe or cell, where an antibody is bound and a complement is activated. C4b binds C2 for cleavage by C1s while C4a stimulates inflammation (anaphylatoxin) (41).

Complement activation is connected with C3 and/or C4 consumption, and therefore the reduction of their concentrations is useful in achieving diagnostic conclusions. Decreased sera C3 and C4 concentrations are reported in SLE patients with membranoproliferative glomerulonephritis and in immune complex disease. In the case of SLE, serum complement concentrations reflect disease activity. Decreased C3 concentrations were reported in acute glomerulonephritis. Isolated decreased C4 levels were reported in hereditary angioneurotic edema and in crioglobulinaemia. Both C3 and C4 are acute phase reactants and, therefore, their increased concentrations can appear in patients with inflammatory diseases. During inflammation they are produced not only in liver, but also in macrophages. Major causes of elevated complement concentrations are: systemic infectious diseases, non-infection chronic inflammatory conditions such as rheumatoid arthritis, physiological conditions such as pregnancy. The detection of elevated complement levels is not particularly valuable for the diagnosis of such conditions. The determination of CRP can be useful. If CRP is elevated, an acute phase response is present and complement activity or complement concentration is not an indicator of an immune complex disease. Hereditary deficiencies have been registered for both C3 and C4. In certain situations, complement activation is linked with intravascular thromboses leading to ischemic tissue injury (42).

Linear deposits of anticardiolipin antibodies of the IgG isotype and granular depositions of C1q, C3

and C4 complement factors were found in subendothelial tissue of deformed valves of APS patients, suggesting that in this condition antiphospholipid antibodies could be involved in immune complex formation by their reaction to a »planted« antigen like β2gpI that has been demonstrated at the valvular level (43). The immune complex deposition induces a mild inflammatory process that ends in vascular proliferation, fibroblast influx, fibrosis and calcification resulting in the valve's thickening, fusion, valve rigidity and its loss of function. The characteristic histopathological lesion in APS is a thrombotic vascular occlusion without clear signs of inflammation. In contrast, mild inflammatory changes were observed in the affected valves of patients with PAPS and in APS within SLE. Control valve obtained from patients with negative findings of antiphospholipid antibodies and samples of control tissues from APS patients do not show such deposits (39, 43, 44). Pope et al. (44) have reported decreased total complement levels with low C3 and C4 in 11 out of 14 patients with APS and valvular heart disease, even though only three out of those 11 patients met the diagnostic criteria for SLE, while others were suffering from PAPS. PAPS patients, as well as the subgroup of PAPS patients whose main manifestation of PAPS were MI, had decreased serum C3 and C4 concentrations in comparison to MI survivors without PAPS. Also, PAPS patients had decreased C3 and C4 concentrations in comparison to patients with PE without PAPS. SLE patients in comparison to PAPS patients had significantly elevated complement consumption, which is manifested by lower C3 concentrations (32).

Haptoglobin

Haptoglobin (HPT) is a glycoprotein composed of four polypeptide chains, two light alpha and two heavy beta chains. Three types of HPT can be differentiated: HPT 1-1, 2-1, 2-2. The physiological function of HPT is to prevent renal losses of hemoglobin, and thus the loss of iron, and this is based on the fact that, unlike hemoglobin, HPT-hemoglobin complex, on account of its high molecular mass, is not glomerularly filtrated.

Both a decrease as well as an increase in HPT concentrations may be clinically significant. Greatly reduced levels are of major significance because they indicate intravascular hemolysis, acute phase response (acute and chronic active inflammation), acute tissue necrosis, malignant tumours, intrahepatic and extrahepatic cholestasis, Hodgkin's disease, nephrotic syndrome, rheumatoid arthritis, iron defficiency anemia, *de novo* synthesis of unknown ethiology, plasmacytoma, amyloidosis. Increased serum concentrations are mostly due to the acute phase function of HPT and are found to occur in conjuction with inflammatory diseases (45).

It was reported that among proteins eluted from human coronary circulation *in vivo*, HPT appears to discriminate between minor and extensive coronary atherosclerosis. The association of HPT elution with coronary atherosclerosis also supports the origin of HPT as being arterial rather than from capillary or venous vessels – an important distinction for markers of arterial pathology. An association between HPT and cardiovascular disease has been described, whereby the HPT 2-2 polymorphism has been associated with increased risk of coronary and peripheral artery disease (46). The beta subunit of HPT is reportedly absent or present in low quantities in the normal intima, but is abundant in fibro-fatty atherosclerotic lesions (47, 48). Previous detection of HPT in the atherosclerotic artery, and its stimulation under conditions of increased shear stress and inflammation, supports the conclusion that in that population HPT is a marker of arterial pathology (atherosclerosis) (46). Statistically significant decreased HPT concentrations were present in PAPS patients, as well as in the subgroup of PAPS patients whose clinical manifestation of PAPS were MI, in comparison to MI survivors without PAPS (32). Further studies are necessary to show the significance of determination of HPT in patients with thrombosis.

C-reactive protein

C-reactive protein (CRP) is a stable analyte that can easily be measured with newly developed high-sensitivity assays (hsCRP). CRP derives its name from its ability to bind the C polysaccharide of *Streptococcus pneumoniae* (49).

Human CRP is calcium-dependent ligand-binding protein which binds to phosphocholine and to different autologous and extrinsic ligands and aggregates or precipitates cellular, particular or molecular structures which bear those ligands. Autologous ligands are native and modified plasma lipoproteins, disrupted cell membranes, different phospholipids, small particles of ribonucleoproteins and apoptotic cells. Extrinsic ligands are glycans, phospholipids and other components of microorganisms such as capsular or somatic components of bacteria, fungi and parasites, as well as plant products. When CRP is bound to its ligand, it is recognized by C1q and strongly activates the classical pathway of complement activation, including C3 and the membrane attack complex (C5 – C9). CRP can also provide a secondary binding site for factor H and in this way regulate the amplification of the alternative pathway of complement activation and C5-convertase (50). Secondary effects of CRP which follow after binding ligands are similar to some features of antibodies, which suggests that under different circumstances, CRP could contribute to host defense against infection, and function as a proinflammatory mediator (51).

Human CRP molecule (RMM = 115 135) has five identical nonglycosylated polypeptide subunits (RMM = 23 027) and each subunit contains approximately 206 amino acid residues. Although it has been shown that CRP is an acute phase reactant and a component of human innate immunity which is enhanced in response to inflammatory stimuli, and a well-known mediator of complement activation, production of adhesive molecules and chemokines, as well as liberation of thrombogenic factors, its biological role still remains unexplained. CRP is very interesting from the aspects of cardiovascular biology and pathology, because it selectively binds to LDL, especially oxLDL and enzymatically modified LDL which have been found in atheromatic plaques, and CRP is deposited in plaques. CRP has a wide range of proinflammatory features which could eventually lead to pathogenesis, progression and complication of atheroma. Predictive value for future cardiovascular diseases is even stronger for CRP than for LDL, and there are evidence which suggest that elevated CRP values identify a person with higher risk, which could not be detected by Framingham risk score (52).

The presence of CRP in atherosclerotic plaques and in all acute MI lesions, as well as binding of CRP to lipoproteins and the ability of CRP to activate the complement, suggest that CRP could contribute to the pathogenesis and to complications of cardiovascular diseases. Bhakdi et al. (53) showed that CRP binds *in vitro* to enzymatically degraded LDL *via* phosphocholine motifs (54), which suggests that enzymatic degradation of LDL is another mechanism which contributes to expression of phosphocholine motifs that leads to binding of CRP.

CRP production which follows myocardial necrosis is a typical acute phase response to cell death and inflammation. CRP colocalizes with membrane attack complex in early atherosclerotic lesions, suggesting that CRP has a role in the formation of atherosclerotic lesions by activation of complement. Different studies revealed heterogeneity in results which cannot be easily explained by methodological differencies. There is evidence which suggests that the strength of the association between CRP levels and the risk of coronary vascular disease might have been overestimated in previous studies in comparison with some novel investigations (55).

Patients with MI have elevated CRP concentrations in comparison to control subjects (32) and future epidemiological investigations are necessary to help in the redefinition of the CRP status as a risk factor for cardiovascular diseases. Binding of CRP to its ligand could activate complement, which leads to C3 deposition in tissues. In animal models of MI, this could enhance the infarcted area. C3 deposition and complement activation in arteria might potentially promote atherogenesis (56). Immunohistochemically, CRP deposits were shown in all acute MI, which were colocalized with activated complement components. *In vivo* depletion of complement reduces the inflammation and size of MI in animal models. Significant part of the final MI size after coronary occlusion is determined by inflammation which is mediated by complement, and CRP is responsible for this complement activation (57). Positive correlation between C3 and CRP concentrations was found in PAPS patients (32). Elevated association of oxLDL with macrophages via CRP-Fcγ-receptor interaction leads to enhanced intake of oxLDL by macrophages. At moderate LDL deposition, this activity of CRP leads to enhanced clearance of oxLDL. At excessed LDL deposition (caused by elevated plasma LDL levels), this mechanism could promote foam cells formation and enhancement of atherosclerosis. In CRP's absence, oxLDL binds to scavenger receptors, and in the CRP's presence, binding of oxLDL represents CRP-Fcγ-receptor interaction. This suggests that CRP influences the oxLDL processing mediated through the interaction oxLDL-CRP-Fcγ-receptor and by inhibition of oxLDL binding to scavenger receptors. Therefore, this could be an important mechanism which determines the extent of atherogenesis.

Annexin A5 and anti-annexin A5 antibodies

An annexin protein has to fulfill two major criteria. First, it must be capable of binding in a calcium-dependent manner to negatively charged phospholipids. Second, it has to contain as a conserved structural element the so-called annexin repeat, a segment of 70 amino acid residues. There are five major annexin groups (A–E), and vertebrate annexins are designated as A1–A13. Deregulations in annexin expression and activity have been correlated with human diseases and the term *annexinopathies* has been coined (58).

Annexin A5 forms two-dimensional crystals on planar lipid bilayers containing negatively charged phospholipids. Annexin A5 was originally described as an anticoagulant protein, and this activity most likely depends on its calcium-regulated binding to anionic phospholipids, possibly those exposed on the surface of activated platelets or endothelial cells. This binding could interfere with the accessibility of such sites for coagulation factors, thereby preventing their local accumulation/activation.

Increased annexin A5 levels were noticed in patients with acute MI or unstable angina, and an enormous expression was seen in the atherosclerotic lesions of coronary arteries (59). Annexin A5 binds to the apical surfaces of placental syncytiotrophoblasts and because of shielding these coagulation-promoting surfaces it could be important for the maintenance of blood flow through the placenta (58). Antiphospholipid antibodies could induce thrombosis by interfering with anticoagulant properties of annexin A5. In

the absence of annexin A5, a complex of antiphospholipid antibodies and their antigens, such as β2gpI or prothrombin, binds to phospholipid bilayers, which reduces the access of coagulation factors to anionic phospholipids. Prolongation of coagulation tests is the result of these interactions because of the limited amount of anionic phospholipids. Nevertheless, when there is a sufficient amount of annexin A5, antiphospholipid antibodies disrupt the ability of annexin A5 to form crystals on phospholipid surfaces and inhibit anticoagulant effects of annexin A5. This condition results in increased availability of anionic phospholipids for coagulation reactions which promote hypercoagulable conditions (60).

Anti-annexin A5 antibodies were reported in APS patients (61). Furthermore, antiprothrombin and anti-annexin A5 antibodies might be more specific for the diagnosis of APS (61). Moreover, anti-annexin A5 antibodies of the IgG isotype were associated with higher incidence of arterial or venous thromboses, intrauterine fetal death and prolongation of aPTT in SLE patients. There are contradictory data regarding the association of anti-annexin A5 antibodies and clinical manifestations of APS. Strong evidence for the functional and clinical significance of those autoantibodies is not available yet.

Although elevated anti-annexin A5 antibody levels were found in patients with different autoimmune diseases, the role of anti-annexin A5 antibodies in PAPS has not yet been explained. Previously, in a study which included 192 patients with SLE and only six patients with PAPS, it was found that the detection of anti-annexin A5 antibodies of the IgG and IgM isotypes was not relevant for the detection of patients at risk for thrombosis (62).

In agreement with the above-mentioned investigation, in a study (32) which included 44 PAPS patients, no correlation between anti-annexin A5 antibodies and arterial or venous thromboses was found. Furthermore, no association was found between anti-annexin A5 antibodies and MI or PE, which were the main complications of arterial and venous thromboses, respectively, in PAPS patients. Moreover, no significant difference in anti-annexin A5 levels among the investigated groups of patients was found, which suggests that those antibodies are not related with thromboses and their complications, but positive correlation was found between concentrations of the IgM isotype of anticardiolipin antibodies, anti-β2gpI antibodies and anti-annexin A5 antibodies of the same isotype.

Prospective studies have shown that the determination of anti-annexin A5 antibodies is not useful for the evaluation of the risk of abortions in early pregnancy of healthy women (63). Some studies have found reduced levels of annexin A5 on placental villi in women with APS, in comparison to placentas obtained from women with abortions in the absence of antiphospholipid antibodies, but data are contradictory. Only PAPS patients with a history of repeated abortions have significantly elevated IgG anti-annexin A5 antibodies concentrations, although this finding must be viewed with caution because it reflects a small number of patients with recurrent abortions (n=6) included in the study (34). Therefore, it is possible that the IgG isotype of anti-annexin A5 antibodies is a predictor for recurrent abortions in PAPS. Also, it is possible that those antibodies occur after one or more abortions, and thus represent an immunological epiphenomenon of abortions.

Conclusion

Considering that elevated oxLDL and anti-oxLDL antibodies concentrations were found in PAPS patients, and also in subgroups of PAPS patients with MI or PE, it could be concluded that those parameters represent additional risk factors which together with other factors may lead to thromboses and their complications in PAPS. Regarding the fact that C3 and C4 concentrations were decreased in PAPS patients and that a positive correlation was found between hsCRP and C3 concentrations, this finding could indicate the potential roles of these parameters as markers of atherosclerosis, which represents the leading cause of morbidity and mortality. HPT and apo(a) concentrations are not independent risk factors for MI in PAPS because lower levels were found in those patients in comparison to MI survivors without PAPS. No significant correlation of anti-anxA5 antibodies and the presence of arterial or venous thromboses or their complications was found, but increased concentrations of the IgG isotype of those antibodies could be a marker for recurrent abortions in PAPS, although this finding should be further investigated on a larger number of patients with this clinical finding. Determination of hsCRP in PAPS patients could not be an adequate parameter which would provide discrimination between patients at increased risk of development and/or recurrence of venous and/or arterial thromboses, nor of their complications, because no statistically significant difference in the concentrations of this parameter was found among PAPS, IM, PE and SLE patients who were included in this study.

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