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Antiphospholipid Antibodies and Methods of their Determination

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Instructions for processing:

Antiphospholipid antibodies represent an intensively researched heterogeneous group of antibodies directed against multiple phospholipids and their complexes with some proteins (e.g. β-2-glycoprotein I, prothrombin). The aim of the theoretically focused bachelor’s thesis is to obtain comprehensive and current information about antiphospholipid antibodies and methods of their determination. An overview of the different groups of antiphospholipid antibodies and the mechanism of their action will be given. Antiphospholipid syndrome (APS) will be described and specified by laboratory criteria, clinical signs such as repeated premature termination of pregnancy and thrombosis will be mentioned. Attention will be paid to diverse possibilities of determining antiphospholipid antibodies in biological fluids.

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Declaration

I hereby declare that I have completed this thesis with the topic of the Antiphospholipid Antibodies and Methods of their Determination independently and I have included a full list of used references.

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………………………
Juta Bulay
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Abstract

This Bachelor’s thesis describes problematic of antiphospholipid antibodies as an intensively researched group of antibodies causing various clinical manifestations such as antiphospholipid syndrome, thrombotic conditions and obstetric complications. Theory section will also summarize current information about antiphospholipid antibody types and their pathogenic functions.

Methodology section will describe various methods of classical spectrum of antiphospholipid antibody determination in biological fluids and newer tendencies of determination in clinical laboratories and researches. Results obtained by ELISA method will be presented in tables and graphs.

Keywords

Antiphospholipid antibodies; antiphospholipid syndrome; aPTT; ELISA; pathogenesis; recurrent pregnancy termination; thrombosis.
Abstrakt

Tato bakalářská práce popisuje problematiku antifosfolipidových protilátek jako intenzivně zkoumanou skupinu protilátek způsobujících různorodé klinické projevy jako: antifosfolipidový syndrom, trombózy a komplikace v těhotenství. Teoretická část bude také obsahovat aktuální informace o typech antifosfolipidových protilátek a jejich patologických účincích.

Metodická část popíše různé metody klasického stanovení antifosfolipidových protilátek v biologických tekutinách a nové možnosti jejich stanovení využívané v klinických laboratořích a výzkumech. Výsledky získané metodou ELISA budou prezentovány v přehledných tabulkách a grafech.

Klíčová slova
Antifosfolipidové protilátky; antifosfolipidový syndrom; aPTT; ELISA; patogeneze; předčasná ukončení gravidity; trombózy.
# Table of Contents

1 Introduction .................................................................................................................................................. 11

2 Current state .............................................................................................................................................. 12

  2.1 Historical background .......................................................................................................................... 13

  2.2 Antigens .................................................................................................................................................. 14

      2.2.1 β2-glycoprotein I .......................................................................................................................... 15

      2.2.2 Prothrombin .................................................................................................................................. 16

  2.3 Antiphospholipid antibodies ................................................................................................................ 16

      2.3.1 Lupus anticoagulant ....................................................................................................................... 17

      2.3.2 Anticardiolipin antibodies .............................................................................................................. 18

      2.3.3 Anti-β2-glycoprotein I .................................................................................................................... 20

      2.3.4 Antiprothrombin antibodies ........................................................................................................... 21

      2.3.5 Phosphatidylethanolamine antibodies ........................................................................................... 22

      2.3.6 Antiphospholipid antibodies against anionic phospholipids (except cardiolipin) ................. 23

      2.3.7 Genetic factors ............................................................................................................................... 24

  2.4 Pathophysiology .................................................................................................................................. 24

      2.4.1 Pathogenic effects of anti-β2GPI antibodies ................................................................................... 25

      2.4.2 Pathogenic effects of anti-prothrombin antibodies ........................................................................ 26

  2.5 Antiphospholipid syndrome ................................................................................................................ 27

      2.5.1 Thrombotic APS ............................................................................................................................. 28

      2.5.2 Obstetrical APS .............................................................................................................................. 30

      2.5.3 Non-criteria manifestations .......................................................................................................... 31

      2.5.4 Catastrophic APS ............................................................................................................................ 32

  2.6 Systemic lupus erythematosus ............................................................................................................ 34
1 INTRODUCTION

Antiphospholipid antibodies (aPL) represent an intensively investigated group of autoantibodies, regularly mentioned in recent researches. This heterogeneous group of antibodies is directed against various phospholipids and their complexes with other proteins such as β2-glycoprotein I (β2GPI) and prothrombin (PT). Due to pathogenic effect of these autoantibodies, their persistent occurrence can cause development of diverse autoimmune diseases (AID), primarily antiphospholipid syndrome (APS) and systemic lupus erythematosus (SLE).

Laboratory determination of aPL plays a significant role in APS diagnosis and early treatment, therefore market offers wide spectrum of methods appropriate for clinical laboratory use. Major determinations apply methods of immunology and hematology, providing reliable results.

Antiphospholipid antibody determination requires summarized knowledge and therefore it is necessary to obtain comprehensive and current information to understand their problematic.
2 CURRENT STATE

The immune system forms several mechanisms to protect the body against various invaders such as bacteria, viruses, parasites and fungi. The human body also uses many barriers (epithelia, blood-brain barrier, etc.) to eliminate the infection. Some diseases, however, can strike against these mechanisms and lead to an abnormal response of the immune system. This response leads body to attack its own tissues or organs causing functional disorder, inflammation or permanent harm on tissue. Such diseases causing this kind of reaction of the immune system are called autoimmune diseases. The activity of the immune system can be suppressed due to genetic factors such as inherited predispositions or environmental factors including infectious invaders. Some of the AID are more frequent than the others, e. g. diabetes mellitus with increased secretion of hormone insulin damaging pancreatic cells; immune-mediated thrombocytopenia characterized by immunoglobulins binding to blood platelets and causing their destruction. Other less common autoimmune diseases are antiphospholipid syndrome (APS) and systemic lupus erythematosus (SLE) caused by cells of the immune system and antibodies. [1] [2]

The antiphospholipid syndrome (APS) is an autoimmune disease characterized by arterial and venous thrombotic conditions with presence of antibodies recognizing phospholipid-binding proteins. APS includes other clinical manifestations such as premature spontaneous pregnancy losses, coronary artery disease, pulmonary emboli or thrombosis, APS can also develop into catastrophic antiphospholipid syndrome (CAPS) which is a rare life-threatening disease caused by disseminated intravascular thrombosis and followed by multiorgan failure. [3]

National Research Council (US) Subcommittee on Immunotoxicology suggests establishing a national registry of the AID to understand their distribution and to classify them. It is important to examine genetic and immune-system markers to recognize patients sensible to autoimmune diseases. These markers of
immunotoxicology could be used in epidemiology to confirm relationship between genetic inheritance, environmental influence and widespread presence of hypersensitivity. [4]

2.1 Historical background

The history of autoimmune antibodies started with a first case of anticoagulants described by Beaumont J.L. in 1954. In early 1960s circulating anticoagulants were revealed in patients suffering from disseminated lupus erythematosus. The vessel thrombosis and recurrent pregnancy losses started to be associated with antiphospholipid antibodies as well. At the same time several healthy patients and the ones suffering from AID received false positive tests for syphilis (i.e. having a positive result in the absence of this disease) as well as coagulation inhibitor lupus anticoagulant (LA) was discovered in their hematology tests. LA was not present in patients having syphilis. Although LA causes anticoagulation in blood tests, its effect in human body is almost reversed: it enhances coagulation causing thrombosis. In the early 1980s were developed several tests for detection of anticardiolipin antibodies (aCL), whose occurrence was also associated with thrombotic conditions and premature miscarriages. In the next years these antibodies were clarified to be similar but not identical, being a marker of APS but also causing various clinical manifestations of the disease. [2] [5]

The widest description of the aPL was developed by Dr. Graham Hughes and his team in 1983-86. This work was a summary of clinical observations and scientific studies which described an association of aPL with both arterial and deep vein thrombosis, neurological diseases such as stroke, pulmonary complications and premature pregnancy losses. He also differed anticardiolipin syndrome stated as a primary APS from systemic lupus erythematosus as a secondary APS. In the early 1980s several workshops were organized, and later, in 1984, historically first international APS meeting in Sapporo, Japan was convened in which classification criteria of APS (Hughes’ syndrome) were formed. [6]
Since then an increasingly wider knowledge about clinical manifestations and types of autoantibodies has led to a much concise definition of APS. [2]

2.2 Antigens

The main target of antiphospholipid antibodies are macromolecular structures bonded to polar lipid components, i.e. phospholipids (PL), which are the major component of cell membranes. Phospholipids appear with different charges, such as negatively charged phosphatidylserine, phosphatidylinositol, phosphatidic acid and cardiolipin, neutral phosphatidylcholine, or zwitteronic (dipolar ionic) phosphatidylethanolamine, as seen in Figure 1. Another important antigenic target are complexes of phospholipids bonded to plasma proteins such as β2-glycoprotein I, prothrombin, protein C, protein S, annexin V and kininogens. [7]

Phospholipids have significant and various biological functions. As a component of cell membranes, they maintain them and control functions of cell surface proteins. They are also responsible for initiation and continuance of coagulation cascade pathways. Phospholipids activate coagulation factors IX and X during the extrinsic pathway, factor X during intrinsic pathway, they transform prothrombin into the active thrombin during common pathway. Although in vivo experiments proved an important role of anionic phospholipids binding antibodies,
thrombotic events in patients are mainly caused by phospholipid-binding plasma proteins. [7]

2.2.1 β2-glycoprotein I

As an autoantigenic target for anti-β2GPI antibodies is recognized β2GPI, also called as apolipoprotein H. This single-chain polypeptide is normally present in human plasma at a concentration of 200 μg/mL. β2GPI consists of five homologous domains recognized as antibody epitopes. Each of domain I–IV consists of 60 amino acid chains with highly frequent prolines, cystines and tryptophans, when each domain bonds to half-cysteine residue with two disulfide bridges. The domain V differs from the previous and consists of 82 amino acid chains and three disulfide bridges, it is also positively charged and responsible for binding negatively charged phospholipids. Schematic structure is can be seen in Figure 2.

Figure 2 - Domain structure of β2-glycoprotein I. A – open conformation, B – circular conformation [9]

β2GPI as a free plasma protein needs to be provoked by antibodies to increase relatively low affinity for binding negatively charged phospholipids. This immunocomplex transforms β2GPI conformation and increases affinity to phospholipid binding sites. This complex interacts with lipoproteins and enzymes,
creating risk of thrombotic condition in patients with higher aPL antibody titers. [10]

β2GPI is a protein has other various functions, main in vitro function is to bind negatively charged phospholipids (cardiolipin, phosphaditylserine, etc.), lipoproteins (oxidized low-density lipoprotein (oxLDL)), heparin, phospholipid membranes of endothelial cells or activated blood platelets. Binding of β2GPI with activated platelets causes their aggregation through coagulation pathway or prothrombinase activity. More recently researches found involvement of β2GPI in apoptotic cell processes, in anticoagulation pathways of protein C and protein S, and even in lipoprotein metabolism of oxLDL. [10]

2.2.2 Prothrombin

Prothrombin (PT), also known as factor II, is a single-chain glycoprotein synthetized in liver and normally present in plasma at a concentration of 100 μg/mL. Hepatic biosynthesis converts PT into 10 γ-carboxyglutamic residues through γ-carboxylation. PT residues and their domains are responsible for calcium-dependent binding with phospholipids. This complex leads to PT transformation into α-thrombin, it’s biologically active form, in presence of prothrombinase complex including activated factor X, factor V, calcium and PL. Afterwards, thrombin causes polymerization of fibrinogen into fibrin and then binds fibrin domains. Activated thrombin also binds thrombomodulin present endothelial cell membranes and triggers protein C to express anticoagulation properties. [11]

2.3 Antiphospholipid antibodies

Antiphospholipid antibodies are very heterogeneous group of immunoglobulins of IgG and/or IgM, rarely IgA isotypes, directed against body tissues recognized as antigens. Action of this group of antibodies is associated with clinical manifestations of antiphospholipid syndrome such as acquired thrombophilic
conditions or premature miscarriages. aPL can be detected as a marker of autoimmune disease, but it also occurs in healthy patients as an accidental laboratory finding after infections. Incidence of aPL in general population rises to 5%, although presence of APS evolves only in small proportion of patients, mainly in elderly ones suffering from chronic diseases. aPL can also affect hematological tests by prolonging prothrombin time or activated partial thromboplastin time (aPTT). It has been widely documented, that the genetic factors shared by patients may participate. Research data are showing a high occurrence of aPL in family members of an APS patients. This research including genetic analyses and studies are suggesting an autosomal-dominant inheritance type of APS. [5] [7] [12] [13]

These antibodies can be divided in several ways: auto- and alloantibodies, drug-induced antibodies, eventually into primary or secondary occurring antibodies (the most common in rheumatic diseases, tumor processes, during bacterial or viral infection). However, the most effective distribution is based on the detection method of antibody binding on specific antigen. According to this method antiphospholipid antibodies are divided into these groups: lupus anticoagulant (LA), anticardiolipin (aCL) antibodies, anti-β2 glycoprotein I antibodies (anti-β2GPI) and other antibodies directed against anionic proteins or coagulation cascade proteins relevant to APS. [5] [13]

### 2.3.1 Lupus anticoagulant

As the heterogeneous group of autoantibodies affecting phospholipid-dependent coagulant reaction in vitro is recognized lupus anticoagulant. Lupus anticoagulant as a term was introduced by Feinstein and Rapaport in 1972 to mark out an inhibitor of coagulation that impairs prothrombinase activation of prothrombin. While performing laboratory immunology tests, LA primarily appeared in plasma of patients suffering from systemic lupus erythematosus. As a sensitivity of laboratory tests increased, LA antibody titers occurred in patients not
having SLE, as well as in healthy patients. In addition, it became apparent that it was not causing bleeding but rather thromboembolic complications. [14] [15]

LA is one of the autoantibodies of immunoglobulin IgG or IgM isotype directed against plasma epitope proteins (β2 glycoprotein I and/or prothrombin), phospholipids (cardiolipin and phosphatidylserine) and other protein complexes with anionic phospholipid surfaces (annexin V, protein C and S). This reaction has been testified by anticoagulation properties of purified anti-β2GPI antibodies in normal plasma. Laboratory tests based on prolonged phospholipid-dependent coagulation provided conclusion, that the action of these antibodies is inhibited by an excess of PL. LA activity and anti-prothrombin antibodies were discovered using a purified system consisting of human PT, coagulation factors (Xa and Va) and calcium. The occurrence of LA in healthy patients can be explained through the strong homology between β2GPI-related peptides and different pathogens (causing common infections), which both induce laboratory findings of LA in patient serum. [15] [16]

There is still no conclusive evidence on how exactly the mechanism of thromboembolic or obstetric conditions works, none of the scientific theories are completely convincing. One of the possibilities states that antibodies responsible for LA in vitro can impede the in vivo phospholipid anticoagulation pathway causing thrombofilic conditions. In addition, presence of LA can be explained by PL-dependent inactivation of coagulation cascade factors Va/VIIIa by the thrombomodulin – protein C – protein S system or by PL-dependent tissue factor (TF) inhibition. [15]

2.3.2 Anticardiolipin antibodies

Another group of antiphospholipid antibodies directed against body structures are anticardiolipin antibodies (aCL) of IgG, IgM and/or IgA isotypes. These autoantibodies are not primarily directed against cardiolipin, appearance of aCL antibodies associated with autoimmune diseases involves the presence of
phospholipid binding protein β2-glycoprotein I. Cardiolipin usually forms a complex with anionic phospholipids, mitochondrial membrane proteins and plasma protein cofactor β2-glycoprotein I. Complex of cardiolipin and β2GPI is considered as a main target for aCL antibodies. [17] [18]

At the beginning of 20th century, serological tests for syphilis used antibody binding to bovine heart cells. Later, this serological antigen was classified as a mitochondrial cardiolipin and still used as a primary antigen in Venereal Disease Research Laboratories (VDRL). Occurrence of positive VDRL tests for syphilis became widespread in patients suffering from SLE, without characteristic clinical syphilis manifestations. Using methods of radioimmunoassay (RIA) led to determination of aCL antibodies, their occurrence was also confirmed by thrombotic conditions of patients with SLE, later in patients with APS. [7]

Such an interest in researching aCL antibodies arose due to the pathophysiology mechanisms against phospholipids in cell membranes resulting antiphospholipid syndrome. This autoimmune disease is described by the risk of arterial and/or venous thrombosis and recurrent fetal losses in a presence of persistent aCL. Later researches from 2006-2007 in two hundred of continual diabetic patients with high risk criteria stated that the aCL immunological mechanisms may start pathological processes of diabetic microangiopathy and diabetic retinopathy via immune complex deposition. Another determination discovered aCL antibodies directed against endothelial antigens possibly causing initiation of vascular injury, which could be considered as a marker of endothelial dysfunction. There is also an association between presence of aCL antibodies and occurrence of vascular occlusive disease. [17]

In widespread clinical laboratory practice, it is necessary to perform testing both IgG and IgM aCL and LA antibodies to diagnose APS. The most common is determination of IgG aCL antibody isotype, appearing in progressive stages of autoimmune diseases. It is not appropriate to determine IgA aCL isotype for screening purposes, but it may be used in chosen cases. The aCL determination test
is positive in up to 80% of patients suffering from APS. However, these antibodies can be positive in disorders such as infectious diseases causing syphilis or Q fever, connective tissue diseases and acquired immune deficiency syndrome (AIDS). Synthesis of aCL can be also provoked by taking medication such as beta-blockers, hydralazine, interferon alfa, phenothiazine, phenytoin or narcotic substance cocaine. [7]

2.3.3 Anti-β2-glycoprotein I

Several independent researches stated, that the main antigenic target for aCL antibodies is complex of β2-glycoprotein I bonded to cardiolipin rather than cardiolipin alone. Nowadays, the β2GPI epitopes bonded to negatively charged phospholipids, are widely recognized as a clinically significant antigenic target for IgG, IgM, IgA anti-β2GPI antibody isotypes and suggested as a main antigen in the autoimmune disease pathogenesis. [10]

β2GPI, also known as apolipoprotein H, consists of five homologous domains, when domain I is the primary binding epitope for aPL, although other domains can also behave as epitopes. To prove clinical effect of anti-domain I antibodies, two similar studies were organized. First study supported role of these antibodies in venous thrombosis more than antibodies targeting other domains. This fact was proven by murine model injected with purified IgG isolated from APS patients. Mice formed bigger thrombus after standardized vessel injury. Second study connected anti-domain I antibodies with premature pregnancy losses. However, these studies need to be supported by additional testing before imparting specific anti-domain I β2GPI antibodies to panel of regular APS screening. [19]

β2GPI also has a natural anticoagulant character which is activated by interaction with phospholipid cell membranes of blood platelets or endothelial cells. This synergy is creating inviting surface for anti-β2GPI autoantibody production which leads to thrombotic conditions. In clinical and laboratory practice it is common to determine IgG anti-β2GPI isotype individually, or along
with aCL and/or LA antibodies for the APS diagnosis. More recently, isolated IgA anti-β2GPI antibody positive testing has been suggested to form a risk of thrombosis in APS apart from regular factors. This antibody isotype was also found in women suffering from premature spontaneous abortions and fetal deaths with negative results for LA and IgG aCL antibodies. Other studies demonstrated higher association of IgA anti-β2GPI antibodies with stroke and thromboembolic conditions rather than IgG or IgM isotypes. It is still preferable to perform other available laboratory tests of IgG and IgM isotypes of anti-β2GPI for the complete applicable diagnosis of APS. [10] [20]

Antiphospholipid antibodies can also recognize oxygenated surfaces in complex with β2GPI epitopes, which is already a complex of protein binding lipid. This bond makes phospholipid presence unnecessary. One of the main oxygenated surfaces creating complex with β2GPI is oxidized low-density lipoprotein of pathological atherosclerotic significance. Apart from thromboembolic events caused by autoantibodies, these complexes may induce development of premature atherosclerotic cardiovascular disease in patients suffering from APS or SLE. [10]

2.3.4 Antiprothrombin antibodies

Although the fact, that only LA, aCL and anti-β2GPI antibodies are included in the official recommendation for APS diagnosis, there are other types of antibodies directed against different phospholipids having similar pathogenesis. [10]

Researches starting from 1959 suggested prothrombin (PT, also known as a factor II) as a cofactor for lupus anticoagulant according to SLE patients suffering from bleeding complications. Several tests in vivo documented quick clearance of PT/anti-PT complexes resulting hypoprothrombinemia in samples with higher LA activity. These results led to describing autoantibodies against phosphatidylserine-prothrombin complex (anti-PS/PT) in patients with positive LA testing. Detection of these autoantibodies is much more specific using complexes of PS and PT together, rather than PT coat alone. Similarity between PT and β2GPI has been
widely described, making PT one of the main antigenic targets for antiphospholipid antibodies. Primary function of IgG anti-PT antibody isotype (in vitro) is to create immune complexes with PT or PL, causing elongation of clotting time. [11]

Several studies tried to find connection between anti-PT and APS clinical symptoms, neither of them found convincing evidence of this relationship. However, IgG isotype antibodies for PT/PS were described as a more specific for the APS diagnosis, probably mainly causing venous thrombosis. Even less convincing is fetal morbidity caused by anti-PT targeting prothrombin. [11]

2.3.5 Phosphatidylethanolamine antibodies

Multiple types of antiphospholipid antibodies were described as a cause of developing APS with various clinical manifestations, relatively newer researches found a relationship between APS symptoms and antibodies primarily directed against phosphatidylethanolamine (PE). However, anti-phosphatidylethanolamine antibodies (aPE) are rather binding complexes of PE and various plasma proteins from the coagulation cascade. Among these proteins are: factor XI, prekallikrein and other high-molecular-weight kininogens. [19]

Clinical studies suggested testing aPE antibodies in patients negative for laboratory criteria of APS, but strongly reminding APS clinical manifestations (pregnancy losses and/or thrombosis). Laboratory testing stated that aPEs were more frequent in women suffering from unexplained recurrent pregnancy loss than in healthy women with known cause of miscarriage. In addition, the most frequent aPL in infertile women was aPE, rising to 67.5% of all aPL-positive non-male patients. Studies from 2001 specified IgG aPE antibodies in women with unsuccessful in vitro fertilization or premature pregnancy losses. Laboratory testing of IgG aCL with IgG aPE or IgG aPE with LA (with positive finding) is used as a prognostic factor of severe pregnancy-induced hypertension. Recent studies using murine model, passively immunized with aPE antibodies, showed reduction
of litter, fetal losses, placental thrombosis, thrombocytopenia, characteristics close to the human APS manifestations. Specificity of a modern testing rises to 99.2% and helps to prevent recurrent abortions, certainly with using appropriate medication in advance. [19] [21]

Other studies focused on describing aPE as a cause of thrombosis, another clinical characteristic of APS. In 1992, aPE antibodies were detected in patient suffering from severe thrombosis and pulmonary embolism, with positive LA finding. Later in 1993, studies reported detection of sole aPE in SLE and thrombotic patients, without LA or aCL antibody finding. In 2001, laboratory tests in stroke patients revealed aPE as a most persistent serological finding (comparing to antiphosphatidylserine antibodies and aCL), These patients were also suspected of APS developing. Laboratory testing found 63% of 40 aPE positive patients, having negative result in regular APS laboratory criteria. Scientists described this type of APS as a seronegative. According to similar clinical manifestations of seronegative and seropositive APS, diagnosis should not be based exceptionally on classical laboratory criteria. However, medical conventions are considering about including aPE as an additional laboratory finding of APS. [19] [21]

2.3.6 Antiphospholipid antibodies against anionic phospholipids (except cardiolipin)

Panel of non-criteria aPLs is constantly expanding and describing new types of antibodies against various phospholipids. This panel includes negatively charged phospholipids such as phosphatic acid (PA), phosphatidylserine (PS) and phosphatidylinositol (PI), which are present in inner or outer cell membrane. [19]

Basic researches stated a convincing role of aPS, aPI and aPA in causing obstetric APS complications. In vitro results proved negative influence of aPS on trophoblast development, formation of placental epithelia, aPS also lowered human chorionic gonadotropin (hCG) levels. Studies also stated aPS antibodies to occur being more specific in APS diagnosis than aCL antibodies, although it appears during infectious diseases in patient serum. aPS are the most researched antibodies from
negatively charged antibody panel in APS, however, International Congress on Antiphospholipid Antibodies did not include aPA, aPI and aPS determination in routine laboratory diagnosis of APS. Adding mentioned autoantibodies into laboratory criteria for APS is still a debatable matter, methods require standardization among laboratories. [19]

2.3.7 Genetic factors

Better knowledge and understanding of aPL led researches to determine genetic factors of APS occurrence. It was found out, that the incidence of HLA-DR7 and -DR5 is increased in patients with aCL. With the implementation of the specific β2GPI-based enzyme-linked immunosorbent assay (ELISA) tests it became clear, that the HLA-DQB1 haplotypes may be causing anti-β2GPI antibody production, which are usually determined with aCL. Another alleles linked to HLA-DR4 haplotype, can be involved in developing of anti-β2GPI antibodies, but these associations mainly depend on different ethnic groups. These assignments are giving a vision of molecular epitope(s) reacting with autoimmune anti-phospholipids. [7]

Several researches reported that the genes in major histocompatibility complex (MHC) regions are causing many autoimmune diseases. It is possible that MHC alleles from class II (HLA-DR and DQ) are included in congenital genetic predispositions for developing LA. This fact was supported by increased laboratory findings of HLA-DQw7, relative to HLA-DR5 and -DR4 haplotypes, in LA-positive patients compared with race-matched normal controls. [7][15]

2.4 Pathophysiology

It has been widely described, that the antiphospholipid antibody appearance is a significant risk factor for thrombosis development. Experimental studies on mice supported these effects of aPL: enhancing platelet activation and aggregation, inducing adhesion molecules and endothelial cells, complement activation and
interruption of inflammatory function. Visual representation of the APS pathogenesis can be observed in Figure 3. It is an assignment of future researches to discover the sequence of these effects, but most likely they are connected. [7] [22]

Despite the fact, that the exact mechanism of thrombosis is still not agreed completely, many studies supported theory about antiphospholipid antibodies triggering protein C resistance in vitro which causes a pro-thrombotic phenotype. However, patients suffering from congenital protein C resistance develop not arterial, but only deep vein thrombosis (DVT), which makes acquired protein C resistance a side reason. [22]

![Visual representation of the APS pathogenesis](image)

**Figure 3 - Pathogenesis of APS [23]**

### 2.4.1 Pathogenic effects of anti-β2GPI antibodies

Many studies and experiments on mice, rats and hamsters provided convincing information about pathophysiological mechanisms applicable on human due to complete similarity of β2-glycoprotein I epitope. However, thrombotic mechanisms are not initiated after the antiphospholipid antibody application, it is necessary to lightly disturb vein structure or apply a low dose of lipopolysaccharide to achieve stronger thrombotic result. This experiment supported suggestion, that antiphospholipid antibodies need to be provoked by vascular injury. [22]
Other studies collected and differed antiphospholipid antibodies from the APS patients into subpopulations to verify stronger thrombotic effect of anti-β2GPI antibodies. These autoantibodies showed increased thrombogenicity especially after bonding to the first domain of β2GPI in rodents in line with the patient condition observation. These studies came to conclusion, that anti-β2GPI antibodies secure interaction between β2GPI and injured cells, including endothelial cells, monocytes, blood platelets, neutrophils, fibroblasts and trophoblasts. The final complex causes cell activation and release of activation markers characteristic for the cell type, which is the main reason for thrombogenicity. Endothelial cells start to release adhesion molecules, monocytes are characteristic for release tissue factor (also called factor III or CD142), activated platelets are producing thromboxane A2, which stimulates platelet aggregation. All these factors induce thrombotic conditions in patients with aPL, although it is not clear, if aPL have higher affinity to the specific cell type. [1] [22]

The complex of β2GPI and injured cells can also activate the complement system (more specifically C3 and C5), which is part of a non-specific innate immune system. Complement system consists of proteins circulating in blood as inactive precursors. The system is activated in cascades, stimulating release of cytokines, attracting phagocytes and activating membrane attack complex to damage pathogen’s membrane. Structure of β2GPI is similar with complement factor proteins, therefore β2GPI can control function of complement factor H, which regulates alternative complement pathway. Recent studies used eculizumab (medication, used as a terminal complement inhibitor) on patient with APS to prevent recurrent thrombotic risk, which makes complement system an important factor of pro-thrombosis phenotype. [7] [22]

2.4.2 Pathogenic effects of anti-prothrombin antibodies

Other studies experimented thrombogenic effect of anti-prothrombin antibodies injected into mice. According to these studies, prothrombin and β2GPI create an
immune complex with phospholipids, making it an important antigenic target for antiphospholipid antibodies. In vitro experiments stated, that monoclonal IgG anti-prothrombin antibodies directed against PT, are affecting damaged endothelial cells, causing tissue TF production. This binding of PT to endothelial cells leads to hypercoagulable condition, resulting thrombogenesis. Recent studies suggested anti-PT antibody inhibiting activity of prothrombinase and tenase complex (FIXa, FVIIIa, phosphatidylserine, Ca²⁺) by competing for the available phospholipid bonding space. In vivo experiments in murine blood sample described thrombus size enlarging, which supported thrombogenic effect of anti-PT antibodies. [11] [22] [24]

2.5 Antiphospholipid syndrome

Antiphospholipid syndrome was described approximately 30 years ago and is characterized by persistent presence of antiphospholipid antibodies with pathogenic features. APS is an immune condition causing various clinical manifestations such as vessel thrombosis, recurrent pregnancy losses or fetal deaths, coronary artery disease, pulmonary emboli or thrombosis, stroke and wide spectrum of skin diseases. International medical conventions created and summarized exact criteria for APS diagnosis and classification. [2] [25]

**Clinical criteria:**

- Thrombosis – at least one arterial, venous or small vessel thrombotic condition within any organ or tissue, confirmed by histopathological examination or applicable imaging method.

- One of the acquired pregnancy conditions:
  1. One or more pregnancy loss of morphologically normal fetus after 10th gestation week; anatomical, genetic and hormonal reasons for abortion are excluded.
  2. One or more premature delivery before 34th gestation week, caused by severe placental insufficiency or severe pre-eclampsia.
3. Three or more spontaneous abortions before 10th gestation week; anatomical, genetic and hormonal reasons for abortion are excluded. [2] [25]

Laboratory criteria (present in two different measurements 12 weeks apart):

- Positive detection of LA in plasma.
- Positive IgG and/or IgM aCL isotype in medium to high titers in serum or plasma.
- Positive IgG and/or IgM anti-β2GPI isotype in serum or plasma. [2] [25]

Although these typical autoantibodies characterize and classify APS, non-criteria antibodies can also be important in clinical diagnosis. Presence of aPL in general population rises to 5%, although APS development occurs mainly in elderly people, in patients taking medications, during lymphoproliferative diseases and after infections (AIDS, syphilis, hepatitis C, malaria etc.). Studies of Antiphospholipid Syndrome for Clinical Trials and International Networking provided approximate statistic evaluation of aPL prevalence causing various clinical signs: 6% in premature abortions, 10% in deep vein thrombosis (DVT), 11% in myocardial infarction and 14% in stroke. [25]

For the complete characteristic, APS was divided into primary and secondary APS. Primary APS occurs in patients without disease associated with aPL production, or evidence of agent causing the production. Secondary APS usually occurs in line with another disease, which probably caused aPL production, therefore approximately 40% of lupus patients could have secondary APS. However, primary and secondary APS can be identical in clinical manifestations and phenotype. [2]

2.5.1 Thrombotic APS

It has been accepted, that aPL are associated with developing thrombotic conditions. However, results of studies may differ from clinical laboratory results due to different detection methods, cutoff values and especially heterogeneity of
aPL isotypes. Another matter is equivalency of clinical manifestations in patients with different antibody results, when antibodies occur in different titers, in single, double or triple positivity. [14]

Results from recent studies suggested to determine multiple positive aPL tests to fully confirm thrombotic effect, although laboratory finding of single positive aPL supports risk of thrombosis development, usually in addition to cardiovascular or autoimmune diseases, hyperlipidemia or arterial hypertension. Experimental studies found stronger thrombotic effect of LA in single positivity tests in comparison to other single aPLs. Clinical research provided data collected from over 7000 patients, when LA positive patients achieved increased risk of stroke and myocardial infarction development, although additional anti-β2GPI positivity had no effect on myocardial infarction, but doubled the risk of stroke and thromboembolic events. Research of single positivity aCL influence did not confirm higher risk of stroke or myocardial infarction development. However, persistent positive aCL titers in SLE patients is a risk factor of thrombotic events, while occasional laboratory finding did not support thrombogenesis. [14] [25]

Multiple positivity, especially in triplet of LA, aCL and anti-β2GPI, has been described as highest risk in developing vascular thrombosis, usually in lower limbs. Study about laboratory accuracy listed the most appropriate combination of aPL isotypes, which can be used for APS laboratory diagnosis, but also in more specified cases of thrombosis and/or pregnancy losses. This profile includes LA, anti-β2GPI and anti-phosphatidylserine/prothrombin, and has the highest precision comparing to other isotype combinations. Multiple antibody positivity causes persistent antibody titers, as well as risk of repeated vascular thrombosis and/or thromboembolism. [14]

Overall, aPL are divided into groups with a risk of thrombosis:

- High-risk properties: LA positivity, triple positivity of LA, aCL and anti-β2GPI, persistent aCL positivity in medium-high concentrations.
• Low-risk properties: occasional positivity of aCL or anti-β2GPI in low-medium concentrations. [14]

Occurrence of aPL positivity in patients under 50 years causes ischemic stroke in 17%. Therefore, British Society of Haematology guidelines on investigation and management of APS included aPL screening in recommended testing for stroke in patients under 50 years. [25]

2.5.2 Obstetrical APS

Obstetrical complications caused by positive aPL were supported by experimental murine models. Studies introduced passive transfer of IgG aPL isotype as a risk factor of intrauterine growth restriction, placental thrombosis and inflammation. These manifestations are lethal and primarily cause recurrent pregnancy morbidity. [14]

For many years early pregnancy losses in aPL positive patients were explained by placental thrombosis and maternal-fetal blood exchange, as a main pathogenic mechanism of aPL. However, histological determination of APS patient placentas did not always show thrombotic changes, but rather inflammatory signs. In addition, maternal blood flow is not found in intervillous space (in vessels between mother and fetus) until 8th week of gestation, excluding thrombosis from causing early miscarriages. Recent studies demonstrated damaging aPL effect on placenta, impeding trophoblast differentiation, activating trophoblast apoptosis, promoting trophoblast to produce adhesion molecules and inhibiting trophoblast angiogenic factor production. These trophoblast mechanisms are affecting maternal endothelial cells, causing pre-eclampsia. [14] [25]

Nowadays, inflammation is included in main causes of pregnancy morbidity in aPL positive patients. Histological determination described neutrophil finding, complement release, secretion of tumor necrosis factor (TNF) in maternal part of placenta. In vivo murine experiments also described complement deficiency as a protective factor against obstetrical manifestations. In vitro studies supported aPL
trophoblast activation, which start to produce interleukin-1β and induce inflammation. [14]

Laboratory risk criteria of obstetric complications in aPL positive patients can differ from thrombotic events criteria. Formally, medium-high titers of aPL are considered as a significant risk of APS manifestations, pregnancy morbidity risk is also described by equivalent international classification criteria. Data from EUROAPS registry stated LA or triple aPL positivity in medium-high titers as a strongest risk of pregnancy complications. However, clinical and laboratory practice revealed low-titer female patients with the similar obstetric complications as high-titer patients. According to these data, studies suggest including low-titer aPL in obstetric complication laboratory criteria. In addition, previous occurrence of autoimmune disease (mainly SLE), thrombotic events, complement reduction, is generally a predictive factor of pregnancy losses. [14] [25]

Non-criteria aPL antibodies such as aPE, aPT/aPS, aPI and anti-A5 were proposed to describe obstetric complications and supported by high-titer laboratory finding in women with pregnancy losses. However, there is not an approved and standardized method among laboratories for determination non-criteria aPL. [14]

2.5.3 Non-criteria manifestations

Besides criteria of APS multisystem thrombotic manifestations, aPL induce diverse multiple organ system diseases. These manifestations include pulmonary emboli, myocardial infarction, livedo reticularis, stroke, heart valve disease, skin ulcers, thrombocytopenia etc. [2]

Heart diseases induced by aPLs are associated with high morbidity and mortality due to atherosclerotic properties of anti-β2GPI and other. These aPLs cause coronary artery disease and unstable angina pectoris due to insufficient blood supply to the heart. Atherosclerosis can also lead to coronary artery thrombosis, provoking classical myocardial infarction. Heart valve disease in high-
titer APS patients often causes heart valve thickening, making proper blood transport impossible. Significant amount of APS and SLE patients develop mitral valve injury causing reversed blood flow, which is lethal for 5% of mentioned patients. [2]

Main sign of APS is the vascular or vessel thrombosis in lower extremities, but approximately third of thrombosis patients suffer from pulmonary emboli. Pulmonary emboli are also associated with pulmonary hypertension in 2-3% of APS patients, which increases arterial lung blood pressure and may lead to lung failure. Rarely, APS and SLE patients may experience pulmonary hemorrhage into lung nodes, causing hemoptysis cough and dyspnea. [2]

Vessel thrombosis causes insufficient blood supply to the dermis and subcutaneous fat, resulting several dermatological manifestations primarily in secondary APS patients. The most common skin disease is livedo reticularis, which occurs in 25% of APS patients, mainly women. Other less frequent manifestation are skin ulcers, characterized by small skin lesions with intra-dermal hemorrhage. Rare manifestation such as superficial skin necrosis occurs in 2% hyper-coagulable APS patients and usually follows other autoimmune or malignant disorders. [2] [25]

Main hematological manifestation is thrombocytopenia, found in 20-40% of APS and SLE patients, often above severe platelet count (lightly above 50,000 per microliter). However, typical bruising and intra-dermal hemorrhage during thrombocytopenia is rarely seen. Another typical hematology laboratory finding in APS patients is prolonged activated partial thromboplastin time. [2] [25]

2.5.4 Catastrophic APS

Catastrophic APS (CAPS) is a severe and condition presented in less than 1% of APS patients, and characterized by multiple thrombosis in small vessels, usually causing multi-organ collapse. CAPS can be lethal and frequently occurs during pregnancy. Classification criteria of CAPS include multiple vessel thrombosis in at
least 3 organs and/or tissues, thrombotic condition development within a week, histopathological evidence of vessel thrombosis in at least 1 organ and/or tissue and laboratory finding of antiphospholipid antibodies LA and/or aCL types. [26]

International registry presented statistics and incidence of 500 CAPS patients. Over 70% of patients were middle-aged female, over 50% of patients suffered from primary APS and around third of patients suffered from SLE or similar autoimmune disease. 50% of patients experienced CAPS as a first occurrence of APS development, majority of patients also presented additional factors of CAPS development such as infections, malignances, contraceptives or pregnancy. CAPS without treatment developed with high mortality of 44%. [25] [26]

Diagnosis of CAPS may be complicated due to similarity with other thrombotic-like conditions. Laboratory findings confirm aPL positivity, primarily in LA and IgG aCL, patients often occur with triple positivity in LA, aCL and anti-β2GPI. Hematological determination confirms thrombocytopenia and prolonged aPTT in two thirds of patients, cell differentiation may determinate schistocytes. [26]

Signs and symptoms are various, but over three quarters of CAPS patients experience renal failure, other frequent manifestations are pulmonary emboli and acute respiratory distress, stroke and myocardial infarction, usual APS skin manifestations such include livedo reticularis, cutaneous ulcers and necrosis. CAPS can occur in early-term pregnancy in 48.2% and is strongly associated with previous recurrent fetal losses. CAPS during pregnancy can develop in many forms such as pre-eclampsia, hemolytic-uremic syndrome or thrombotic thrombocytopenic purpura, all related to hematological disorders. [25] [26]

Early diagnosis and treatment of CAPS should be set in patients with previous thrombotic conditions, diagnosed APS or other autoimmune diseases, in female patients with recurrent pregnancy losses. It is also important to identify additional factors such as infectious agents to predict CAPS development. Laboratory determination should include complete blood count with platelet count,
coagulation tests, immunological aPL determination, occasionally renal or liver functional tests. [26]

2.6 Systemic lupus erythematosus

SLE and APS were historically developed together, although later it was found that this combination is rather rare and occur in 20% of patients. SLE is described as a complex chronic autoimmune disease affecting multiple organ systems. It is characterized by aPL autoantibody production, complement activation and deposition of immunocomplexes. SLE was associated with developing thrombotic conditions independently, but 40% of SLE patients were diagnosed for aPL positivity. Statistics showed, that 9.1% of 1000 SLE aPL positive patients experienced thrombotic events, in 1.8% this condition caused death. It is also known that SLE and antiphospholipid antibody positivity (secondary APS) cause an increased risk of thrombosis comparing to single antibody positivity (primary APS), although SLE patients can still develop thrombosis with aPL negativity. Table 1 describes main thrombotic and obstetric manifestations related to APS associated with SLE and primary APS. [27] [28] [29]

<table>
<thead>
<tr>
<th>Table 1 - Manifestations associated with APS and SLE [29]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thrombotic manifestations</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Superficial thrombophlebitis</td>
</tr>
<tr>
<td>Deep vein thrombosis</td>
</tr>
<tr>
<td>Stroke</td>
</tr>
<tr>
<td>Transient ischemic attacks</td>
</tr>
<tr>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>Unstable angina</td>
</tr>
<tr>
<td>Pulmonary embolism</td>
</tr>
<tr>
<td>Glomerular thrombosis</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Pre-eclampsia/eclampsia</td>
</tr>
<tr>
<td>Early pregnancy loss (&lt;10 weeks)</td>
</tr>
<tr>
<td>Late pregnancy loss (&gt;10 weeks)</td>
</tr>
<tr>
<td>Live birth</td>
</tr>
<tr>
<td>Live birth with prematurity</td>
</tr>
<tr>
<td>Live birth with intrauterine growth restriction</td>
</tr>
</tbody>
</table>

SLE may occur in cohorts with genetic predisposition or may follow manifestations such as renal injury, cardiovascular diseases, infections and malignances, adding relevant risk of lethal outcome. This disease occurs primarily in young or middle-aged women, more often in African, Hispanic and Asian
population, being severe without early treatment. Although majority of SLE patients are female in ratio of 9:1 comparing to men, male patients appear with the same symptom severity. According to several studies and observations, female patients tend to develop arthritis and livedo reticularis, male patients were often diagnosed with arterial thrombosis in lower limbs, myocardial infarction and epilepsy. Apart from genetic predisposition and gender, predictive factors are age, disease activity and duration, hormonal changes, medication use (e.g. corticoids, hydralazine). [2] [27] [29]

2.6.1 Laboratory profile

Occurrence of aPL in SLE patients leads to pro-thrombotic conditions by activating prothrombotic and proinflammatory properties of endothelial cells. Therefore, thrombotic risk is described by aPL type, titer and their persistency. Similarly to APS laboratory criteria, multiple aPL positivity is considered as an increased thrombotic risk, especially triple positivity of LA, aCL and anti-β2-GPI. As a single positive, LA is considered as a most important antibody in thrombosis development, as well as medium-high persistent aCL titers. Among SLE patients, laboratory findings detect positive LA in 22% and aCL in 45% of antibody determinations. As a weak risk of thrombosis is considered single positivity of anti-β2GPI and occasional aCL findings. [29]

2.6.2 Pathogenesis mechanisms

Pathogenesis of SLE triggers almost every immune system mechanism, primarily affecting endothelial cells, causing thrombosis development. Pathogenic mechanism involves nuclear antigens, hyper-responsive B and T cells, which create basis for antibody formation. Endothelial dysfunction is caused by antibody formation, cell apoptosis defect, excessive lipoprotein and neutrophil function. Inflammatory processes are caused by SLE pro-inflammatory macrophages. [22]
Similarly to APS, thrombotic mechanisms can be triggered by vascular injury, which is considered as a primary cause of arterial thrombosis development. Therefore, SLE is described by both arterial and venous thrombosis. [22]

2.7 Treatment

Therapy of APS patients may differ due to condition severity. Main aim of the therapy is eliminating aPL using high-dosage steroid administration, immunosuppression and plasma exchange during severe conditions. However, 1-3 week pauses in medication cause rapid increase of antibody titers, therefore immunotherapy of usual APS is not indicated. Increased titers of aPL within 6 months after treatment cause higher risk of recurrent thrombosis, therefore patients with previous thrombotic events are referred to a life-long anticoagulant therapy. [30]

In case of occasional aPL finding, of first thrombotic event or low antibody titers, antiaggregant medication is administered within 3-6 months, attention should be paid to predictive thrombosis factors such as infections, oral contraceptives or other medication. [30]

Patients with definite APS diagnosis receive oral anticoagulants such as heparin, later accompanied or substituted with warfarin. Warfarin can also be taken with low-dose aspirin, low-molecular-weight heparin, hydroxychloroquine which prevents thrombotic and inflammatory events in SLE, statins which decreases risk of venous thromboembolism. Lately, clinical studies suggest including direct oral anticoagulants, being alternatives to vitamin K antagonists. [25] [30]

Obstetric APS patients receive low-dose aspirin and low-molecular-weight heparin during pregnancy. SLE and APS patients often receive more pregnancy-compatible medication such as corticosteroids and hydroxychloroquine. Warfarin is not considered as a medication during pregnancy because of its teratogenic effect. [25]
2.7.1 Treatment of CAPS

Life-threatening condition as CAPS requires combined and more aggressive treatment including anticoagulation with heparin, intravenous immunoglobulins, plasma exchange, high-dose intravenous glucocorticoids. Anticoagulation and anti-inflammation therapy should be started with intravenous unfractionated heparin dose, in case of any later intervention is required. Heparin administration should be continued in 7-10 days, later substituted with low-molecular-weight heparin if needed. Anticoagulant therapy is usually accompanied with immunosuppressive corticosteroids, decreasing the risk of aPL binding and thrombogenesis. In extremely severe conditions, intravenous immunoglobulins and plasma exchange are required. Immunoglobulins inactivate pathogenic antibodies and cytokines, while plasma exchange removes pathogenic mediators. CAPS can be also effectively treated with eculizumab, which inactivates complement protein C5 and prevents its pathogenic effect. [25] [26] [30]
3 AIM OF THESIS

The aim of this theoretically focused bachelor's thesis is to present current information about intensively researched antiphospholipid antibodies, their importance in clinical diagnosis and to summarize various methods of their determination in clinical laboratories.

Theory section will use recent medical sources to give an overview of different antiphospholipid antibody groups and isotypes, describing their antigens, mechanisms of action and pathogenesis. Theory will also mention autoimmune disease such as antiphospholipid syndrome with laboratory criteria description, which are necessary for clinical diagnosis. Clinical signs such as thrombotic conditions and recurrent termination of pregnancy will be described.

Attention will be paid to diverse possibilities of laboratory antiphospholipid antibody determination in biological fluids. Laboratory methods such as ELISA, aPTT will be described, as well as recent advances in antibody determination.
4 METHODOLOGY

Laboratory determination of antiphospholipid antibodies plays the most important role in APS diagnosis and early treatment. Classification criteria of APS including one or more unexplained thrombotic events, one or more pregnancy losses or fetal deaths, thrombocytopenia or prolonged clotting assay, should be considered as an indication for antiphospholipid antibody testing. Therefore, combination of clinical manifestations and medium to high-titer persistent antibody determination is used as a routine sign for APS diagnosis. However, it is important to distinguish patients with similar clinical criteria and occasional, but not pathogenic aPL, according to circumstances. [25]

Laboratory criteria consists of two indirect ELISAs with detection of antibodies against cardiolipin or β2GPI and LA determination. Positive laboratory APS diagnosis consists of two independent ELISAs of IgG/M aCL and/or IgG/M anti-β2GPI and/or LA determination. Two determinations are performed 12 or more weeks apart and require persistent antibody titer. Time interval helps to exclude occasional non-pathogenic antibody finding after infections, which are not necessarily related to APS clinical manifestations. LA determination consists of two clotting assays, factor Xa-dependent dilute Russell venom viper time (dRVVT) and more common aPTT, both parameters are prolonged in case of positive LA finding. [24][25]

Several laboratories use ELISA testing to detect non-criteria antibodies against phosphatidylethanolamine, phosphatidylserine, some proteins from coagulation cascade such as prothrombin, or on specific domains (DI) of β2GPI. Recently, researches used ELISA for determination of IgA aCL associated with APS. However, these specialized methods require expensive commercial kits and highly purified reagents, and yet are not validated in routine clinical laboratories. [25]

Apart from classical laboratory methods such as ELISA and aPTT, market provides variety of other methods appropriate for aPL determination. Recently
introduced chemiluminescence and fluorescence enzyme immunoassays are automated methods, which can decrease variability among laboratories. The new technology of aPL chemiluminescence provides good sensitivity and specificity, which helps in reliable identification of APS patients. Chemiluminescence method requires immunoanalyzer, which uses two-step immunoassay method with chemiluminescent reaction measured as a light signal. The concentration of specific antibodies in patient sample is proportional to generated light signal. [31] [32]

Other diseases with symptoms close to APS manifestations should be excluded by additional testing. Standard blood testing (complete blood count, blood chemistry tests, blood enzyme tests) can reveal alternative cause of aPL positivity due to viral or bacterial infections, malignancies, coagulopathies or patient medication. Erythrocyte sedimentation rate can point out inflammatory processes. Renal dysfunction can also induce aPL production, therefore renal function testing can exclude APS. Immunology tests on antinuclear antibody, extractable nuclear antigen and complement 3/4 antibodies can reveal SLE as a cause of aPL production. [25]

4.1 Enzyme-linked immunosorbent assay (ELISA)

Perhaps, the most used methods in immunology laboratory are enzyme immunotests, including enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA). These quantitative analytical methods provide precise measurement of very low-concentration molecules such as peptides, proteins, hormones, vitamins, etc. in various biological fluids and are standard methods in clinical diagnosis or research. [33]

Basic principles of EIA and ELISA are similar, both are using specific reaction between antigen and antibody in presence of enzyme bonding this immunocomplex. Antigen-antibody reaction is visualized by reaction of enzyme-linked conjugate and enzyme substrate causing color change of immunocomplex, making it usable for spectrophotometer measurement. [33]
4.1.1 Minimal requirements on antiphospholipid ELISA determination

Despite long-term application and standardization of ELISA method in laboratories, results can be affected by using different kits, working protocols or homemade assays. Inter-laboratory variability in antiphospholipid ELISA determination can cause significant differences in wide, sometimes international studies. Therefore, European experts on APS matter classified following minimal requirements to improve accuracy of antiphospholipid (especially aCL) ELISAs. [34]

- Despite relatively high expenses on kits and reagents, European aPL forum suggests performing determination in duplicate.
- Commercial kits usually provide their own cutoff values neglecting additional factors such as age or infections. Therefore, committee suggests determination of individual cutoff level in each laboratory based on 50-100 samples of normal subjects.
- Committee also suggests colligation of aPLs units and calculating cutoff values in percentiles.
- External control is an essential in laboratory practice, therefore, committee suggests including them in two concentration levels: one under cutoff and one medium positive. [34]

4.1.2 Types of ELISA

Enzymatic immunoassay can be divided into homogeneous and heterogeneous general groups. Homogenous enzymatic assays use enzymes, which become inactivated after binding on antibody, therefore washing is unnecessary. This method is rarely used due to high pricing and low sensitivity. Heterogeneous enzymatic immunoassays are widely used in routine laboratory practice, mainly because of the high sensitivity. Main difference is using washing mechanism as a middle step to prevent bonding of any other unwanted molecule. Heterogenous ELISA is used in detection of specific antibodies and antigens, although variety of
these molecules require specific types of this method described below in Figure 4 - Types of ELISA. [33]

![Types of ELISA](image)

**TYPES OF ELISA**

HOMOGENOUS (For Special Use)  
HETEROGENOUS (For General Use)

- Direct  
- Indirect  
- Sandwich  
- Competitive

Schematic principle is described in Figure 5. [33]

Direct ELISA was developed in 1971 for antigen/antibody screening purposes and was a starting point for developing other types of ELISA. This method is suitable for high molecule-weight antigen determination. Direct ELISA require microplates previously coated with antigen/antibody, enzyme-labeled antibody and washing procedures after each incubation step to remove any excess antigen/antibody. Visualized signal is provided by the appropriate enzyme substrate in complex with enzyme conjugate, and is measured in the spectrophotometer. Final concentration is proportional to coloration intensity.

![Direct ELISA](image)

Figure 4 - Types of ELISA [33]

Figure 5 - Direct ELISA [33]
Development of indirect ELISA was based on knowledge about widely applied direct ELISA type. This method requires wells coated with antigen, where examined serum is added. During incubation time, antigen binds antibody, creating immunocomplex. Recognition of this complex is possible after adding secondary antibody tagged with enzyme. Color visualization is provided by adding enzyme substrate, final concentration is measured in the spectrophotometer and is proportional to coloration intensity. Schematic principle is described in Figure 6. [33]

![Indirect ELISA](image)

**Figure 6 - Indirect ELISA [35]**

### 4.1.3 Principle of ELISA determination

The process of ELISA determination takes place in a solid phase of carriers, usually microplates made of polystyrene, polyvinyl or polypropylene, or similar material allowing antigens/antibodies to attach. The material should not be adhesive for unwanted molecules. Attached antigen/antibody reacts with enzyme-conjugate, this complex later reacts with substrate to provide color change. The most common enzymes used in clinical laboratories are: beta galactosidase, glucose oxidase, peroxidase or alkaline phosphatase with different conjugates. The most used enzyme is peroxidase with 5 amino salicylic acid or ortho-phenylenediamine substrate, causing brown color in case of positive finding. Every step of ELISA determination is followed by incubation (time highly depends on determined antigens/antibodies) and washing. The specific enzyme-substrate reaction is completed within 30-60 min and is stopped by sodium hydroxide (NaOH), hydrochloric acid (HCl) or sulfuric acid (H₂SO₄). To complete ELISA determination,
results of concentrations are measured on a spectrophotometer at 400-600 nm wavelengths depending on properties of conjugate. [33]

4.1.4 ELISA determination of IgG aCL

During practical determination of aCL antibodies I had an opportunity to participate in Department of Immunology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, using ORGENTEC Diagnostika GmbH kit (Mainz, Germany) designed for quantitative determination of IgG/IgM anticardiolipin antibodies in patient serum or plasma. All the following results were determined using kit for IgG aCL antibodies. Principle of this determination is based on indirect enzymatic immunoassay and require microplates coated with β2GPI and highly purified cardiolipin. Anticardiolipin antibodies in patient serum sample bind to the antigen (cardiolipin) in the microwells. After incubation, washing procedure removes any unbound and unspecifically bound serum elements. Added enzyme conjugate binds antigen-antibody immunocomplexes. After second incubation, washing procedure removes any unbound enzyme conjugate. Added substrate solution binds enzyme conjugate causing blue coloration of the product in wells. Before quantitative measurement, reaction is stopped by hydrochloric acid creating yellow product in wells. Concentration of antigen-antibody product correlates with the intensity of yellow color measured in reader (spectrophotometer) at 450 nm. [36]

Contents of the kit:

- Microplate coated with cardiolipin and β2GPI (consisting of 12 modules of 8 wells each);
- six calibrators 1,5 ml with IgG anticardiolipin antibody concentration range (0 U/ml, 7,5 U/ml, 15 U/ml, 30 U/ml, 60 U/ml, 120 U/ml) (previously diluted);
- positive and negative controls 1,5 ml containing cardiolipin antibody (previously diluted);
- enzyme conjugate 15 ml, animal immunoglobulin against human IgG tagged with peroxidase;
- sample diluent 15 ml (5x concentrated);
- TMB substrate 15 ml (previously diluted);
- stop solution 15 ml (1M HCl, previously diluted);
- washing buffer 20 ml (contains Tris, detergent, sodium azide, 50x concentrated). [36]

Materials required:
- Microplate reader (spectrophotometer), computer with an appropriate software;
- laboratory timing device;
- pipettes for 10-200 μl;
- vortex mixer;
- measuring calibrated cylinder for 1000 ml and 100 ml;
- distilled or deionized water;
- plastic container. [36]

Preparation of reagents:
- Wash buffer is prepared by diluting one vial of buffered wash solution concentrate (50x) with distilled or deionized water to a calibrated cylinder of 1000 ml.
- Sample buffer is prepared by diluting one vial of sample buffer concentrate (5x) with distilled or deionized water to a calibrated cylinder of 100 ml.
- Patient samples are diluted in 1:100 ratio before the assay. Prediluted sample buffer (990 μl) is added to patient serum (10 μl) in polystyrene tube. It is necessary to mix the diluted serum on vortex.
- Calibrators and controls are ready to use without dilution. [36]

Test procedure:
1) Prepare enough microplates for calibrators, controls and patient samples.
2) Pipette 100 μl of calibrators, control and patient samples into the wells, following pipetting scheme described in Figure 7.

3) Incubate for 30 minutes at room temperature (20-28 °C).

4) Discard the contents of the microwells and wash 3 times with 300 μl of wash solution.

5) Dispense 100 μl of enzyme conjugate into each well.

6) Incubate for 15 minutes at room temperature.

7) Discard the contents of the microwells and wash 3 times with 300 μl of wash solution.

8) Dispense 100 μl of TMB substrate solution into each well.

9) Incubate for 15 minutes at room temperature.

10) Add 100 μl of stop solution (1 M HCl) into each well.

11) Incubate for 5 minutes at room temperature.

12) Read the optical density within 30 minutes at 450 nm and calculate the results. [36]

![Figure 7 - Recommended pipetting scheme, individual numbers represent patients [36]](image)

4.1.5 Calibration curve of IgG aCL ELISA

Calculation of the results is provided by creating a calibration curve. For quantitative results plot the optical density (absorbance) of each calibrator versus the calibrator concentration. The concentration of patient samples may be
estimated due to calibration curve interpolation. The calculation range of this IgG aCL ELISA determination is 0 – 120 GPL-U/ml. [37]

Table 2 - Concentration and absorbance of calibrators [37]

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>Concentration [GPL-U/ml]</th>
<th>Optical density/Absorbance A at 450 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>0.018</td>
</tr>
<tr>
<td>B</td>
<td>7.5</td>
<td>0.168</td>
</tr>
<tr>
<td>C</td>
<td>15</td>
<td>0.316</td>
</tr>
<tr>
<td>D</td>
<td>30</td>
<td>0.577</td>
</tr>
<tr>
<td>E</td>
<td>60</td>
<td>1.039</td>
</tr>
<tr>
<td>F</td>
<td>120</td>
<td>1.852</td>
</tr>
</tbody>
</table>

Figure 8 - Graph of the calibration curve for IgG aCL determination

4.2 Activated partial thromboplastin time (aPTT)

Most important antiphospholipid antibody affecting one or more in vitro coagulation cascade reactions is lupus anticoagulant. Diagnosis of APS and long-term treatment with anticoagulant medication provide a risk of recurrent thrombosis, therefore appropriate diagnosis of LA in APS patients is necessary. Recently, International Society on Thrombosis and Haemostasis (ISTH)
summarized criteria for the laboratory identification of LA. ISTH recommended performing two or more mixed screening tests, including dRVVT and sensitive aPTT. Prolonged clotting assays can primarily indicate positive LA. These results should be supported and confirmed with additional phospholipid-dependent coagulation assays such as prothrombin time, silica clotting time, index of circulating anticoagulant or kaolin clotting time in SLE factor deficient patients. Certain medication such as rivaroxaban or dabigatran (similar effect as warfarin) can result prolonged aPTT without LA positivity, these observations should be taken in account during clinical diagnosis. [9] [38]

4.2.1 aPTT determination

Determination of aPTT is a routine component of hematological screening tests used to detect intrinsic clotting factors (VIII, IX, XI, XII) deficiency and presence of thrombin inhibitor lupus anticoagulant. [39]

I had an opportunity to assist during practical determination of aPTT in Department of Hematology and Blood Transfusion, Military University Hospital Prague, using reagents aPTT-SP (United States) in closed system IL measured on hematological analyzer ACL TOP 500 CTS. aPTT is most commonly measured in patient plasma collected in blue tube containing citrate. Analyzer aPTT measurement starts with adding partial thromboplastin to phospholipids, silica or kaolin and calcium chloride (CaCl₂). In presence of calcium, partial thromboplastin activates coagulation factors, particularly intrinsic coagulation cascade, which requires transformation of prothrombin to thrombin, i.e. plasma clotting. Intrinsic coagulation cascade is regulated by clotting elements such as factor II, V, VIII, IX, X, XI, XII, fibrinogen, prekallikrein or kininogens. Produced thrombin activates transformation of fibrinogen to fibrin. Fibrin clot is measured by optical detection in automatized analyzer. Summarized scheme is described in Figure 9. [40]
Results are measured in seconds, recommended reference range is 24.0 to 36.0 seconds. Critical values ranging to 100 seconds are immediately reported to appropriate department. Shortened aPTT is not considered as clinically relevant, although critical values may indicate thromboembolic conditions. Apart from LA positivity, prolonged aPTT may indicate coagulation factors insufficiency or presence of their inhibitor, sepsis, use of heparin (usually as sample contamination). [40]

Contents of the kit:

- Five vials of Hemosil aPTT-SP reagent 9 ml;
- five vials of Hemosil CaCl₂ 8 ml;
- ten vials of Hemosil Normal Control Assayed 1 ml (lyophilized plasma);
- ten vials of Hemosil Low (High) Abnormal Control Assayed 1 ml (lyophilized plasma with declared values).

Materials required:

- Analyzer coagulometer ACL TOP 500 CTS;
- original cuvettes and stands;
- uncooled centrifuge;
- calibrated automatic pipettes.
Preparation of reagents:

- Reagents are ready to use without diluting. It is necessary to lightly mix vials and temper to a room temperature.
- Normal and Low (High) controls are prepared by diluting one vial 1 ml in demineralized water. It is necessary to lightly mix vial and temper 30 minutes at room temperature. [40]

Test procedure:

1) Sample needs to be centrifuged and correctly identified.
2) Test tube with patient plasma needs to be placed in analyzer.
3) Methods and measurement of aPTT should be regulated according to technical standard operation procedure of analyzer.
4) Adding of CaCl₂ reagent starts measurement of optical time. Any sample or reagent dosage, incubation time and measurement time are programmed in analyzer by manufacturer and distributor. [40]
5 RESULTS

All the results were kindly provided by Department of Immunology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, and they will be reworked as tables and graphs. The results were collected using IgG Cardiolipin ELISA ORGENTEC Diagnostika GmbH kit (Mainz, Germany) designed for quantitative determination of antiphospholipid antibodies.

Validation of the results is regulated by kit distributor and is described in the Certificate of Analysis enclosed to each test kit. Test results are valid if the optical densities at 450 nm for calibrators and controls comply with the reference ranges stated in the Certificate of Analysis. If these quality control criteria were not accomplished, result is invalid and determination should be repeated.

5.1 Results of the IgG aCL ELISA

The results provided by Department of Immunology summarized number of IgG aCL determinations from 2012 – 2017 showing numbers of negative and positive patients ranging in different aCL titers. Table 3 below describes IgG aCL positive and negative control ranging applicable on patient result evaluation. Negative control result includes patients with low antibody titer <10 GPL-U/ml due to low diagnostic value and is not considered as a relevant laboratory finding.

Table 3 - Expected results of IgG aCL controls [37]

<table>
<thead>
<tr>
<th>Control</th>
<th>IgG aCL target [GPL-U/ml]</th>
<th>IgG aCL range [GPL-U/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>50</td>
<td>40 – 60</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>&lt; 10</td>
</tr>
</tbody>
</table>

Following patient results, I have compiled graphs describing IgG aCL development in period from 2012-2017. Figure 10 represents determination development and describes reduction of requested determinations in male and female patients. Figure 11 represents occurrence of positive patients ranging from
>10 GPL-U/ml. In spite the reduction of determinations, number of positive patients increased in 2013 and stayed approximately persistent until 2017.

**Figure 10 - Requested determinations of aCL in years 2012-2017**

**Figure 11 - Development of aCL positivity in years 2012-2017**

Number of positive male and female patients from previous Figure 11 is expressed in percentage of positive male and female patients in Figure 12. Reduction of requested examinations rather increased incidence of positive patients from examined cohort. Percentage of positive laboratory findings increased from 1.397% in 2012, to 2.808% in 2013 and up to 4.135% in 2017.
Figure 12 - Development of aCL positivity expressed in percentage in years 2012-2017

Following Figure 13 describes occurrence of positive male and female patients in 2017 ranging by aCL concentrations. Ranges of aCL concentrations are divided into 11-20 GPL-U/ml, 21-50 GPL-U/ml, 51->100 GPL-U/ml represented by columns. Majority of patients (166 male and female) with titers <11 GPL-U/ml are not included in graph according to negative results stated in the kit. Low antibody titer ranging from 21-50 GPL-U/ml was equal in male and female patients, although medium-high titers from >50 GPL-U/ml showed prevalence of positive aCL in female patients.

Figure 13 – Occurrence of positive male and female patients in 2017
Following Figure 14 represents positive patients with detected IgG aCL positivity range. Considering the fact, that standard operation procedure provided by ORGENTEC Diagnostika GmbH kit (Mainz, Germany) consider negative result as <10 GPL-U/ml antibody titer, graph included only patients with >11 GPL-U/ml. Ranges of aCL concentrations are divided from 11-20 GPL-U/ml, 21-50 GPL-U/ml, 51-100 GPL-U/ml and >100 GPL-U/ml represented by columns.

Figure 14 – Development of positive aCL patients
6 DISCUSSION

Antiphospholipid antibody problematic is widely mentioned in recent researches. Such an interest is given especially due to accretive aPL incidence since the classification criteria were summarized. Low titer of aPL can be determined in a tenth of healthy adults and up to 1% of the population is diagnosed with medium-high titer aPL positivity. Antiphospholipid antibodies were found in a tenth of stroke patients, with almost 30% incidence in young stroke patients. These antibodies are also found in a fifth of female patients having three or more recurrent pregnancy losses, causing placental thrombosis and inflammations. aPL were also found in one-seventh of patients with recurrent venous thrombosis. Described epidemiology is a significant reason for reliable identification of APS patients, precise laboratory determination and correct early treatment of the symptoms. [42]

Comprehensive knowledge about antiphospholipid antibodies and methods of their determination is an essential part of autoimmune disease diagnosis. Clinical studies and research regularly provide new methods and advanced techniques in aPL determination. However, International Hematological and APS conventions still recommend validated and standardized methods such as ELISA and aPTT. [25]

According to the results provided by Department of Immunology about aCL determination it is clear, that percentage of patients with positive aPL had increased in years 2012-2017. This development can assume catchment of higher percentage of aPL positive patients in following years. Laboratory methods as ELISA still require qualified laboratory staff and a relatively long working process, which can postpone early diagnosis (especially in extreme cases of CAPS or higher number of patient samples). Therefore, future laboratory development supposes wider implication of automatized methods. Recent innovations include chemiluminescence and fluorescence enzyme immunoassays, which can increase laboratory reproducibility and decrease interlaboratory variability. It is a matter of
future solutions, whether International Hematological and APS conventions will accept these methods and include them in laboratory criteria of APS. [31] [32]

Provided results also showed reduced number of requested aCL determinations in years 2012-2017. Preliminary clinical determination of patient plays an important role in preanalytical phase of laboratory requests. Detailed anamnesis of patient condition can predict diagnosis and constrict number of patients in need for aPL ELISA determination.

Although criteria laboratory methods cover majority of APS patients, some of the patients show similar manifestations without criteria aPL positivity. These patients require non-criteria antibody testing including anti-prothrombin, anti-phosphatidylethanolamine etc., but mentioned specialized ELISA methods are not standardized among laboratories and quite inconsistent. Additionally, it is inconvenient for routine laboratories to perform such specialized methods due to lower incidence of non-criteria antibodies in patients. For example, Department of Immunology, Motol University Hospital, as one of the biggest immunology centers in Czech Republic, uses wide spectrum of methods and owns unique analyzers. However, the only antiphospholipid antibody testing is anticardiolipin ELISA, which is performed once a week. Certainly, this solution is understandable due to an economy reasons, however rare APS patients showing aCL negativity will stay undiagnosed. Recent researches with non-criteria antibody cohort recommended implication of non-criteria antibodies in APS laboratory criteria. It is a matter of debate, whether International conventions will find a solution of standardization mentioned methods. [10] [19]

Antiphospholipid antibody problematic is an indeed widespread matter in immunology clinical practice. Involving variety of manifestations, APS diagnosis requires comprehensive knowledge and experience from clinician, as well as precise processing from medical laboratory technicians.
7 CONCLUSION

This bachelor’s thesis presented summarized theoretical information about antiphospholipid antibodies causing diverse clinical manifestations and list of determination methods. Theoretical information can serve as a guide through antiphospholipid antibody problematic, involving full characteristic of antigens, antibodies, their functions and pathogenetic mechanisms. Attention was paid to list main autoimmune diseases such as antiphospholipid syndrome and systemic lupus erythematosus with laboratory and clinical profile. It was also important to mention primary treatment methods, which save lives in extreme cases such as CAPS.

For a medical laboratory technician branch, it is necessary to have a comprehensive overview of laboratory methods and advances in trends slowly occupying the market. The emphasis has been placed on ELISA and aPTT methods used in a routine service for aPL determination. Precise analytical phase is an essential component of clinical diagnosis, therefore experience and knowledge of laboratory technicians can significantly help in determination of APS patients.
## 8 LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>aCL</td>
<td>anti-cardiolipin antibody</td>
</tr>
<tr>
<td>AID</td>
<td>autoimmune disease</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>aPL</td>
<td>antiphospholipid antibody</td>
</tr>
<tr>
<td>APS</td>
<td>antiphospholipid syndrome</td>
</tr>
<tr>
<td>β2GPI</td>
<td>beta-2 glycoprotein I</td>
</tr>
<tr>
<td>CAPS</td>
<td>catastrophic antiphospholipid syndrome</td>
</tr>
<tr>
<td>dRVVT</td>
<td>dilute Russell venom viper time</td>
</tr>
<tr>
<td>DVT</td>
<td>deep vein thrombosis</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>ISTH</td>
<td>International Society on Thrombosis and Haemostasis</td>
</tr>
<tr>
<td>LA</td>
<td>lupus anticoagulant</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>oxLDL</td>
<td>oxidized low-density lipoprotein</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatic acid</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PL</td>
<td>phospholipid</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>PT</td>
<td>prothrombin</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>TF</td>
<td>tissue factor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>VDRL</td>
<td>Venereal Disease Research Laboratories</td>
</tr>
</tbody>
</table>
9 REFERENCES


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10 LIST OF FIGURES

Figure 1 – Different charge phospholipid representation [8] .......................................... 14
Figure 2 - Domain structure of β2-glycoprotein I. A – open conformation, B – circular conformation [9] ........................................................................................................... 15
Figure 3 - Pathogenesis of APS [23] .................................................................................... 25
Figure 4 - Types of ELISA [33] ............................................................................................ 42
Figure 5 - Direct ELISA [35] ............................................................................................... 42
Figure 6 - Indirect ELISA [35] ............................................................................................ 43
Figure 7 - Recommended pipetting scheme, individual numbers represent patients [36] .............................................................................................................................................. 46
Figure 8 - Graph of the calibration curve for IgG aCL determination ................................. 47
Figure 9 - Scheme of aPTT determination [41] .................................................................. 49
Figure 10 - Requested determinations of aCL in years 2012-2017 ..................................... 52
Figure 11 - Development of aCL positivity in years 2012-2017 ....................................... 52
Figure 12 - Development of aCL positivity expressed in percentage in years 2012-2017 ......................................................................................................................................... 53
Figure 13 – Occurrence of positive male and female patients in 2017 ............................... 53
Figure 14 – Development of positive aCL patients ............................................................. 54
11 LIST OF TABLES

Table 1 - Manifestations associated with APS and SLE [29] .............................................. 34
Table 2 - Concentration and absorbance of calibrators [37] ................................................. 47
Table 3 - Expected results of IgG aCL controls [37] .............................................................. 51
12 LIST OF ATTACHMENTS

Attachment 1 – Standardní operační postup – metodický č. IISOPM_8IMUNO126/2008-2, Protilátky IgG proti kardiolipinu (ACA) - kvantitativně