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Genetics of Antiphospholipid Syndrome

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1. Introduction

Antiphospholipid Syndrome (APS), also known as Hughes Syndrome in honor of the doctor who first described it, is an autoimmune disease characterized by clinical manifestations such as vascular thrombosis (both arterial and venous), and/or recurrent pregnancy loss along with the presence of persistently elevated antiphospholipid antibodies (aPL) titers in serum (Bertolaccini et al., 2006). The etiology of APS, however, is still unknown. Incidence of disease remains unknown; however, the reported prevalence of aPL in the general population is low (1-4.5%) and increases with age (http://www.orpha.net/data/patho/GB/uk-APS.pdf in Orphanet, INSERM MIM nº 107320). APS can involve almost any organ system, including a wide range of clinical manifestations. The clinical involvement of different organs and systems poses the question of whether the syndrome should be considered a true systemic autoimmune disease, rather than an acquired autoimmune coagulopathy (Shoenfeld et al., 2008). Patients with this syndrome often have systemic lupus erythematosus (SLE) or related autoimmune diseases. In this case, we refer to the disease as secondary APS. The syndrome may also occur in the absence of such diseases and it is then known as primary APS.

Most autoimmunes diseases (AID) have a genetic background, but this hereditary component is not as obvious as diseases which are transferred from a parent to his children in half of quarter of cases. On the other hand, it is relatively common that members of the same family will have different AID. The conclusions of the genetic research in the field of primary APS are that this syndrome is significantly different in its genetic aspects from Systemic Lupus Erythematosus (SLE) (even though secondary APS might occur during lupus). Like other autoimmune diseases, APS is a complex multifactorial and polygenic disorder caused by interactions between multiple genes of small to modest effect in combination with environmental factors. Although complex disorders often cluster in families, they do not have a clear-cut pattern of inheritance. This makes it difficult to determine a person's risk of inheriting or passing on these disorders (Horita et al., 2004).

There is little doubt as to the pathogenic role of aPL in determining the clinical manifestations of APS, even if their mechanism of action has not been fully clarified. Although the pathogenic role of aPL is widely accepted, the fact that aPL induces

thrombotic events only occasionally suggests the need for a "seconf hit" to display the thrombogenic effect. The question of whether other environmental triggers or a genetic individual susceptibility can behave as a second hit is still open. Despite much research effort over the last 25 years, we do not know how aPL increases the risk of thrombosis and recurrent fetal loss in patients with APS. Many theories have been proposed to explain the increased thrombotic tendency in patients with aPL, but unfortunately none of them has been proven by convincing evidence.

2. Antiphospholipid antibody syndrome: Phenotype assessment

In the decades following the recognition of APS as a distinct entity, there came increasing calls for a consensus on the criteria required for accurate diagnosis of these patients. In response, an International workshop was held in Sapporo, Japan (Wilson et al., 1999) with the sole aim of producing classification criteria that would allow further investigation and study of the syndrome. This expert workshop's result was a group of criteria divided into clinical and laboratory findings. The stated requirements were that patients must meet at least one clinical and one laboratory criteria in order to be classified as having APS. The clinical manifestations focused on vascular thrombosis and obstetric complications whilst the laboratory criteria required the presence of either Lupus Anticoagulant (LAC) or Anticardiolipin antibodies (aCL). These antibodies had to be present on two separate testings at least six weeks apart. The Sapporo criteria were afterwards revised and updated in 2006. These updates resulted in two important amendments. Firstly, the addition of a new laboratory criteria, anti-beta 2 glycoprotein-I antibody. This antibody is now recognised as being crucial to APS pathogenesis and is in fact an independent risk factor for thromboses. Secondly, it was advised that the time delay between serological testing should be extended to twelve weeks instead of the original six so as to avoid positive results caused by transient rises in autoantibody titres. It should be noted that these criteria were not developed with clinical situations in mind but were specifically aimed at encouraging clinical trials in the area. Despite this, there remains no alternative for clinicians who simply need accurate guidance in providing valid diagnoses for their patients. As previously mentioned, the potential APS clinical manifestations are numerous and widespread. This can be illustrated by listing a myriad of clinical specialities that can be involved in a patient's management-rheumatology, neurology, cardiology, nephrology, endocrinology, gastroenterology, dermatology, surgery, haematology, intensive care, and obstetrics. The hallmarks of APS, as defined by the Sapporo criteria, are however limited to thromboses and obstetric complications.

Defining the phenotype correctly is an important issue in genetic studies of complex diseases as autoimmune diseases. In APS it appears quite difficult, since several clinical entities coexist. Many epidemiological and genetic studies assess the APS phenotype based on clinical criteria of thromboembolism or pregnancy morbidity, and laboratory findings of medium or high titers of antiphospholipid antibodies that are present on two or more occasions at least 12 weeks apart (Miyakis et al., 2006). These international consensus criteria were designed to facilitate clinical studies of treatment and causation in APS and were not intended to be diagnostic criteria for clinical practice. Nonetheless, these criteria can be useful to assess the applicability of the results of clinical trials to an individual patient or even for genetic studies in other populations. It is notable that patients with APS may have

other clinical characteristics, including thrombocytopenia; livedo reticularis, valvular heart lesions and nephropathy, but these features are not formally part of the consensus diagnostic criteria. Similarly, these patients may have antiphospholipid antibodies other than LA, aCL and anti-β2-GPI, including antibodies against prothrombin and other phospholipids and proteins that are not included in the current consensus criteria. Another problem in defining the APS phenotype is that the clinical expression of disease may vary over time even within an individual, especially at older age. Thus, lack of a gold standard to diagnose APS, (variable clinical expression, variable age at onset, and variable progression of disease activity during lifetime) are providing some difficulties in the studies on the genetics of APS.

3. HLA and genetic susceptibility to antiphospholipid syndrome

The genetic predisposition for APS is partially explained in part by markers called Human Leukocyte Antigens (HLA). Some of these HLA molecules are associated to the presence of aPL (Horita et al., 2004): HLA-DR4, -DR7, -DRw53, and DQB1*0302 are associated with the presence of aCL that has been demonstrated in primary APS and can also be found in SLE, a disease with a completely different pattern of HLA allele association (HLA-DR2, -DR3, -DRw52). Therefore, it can be argued that both DR4 and DR7 are independently associated with aCL. According to published results, it seems that DRB1*0402 and DRB1*0403 are slightly more important than DR7 and that the association with DRw53 is only apparent because patients typing positive for DRw53 possess haplotypes that also contain either DR4 or DR7. Furthermore, it is hard to discriminate whether aCL and anti-beta-2GPI antibodies are more strongly associated with DR alleles or DQ alleles, because they are often in strong linkage disequilibrium. Alternatively, these alleles may be apparent only because of their linkage disequilibrium with an as yet unidentified primarily involved HLA locus, or they could act in cooperation with other genes, possibly even outside the MHC. For instance, some reports indicate that aCL are associated with C4A or C4B null alleles (Galeazzi et al., 2000). In addition, the different aPL (anticardiolipin antibodies, lupus anticoagulant, anti-β_2GPI antibodies, antiphosphatidylserine/prothrombin antibodies) show similar HLA association, again independent of the clinical context (primary APS or SLE), and across various ethnic groups.

The genetic findings in the research of APS can explain only partially the development of APS, and like in other AID, disease occurrence depends both on hereditary factors and environmental factors. There is increased prevalence of APS among family members, even though the genetic background of the syndrome is not completely understood. Yet, the chances of a family member of an APS patient also to develop APS are low. Furthermore, aPL are a heterogenous family of autoantibodies. Their presence is not always associated with the clinical manifestations of APS and even in experimental animal models not all aPL are of pathogenetic significance (Sebastiani et al., 2003). Many authors favour the hypothesis that the association of APS with HLA alleles is a consequence of the association of aPL with HLA alleles; therefore, it is reasonable to think that, like in SLE, HLA alleles account only in part for the genetic susceptibility to develop APS. In fact, it appears that some HLA alleles only determine the risk of susceptibility to producce aPL, and this is independent of the clinical context. Other genes, even outside the MHC, give their contribution to the development of this syndrome. For example, it has been shown that a polymorphism in

domain 5 of beta-2 GPI, (a valine instead of a leucine at position 247), is correlated with anti-beta2-GPI antibodies production in patients with primary APS (Hirose et al., 1999); (Atsumi et al., 1999). This replacement of one aminoacid in the structure of beta2-GPI could turn this molecule immunogenic and may induce the production of anti-beta-2GPI autoantibodies.

As we will see, additional genetic risk factors for thrombosis have been described in patients with APS (Factor V Leiden, Methylenetetrahydrofolate-reductase (MTHFR), Homocysteine, Protein C or Protein S deficiency, Adquired Activated Protein C Resistence). The role of these genetically determined factors in APS is not completely clarified, but it appears that they can act as additional (to aPL) thrombogenic risk factors. In conclusion, immunogenetic studies suggest that APS is an independent entity from SLE, even if it can appear in the course of this latter disease. A genetic predisposition to APS can be at least in part explained with an influence of certain HLA alleles. However, these alleles could only be apparent because of their linkage disequilibrium with an as yet unidentified primarily involved HLA-locus, or they could act in cooperation with other genes, even residing outside the MHC. A recent advance in the field of molecular genetics has led to a better understanding of the genes predisposing to APS in both humans and laboratory animal models. The search for a more strongly associated polymorphim is actively pursued whenever new loci are identified in the HLA region. Identification of many more susceptibility genes provides key insights into the pathogenesis of APS, making new prophylactic and therapeutic approaches feasible.

3.1 HLA alleles, antiphospholipid antibodies and genetic susceptibility to the antiphospholipid syndrome

Like many other autoimmune diseases, this syndrome arises in a predisposed subject after antigenic stimuli from various sources. Proof of the genetic predisposition of APS lies in the observation of familial clustering of cases, greater prevalence of aPL in the serum of subjects sharing the same descent of patients, animal models (mice), and associations with HLA alleles. Many autoimmune diseases are associated with genes in the HLA region. In some autoimmune disorders, such as SLE, HLA antigens seem to be associated with specific autoantibodies, including anticardiolipin (aCL) and anti-beta-2 glycoprotein I (anti-beta2 GPI), rather than with the disease itself (Smolen et al., 1987); (Lulli et al., 1991). Thus, it appears that HLA genes may influence not only the expression of autoimmune diseases, but also the production of autoantibodies that can be found in these diseases. Many researchers in the field of immunogenetics have investigated possible associations between APS or the various antibodies directed against negatively charged phospholipids and HLA genes or their products. However, there is increasing evidence that aPL represent a heterogeneous group of antibodies, which includes lupus anticoagulant (LAC), aCL, anti-b2-GPI, antibodies to prothrombin, annexin V, phosphatidylethanolamine, phosphatidylserine and other oxidized phospholipids. Thus, it appears evident that the spectrum of associations with HLA alleles in APS might become clearer if more specific autoantibody subgroups are studied. As stated before, APS may exist both as a primary condition as well as in the setting of another autoimmune disease (mainly SLE), and this implies possible differences in the association with HLA alleles. The presence of aPL is not always associated with the clinical manifestations of APS, and even in experimental animal models not all aPLs are of pathogenetic significance. Some aPL bind preferentially to anionic phospholipids, whereas as others react with zwitterionic phospholipids, and their binding can be either enhanced or depressed by beta2-

GPI, depending on the source of aPL. Therefore, what we call ''antiphospholipid antibodies'' may comprise a group of antibodies whose unique common feature is their reactivity against phospholipids, but with different specificities and different HLA associations.

The question of whether a genetic predisposition to develop APS and to produce aPL exists can be examined both in animal models and in humans (Ahmed et al., 1993). It thus appears that the genetic background of mice can influence the production of aPL, and this production can be modulated by hormones. Nevertheless, it has not been clarified whether aPL are constitutively expressed by mice or induced by antigenic stimulation (Hashimoto et al., 1992). In humans, the contribution of immunogenetics to the development of aPL and APS has been addressed mainly by family-based studies and by population-based studies looking at the association with the HLA region.

3.1.1 Family-based studies

Familial occurrence of aPL with or without clinical evidence of APS has been documented since 1980. A genetic basis for aPL was suggested for the first time when a familial clustering of chronic false-positive syphilis test individuals were detected (McGhee-Harvey, 1966); in this case, aPL were observed many years before overt autoimmune disease developed. Familial occurrence of lupus anticoagulant (LA) was first described in three sets of siblings, two of which had more than one clinically affected member (Exner et al., 1980). Subsequent studies have reported that first-degree relatives of patients with SLE or primary APS have a higher incidence of aCL antibodies, suggesting a genetic predisposition to the development of these antibodies (Mackworth-Young et al., 1987); (Goldberg et al., 1995). Identification of several pedigrees with an increased frequency of aPL antibodies and the associated clinical manifestations further support the familial form of APS. In one of these studies, a large kindred in which nine individuals had aPL antibodies was described. Associated clinical manifestations included stroke, deep venous thrombosis, and recurrent abortions (Ford et al., 1990). A study also have described a family, including identical twins and their mother, in which all members had SLE and presented with different manifestations of APS. The mother and the twins shared the HLA haplotype that included DR4, DRw53 and DQw7, whereas C4A or C4B deficiencies could not be implicated in the autoimmune process (May et al., 1993). In another study, pedigrees with more than one affected member were examined for possible modes of inheritance and linkage to potential candidate genes (Goel et al., 1999). Thirty out of 101 family members from 7 families met diagnostic criteria for the syndrome. Segregation studies rejected environment and autosomal recessive models, and the data were fitted best by a dominant or codominant model. However, linkage analysis showed independent segregation of APS and several candidate genes.

In conclusion, family studies suggest a genetic predisposition to APS, either when it presents as a primary condition or when it is seen in the context of SLE. It appears that this genetic predisposition is in part accounted for the HLA system, the most consistent associations being those with DR4 and DRw53. Furthermore, it appears that LA and aCL are both associated with the same HLA antigens.

3.1.2 Population-based studies

In a study of primary APS and HLA associations, HLA-DQw7 (DQB1*0301 allele) was significantly associated with disease. All patients with DQw7 were HLA-DR4 or -DR5-positive

(Arnett et al., 1991). Asherson et al., reported on 13 English patients with primary APS, in which both HLA class I and HLA class II genes were examined by molecular methods (Asherson et al., 1992). They found that significant differences were limited to the HLA class II region. In fact, DR4 and DRw53 were found with increased frequency in patients compared with controls, whereas DR3 was absent in all patients. No significant associations between any DQB alleles or C4 or 21-hydroxylase gene polymorphisms and primary APS were found, and the prevalence of DQw7 was not significantly increased in patients.

More recently, Caliz et al., found that the haplotypes DQB1*0301/4-DQA1*0301/2-DRB1*04 and DQB1*0604/5/6/7/9-DQA1*0102-DRB1*1302 were more frequent in 53 white British patients with primary APS than in controls (Caliz et al., 2001). The most striking association was found between DQB1*0604/5/6/7/9-DQA1*0102-DRB1*1302 and the presence of anti-b2GPI antibodies in primary APS.

The DQB1*0301/DQA1*0301/DRB1*04 haplotype was also associated with antiphosphatidylserine/prothrombin autoantibodies (Bertolaccini et al., 2000). In another study on the same patients, Bertolaccini et al., evaluated the role of tumor necrosis factor-alpha (TNF-α), an immunomodulatory cytokine with prothrombotic action, encoded at the TNFA locus in the HLA class III region (Bertolaccini et al., 2001). They found significantly higher plasma TNF-alpha levels in patients with APS when compared with controls. In addition, they found a strong association between TNFA-238A polymorphism and APS, and a possible association of the TNFA-238A-DQB1*0303-DRB1*0701 haplotype with APS. However, they failed to demonstrate correlation between TNFA-238A and plasma TNF-alpha levels, suggesting that this polymorphism is not implicated in the elevation of TNF-alpha levels found in APS. It is possible that TNFA-238A polymorphisms associated with APS because of its linkage with the DRB1*0701-DQB1*0303 haplotype.

Another study reports the association of HLA-DR5 with primary APS in Mexican patients (Vargas-Alarcon et al., 1995). To assess whether the HLA profile of patients presenting with primary APS is different from that of patients with secondary APS, Freitas et al., studied 123 patients, 34 of whom presented primary APS and 35 secondary APS due to SLE, 54 SLE patients without APS, and 166 controls. Compared with controls, primary APS patients exhibited a non-significantly increased frequency of DRw53-associated alleles, and secondary APS patients presented an increased frequency of HLA-DRB1*03 alleles. In addition, HLA-DRB1*03 alleles were over-represented in secondary APS patients presenting aCL, in SLE patients as a whole, and in SLE patients without APS. Taken together, their results suggest that the HLA class II profile of primary APS is different from that of secondary APS (Freitas et al., 2004). Sánchez et al., examined the susceptibility of the polymorphisms at the HLA-DM locus (whose products are involved in the antigen processing pathway of HLA class II restricted antigen presentation) to aPL production in a white British population, and observed the skewed distribution of DMA alleles, including the increase of DMA*0102 in patients with aPL. However, this association could simply reflect the strong linkage disequilibrium between HLA-DM alleles and HLA-class II alleles (Sanchez et al., 2004).

Thus, population studies suggest that HLA genes have a role in conferring susceptibility to develop primary APS. DRB1*04, DR7, DRw53, DQB1*0301/4, DQB1*0604/5/6/7/9, DQA1*0102, and DQA1*0301/2, seem to be the relevant loci. HLA-DR4 seems to be more important in Anglo-Saxon populations, whereas DR7 emerges in populations of Latin

origin. Results of those studies in which HLA polymorphisms have been investigated by molecular methods overlap those obtained by serological typing. It is difficult to discriminate whether DR loci contribute to this genetic susceptibility more than DQ loci, because they are in strong linkage disequilibrium.

4. Prothrombotic risk factors in antiphospholipid syndrome

Antiphospholipid antibodies (aPL) are related to thrombosis in APS and thromboses are, together with obstetric complications, the main clinical manifestations of APS. Numerous pathophysiological mechanisms have been suggested to explain thrombotic events in APS, in both arterial and venous territories, involving cellular mechanisms, plasma coagulation regulatory proteins, and fibrinolysis. However, although there is a clear epidemiological association, not all aPL-positive individuals experience such complications. The heterogeneity of thrombotic manifestations in APS suggests that other additional factors may contribute to determine the ''prothrombotic profile'' in these patients: First, several characteristics of aPL, such as the concentration, class/subclass, affinity, or charge, and several characteristics of the antigens, such as the concentration, size, location, or charge, may influence whether aPL will act as prothrombotic "in vivo" (Roubey, 1996); second, oral contraception, pregnancy, surgery, trauma, smoking, immobilization, and other environmental causes can modify the thrombotic risk; and third, individual patient variability due to a predetermined genetic profile can modulate the effect of aPL on hemostasis.

Thrombophilia can be (inherited) congenital, which is supposed to result from the interaction of multiple genetic backgrounds with environmental or acquired factors, such as aPLs or hyperhomocysteinemia, causing together the thrombus growth. There are two levels of prothombotic genetic characteristics potentially related to the clinical expression of APS, the major alterations consisting of deficiencies or polymorphisms clearly related to thrombosis (mainly related to venous thromboembolism), which are for this reason included in the usual thrombophilia test profiles, and a series of polymorphisms that have a little prothrombotic role on their own, but can significantly modify the effect of aPL on hemostasis.

The main genetic thrombophilic conditions in APS include: Major and minor thrombophilic defects.

5. Major thrombophilia alterations in antiphospholipid syndrome

Results of early genetic studies have established that two types of genetic thrombophilic defects cause venous thrombosis: loss-of-function mutations (Antithrombin [SERPINC1], Protein C [PROC] and Protein S [PROS1] deficiencies) and gain-of-function mutations (Factor V Leiden [F5] G1691A and Prothrombin [F2] G20210A mutation) (Reitsma et al., 2007). Combined, these loss and gain-of-function mutations account for about half of the genetic thrombosis risk. Therefore, there is every reason to believe that additional genetic causes of thrombosis remain to be discovered, and many attempts have been made and are still being made to identify them.

Congenital deficiencies of antithrombin and protein C are very uncommon and for this reason, the number of patients with these deficiencies is too small to allow an accurate assessment of the associated risk of thrombosis in APS (Brouwer et al., 2004). By contrast,

low levels of free protein S are seen in a high number of APS patients, suggesting an acquired origin like high level of C4-binding protein. Although it is likely that these genetic defects may increase the risk of venous thromboembolism in patients with APS, only little is known about its possible interactions (Brouwer et al., 2004).

Anecdotically, a single patient suffering from recurrent thrombosis with type II plasminogen deficiency (a controversial thrombophilia factor deficiency) associated with APS and SLE has been described (Iguchi et al., 2002). This case may be coincidental or it may be that the plasminogen deficiency increased the thrombotic tendency of APS in this patient.

5.1 Antithrombin deficiency

Family studies in kindreds with antithrombin deficiency have revealed that antithrombin deficiency probably confers a higher risk of thrombosis than protein C or protein S deficiencies (Lane et al., 1996). The reported incidence of antithrombin deficiency in the general population varies from 0.17-0.20 % as revised by De Stefano *et al.* (De Stefano et al., 1996) which amounts to one-tenth of that for protein C deficiency. Despite this, it has 1-2% prevalence in patients with thrombosis (as against 2.5% for Protein C deficient patients). Thus, Antithrombin III deficiency appears to confer a higher thrombotic risk than Protein C and S deficiencies (Van Cott et al., 1998).

5.1.1 Antithrombin deficiency and risk of thrombosis

Affected patients have antithrombin levels between 40%–60% of normal and 70% of those affected experience thromboembolic events before the age of 50. Thrombotic episodes are rare before puberty in AT-deficient individuals. They start to occur with some frequency after puberty, with the risk increasing substantially with advancing age (Khan et al., 2006). In 1994, Tait et al. studied 9,669 blood donors in Scotland by taking blood samples and monitoring the donors for two years. Of the study participants, 107 were found to have an initial level of AT< 83 IU/dl. They were retested, and eventually, 16 donors where found to have congenital AT deficiency. The study suggested a prevalence of 1/600 or 1.65 per 1000 (95% CI of 0.95–2.27 per 1000). Two of the affected individuals had Type I deficiency while the other 14 had Type II. The study showed an overall prevalence of 1/4,400 for Type I AT deficiency and 1/630 for type II with an overall prevalence of AT deficiency at 1/630 in the population studied. It all brins up the important issue of whether AT deficiency itself, or in combination with other factors, would make a thromboembolic event likely in these individuals (Tait et al., 1994). Another challenge when testing for AT without other clinical information is that levels of antithrombin can be reduced in protein-losing states. Marked reductions in antithrombin levels occur in patients with liver disease as well as severe malnutrition and these may cause skewed results (Hoffman, 2000).

Using only an immunoassay for measuring plasma levels of AT, initial estimates of the prevalence of AT deficiency in the general Scottish population was 1 in 2,000 to 5,000. However, studies employing functional assays that measure AT-heparin cofactor activity have found that the prevalence of AT deficiency in the Scottish population is 1 in 250 to 500 (Tait et al., 1994). The majority of AT-deficient patients identified in these studies did not have a personal or familial history of thrombosis and had a type II defect with mutations at the heparin binding site. Among patients with a first thrombotic event, the prevalence of

hereditary AT deficiency is approximately 0.5 to 1 %, being less common than factor V Leiden, the prothrombin gene mutation, or protein S/protein C deficiencies. A recent study showed that homozygous children of consanguineous parents who were asymptomatic carriers developed severe venous or arterial thrombosis in association with plasma AT-heparin cofactor levels below 10 percent of normal (Picard et al., 2003).

The thrombotic risk associated with AT deficiency, as with other inherited thrombophilias, has been assessed in two ways: evaluation of patients with deep venous thrombosis and evaluation of families with thrombophilia. In a Spanish study of 2,132 consecutive unselected patients with venous thromboembolism 12.9 % had an anticoagulant protein deficiency (7.3% of protein S, 3.2% of protein C, and 0.5% of antithrombin). Similar findings were noted in a series of 277 Dutch patients with deep venous thrombosis: 8.3% had an isolated deficiency of antithrombin, protein C, protein S, or plasminogen compared to 2.2% of controls subjects (Heijboer et al., 1990). In a study in 2001, five children from three Austrian families had a homozygous antithrombin deficiency type II affecting the heparin binding site (99 Leu → Phe mutation). Four children had severe spontaneous thromboembolic events (deep leg or caval vein thrombosis, ischemic stroke) at one week, 3 months, 13 and 14 years of age. The fifth patient, a 17 year-old boy, was asymptomatic (Kuhle et al., 2001).

The absolute risk of thrombosis among patients with inherited thrombophilia was evaluated by Martinelli et al. in 1998 in an Italian cohort study of 150 pedigrees consisting of 1,213 individuals. The study compared the risk for thrombosis in individuals with inherited thrombophilia due to factor V Leiden, antithrombin, protein C, and protein S deficiency (Martinelli et al., 1998). The lifetime probability of developing thrombosis compared to those with no defect was 8.5 times higher for carriers of protein S deficiency, 8.1 for type I antithrombin deficiency, 7.3 for protein C deficiency, and 2.2 for factor V Leiden. The selection of patients was not solely based on their registration at the thrombosis centers in Milan or Rome but also required individuals to provide clinical evidence of thrombotic events; hence, this fact increases the validity of the study.

5.2 Protein C deficiency

Protein C deficiency is less common than either the factor V Leiden or the prothrombin G20210A gene mutation with prevalence in Caucasians estimated to be 0.2–0.5% (Rosendaal, 1999). Protein C deficiency is inherited in an autosomal dominant manner and is associated with familial venous thrombosis. The gene for protein C is located on chromosome 2 (2q13–14) and appears to be closely related to the gene for factor IX (Foster et al., 1985). The primary effect of activated protein C (APC) is to inactivate coagulation factors Va and VIIIa, which are necessary for efficient thrombin generation and factor X activation (Clouse et al., 1986). The inhibitory effect of APC is markedly enhanced by protein S, another vitamin K-dependent protein. Two major subtypes of heterozygous protein C deficiency (Type I and Type II) have been delineated using immunologic and functional assays. Over 160 different gene abnormalities have been associated with the two subtypes (Reitsma et al., 1995).

Type I deficiency – The type I deficiency state is more common. Most affected patients are heterozygous, having a reduced plasma protein C concentration at approximately 50 percent of normal in both immunologic and functional assays (Broekmans et al., 1985). More

than half of the mutations identified so far are missense and nonsense mutations. Other types of mutations include promoter mutations, splice site abnormalities. In frame deletions, frameshift deletions, in-frame insertions, and frameshift insertions (Reitsma et al., 1995). There is marked phenotypic variability among patients with heterozygous type I protein C deficiency. Similar mutations have been found among symptomatic and asymptomatic individuals. This finding suggests that the nature of the protein C gene defect alone does not explain the phenotypic variability.

Type II deficiency – Individuals with the type II deficiency state have normal plasma protein C antigen levels with decreased functional activity. A variety of different point mutations affecting protein function have been identified in this disorder (Reitsma et al., 1995).

Although the clinical manifestations of protein C are similar to those of antithrombin deficiency, there are some unique features of protein C deficiency. In a study of 11 infants in Denver (Colorado, U.S.), Manco-Johnson et al. suggested that homozygotes can develop a severe thrombotic tendency in infancy characterized as purpura fulminans (Manco-Johnson et al., 1988). Heterozygotes for protein C deficiency have an increased risk of developing warfarin-induced skin necrosis (Chan et al., 2000). Protein C deficiency has been implicated in adverse pregnancy outcomes such as deep venous thrombosis (DVT), preeclampsia, intrauterine growth restriction, and recurrent pregnancy loss (Greer, 2003). Family studies from the Netherlands and the US have shown that family members who are PC deficient are at an 8–10 fold increased risk of venous thrombosis, and, by age 40, 50% or more will have experienced a thrombotic event (Bovill et al., 1989); (Broekmans, 1985). The initial episode of venous thromboembolism in patients with protein C deficiency is apparently spontaneous in approximately 70 % of cases. The remainder of the cases suggests that other genetic or acquired factors are involved in the presentation of thrombotic events in this population. Further studies in the Netherlands showed that most patients are asymptomatic until their early twenties, with increasing numbers experiencing thrombotic events as they reach the age of 50 (Lensen et al., 1996). Lensen et al. concluded that the median age at onset for a thrombotic event and the risk of thrombosis is similar in both protein C deficiency and factor V Leiden (APC resistance). Approximately 60% of affected individuals develop recurrent venous thrombosis and about 40% have signs of pulmonary embolism.

The first case-control study looking at protein C deficiency was conducted by Heijboer et al. in 1990 that performed a study on 277 Dutch patients and 138 controls. The overall prevalence of protein C deficiency in the patients with venous thrombosis was 8.3% (23 of 277 patients) (95% CI 5.4–12.4), as compared with 2.2% in the controls (i.e. 3 of 138 subjects 95% CI 0.5–6.1; $P < 0.05$). Interestingly, the relative risk (RR) estimate for thrombosis given protein C deficiency was also close to 7 as in the case of the family studies in Netherlands.

Both the work done by Heijboer et al. and a subsequent study by Tait et al. estimated the population prevalence of heterozygous protein C deficiency at 0.2% (Tait et al., 1994). The relative risk for thrombosis among patients with protein C deficiency was also carried out by Koster et al. involving 474 consecutive outpatients at anticoagulation clinics who were excluded by age (older than 70) and known malignancy. The patients were asked to find their own controls by sex, age and no known thrombotic disorder. The study demonstrated the relative risk of thrombosis with protein C deficiency to be at least 3.1 (95% CI 1.4–7) with re-evaluation of patients with low levels increasing the RR to closer to 4 (Koster et al., 1993).

There is marked variability in risk among families with protein C deficiency that cannot be explained by the genetic defect itself. In severely affected families, as many as 75% of protein C-deficient individuals experience one or more thrombotic events (Broekmans et al., 1983); in other families, the thrombosis rate is much lower. A risk factor for more severe disease is the presence of a second thrombotic defect, particularly factor V Leiden as will be discussed in more detail later on in this chapter.

5.3 Protein S deficiency

Protein S serves as a cofactor for activated protein C. There are two homologous genes for protein S: PROS1 and PROS2, which both map to chromosome 3 (Schmidel et al., 1990). In 2000, Gandrille et al. identified 15 point mutations and 3 polymorphisms among 19 French patients affected of thrombosis; since then, mutations have been identified in 70% of protein S deficient probands and a database of known protein S gene mutations has been published (Gandrille et al., 2000). As in the case of other inherited thrombophilias, there seem to be few reports of large deletions causing the disorder. Three phenotypes of protein S deficiency have been defined on the basis of total protein S antigen concentrations, free protein S concentrations, and protein S functional activity:

Type I – The classic type of protein S deficiency is associated with a decreased level of total S antigen (approximately 50% of normal), and marked reductions in free protein S antigen and protein S functional activity (Simmonds et al., 1996).

Type II – This type of protein S deficiency is characterized by normal total and free protein S levels, but diminished protein S functional activity. Interestingly, all five mutations originally described in these patients were missense mutations located in the N-terminal end of the protein S molecule, which includes the domains that interact with activated protein C (Gandrille et al., 1995).

Type III – Also known as type IIa, this is characterized by total protein S antigen measurements in the normal range and selectively reduced levels of free protein S and protein S functional activity to less than approximately 40% of normal (Zoller et al., 1994). Interestingly, a case-control study by Zoller in 1995 involving 327 Swedish families showed that type I and type III are phenotypic variants of the same genetic disease.

Protein S deficiency is inherited in an autosomal dominant manner and is at least as common as antithrombin and protein C deficiency (Heijboer et al., 1990). The clinical manifestations are similar to those seen with antithrombin and protein C deficiency. Thrombosis occurs in heterozygotes whose levels of functional protein S are in the range of 15–50% of normal.

The prevalence of familial deficiency of protein S type I among Caucasians, estimated from a large cohort of healthy blood donors from the West of Scotland, is in the range of 0.03 to 0.13 percent (Dykes et al., 2001). The prevalence is much higher among individuals with established thrombophilia.

In a Spanish study of 2,132 consecutive unselected patients with venous thromboembolism, 7.3% had protein S deficiency (Mateo et al., 1997). Based on these and other studies, Martinelli et al. estimated that the life-time probability of developing thrombosis among carriers of protein S deficiency was 8.5 times higher compared to those without thrombosis events.

In 1987, Engesser and colleagues conducted a study on 12 Swedish families with 136 members and found 71 of them to be heterozygous for Type I protein S deficiency. 55% of those who carried the defect were found to have had a thrombotic event and 77% of those were recurrent. About half of the cases were precipitated by another condition. They also showed that in phenotypic protein S deficient families, the likelihood that affected family members remain thrombosis-free at 45 years of age was 35% to 50%. This study showed a difference in rates between men and women but was not able to provide an adequate explanation in terms of difference in risk factors between the two sexes (Engesser et al., 1987).

Another study examined the incidence of thrombosis in carriers in a Swedish family with a known missense mutation ($Gly^{295}Val$) (Simmonds et al., 1998). Simmonds studied 122 members in a family, with 44 of the members previously characterized for the specific gene defect in protein S. The study showed little thrombotic risk before the age of 15 years. On the other hand, the likelihood of being free of thrombosis by age 30 was only 50% compared to 97% in normal family members. The odds ratio for thrombosis in affected subjects was 11.5, and the study showed that measurement of free protein S antigen levels was predictive of the mutation and deficiency. In a UK based family study with 28 index patients with protein S deficiency, first degree relatives with the PROS1 gene defect had a five-fold higher risk of thrombosis than those with a normal gene and no other apparent thrombophilia (Makris et al., 2000).

Overall, both family and cohort studies demonstrate that like other thrombophilic disorders, heterozygous protein S deficiency usually manifests in adulthood with a thromboembolic event. When present with other thrombophilias or when present in the homozygous form, protein S usually presents in neonates with purpura fulminans (Mahasandana et al., 1996).

5.4 Factor V Leiden (F5) G1691A polymorphism

In 1994, Bertina et al. first described a defect in the factor V gene that makes it less susceptible to inactivation by activated protein C (APC) (Bertina et al., 1994). The following year, Kalafatis et al. showed that the mechanism of inactivation of the membrane bound profactor Va is an ordered event. Factor Va is sequentially cleaved at Arg^{506} and at Arg^{306} and Arg^{679} by activated protein C (Kalafatis et al., 1995). They suggested that the peptide bond cleavage at Arg^{506} facilitates the exposure of the subsequent cleavage sites at Arg^{306} and Arg^{679}. At around the same time, Shen and Dahlback et al. showed that factor V is also a cofactor in the inactivation of factor VIIIa by APC (Shen et al., 1994). The understanding of factor V inactivation was almost immediately followed by reports on how activated protein C in patients' plasma failed to prolong the activated partial thromboplastin time, hence the term "activated protein C resistance" was developed (Koster et al., 1993). Further studies have shown that most patients with activated protein C resistance have a factor V allele that is resistant to the proteolytic effect of protein C. A transition (guanine to adenine) at nucleotide 1691 (G1691) results in the replacement of arginine by glutamine. This gene product, called factor V Leiden, also known as factor V Q^{506} or $Arg^{506}Gln$, is named after the city in the Netherlands that it was first identified in. Factor V Leiden is a variant of the normal gene and is not susceptible to cleavage at position 506 by activated protein C. The consequence

of this is a hypercoagulable state as more factors Va is available within the prothrombinase complex, thereby increasing the generation of thrombin. Factor V is also thought to be a cofactor, along with protein S, in supporting the role of activated protein C in the degradation of factors Va and VIIIa. Thus, lack of this cleavage product decreases the anticoagulant activity of activated protein C. Several mutations at the Arg^{306} residue in factor V have been described in patients with a history of thrombosis. These include replacement of Arg^{306} with threonine (factor V Cambridge) (Williamson et al., 1998) or with glycine (in Hong Kong Chinese) (Chan et al., 1998).

Occasionally, patients have been described who have heterozygous APC resistance due to the factor V Leiden mutation and type I factor V deficiency (Guasch et al., 1997). The plasma of these individuals manifests severe APC resistance in activated partial thromboplastin time assays, similar to that seen in patients with homozygous factor V Leiden. These patients appear to be more thrombosis prone than their heterozygous relatives with factor V Leiden alone, suggesting that the clinical phenotype is similar to patients who are homozygous for factor V Leiden.

5.4.1 Factor V Leiden mutation and risk of thrombosis

There are multiple studies showing evidence for factor V Leiden as a cause of deep vein thrombosis (DVT) among the Caucasian population. The major clinical manifestation is deep vein thrombosis with or without pulmonary embolism. There is also evidence that the factor V Leiden mutation, presumably due to thrombosis of placental vessels, may play a role in some cases of unexplained recurrent pregnancy loss (Ridker et al., 1997). Svennson and Dahlback et al. studied 34 families with the Factor V506 Arg to Gln mutation and found an increased lifetime risk of venous thrombosis. By age 50, at least 25% of those affected had experienced at least one thrombotic event.

The Leiden Thrombophilia Study by Koster et al. in the Netherlands provided a population-based case-control study to assess the prevalence of this disorder. APC resistance was found in 21% of those who had a history of thromboembolism compared with 5% of controls. Overall, the relative risk for a thromboembolic event was increased seven-fold in heterozygous individuals. They further studied individuals who were homozygous for factor V Leiden mutation and found an 80-fold increase in the lifetime risk for a thrombotic problem. It was subsequently estimated that homozygous individuals can be expected to experience at least one venous thromboembolic event in their lifetime (Koster et al., 1993). This is supported by a study of 306 family members from 50 Swedish families, which found 40% of homozygotes who had an episode of venous thrombosis by age 33, compared to 20% of heterozygotes and 8% of normals. Ridker et al. published a study in 1997 based on 4,047 American men and women The study found a 12% incidence of heterozygosity for the factor V Leiden mutation in patients with a first confirmed DVT or pulmonary embolism compared with 6% in controls (Ridker et al., 1997). The incidence reached 26% in men over the age of 60 with no identifiable precipitating factors.

In conclusion, Factor V Leiden seems to have a milder effect on the development of thrombosis in patients with APS compared with that seen in the general population due to the strong effect of aPL, but Factor V Leiden may in several patients increase the thrombogenic effect of aPL.

5.5 Prothrombin (F2) G20210A mutation

In 1996, Poort et al. described a single aminoacid genetic variation in the 3' untranslated region of the gene that codes for prothrombin. Prothrombin (factor II) is the precursor to thrombin, the end-product of the coagulation cascade. Prothrombin has procoagulant, anticoagulant and antifibrinolytic activities and thus a disorder involving prothrombin results in multiple imbalances in hemostasis. A report published in 1996 based on 28 families from the Netherlands with established venous thromboembolism identified a substitution of guanine to adenine at nucleotide 20210 in the 3'-UTR of the prothrombin gene (Poort et al., 1996). Linkage studies performed in 397 individuals from 21 Spanish families have provided further evidence that a quantitative trait locus (G20210) in the prothrombin gene influences prothrombin activity levels and susceptibility to thrombosis (Soria et al., 2000). The single base pair substitution in F2, termed F2 G20210A, is one of the most common genetic alterations described in thrombophilia. Its prevalence is higher in patients with venous thrombosis (6–18%) when compared with the general population (1–3%) (Poort et al., 1996).

5.5.1 Prothrombin gene mutation and risk of thrombosis

The prothrombin G20210A gene mutation is a common polymorphism associated with an elevated risk of deep venous thrombosis, although to a lesser degree than factor V Leiden is. The Leiden Thrombophilia Study, (a population-based study) demonstrated a prevalence of the prothrombin 20210A allele among healthy carriers of 6.2% among venous thrombosis patients and between 2% - 4% among healthy matched controls.

The initial studies did not show an increased risk of thrombosis related to the G20210A polymorphism in the prothrombin gene in APS patients of Caucassian (Bentolila et al., 1997); (Bertolaccini et al., 1998) or Mexican mestizo origin (Ruiz-Arguelles et al., 1999). However, from the first case of SLE-associated APS in a young female homozygous for the 20210A allele in the prothrombin gene who developed venous thrombosis while taking oral contraceptives (Sivera et al., 2000), several subsequent studies have demonstrated an association between the prothrombin G20210A polymorphim and thrombosis in APS patients. Torresan et al. found in 30 Brazilian patients with APS and thrombosis a higher prevalence of the 20210A allele of the prothrombin gene when compared with controls individuals (5% vs. 0,7%) (Torresan et al., 2000). Similarly, Forastiero et al. found in 105 Caucassian consecutive unselected patients with aPL grouped as having APS (n= 69) and not having APS (n= 36) that the 20210A allele was significantly more frequent in APS patients than in healthy controls subjects (8,7% vs. 2%) (Forastiero et al., 2001). Brouwer et al. in a cohort of 144 consecutive patients with SLE, found that the 20210A allele of the prothrombin gene was an independent risk factor for venous thromboembolism that when presented together with aPL and it increased the risk 30-fold (Brouwer et al., 2004).

Other studies, however, have shown no relationship between prothrombin G20210A polymorphim and thrombosis in APS. In this study of Galli et al. the prevalence of the G20210A polymorphim was evaluated in 145 Caucassian aPL-positive patients and they found no association between the 20210A allele with venous thrombosis (Galli et al., 2000). Similarly, in 157 aPL-positive patients (44% with previous thrombosis), the G20210A polymorphism was not associated with thrombosis (Chopra et al., 2002). Finally, in a recent study (Sallai et al., 2007) in 105 SLE patients, the prothrombin G20210A polymorphism was not associated with thrombosis risk.

6. Minor thrombophilia alterations in antiphospholipid syndrome

Several polymorphisms have been postulated to have the potential to modify the prothrombotic risk in aPL-positive patients. Some of these polymorphisms affect proteins directly related to aPLs, others are related to normal hemostasis components, and, finally, others are related to immune or inflammatory pathways.

6.1 Beta2-glycoprotein I gene polymorphisms

Among the targets of aPL, beta2-glycoprotein I (beta2-GPI), which bears the epitopes for aCL antibody binding, has been extensively studied. It is a glycoprotein of around 50 kDa, found in plasma at a concentration of approximately 200 μg/ml, which makes it one of the most abundant proteins in human serum, second only to fibrinogen among the plasma proteins involved in clotting (McNeil et al., 1990). A member of the short consensus-repeat protein family, beta2-GPI is characterized by five 'sushi-domains'. The fifth sushi-domain contains the binding site to phospholipid, and it attaches to activated cellular surfaces (Wurm, 1984). Although its physiological role is not known, 'in vitro' data suggest that beta2-GPI may play a role in coagulation. It binds to anionic phospholipids and inhibits the contact phase of the intrinsic blood coagulation pathway (Schousboe, 1985), adenosine diphosphate–dependent platelet aggregation (Nimpf et al., 1987), and the prothrombinase activity of human platelets (Nimpf et al., 1986). Although these data imply an anticoagulant role for beta2-GPI, deficiency of this protein is not a clear risk factor for thrombosis. The plasma level of this protein is under genetic control (Cleve, 1968). A study of familial thrombophilia demonstrated that heterozygous partial beta2-GPI deficiency is not associated with the risk of thrombosis (Bancsi et al., 1992). Patients with APS appear to have normal or somewhat elevated levels of beta2-GPI (De Benedetti et al., 1992); (Galli et al., 1992).

Neo- or cryptic- epitopes expressed on beta2-GPI as a result of the beta2-GPI/phopholipids interaction could be a potential antigenic target for the autoimmune type of aCL antibodies. The human beta2-GPI gene (ITGA2B) is mapped on chromosome 17q23-qter with four major polymorphisms (Ser^{88}/Asn, Leu^{247}/Val, Cys^{306}/Gly and Trp^{316}/Ser). Alterations in beta2-GPI properties related to these polymorphisms are not defined. The Leu^{247}/Val polymorphism locates in domain 5 of beta2-GPI which is a potential epitope site for anti-beta2-GPI antibodies (Ichikawa et al., 1994). As a result, polymorphisms on or near the phospholipid binding site or the antigenic site can affect autoantibody production. Hirose et al. (Hirose et al., 1999) found that the valine 247 allele was more frequently detected in Asian patients with APS than in matched normal individuals. Furthermore, it reported an association between the Val^{247}/Val homozygous genotype and the presence of anti-beta2GPI antibodies only among Asian patients with APS. The authors found no evidence of an increased risk of thrombosis in this Asian population. In this same line, we performed a study comparing the distribution of polymorphisms at codons 247 (Val^{247}Leu) and 316 (Trp^{316}Ser) of the β2-GPI gene in a Caucasian Spanish population of Primary APS patients and healthy controls subjects, and then making correlations with the development of anti-β2GPI antibodies and other aPL and associated clinical manifestations. In total, 57 primary APS patients and 100 control subjects were included in our study. In the analysis of Val^{247}Leu polymorphism, alleles (V and L) and genotypes (V/V, V/L, L/L) analyses were similarly distributed in PAPS patients and controls (P= 0.66 and P= 0.22, respectively).

Regarding Trp^{316}Ser polymorphism, we found a higher percentage of patients with respect to controls subjects expressing S allele (11.4% vs. 5%, P= 0.02) and T/S genotype (22.8% vs. 10%, P= 0.02). However, when we compared T/T and T/S genotypes in primary APS patients, we found no differences regarding generation of anti-β2GPI antibodies, other aPLs and clinical manifestations favoring any genotype. Our findings suggest that among Spanish Caucasians, polymorphisms at codon 247 (Val^{247}Leu) do not seem to influence PAPS pathogenesis. On the contrary, polymorphisms at codon 316 (Trp^{316}Ser), by means of an increased S allele and T/S genotype presence in Spanish Caucasian patients, might play a role in the pathogenic development of primary APS, although it mechanism would not involve an increased production of anti-beta2-GPI antibodies and other aPLs (Pardos-Gea et al., 2011).

6.2 Tissue factor pathway inhibitor (TFPI T-33C; C-399T)

Several polymorphisms within the tissue factor pathway inhibitor gene (TFPI) may determine TFPI expression and increase the risk of venous thromboembolism (VTE) in predisposed individuals. Lincz et al. (Lincz et al., 2007) tested this hypothesis by comparing TFPI activity and the frequency of common TFPI polymorphisms (T-33C, C-399T and T-287C) or Factor V Leiden in patients with APS who had a history of VTE and compared them with those without VTE and also with normal control individuals. They found that only APS patients with a history of venous thrombosis had TFPI activity levels significantly different from control individuals (1.77 ± 0.60 vs. 0.77 ± 0.19 U/ml; p= 0.0001), and this was associated with inheritance of the TFPI -33C allele (1.70 ± 0.72 U/ml for TC/CC genotypes vs. 0.97 ± 0.56 U/ml for TT; p= 0.01). Multivariate analysis of APS and FVL patients revealed that the greatest independent contributor to VTE was TFPI activity, while inheritance of either the TFPI-33C or -399T alleles each increased the odds of VTE by nearly 13 times. These results indicate that the TFPI T-33C and C-399T polymorphisms are significantly associated with venous thrombosis in the presence of other risk factors, especially in APS, and may be clinically relevant in patients who are prone to hypercoagulability.

6.3 Methylenetetrahydrofolate-reductase (MTHFR) C677T/A1298C

The C677T methylenetetrahydrofolate reductase (MTHFR) polymorphism (thermolabile variant 677TT) has been shown to exert a potential effect on plasma homocysteine levels. At present, this polymorphism is not considered *per se* to be a risk factor for thrombosis (Bertina, 2001) and is recommended to exclude C677T MTHFR polymorphism from the analysis of multiple thrombophilic genotypes. In a series of 152 aPL-positive patients, Galli et al. (Galli et al., 2000) did not find an association between the C677T MTHFR polymorphism alone or in combination with either Factor V Leiden or G20210A prothrombin polymorphisms and thrombosis. Similar results were obtained by Torresan et al. (Torresan et al., 2000) in 30 patients with APS in whom no significant variation was found between the patient group and the controls regarding the prevalence of homozygotes for the mutated 677T allele (2.5% vs. 5.4%), and by Forastiero et al. (Forastiero et al., 2001) in 105 aPL- positive patients in whom the frequencies of the C677T MTHFR alleles were not different either between the aPL groups and normal controls or between APS and non-APS groups. In addition, cerebrovascular disease was not related to homozygous or heterozygous C677T MTHFR polymorphism in 44 primary APS patients (Kalashnikova et

al., 2005). Finally, Ames et al. (Ames et al., 2001) also observed that the C677T MTHFR polymorphism was not related to venous thrombosis in 49 aPL-positive subjects, but the homozygous 677TT patients had lower mean age at first event and suffered an increased average number of events per person.

6.4 Thrombomodulin THMD Ala455Val

Thrombomodulin is an integral membrane protein of endothelial cells and monocytes. When thrombin binds to thrombomodulin, it loses its procoagulant activity, but becomes capable of activating PC. Therefore, a hereditary deficiency of thrombomodulin may very well play a role as a risk factor for thrombotic disease. A number of missense mutations are currently known in the thrombomodulin gene (THMD) of patients with venous thrombosis (Norlund et al., 1997). A particular aminoacid dimorphism, $Ala^{455}Val$, was found with frequencies of 0.81/0.18 in Caucasian patients who suffered from thrombophilia (van der Velden et al., 1991). The potential role of THMD mutations in myocardial infarction recently received further support from the documentation of a frame-shift mutation in a family with arterial disease (Kunz et al., 2000).

6.5 Protein C receptor (PROC), and endothelian (EPCR) (PROC 4031ins23)

The recently discovered endothelial protein C receptor (EPCR) on endothelial cells is another important regulator of the PC anticoagulant pathway. A 23-bp insertion at position 4,031 in exon 3 (4031ins23) of the gene encoding EPCR (PROCR) has been identified that may predispose patients to deep venous thrombosis (Biguzzi et al., 1998). Furthermore, mutations in PROCR and THMD have been associated with late fetal loss (Franchi et al., 2001). Given that EPCR plays a role in the anticoagulation system and in placental development, a study from Spain (Hurtado et al., 2004) hypothesized that anti-EPCR autoantibodies may be involved in clinical manifestations of APS and in fetal loss. They found that both IgM and IgG anti-EPCR serum levels were higher among APS patients than in controls (57 vs. 45 AU and 75 vs. 72 AU, respectively). They concluded that anti-EPCR autoantibodies could be detected in APS patients and constituted an independent risk factor for a first fetal death episode for anti-EPCR antibodies IgM (OR: 23; CI 2.0–266.3) and IgG (OR: 6.8; CI 1.2–38.4).

6.6 Polymorphisms in platelet glycoproteins

A Spanish study (Jiménez et al., 2008) analysed the genetic polymorphisms in platelet glycoproteins (GP) Ib-alpha (GP1BA), Ia/IIa (ITGA2) complex and IIb/IIIa (ITGA2B) complex and their correlation with the development of arterial thrombosis and preclinical atherosclerosis in patients with APS or with SLE. Thrombotic events were assessed clinically and confirmed by objective methods. They found a significant correlation between the 807 T/T genotype of ITGA2 and arterial thrombosis (22% in APS patients vs. 7% in controls, P= 0.04; OR: 3.59, CI 1.20–10.79). The variable number tamden repeat (VNTR) GP1BA and PIA1/2 ITGA2B polymorphisms were not associated with arterial thrombosis in patients with APS when they were individually analysed. The coexistence of both ITGA2 807T and ITGA2B PIA2 alleles increased the arterial thrombosis risk (28% vs. 7%, P=0.005; OR: 4.84, CI 1.67–13.96). Interestingly, the coexistence of ITGA2 807T and ITGA2B PIA2 was associated with the presence of carotid plaque (35% vs. 4%, P=0.002; OR: 12.92, CI 2.39–69.81). They

concluded that the T/T genotype of the ITGA2 807C/T polymorphism may be an additional risk factor for the development of arterial thrombosis in APS.

6.7 Polymorphisms in platelet Fcγreceptor IIA

Platelet Fc gamma-receptor IIA (FcγRIIA; CD32) molecules are essential for the effect of aPL against beta2-GPI by causing platelet activation, thromboxane A2 generation, and granule release after their binding to the Fc fragments of aPL. Human FcγRIIA reacts best with IgG subclasses 1 and 3, but weakly with subclass 2, which includes the majority of the anti-beta2-GPI seen in autoimmune patients (Arvieux et al., 1994). The His^{131} allele of the $His^{131}Arg$ polymorphism of the FcγRIIA gene reacts much more efficiently than the Arg^{131} allele to IgG subclass 2. Carlsson & Atsumi et al. (Carlsson et al., 1998); (Atsumi et al., 1998) tested whether patients with the His131 allele of the FcγRIIA polymorphism may be at higher risk for developing thrombosis by this platelet activation mechanism. Carlsson et al. and Atsumi et al. studied 100 white patients with aPL and they found that none of the clinical manifestations of primary APS (arterial or venous thrombosis, recurrent pregnancy loss, and thrombocytopenia) was significantly correlated with the $His^{131}Arg$ FcγRIIA polymorphism. However, in a more recent meta-analysis (Karassa et al., 2003) a significant increase in Arg^{131} homozygosity was found in APS patients and the authors suggested a complex genetic background underlying the relationship between the FcγRIIA $Arg^{131}His$ polymorphism and APS as a composite of two different and opposing influences with regard to susceptibility. Unfortunately, the number of APS patients with specific clinical manifestations was too small to reliably assess the effect of the FcγRIIA polymorphism on the risk of vascular thromboses or other APS-related features. More recently (Schallmoser et al., 2005), the $Arg^{131}His$ FcγRIIA polymorphism was evaluated in 73 aPL-positive patients (47 with thrombosis) and an increased frequency of heterozygous patients was associated with thrombosis (OR: 6.76). In this study heterozygosity, rather than Arg^{131} homozygosity, was linked to the clinical manifestations of APS. The authors explained these data by the dual function of the FcγRIIA, namely binding of antibodies to platelets and thereby their activation, and, on the other hand, clearance of antibody-coated platelets by the phagocyte system.

6.8 Tissue plasminogen activator (PLAT) (Alu I/D) and Type-1 plasminogen activator inhibitor (SERPINE1 [4G/5G])

Impaired fibrinolytical outcomes may be one of the pathogenic factors for thrombotic events in patients with antiphospholipid antibodies (aPL). Yasuda et al. investigated the consequences of gene polymorphisms of tissue plasminogen activator (PLAT) and plasminogen activator inhibitor-1 (SERPINE1) in patients positive for aPL (Yasuda et al., 2002). Seventy-seven Japanese and 82 British patients with aPL were examined for an Alu-repeat insertion (I)/deletion (D) polymorphism of PLAT and for the 4G/5G polymorphism in the SERPINE1 promoter. Correlations between these polymorphisms and clinical symptoms of APS (arterial thrombosis, venous thrombosis, miscarriage) were analysed. No significant differences in the allele frequencies of these genes between patients and controls were found. There was no significant correlation between these gene polymorphisms and clinical symptoms of APS in patients with aPL antibodies. Therefore, polymorphisms of PLAT or SERPINE1 probably do not significantly influence the risk of arterial/venous thrombosis, or pregnancy morbidity in patients with aPL antibodies.

The effect of the 4G/5G polymorphism of SERPINE1 on the risk of venous thromboembolism (VTE) remains controversial. In a recent meta-analysis, Tsantes et al. investigated the association between the SERPINE1 4G/5G polymorphism and the risk of venous thromboembolism (VTE) in 18 papers; it included patients without another known risk factor, and comprised 2,644 cases and 3,739 controls. Based on their findings, the SERPINE1 4G allele appears to increase the risk of venous thrombosis, particularly in subjects with other genetic thrombophilic defects (Tsantes et al., 2007).

6.9 Coagulation factor XIII, A subunit (F13A1) (Val34Leu)

Diz-Kucukkaya et al. found that a polymorphism ($Val^{34}Leu$) in the factor XIIIA gene (F13A1) decreased the risk of both arterial and venous thrombosis (Diz-Kucukkaya et al., 2007). Nevertheless, the results showed that the F13A1 Leu^{34} allele had no protective effect in the development of thrombosis in patients with APS. On the contrary, De la Red et al. found that this polymorphism was associated with a higher risk of thrombosis in patients with the presence of both aPL antibodies and high fibrinogen levels (de la Red et al., 2009). They found no significant differences in F13A1 Leu^{34} allele frequencies between primary APS, APS/SLE, SLE-aPL and asymptomatic-aPL patients, or between patients with and without thrombosis. In this study, the F13A1 Leu^{34} allele seemed to have a protective effect on the development of thrombosis in patients with aPL antibodies, but only in those patients with high plasma fibrinogen values.

6.10 Annexin 5 (ANXA5 -1C – T)

Another study (de Laat et al., 2006) on the ANXA5 polymorphism (ANXA5 -1C–T) and the presence of antiannexin A5 antibodies in APS concluded that the detection of anti-annexin A5 antibodies does not seem relevant for estimating the risk for thrombosis or miscarriage in APS. The -1C–T mutation was an independent risk factor for miscarriage which is independent of APS.

6.11 P-selectin glycoprotein ligand-1 (SELPG) gene polymorphisms

Diz-Kucukkaya et al. studied the gene encoding P-selectin glycoprotein ligand-1 (SELPG) and showed that a variable number tandem repeats (VNTR) polymorphism on this gene is a significant determinant of thrombosis predisposition in patients with APS. Furthermore, this risk appears to correlate better with the combination of alleles inherited rather than with the presence of any particular allele (Diz-Kucukkaya et al., 2007).

6.12 CD40 ligand (CD154) gene polymorphisms

Increased levels of soluble CD154 have been described in various inflammatory disorders, particularly SLE. A polymorphic CA repeat sequence has been identified in the 3-UTR of the CD154 gene. The larger alleles of this CA repeat are more frequent in SLE patients and are also associated with a prolonged protein expression on T lymphocytes (Citores et al., 2004). In a recent study in 107 aPL-positive patients, Bugert et al. found than the CA repeat polymorphism in the 3-UTR of CD154 was associated with the development of arterial thrombosis (applying the dominant model and considering CD154 genotype exclusively containing alleles with 24 CA repeats, OR: 4.04) but not with venous thrombosis (Bugert et al., 2007).

6.13 TNF-alpha gene polymorphisms

Bertolaccini et al. explored in 83 Caucasoid patients with APS the possible involvement of the proinflammatory and prothrombotic cytokine tumor necrosis factor-alpha (TNF-α) and observed that the presence of the -238*A genotype in the promoter region of the TNF-alpha gene was more frequent in APS patients with arterial thrombosis and pregnancy loss than in controls (OR: 3.7 [95% CI 1.37-10.1], p= 0.007 and OR: 3.95 [95% CI 1.3-11.7], p= 0.01; respectively). HLA-DQB1*0303-DRB1*0701 haplotype was associated with TNFA -238*A in the control group (OR 96.0 [95% CI 9.6-959], p <0.0001) as well as in APS patient's group (OR 54.2 [95% CI 9.6-306.5], p <0.0001) (Bertolaccini et al., 2001).

6.14 Angiotensin-converting enzyme (ACE) gene polymorphisms

Evaluating the reported association between the D allele of the insertion (I)/deletion (D) polymorphism in the ACE gene and the occurrence of arterial thrombosis in coronary heart disease and stroke, Lewis et al. studied in 93 patients with APS whether this polymorphism could be an additional risk factor for arterial thrombosis. The distribution of the alleles was not significantly different between the patients with a history of arterial thrombosis and those without, although an unexpected skewing from DD to II was seen in patients older than 45 years in association with arterial thrombosis (Lewis et al., 2000).

6.15 Mannose-binding lectin (MBL) gene polymorphisms

Innate immunity is the first-line defense against pathogens. Among the components of innate immunity, mannose-binding lectin (MBL) and toll-like receptor 4 polymorphisms have been related to APS clinical manifestations. MBL is a liver-derived serum protein that binds to sugars on the surface of pathogenic microorganisms and triggers complement. Serum levels of MBL are associated with MBL gene polymorphisms. In 91 Caucassian patients with SLE MBL variant alleles were evaluated and a statistically significant association was found between the deficient homozygous 0/0 MBL genotype and the development of arterial thrombosis (OR: 5.8), but not venous thrombosis, mainly due to the strong association between this genotype and myocardial infarction (Ohlenschlaeger et al., 2004). However, MBL polymorphisms were not specifically evaluated in the APS subgroup. Font et al. studied MBL polymorphisms in a series of 114 Caucassian SLE patients (Font et al., 2007) and found that MBL-low genotypes showed a closer association with venous rather than arterial thrombosis. This fact probably is due to the different MBL alleles analyzed (0/XA and XA/XA were also included as deficient alleles) and/or to the varying prevalence of thrombotic events. In addition, in 53 patients with SLE, Seelen et al. reported that the presence of aCL was significantly associated with the variant alleles of MBL gene polymorphisms and they hypothesized that an enhanced production of autoantibodies may be related to disturbed clearance of apoptotic material due to impaired MBL function (Seelen et al., 2005). Additional contradictory results have been found in non-APS patients. In several different ethnic origin SLE patients, Calvo-Alen et al. found no differences in arterial thrombosis in patients homozygous for MBL-deficient alleles compared with non-SLE individuals (Calvo-Alen et al., 2006). Similar results were seen within ethnic groups, except for Caucassian patients in whom a statistically significant higher frequency of MBL-deficient alleles was found in those with cerebrovascular events. In a Japanese population, Takahashi et al. did not find a relationship between MBL alleles and the risk of arterial

thrombosis. These results point out the need to bear in mind the importance of the differences in the genetic substrate among the ethnic group when evaluating the influence of genetic polymorphisms in APS clinical expression (Takahashi et al., 2005).

6.16 Toll-like receptor 4 (TLR4) polymorphisms

TLR4 belongs to the family of transmembrane receptors whose activation leads to induction of various genes and production of proinflammatory cytokines. Polymorphisms within TLR4 genes result in an altered susceptibility to infectious or inflammatory diseases. In 110 Caucassian patients with APS with arterial and/or venous thrombosis, Pierangeli et al. evaluated whether the two co-segregating TLR4 polymorphisms $Asp^{299}Gly$ and $Thr^{399}Ile$ are involved in aPL-mediated thrombosis. This study showed that the frequency of TLR4 Gly^{299} and Ile^{399} alleles in APS patients was significantly reduced in comparison to healthy controls (Pierangeli et al., 2007).

7. Limitations of genetics studies in antiphospholipid syndrome

Interpretation of the results from epidemiological and genetic studies in various ethnic populations is quite difficult for the following reasons:

1. Although the enzyme linked immunosorbent assay (ELISA) for aCL antibodies and LAC testing has been extensively standardised, significant variation between laboratories still remains. The precise cut-off points for positive/negative results vary among laboratories over the world.
2. Clinical heterogeneity: the clinical definition of APS has varied among studies. Some patients with APS also manifest SLE, and constitute a heterogeneous population, making it difficult to analyse the role of a single factor. With the publication of the Sapporo criteria for the preliminary classification criteria for definite APS this problem will be solved with studies done on more uniform patient groups.
3. Interethnic variation in the associations of aPL with thrombosis or pregnancy loss must also take into account the multiple risk factors that exist in most populations for these complications. Possibly, variation in such collateral risk factors—for example, drug use or genetic risk factors for thrombosis may influence complication rates associated with aPL in various populations. For instance, in Lebanon, a high prevalence of prothrombin G20210A and factor V Leiden mutations exist. These factors will increase the thrombotic risk, especially in patients with aPL.
4. Disease activity: the level of disease activity is an important factor to control for in future studies. In early studies in the African-American clinic population in New Orleans it was found that IgG aCL were present in 27% of patients with SLE during periods of disease activity, compared with only 5% of patients with SLE during periods of less active SLE (Wilson et al. 1988).
5. Geographical migration: with the current increasing geographical migration and intermingling across geographical and ethnic groups, it is important to consider these variables in the interpretation of future studies.

8. Future directions

One of the first questions that come to mind when a patient receives the diagnosis of APS is whether we can predict which patients with aPL antibodies will develop thromboembolic

events or obstetric complications during her/him life-time as well an if there is an inherited predisposition to developing APS. There is obviously the most important question: "what is the course of the disease?" Identifying and understanding the causes of APS will likely lead to the identification of the major risk factors, the design of prevention strategies of thrombosis and obstetric complications and the development of targeted therapies with increased efficacy and minimal toxicity. No one knows the precise causes of APS (and there will likely be many that may differ among individuals), but there is strong evidence supporting a role for both genetics and infectious and non-infectious environmental factors. Current research has played an important role in the discovery of potential factors that influence its susceptibility. Ongoing research should lead to the further identification and understanding of both genetic and acquired factors that play a role in the development of APS. At this moment several 'Genome-Wide Association Studies' (GWAS) are underway in other autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, multiples sclerosis, Type 1 diabetes, and so on in which up to a million SNPs are tested in association studies. It is likely that these will add to the list of common and weak genetic risk factors. Individually, these risk factors have no clinical utility at all. It is possible; however, that comprehensive knowledge of all genetic risk factors in an individual may lead to relevant risks.

Nevertheless, it should be noted, that many acquired risk factors confer much higher risks that the genetic variants associated with thrombosis and obstetric complications in patients with APS. As these are often transient, they offer the best opportunity to reduce the burden of thrombosis and recurrent pregnancy losses by improved and individualized anticoagulant prophylaxis.

Family studies with GWAS using microsatellites are ongoing, and in the near future we will probably know which are the other DNA regions containing the susceptibility loci for APS.

The APS Clinical Research Task Force (CRTF) was one of the six Task Forces developed by the 13th International Congress on Antiphospholipid Antibodies organization committee recently held in Galveston, Texas, USA in April 2010 (Erkan et al., 2011) with the purpose of:

a. Evaluating the limitations of APS clinical research and developing guidelines for researchers to help improve the quality of APS research; and
b. Prioritizing the ideas for a well-designed multicenter clinical trial and discussing the pragmatics of getting such a trial done.

Following a systemic working algorithm, the Task Force identified five major issues that impede APS clinical research and the ability to develop evidence-based recommendations for the management of aPL-positive patients:

1. The aPLs detection has been based on partially or non-standardized tests, and clinical (and basic) APS research studies have included patients with heterogeneous aPL profiles with different clinical event risks;
2. Clinical (and basic) APS research studies have included a heterogeneous group of patients with different aPL-related manifestations (some controversial);
3. Thrombosis and/or pregnancy loss risk stratification and quantification are rarely incorporated in APS clinical research;
4. Most APS clinical studies include patients with single positive aPL results and/or low-titer aPLs ELISA results; furthermore, study designs are mostly retrospective and not

population based, with limited number of prospective and/or controlled population studies; and

5. Lack of the understanding of the particular mechanisms of aPL-mediated clinical events limits the optimal clinical study design.

The Task Force stated that there is an urgent need for a truly international collaboration approach to design and conduct well-designed prospective large-scale multicenter clinical trials of patients with persistent and clinically significant aPL profiles.

Notwithstanding the progress made over the last 15 years, APS remains poorly understood and have attracted the interest of many medical specialties including internal medicine, haematology, clinical immunology, rheumatology, and gynaecology.

9. Conclusions

APS is still seen as a rather obscure disease despite extensive research; this view is mainly the result of the unreliability of the current assays for detecting the presence of aPL. The consequences are a poor correlation between serological markers and clinical manifestations, and a lack of clarity about the pathogenetic mechanism causing the syndrome. Genetic susceptibility related to aPL and APS has been extensively examined during the last years. However, it has been difficult to determine genetic risk factors because of the heterogeneity in the antigen specificity and the pathogenesis of clinical manifestations related to APS. It is becoming increasingly clear that interactions between more than one genetic abnormality or between a genetic factor and environment components determine whether and when an individual will suffer from thrombosis. Given the fact that APS is characterized mainly by the presence of thromboembolic events, it seems perfectly plausible that several genetic factors may also be involved in its pathophysiology. Genome-wide linkage analysis and larger cohort cases-controls association studies, as well as multicenter international collaborations, would be useful to obtain a better understanding of the genetic predisposition which produces aPL and leads to the development of the clinical features of APS. As more genes responsible for these effects can be identified, a clearer understanding of the pathogenesis of this syndrome will be achieved, which undoubtedly will lead to more useful and safer therapeutic strategies.

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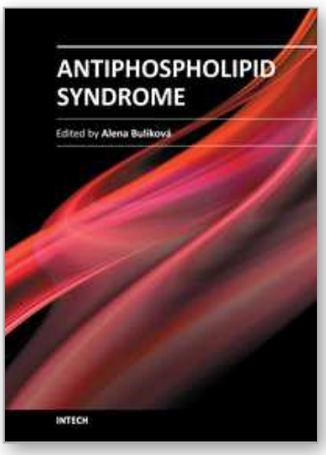

Antiphospholipid Syndrome
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The antiphospholipid syndrome has been described for the first time by Graham Hughes in 1983 as a condition connected with thromboses or foetal losses and antiphospholipid antibodies presence. Form that time there has been a great progress in knowledge, including antiphospholipid antibodies characterisation, their probable and also possible action, clinical manifestations, laboratory detection and treatment possibilities . This book provides a wide spectrum of clinical manifestations through Chapters written by well known researchers and clinicians with a great practical experience in management of diagnostics or treatment of antiphospholipid antibodies' presence.

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