The role of anti-phospholipid antibodies in autoimmune reproductive failure

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Abstract

Anti-phospholipid antibodies (aPL) are autoantibodies that are associated with thrombosis and a range of pregnancy complications including recurrent pregnancy loss and pre-eclampsia. The three clinically relevant, well-characterized aPL are anti-cardiolipin antibodies, lupus anticoagulant and anti-beta-2-glycoprotein I (β_2 GPI) antibodies. aPL do not bind directly to phospholipids but instead bind to a plasma-binding 'cofactor'. The most extensively studied cofactor is β_2 GPI, whose role in pregnancy is not fully elucidated. Although the pathogenicity of aPL in recurrent pregnancy loss is well established in humans and animal models, the association of aPL with infertility does not appear to be causative. aPL may exert their detrimental effects during pregnancy by directly binding trophoblast cells of the placenta, altering trophoblast signalling, proliferation, invasion and secretion of hormones and cytokines, and by increasing apoptosis. Heparin is commonly used to treat pregnant women with aPL; however, as thrombotic events do not occur in the placentae of all women with aPL, it may exert a protective effect by preventing the binding of aPL to β_2 GPI or by acting through non-thrombotic pathways. The aim of this review is to present evidence summarizing the current understanding of this field.

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Anti-phospholipid antibodies: introduction and background

Anti-phospholipid antibodies (aPL) comprise a heterogeneous family of autoantibodies that are associated with thrombosis and pregnancy mortality, particularly recurrent pregnancy loss. There are three clinically relevant aPL: i) lupus anticoagulants (LA) that are detected in serum using *in vitro* coagulation assays; ii) anti-cardiolipin antibodies (aCL) that are detected in serum or plasma by ELISA and iii) anti-beta-2-glycoprotein I (β_2 GPI) antibodies, which are also detected in serum or plasma by ELISA. Although ELISA is the most common method of detection of aCL and anti- β_2 GPI antibodies, other methods such as RIA, fluoro-enzyme immunoassay, multiplexed immunoassay and chemiluminescent immunoassay may also be used to detect aPL positivity (Forastiero *et al.* 2014). Laboratory criteria for the detection of aPL require detection of LA, aCL or anti- β_2 GPI on two or more occasions at least 12 weeks apart (Miyakis *et al.* 2006). It is necessary to conduct repeat tests as some infections are associated with transient false-positive results, especially in aCL tests.

Historically, aPL were first identified in patients with biologically false-positive tests for syphilis (Asherson

& Cervera 1993). These patients were found to have autoantibodies that produced a false-positive syphilitic 'reagin' test and the antigen in the reagin test is the negatively charged phospholipid, cardiolipin (Pangborn 1941). LA were initially identified in patients with the autoimmune disease systemic lupus erythematosus (SLE). LA were also found to be autoantibodies that reacted with negatively charged phospholipids, and in doing so prolonged the *in vitro* clotting time in phospholipid-dependent coagulation assays, such as the activated partial thromboplastin time and the dilute Russel Viper Venom time. Paradoxically, *in vivo*, LA are associated with thrombotic disease (Love & Santoro 1990, Galarza-Maldonado *et al.* 2012). Historically, it was noted that several patients with LA also had biologically false-positive tests for syphilis, suggesting that these may be the same autoantibodies. In an attempt to improve the clinical utility and efficiency for these two aPL tests, solid-phase immunoassays (especially ELISAs) using cardiolipin as the antigen were produced. Some but not all LAs also demonstrate aCL activity and *vice versa*. Although the traditional view was that aPL bind to negatively charged anionic phospholipids, it is evident that the term 'anti-phospholipid antibodies' is a misnomer, and aPL

do not bind directly to phospholipids, but rather to a complex containing a negatively charged phospholipid and a phospholipid-binding 'cofactor' (McIntyre *et al.* 1997, de Laat *et al.* 2004, 2007). The most widely studied of these cofactors is β_2 GPI. Indeed, it has been shown that many, but not all, autoantibodies that are detected in anti-cardiolipin ELISAs can react directly with β_2 GPI, in the absence of phospholipid, provided that a suitable negatively charged surface is available to immobilize the β_2 GPI. Immobilization of the β_2 GPI on such a surface facilitates the binding of aPL in one of two ways: either binding to the negative surface induces a conformational change in the protein that facilitates binding of aPL to a previously hidden cryptic epitope or the negative surface allows clustering of the β_2 GPI that facilitates high-affinity binding of aPL or a combination of these two mechanisms (Fig. 1).

The relationship between LAs, aCL and anti- β_2 GPI antibodies varies between patients and specific clones of antibody. Patients may have only one of the three aPL, or any combination of two or three of them, and the relative importance of these three antibodies is not always clear. However, there is growing acceptance that LAs are strongly correlated with disease and that patients who are positive for all three aPL are at the most risk of disease (Ruffatti *et al.* 2011, Galli 2012, Roggenbuck *et al.* 2012). LA correlates better with thrombosis, pregnancy morbidity and thrombosis in SLE than do aCL. Detection of LA in a patient with SLE predicts a 50% chance of a thrombotic event over 20 years follow-up. IgG/IgM isotype aCL antibodies, but especially those of the IgG isotype, are frequently associated with thrombosis and pregnancy failure.

Figure 1 The antigenicity of anti-phospholipid antibodies (aPL). aPL bind to β_2 GPI but only when this protein is immobilized on a suitable negatively charged surface. Such surfaces include the negatively charged phospholipids, cardiolipin and phosphatidylserine. Irradiated polystyrene, as found in many high-protein-binding ELISA plates is also a suitable negatively charged surface. In the circulation, *in vivo*, high levels of both aPL and β_2 GPI can coexist, as the circulating β_2 GPI is not antigenic for aPL. If negatively charged phosphatidylserine becomes exposed to the circulation, for example after an injury, β_2 GPI can bind to the phosphatidylserine and becomes antigenic for aPL either by exposing a cryptic epitope or via antigen-clustering (or a combination of the two).

A recent systematic review indicated that IgG isotype aCL were approximately four times more prevalent than IgM isotype aCL in patients with anti-phospholipid antibody syndrome (APS; Rodriguez-Garcia *et al.* 2015). This review also suggests that IgA isotype aCL are relatively common in patients with APS (20%) and that IgA isotype anti- β_2 GPI antibodies were even more common in these patients (56%). IgG and IgM anti- β_2 GPI are independent risk factors for thrombosis and pregnancy complications (Miyakis *et al.* 2006).

The APS

With the increased availability of simple tests, it became apparent that aPL identified a subset of patients with SLE who had either thrombotic disease, pregnancy complications or both. It also became apparent that there were a large number of patients, especially women with pregnancy complications, who had aPL but did not have a defined autoimmune disease or systemic thrombotic disease. Consequently, the APS was defined to describe patients with one or more aPL in the presence of thrombotic disease and/or pregnancy morbidity. The first criteria for classification of APS were developed in Sapporo, Japan, in 1990 and are referred to as the 'Sapporo criteria'. These criteria were revised in 2006 at the Eleventh International Congress of Antiphospholipid Antibodies, and these revised Sapporo criteria remain the most recent international consensus for the diagnosis of APS (Miyakis *et al.* 2006). Patients can have primary APS in the absence of other disease, or APS secondary to SLE or other pre-existing autoimmune conditions.

Anti- β_2 GPI antibodies

In recent years in an attempt to improve the diagnostic specificity of aPL testing, there has been a focus on attempting to define specific epitopes within β_2 GPI with which disease-causing pathogenic antibodies react. Structurally, β_2 GPI contains five domains of ~60 amino acids that are referred to as short consensus repeats (SCRs) or sushi domains. It has been suggested that the first SCR domain (domain 1) contains an epitope with which particularly pathogenic aPL react (Iverson *et al.* 1998, de Laat *et al.* 2009). An international multicentre evaluation of 477 patients established that a strong association existed between anti- β_2 GPI-domain I antibodies and venous thrombosis, and to a lesser extent pregnancy complications (de Laat *et al.* 2009). In another recent study, Andreoli *et al.* evaluated the domain specificity profile of anti- β_2 GPI antibodies in 159 subjects with persistently positive-, medium- or high-titre anti- β_2 GPI IgG. The prevalence of anti- β_2 GPI domain 1 antibodies was 73% in patients with thrombosis, and 64% in patients with obstetric APS. Anti-domain 4 and 5 antibodies were present in 19% and 16% of thrombotic and obstetric APS respectively (Andreoli *et al.* 2015). Although it is

clear that anti-domain 1 antibodies are important in the pathogenesis of the systemic thrombotic manifestation of APS, it is much less clear that these antibodies have a particular role in obstetric APS (de Jesus *et al.* 2014). It is abundantly clear that many women with obstetric as well as other complications of the APS have antibodies that react with other SCR domains of β_2 GPI (de Jesus *et al.* 2014, Poulton *et al.* 2015).

Non-consensus aPL

It is important to acknowledge that other autoantibodies that recognise negatively charged phospholipids or phospholipid-binding proteins, including antibodies against phosphatidylserine, phosphatidylinositol and phosphatidic acid, the zwitterionic phospholipid phosphatidylethanolamine, or the proteins annexin V, protein C, protein S, thrombomodulin and oxidised LDL, have been reported (Gharavi *et al.* 1987, Oosting *et al.* 1993, Horkko *et al.* 1996, Rand *et al.* 1998, Atsumi *et al.* 2000, Miyakis *et al.* 2006, Hirakawa *et al.* 2012). As phosphatidylserine, such as cardiolipin, is a negatively charged phospholipid, it can also bind to β_2 GPI, as does oxidised LDL, and it is possible that at least some of the non-consensus aPL are the result of cross-reactive antibodies that will behave as anti- β_2 GPI or aCL or anti phosphatidylserine (Wu *et al.* 1999). Although all these antibodies may be related to aPL, they are not included in the consensus criteria for the diagnosis of APS because their clinical significance is unclear (Bertolaccini *et al.* 2011).

aPL and obstetric diseases

Estimates of the prevalence of aPL in the general obstetric population range from 1 to 7% (Faden *et al.* 1997); however, most estimates are closer to 1–2%. In contrast, aPL have been reported to be more common in women with stillbirths, recurrent miscarriages, pre-eclampsia and intrauterine growth restriction (IUGR). Approximately 7–25% of unexplained recurrent miscarriage may be due to the presence of aPL (Drakeley *et al.* 1998) along with 3.8% of stillbirths and 13.7% of IUGR (Silver *et al.* 2013, Cervera *et al.* 2015). The reported occurrence of aPL in pre-eclampsia is highly variable, ranging from 11% to 61% (Branch *et al.* 1989, Yasuda *et al.* 1995, Katano *et al.* 1996); however, one group has shown that aPL are one of the strongest maternal risk factors for developing pre-eclampsia, increasing a woman's risk approximately ten-fold (Duckitt & Harrington 2005).

The revised Sapporo criteria define the obstetric manifestations of APS as follows: i) the occurrence of recurrent spontaneous abortion (miscarriage) before the tenth week of gestation, ii) unexplained fetal loss after the tenth week of gestation or iii) the development of pre-eclampsia/eclampsia or placental insufficiency before the 34th week of gestation (Miyakis *et al.* 2006).

Recently, the Taskforce on Obstetric Antiphospholipid Syndrome has systematically reviewed the relevant literature and confirmed the association of aPL with these complications of pregnancy (de Jesus *et al.* 2014).

The first demonstration that aPL are directly pathogenic in pregnancy came in 1990 with the passive transfer of human aPL into pregnant mice (Rote *et al.* 1990). This resulted in fetal demise, and since then several groups, using either passive transfer of aPL or immunization with β_2 GPI, have confirmed that aPL cause fetal demise and growth restriction equivalent to stillbirth/miscarriage and placental insufficiency using murine models (Bakimer *et al.* 1992, Blank *et al.* 1994). Moreover, a role for β_2 GPI has also been confirmed in the development of aPL-mediated pregnancy loss (Robertson *et al.* 2004). Until recently, whether aPL cause pre-eclampsia, a human pregnancy-specific disease characterized by new-onset hypertension in the second half of pregnancy, had not been demonstrated. However, when pregnant mice are immunized with β_2 GPI, aPL titres increase and the mice exhibit pre-eclampsia-like symptoms such as hypertension and proteinuria (Ding *et al.* 2014). How aPL cause the pregnancy complications with which they are associated remains unclear. In both women and men, aPL are associated with systemic thrombotic disease, and that aPL can also induce thrombosis has also been demonstrated in animal models where transfer of human aPL to mice results in excess thrombosis, but usually only following an induced vascular insult. It is now very clear that thrombosis is not the primary mechanism by which aPL induce obstetric complications (Viall & Chamley 2015); although thrombosis may be seen in a small proportion of placentae from late fetal deaths, uteroplacental thrombosis is not a feature of early gestation losses (Viall & Chamley 2015).

Before the widespread access to the ELISA-type aPL assays, women with these antibodies were usually identified because of a poor obstetric history, specifically late fetal death, which was frequently accompanied by placentae with evidence of extensive infarction (Out *et al.* 1991). Many, if not most of these, women also had classical autoimmune diseases and systemic thrombotic complications. This finding, coupled with the known association of aPL with thrombotic disease, leads to the reasonable hypothesis that aPL-mediated fetal demise was due to thrombosis in the uterine spiral arteries that supply maternal blood to the placenta, or in the intervillous spaces of the placenta (the original basis for the use of anticoagulant therapies such as heparin and low-dose aspirin (LDA; Ginsberg *et al.* 1995). However, with easy access to tests for aPL, a large number of women have been identified with aPL who do not have systemic thrombotic disease and whose placentae were not characterized by thrombotic lesions/infarction (Out *et al.* 1991, Salafia & Cowchock 1997). This led to the suggestion that obstetric manifestations of APS are not mediated primarily by thrombosis, but rather by direct

effects of aPL on the trophoblast cells of the placenta. This was further supported by the finding that β_2 GPI is synthesised by the trophoblast and is thus endogenously localized to the syncytiotrophoblast, cytotrophoblast and extravillous trophoblast populations (Chamley 1997). Indeed, a recent systematic review concluded that only one-third of women with aPL demonstrate signs of uteroplacental thrombosis (Viall & Chamley 2015).

There is no causative relationship between infertility and aPL

The incidence of aPL is reported to be 22 and 30%, respectively, in infertile women and women with recurrent IVF failure (Buckingham & Chamley 2009). However, due to a lack of well-designed studies, and lack of concordance between antibodies detected in each study, the causative nature of this association has not been established. A systematic review of the literature analysed the association of aPL and IVF outcome and showed that 13 of 29 studies reported a higher prevalence of aPL in infertile women. However, most of the studies used non-standardized, non-criteria aPL tests. Importantly, the presence of aPL was not found to influence IVF outcome, and treatment of aPL-positive patients undergoing IVF was not found to be beneficial. The Taskforce on Obstetric Antiphospholipid Syndrome also recently reviewed this topic and concluded that there is little evidence that aPL affect negatively on fertility and that infertility should not be included in the criteria for APS (de Jesus *et al.* 2014).

How do aPL cause pregnancy complications?

aPL induce characteristic histologic lesions in the placenta

If we are to understand how aPL induce pregnancy complications via direct effects on placental trophoblasts, it might be expected that we would have a clearer understanding of the pathologic lesions these antibodies induce in the placenta of affected pregnancies. In the past 30 or so years, there have been a number of small studies reporting histopathologic lesions in placentae from women with aPL; however, there has been no overview of these studies. A recent systematic review of the literature clarified which lesions are associated with aPL and reported that there is a 'fingerprint' of five common pathologic features in the placentae of women with aPL (Viall & Chamley 2015). These five lesions are as follows: i) placental infarction (found only in later gestation placentae), ii) impaired remodelling of uterine spiral arteries, iii) decidual inflammation, iv) increased syncytial knots in the syncytiotrophoblast and v) decreased vasculosyncytial membranes (Viall & Chamley 2015). Thrombotic lesions are not common in the placentae of women with aPL and were

strikingly absent from placentae of early gestational losses, confirming that thrombosis is not a primary pathophysiological mechanism in these pregnancies (Salafia & Cowchock 1997).

Structure of the human placenta and the role of trophoblasts

It is now widely accepted that aPL act directly upon trophoblasts of the placenta to induce pregnancy complications in women, and indeed, these antibodies can be eluted from affected placentae (Chamley *et al.* 1993). The anatomy of the human materno-fetal interface is quite unique and differs significantly from that in commonly used laboratory animals such as rodents. The human placenta has a villous or tree-like branching structure. The body of the human placenta is covered on its maternal-facing aspect by a single multinucleated cell, the syncytiotrophoblast, which is bathed in maternal blood and has a surface area of 11–13 m² at term (Mayhew 2008). Therefore, the syncytiotrophoblast acts as both a barrier between the maternal and fetal organisms and also the link between the two. The syncytiotrophoblast produces vast quantities of hormones and other factors that are crucial to the success of pregnancy; yet, it is not mitotically active and is formed and replenished by fusion of underlying villous cytotrophoblasts. Growing out from points of contact between the maternal uterine decidua and the villous placenta are columns of extravillous trophoblasts (Fig. 2). The extravillous trophoblasts most proximal to the placenta can proliferate, but as the cells migrate away from the villous surface, they lose their ability to proliferate and instead gain an invasive phenotype. These extravillous trophoblasts migrate into the decidua where they invade and transform the uterine spiral arteries that supply maternal blood to the placenta (Fig. 2). The extravillous trophoblasts erode the musculo-elastic walls of the spiral arteries such that these vessels are transformed from narrow-bore resistance arteries to large-diameter tubes that lack the capacity to respond to vaso-constricting stimuli. This transformation of the spiral arteries is necessary to allow the large and uninterrupted supply of maternal blood into the placenta that is required for increasing fetal growth in the later part of pregnancy. As a terminal phase of differentiation, at least some of the extravillous trophoblasts from early gestation form multinucleated placental bed giant cells, the function of which is unknown, but are likely to be important sources of hormones (Pijnenborg *et al.* 2006). aPL have been reported to affect the function of each of these populations of trophoblasts *in vitro*.

In vitro effects of aPL on trophoblasts

Several *in vitro* studies have been conducted to determine the mechanisms by which aPL interact with

Figure 2 Schematic indicating reported effects of anti-phospholipid antibodies on trophoblast. A first-trimester human villous showing interactions of aPL (A) with both villous and extravillous trophoblasts. Extravillous trophoblast (solid arrows) grows out from anchoring villi into the uterine decidua to transform the spiral arteries allowing increased blood flow to the placenta. The multinucleated syncytiotrophoblast (hollow arrows) covers the entire surface of the placenta and is bathed in maternal blood. The syncytiotrophoblast is a major source of secreted factors, such as hCG and cytokines. Villous cytotrophoblasts (short arrows) are the proliferating progenitor cells for the syncytiotrophoblast and for the extravillous trophoblasts. Interactions of aPL reduce the various functionalities of each of these different trophoblast subpopulations. As aPL are a heterogeneous family of antibodies, the specific effects of aPL on trophoblasts are likely to be variable depending on the exact mix of epitope specificities and affinities of antibodies present.

and directly affect trophoblast function. These studies were systematically reviewed recently by Tong *et al.* (2015). That systematic review showed that there is strong *in vitro* evidence that aPL detrimentally affect trophoblast syncytialization, viability and invasion, and this *in vitro* data support the lesions seen in the placentae of aPL-affected pregnancies (Viall & Chamley 2015). Furthermore, aPL also disrupt molecular signalling mechanisms in trophoblasts and increase inflammation and coagulation on the cell surface.

Trophoblast proliferation, migration and invasion

aPL may negatively affect trophoblast viability and function by reducing proliferation and invasiveness. The majority of studies have examined the effects of polyclonal aPL isolated from patient sera, as well as murine monoclonal aPL on placental explants or cell lines (Yacobi *et al.* 2002, Ornoy *et al.* 2003, Bose *et al.* 2004, Schwartz *et al.* 2007). Studies examining the mechanism of reduced migration showed that aPL mediate this effect possibly through the down-regulation of interleukin 6 (IL6; Mulla *et al.* 2010) and the up-regulation of TIMP2 (Albert *et al.* 2014), all

independently of Toll-like receptor 4 (TLR4). Another study, however, has implicated TLR4 in reduced trophoblast migration by aPL (Poulton *et al.* 2015). More recently, apolipoprotein E receptor 2 (ApoER2) has been implicated in aPL-reduced trophoblast migration (Ulrich *et al.* 2016). Others, however, have suggested that aPL binding directly to trophoblasts via adhered β_2 GPI reduce invasion by altering the repertoire of cell adhesion molecules (Di Simone *et al.* 2000a, 2002). A consequence of this reduced migratory response has been demonstrated using an *in vitro* matrigel model of spiral artery transformation. In this system, aPL and sera from APS patients with pregnancy morbidity disrupt the normal trophoblast–endothelial cell interactions (Alvarez *et al.* 2015).

aPL have also been shown to inhibit the proliferation of trophoblasts and the final differentiation of extravillous trophoblasts into giant multinucleated cells *in vitro* (Quenby *et al.* 2005). Reduced proliferation of cytotrophoblasts in response to aPL would have at least two consequences. i) As villous cytotrophoblasts are the proliferating progenitors of the syncytiotrophoblast, reducing the proliferation of villous trophoblasts may limit the growth and repair of the syncytiotrophoblast, resulting in reduced placental transport and barrier functions. ii) Proliferating cytotrophoblasts are also progenitors for extravillous trophoblasts, and thus, reduced trophoblast proliferation would lead to a limited pool of extravillous trophoblasts, resulting in inadequate invasion of the spiral arteries, underperfusion, oxidative stress and ischaemia–reperfusion injury in the placenta. These, in turn, could contribute to the early pregnancy loss, pre-eclampsia and IUGR that are often seen in women with aPL (Tong *et al.* 2015). The same consequences would result from an aPL-induced reduction in invasiveness of extravillous trophoblasts.

Trophoblast death

Several *in vitro* studies have reported an increase in trophoblast death in response to aPL using either term trophoblasts or first-trimester trophoblasts (Di Simone *et al.* 2001, Yacobi *et al.* 2002, Ornoy *et al.* 2003, Schwartz *et al.* 2007, Chen *et al.* 2009, Mulla *et al.* 2009). Cytotrophoblasts isolated from term placentae (Di Simone *et al.* 2001, 2006) have been shown to alter their expression of the apoptotic regulators Bax and Bcl2 in response to aPL, but without overt signs of cell death (Di Simone *et al.* 2006). The final stage in the normal life cycle of the multinucleated syncytiotrophoblast is thought to be that in localised regions, programmed cell death is initiated, followed by the extrusion of proapoptotic or apoptotic nuclei in large multinucleated vesicles called syncytial nuclear aggregates (SNAs; Mayhew 2008, Askelund & Chamley 2011). These SNAs are then deported via the blood to the maternal lungs where they become trapped in small

vessels and are cleared, most likely, by pulmonary endothelial cells. There is evidence that aPL alter the nature of cell death in the syncytiotrophoblast to a more necrotic process, evidenced by reduced expression of executioner caspases 3 and 7 (Chen *et al.* 2012), as well as reduced expression of the protective apoptotic regulator TRAIL in placental explants in response to aPL (Pantham *et al.* 2012). We have recently demonstrated that aPL are internalized into the syncytiotrophoblast of both first-trimester and term placentae via an antigen-specific receptor-mediated process (Viall *et al.* 2013). Once inside the syncytiotrophoblast, aPL bind to mitochondria and disrupt mitochondrial function and also induce the release of proapoptotic cytochrome c from the mitochondria (Viall *et al.* 2013, Pantham *et al.* 2015a). Consequently, there is an increase in the number of SNAs extruded from the syncytiotrophoblast (Chen *et al.* 2009, Pantham *et al.* 2015a). The proteome of SNAs from aPL-treated placentae is altered compared with that of SNAs from control antibody-treated placentae with notable changes in mitochondria-related proteins (Pantham *et al.* 2015a). In contrast to the SNAs from normal placentae, which prevent the activation of endothelial cells, the SNAs released from aPL-treated first-trimester placentae activate endothelial cells, with the likelihood that the changes in both the number and nature of SNAs contributes to the maternal endothelial cell activation, which is a hallmark of pre-eclampsia (Viall *et al.* 2013). Metabolomic analysis of the culture media from first-trimester placental explants treated with aPL has demonstrated an increase in ceramide metabolites, which play key roles in cell death by suppressing protein kinase C-epsilon, which is also found to be reduced in response to aPL (Pantham *et al.* 2015b). It appears likely that aPL alter cell death processes in many if not all trophoblast populations in the human placenta. Increased death in the syncytiotrophoblast is likely to contribute to increased production of SNAs, whereas increased death of extravillous trophoblasts is likely to reduce the pool of these cells available to modify the spiral arteries (Quenby *et al.* 2005).

Cytokine and hormone production

The deciduae of women with APS show signs of inflammation (Tong *et al.* 2015). *In vitro* studies suggest that aPL may alter the cytokine and hormonal milieu produced by trophoblasts, leading to a pro-inflammatory environment at the maternal-placental interface and altered trophoblast function (Abrahams 2009). It was shown nearly two decades ago that aPL reduce the basal and GnRH-induced production of human chorionic gonadotropin (hCG; Katsuragawa *et al.* 1997, Di Simone *et al.* 2000b, Schwartz *et al.* 2007). Recent evidence suggests that this aPL-mediated reduction in hCG secretion by trophoblasts is mediated via TLR4 (Marchetti *et al.* 2014). Production of hCG is usually considered to

be a marker of syncytiotrophoblast endocrine function, as this cell is the major source of hCG; however, it has been shown that hyperglycosylated hCG can be secreted by, and promotes, extravillous trophoblast invasion (Fournier *et al.* 2015). Thus, the reductions in hCG secretion induced by aPL may reflect adverse effects of the antibodies on both syncytiotrophoblast and extravillous trophoblast functions. Production of IL3 may be reduced in women with aPL-mediated fetal loss and IL3 has been shown to overcome the inhibitory effects of aPL on trophoblast invasion and hCG secretion, but not trophoblast proliferation (Di Simone *et al.* 2000a, Chamley *et al.* 2001). *In vitro* studies utilizing first-trimester trophoblasts and murine monoclonal aPL have demonstrated that aPL (both murine mAb and patient-derived aPL) increase production of IL8, IL1β, monocyte chemoattractant protein 1 (MCP1) and GROα in a TLR4-dependent manner. Furthermore, downstream of TLR4, IL1β secretion is mediated by the induction of endogenous uric acid, which in turn activates the Nod-like receptor, Nalp3, leading to Nalp3/ASC/caspase 1 inflammasome activation and subsequent IL1β processing and secretion (Mulla *et al.* 2013a). These aPL-induced changes could contribute to the reduced placental invasion and pro-inflammatory profile reported in pregnancies affected by aPL (Abrahams 2009). Reported changes in trophoblast/placental cytokine production induced by aPL are summarized in Table 1.

Coagulation

There has been a considerable amount of research into the mechanisms of coagulation and thrombosis leading to placental infarction due to aPL. aPL may cause coagulation in the proximity of the placenta by reducing the expression of annexin A5 on placental villi (Rand *et al.* 2006). Annexin A5, also known as placental anticoagulant protein-I and vascular anticoagulant-α, is thought to function as a potent anticoagulant by competing with coagulation factors for binding sites on anionic phospholipids such as phosphatidylserine.

Table 1 Reported *in vitro* effects of aPL on cytokine production by trophoblast/placentae.

	Effects of aPL treatment on cytokine production by trophoblast/placenta <i>in vitro</i>
Mulla <i>et al.</i> (2009)	Increased IL8, MCP1, GROα and IL1β Did not affect IL2, 4, 5, 6, 7, 10, 12, 13, 17, G-CSF, GM-CSF, IFNγ, MIP1α, RANTES and TNFα
Di Simone <i>et al.</i> (2010)	Decreased HB-EGF expression
Mulla <i>et al.</i> (2010)	Decreased IL6
Carroll <i>et al.</i> (2011)	Increased VEGF, PIGF, sFlt1 and sEng ^a
Ichikawa <i>et al.</i> (2011)	No effect on VEGF or sFlt-1, decreased PIGF
Iwasawa <i>et al.</i> (2012)	Increased IL12
Mulla <i>et al.</i> (2013)	Increased IL1β
Albert <i>et al.</i> (2014)	Increased TIMP2

^aEffect observed with only one of the two aPL tested.

Annexin A5 is constitutively expressed on the apical surface of syncytiotrophoblast and may be necessary to maintain vascular homeostasis and blood fluidity, functioning as an 'anticoagulant shield' in the placenta during pregnancy. Disruption of this shield due to aPL has been observed in placental villi from women with APS, as well as in trophoblasts cultured with aPL *in vitro* (Krikun *et al.* 1994, Rand & Wu 1999, Rand *et al.* 1994, 1997, 1998, 2003, 2004, 2006). It has therefore been postulated that the disruption of the annexin A5 anticoagulant shield in the placenta results in accelerated coagulation and thrombosis at the placental surface, causing recurrent fetal loss and fetal growth restriction in pregnancies with aPL, and this is supported by data from an annexin A5 knockout mouse model (Rand *et al.* 1994, 2006, Ueki *et al.* 2012). However, the disruption of the annexin A5 shield is not a universal feature of placentae from pregnancies complicated by aPL (Lakasing *et al.* 1999). Furthermore, enhanced coagulation at the placental surface is not a common histopathologic feature of pregnancies affected by aPL (Tong *et al.* 2015), and thus, it is unclear how important disruption of the annexin A5 anticoagulant shield is in the pathogenesis of obstetric APS.

Receptors that mediate the effects of aPL in the placenta

Although it was originally thought likely that aPL reacted with phospholipids in the cell membrane of target cells, it now seems much more likely that the interaction of aPL with cells is predominantly via cell surface receptors. Several receptors have been implicated in aPL signalling including TLRs 2 and 4, which have lipid-based ligands, as well as lipoprotein receptors with which β_2 GPI interacts. The ability of aPL to activate these TLRs may arise because β_2 GPI shares molecular mimicry with bacterial products such as lipopolysaccharide (Blank & Shoenfeld 2004, Sorice *et al.* 2007). Interactions of aPL with endothelial cells have been shown to be mediated by TLR4, and when they have invaded the spiral arteries, extravillous trophoblasts take on the functions and some feature of endothelial cells. Although the effects of aPL on extravillous trophoblasts have been shown to be mediated by TLR4 (Mulla *et al.* 2009), there is some disagreement as to whether the intracellular signalling is mediated primarily by the MyD88 or TRIM/TRAF pathways (Poulton *et al.* 2015). It has also recently been shown that aPL are rapidly taken up specifically into the syncytiotrophoblast by a receptor-mediated mechanism. The receptor responsible for this uptake has not yet been specifically identified; however, it was shown that this was not typical antibody transport and was not mediated by an Fc-receptor. The receptor appeared to be a member of the LDL receptor family, as its effects were blocked by receptor-associated protein (Viall *et al.* 2013). Indeed, in other work, the LDL receptor family

member ApoER2 has been shown to mediate changes in trophoblast function and pregnancy complications induced by aPL (Ulrich *et al.* 2016).

Lessons from animal models

The pathogenicity of aPL in pregnancy has been well established in murine models of APS. Infusion of aCL derived from humans or mice into pregnant mice resulted in lower fecundity, increased resorption of embryos, reduced number of embryos per pregnancy and lower fetal and placental weight compared with controls in several studies (Gharavi *et al.* 1989, Blank *et al.* 1991, Suzuki *et al.* 1996). Mice injected with aPL show signs of placental thrombosis, decidual necrosis and infarction, which may play a role in fetal loss and pregnancy failure (Branch *et al.* 1990). Mice in which β_2 GPI has been knocked out are fertile but have reduced fetal and placental weights, indicating that, while necessary for optimal fetal growth and development, β_2 GPI is not essential to pregnancy, at least in mice (Robertson *et al.* 2004).

The mechanism by which aPL induce fetal demise in mice has been clearly established in series of elegant experiments (Holers *et al.* 2002, Girardi *et al.* 2003, 2004). Using a combination of knockout mice, recombinant proteins and neutralising antibodies, it has been shown that aPL activate the complement cascade resulting in the recruitment of neutrophils to the implantation site. These neutrophils, in conjunction with the complement system and the pro-inflammatory protein tissue factor, result in the destruction of affected embryos. Complement component C3, deposited in the remains of implantation sites, is a marker of this process. Although this mechanism is clearly established in mice, it is much less clear how much of a role the complement system plays in fetal demise in women. There is limited evidence for deposition of C3 in the placentae of women with aPL (Out *et al.* 1991, Viall & Chamley 2015). However, one study has reported increased complement deposition in the trophoblast cytoplasm (C4d and C3b), trophoblastic cell and basement membrane (C4d), and extravillous trophoblasts (C4d) of patients with aPL, compared with control patients (Shamonki *et al.* 2007).

The complement cascade has also been associated with the development of pre-eclampsia, which is one of the clinical manifestations of obstetric APS (Haeger *et al.* 1992). One recent prospective study demonstrated that seven out of 40 patients who had aPL and developed pre-eclampsia displayed mutations in complement regulatory proteins, suggesting that complement activation may play a role in initiating pre-eclampsia in the presence of aPL in some women (Salmon *et al.* 2011). The mechanistic differences in how aPL induce obstetric disease between mice and women may relate to the quite different anatomical structures of

the materno–fetal interface between these species and also to the mechanisms and proteins responsible for the regulation of complement activation that differs at the materno–fetal interface in mice and women. In mice, a single molecule, CRRY1, is the major regulator of complement, whereas in women, three proteins CD46, CD55 and CD59 are present to prevent complement activation.

Treatments

Owing to the theory that aPL cause fetal loss mainly by inducing thrombosis and placental infarction, heparin has been utilized to treat women with APS. However, as most placentae of women with aPL do not show signs of thrombosis, heparin likely acts via another mechanism to prevent fetal loss (Tong *et al.* 2015). *In vitro* studies suggest that heparin prevents the binding of polyclonal and monoclonal aPL to trophoblasts and may inhibit the binding of β_2 GPI to anionic phospholipids. Heparin may also exert a protective effect on trophoblasts by antagonizing the effect of aPL on trophoblast death, syncytialization, complement activation and inflammation (Di Simone *et al.* 1997, Bose *et al.* 2004, Girardi *et al.* 2004, Mulla *et al.* 2009, 2010). That heparin acts via a non-anticoagulant mechanism is also supported by the observation that clinical trials employing unfractionated heparin show a protective effect against aPL, whereas trials employing low-molecular-weight heparin (LMWH) have less convincing results (Farquharson *et al.* 2002, Rai & Regan 2002, Noble *et al.* 2005, de Jesus *et al.* 2014). In a recent meta-analysis, Ziakas *et al.* concluded that heparin plus LDA is not more effective than LDA alone in treating women with APS-related fetal deaths but at least one of the trials included in that meta-analysis included a mixed population of patients only some of whom had APS (Ziakas *et al.* 2010). Moreover, a number of studies have shown that heparin promotes the placental release of sFlt1 (Carroll *et al.* 2011, Rosenberg *et al.* 2011), an anti-angiogenic factor associated with pre-eclampsia (Maynard *et al.* 2003). However, *in vitro* studies indicate that vitamin D may reverse this LMWH-induced trophoblast sFlt1 secretion (Gysler *et al.* 2015). The effects of different therapies used in clinical trials to treat women with obstetric APS have been systematically reviewed (White *et al.* 2004). Ten randomized or quasi-randomized, controlled trials were assessed. Aspirin alone (three trials) had no effect on pregnancy loss, whereas heparin in combination with aspirin (two trials, 140 patients) reduced pregnancy loss in women with APS by 54%. Prednisone in combination with aspirin increased prematurity but did not reduce fetal loss at high dose. Low-dose prednisone administered up to 14 weeks of gestation to women with previous aPL-related pregnancy loss increased the live birth rate from

4 to 61%, and this option warrants further assessment (Bramham *et al.* 2011).

Anti-malarial and anti-inflammatory drugs have also been utilized to prevent APS-mediated fetal loss. The effects of hydroxychloroquine on trophoblasts treated with aPL have been investigated in six studies (Tong *et al.* 2015). Hydroxychloroquine has been found to antagonize the effects of aPL on annexin A5 and coagulation on the surface of trophoblasts. It also prevents the effects of aPL on trophoblast migration, invasion, hCG secretion and fusion (Albert *et al.* 2014). Chloroquine also reduces aPL internalization in first-trimester placental explants (Albert *et al.* 2014). Anti-inflammatory drugs such as indomethacin antagonize trophoblast prostacyclin and thromboxane production, whereas betamethasone prevents the effects of aPL on hCG secretion. IL3 has been used in some studies to prevent fetal loss and appears to reverse the effect of aPL on syncytialization, trophoblast invasion and hCG secretion (Chamley *et al.* 2001).

Although pravastatin reduces fetal loss in a murine model of APS, its effect on humans is unknown. *In vitro*, pravastatin had no effect on aPL-mediated changes in trophoblast inflammatory mediators and invasion and may, therefore, not be as beneficial in human pregnancies complicated by APS (Odiari *et al.* 2012). However, preliminary studies suggest that it might be useful at stabilizing the clinical and biochemical features of preterm pre-eclampsia (Brownfoot *et al.* 2015). That said, in a case study, a patient with a history of pre-eclampsia, thrombosis, and APS, presenting with pre-eclampsia at 23 weeks of gestation in her second pregnancy was treated with pravastatin, and this resulted in marked clinical improvement and successful pregnancy outcome (Lefkou *et al.* 2014). There is a clear need for better therapeutic approaches and alternative explanations for why existing therapies, such as heparin and LDA, appear to have positive effects in pregnancies affected by aPL. *In vitro*, as well as *in vivo*, studies may help to inform us. For example, in a recent study, the aspirin-triggered lipoxin, 15-*epi*-lipoxin A4, reversed the negative effect of aPL and sera from APS patients on trophoblast migration and trophoblast–endothelial cell interactions (Alvarez *et al.* 2015).

Conclusions

Although there have been great advances in our understanding of aPL, the mechanisms by which they lead to obstetric disease still remain unclear. Standardization of detection assays and better study design will allow for directly comparable studies and better reproducibility between studies. It has become clear that aPL interact directly with all populations of human trophoblasts, adversely altering their function, although murine models confirm that these antibodies

are pathogenic and not just markers of a disease process. Current therapies, such as heparin, have been applied apparently successfully to prevent recurrent miscarriage caused by aPL; however, the evidence is not entirely conclusive and the optimal therapy to prevent the poor obstetric outcomes associated with these antibodies remains to be determined. Ongoing and future research will hopefully identify the pathways by which aPL exert their detrimental effects during pregnancy to allow the identification of better therapeutic approaches and predictors of pregnancy outcomes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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