AUTOANTIBODIES AGAINST EPCR ARE FOUND IN ANTIPHOSPHOLIPID SYNDROME AND ARE A RISK FACTOR FOR FOETAL DEATH

SHORT TITLE: Anti-EPCR autoantibodies in foetal death

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ABSTRACT

The antiphospholipid syndrome (APS) is associated with thrombosis and foetal death but the pathological mechanisms are poorly understood. Since endothelial protein C receptor (EPCR) plays a role in the anticoagulant system and in placental development, we hypothesised that anti-EPCR autoantibodies may be involved in clinical manifestations of APS and in foetal loss.

The levels of IgM and IgG anti-EPCR autoantibodies were analysed by ELISA in 43 patients with APS and 43 controls. Anti-EPCR levels were higher in APS patients than in controls. Interestingly, one of the IgM anti-EPCR autoantibodies inhibited the generation of activated protein C on endothelium.

Since markedly high anti-EPCR levels were found in women with foetal death, 87 patients with a first episode of unexplained foetal death were subsequently analysed and their anti-EPCR levels compared with 87 matched controls. We found that anti-EPCR autoantibodies constitute an independent risk factor for a first foetal death episode: the adjusted odds ratios (OR) for anti-EPCR autoantibodies above the 95th percentile were 23.0 [95% confidence interval (CI): 2.0-266.3] for IgM and 6.8 [95% CI: 1.2-38.4] for IgG.

Anti-EPCR autoantibodies can be detected in APS patients and are independent risk factors for foetal death.
INTRODUCTION

Antiphospholipid syndrome (APS) is characterised by vascular thrombosis and complications of pregnancy (foetal death, premature birth or multiple spontaneous abortions) associated with the presence of antiphospholipid antibodies (APL). These antibodies are heterogeneous and recognise a variety of combinations of phospholipids, phospholipid-binding proteins or both. The most commonly detected subgroups of antiphospholipid antibodies are lupus anticoagulant antibodies (LAC) and anticardiolipin antibodies (aCL). Other antiphospholipid antibodies are directed against phospholipids other than cardiolipin, or against phospholipid-binding proteins like β2-glycoprotein I or annexin V. However, the mechanisms linking the presence of APL with thrombosis and pregnancy complications such as foetal death are poorly understood.

Activated protein C (APC) is a major regulatory protein of the coagulation cascade. Protein C (PC), the zymogen of APC, is activated by thrombin bound to thrombomodulin on cell surfaces. APC, working in concert with its non-enzymatic cofactor protein S, exerts its anticoagulant function by proteolysing activated factors V and VIII. Genetic and acquired defects of thrombomodulin, PC and protein S have been identified in patients with venous and/or arterial thrombosis, and antibodies against these molecules have been related with APS. Endothelial PC/APC receptor (EPCR) is an endothelial cell membrane glycoprotein which is tightly bound to a phospholipid and binds PC and APC. EPCR is mainly expressed in large vessels, especially arteries, and in the syncytiotrophoblast as well. PC binding to EPCR notably enhances its activation by the thrombin-thrombomodulin complex. APC binding to EPCR allows APC-dependent tethering of protease activated receptor-1 which blocks the apoptosis of endothelial and probably placental cells.
addition, growing evidence supports a role for EPCR in pregnancy maintenance since EPCR-knockout mice experience placental thrombosis and early embryonic mortality.\textsuperscript{15} This fact, together with the nature of EPCR as a phospholipid-binding protein, its function as anticoagulant and cell-protector, its involvement in the development of placenta and its localisation on the surface of vessels and placenta, make this molecule a possible target for APL associated with foetal death and thrombotic disease.

We investigated the presence of anti-EPCR autoantibodies in patients with APS. The results obtained made us suspect that a relationship between these antibodies and foetal death may exist. For this reason we subsequently studied the association of anti-EPCR autoantibodies with first foetal death in a matched case-control study. We also studied the effect of these autoantibodies on APC generation on the endothelial surface. Our results support that anti-EPCR autoantibodies constitute a risk factor for a first foetal death episode. Impaired PC activation on cell surfaces could be one of the ways by which these antibodies exert their pathological effects.
METHODS

APS patients and controls
Cases were 43 consecutive patients [17-67 years old (median age: 43 years), 39 women and 4 men] diagnosed with APS according to international criteria (16, 17) between February 1998 and March 2002 at the St Thomas’ Hospital, London, UK. All of them were identified by having LAC and a personal history of venous thrombosis (n = 17), arterial thrombosis (n = 13) or both (n = 13). Serum samples were drawn at least 3 months after the last thrombotic episode and stored at −80 ºC until tested for anti-EPCR autoantibodies. The control group was made up of 43 healthy volunteers with no history of thrombosis or LAC. All patients and controls had given their informed consent for participation in the study.

Women with foetal death and controls
A matched case-control study on foetal death was carried out at the University Hospital of Nîmes, France, and in clinics belonging to the same obstetric network, between September 1996 and September 2002. This study was part of the Mediterranean Abnormal Pregnancy Study Program which led to the previously published Nîmes Obstetricians and Haematologist Study (NOHA) studies.18

We included as cases 87 consecutive women, 19-31 years old (median age: 27 years), with a first episode of foetal death from the 10th week of amenorrhea that had occurred during their last pregnancy. Reasons for exclusion were: any thrombotic antecedents, any chromosomal abnormality in the conceptus karyotype, any morphologic malformation in the foetus, chronic infectious disease or any known systemic disease, any antecedents of other type of pregnancy with poor outcome (early embryo loss before the 10th week of gestation, eclampsia, intra-uterine foetal growth restriction), diabetes mellitus or lack of adherence to study procedures. The foetal death had
occurred during the first pregnancy in 58 women, during the second one in 21 women and during the third one in the remaining eight women; between the 10th and the 22nd weeks in 75 women and between the 22nd and the 36th weeks in the remaining 12 women.

A control group of 87 healthy mothers, matched for age, number of pregnancies, and time elapsed since the end of the last pregnancy, all negative for thrombotic history and chronic infectious diseases, was simultaneously recruited during the same period of time as patients, among consecutive women attending the outpatient Department of Gynaecology at the same hospital for a general medical examination.

The study was approved by the Ethics Committee and informed consent was obtained from all participants. Inclusion of cases and controls, and blood collection took place at least 6 months after foetal death. Blood samples were collected, processed, and stored at –80 °C, according to standard procedures.

**Expression of soluble EPCR**

We amplified the soluble EPCR (sEPCR) sequence, comprising the extracellular domain without its signal peptide and transmembrane and intracellular domains [residues 1-193, mature protein numbering] by polymerase chain reaction with primers which added Cla I and Not I restriction sites at the 5’ and 3’ ends respectively:

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agcttgccatatcgaggcaagcgcctcagatg
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and

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tattctatgcggccgccgaagtgtaggagcggcttg
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These modifications allowed us to ligate the sEPCR sequence into the Cla and Not sites of the pPICZαC (Invitrogen, Paisley, UK) following the Saccharomyces cerevisiae α-factor secretion signal that allows for efficient secretion of many proteins from Pichia pastoris. The insert was cloned in frame with a myc epitope and a six His tag present in the vector in order to express the sEPCR fused with myc and six His at the C-terminal end. Yeast was transformed with the linearized vector, thus integrating the sEPCR
coding sequence into the endogenous methanol-responsive promoter by homologous recombination. As the pPICZαC vector encodes for the zeocin resistance gene, zeocin resistant colonies of Pichia pastoris were screened for sEPCR expression induced by 1% methanol. The most efficient sEPCR-expressing colony was selected for large-scale production.

**Purification of recombinant sEPCR**

Recombinant sEPCR was purified from culture supernatants using a three-step purification. The sample was loaded onto a 5 mL Hitrap chelating-HP column (Amersham Biosciences, Little Chalfont, UK) loaded with copper. The bound fraction was eluted with 50 mM EDTA, dialysed against 20 mM Tris-HCl (pH 7.5) and then subjected to anion exchange using a Resource Q column (Amersham Biosciences) and a salt gradient from 0 to 300 mM NaCl over 20 column volumes. sEPCR-containing eluted fractions were applied to a Superdex 75-HR10/30 column (Amersham Biosciences). The concentration of purified protein was determined using the BCA total protein assay (Pierce, Rockford, IL, USA).

**ELISA for the determination of anti-EPCR autoantibodies**

96-well microplates (Costar, Acton, MA, USA) were coated with 0.15μg anti-myc monoclonal antibody (Mo Ab) (Invitrogen) to anchor sEPCR preserving its extracellular epitopes [direct sEPCR binding to wells occludes several epitopes]^{11}. After washing with TBS (20 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.4), non-specific binding sites were blocked with 3% (w/v) bovine serum albumin (BSA) in TBS. 100 μL/well of 1 μg/mL recombinant sEPCR in TBS supplemented with 1% BSA (TBS-BSA) were incubated at room temperature (RT) for 4 hours. Subsequently, 100 μL of a 1:100 dilution of the sample (plasma or serum) in TBS-BSA was incubated overnight at 4 °C. Parallel blank wells were included with TBS-BSA without plasma or serum. Bound
IgM and IgG anti-EPCR autoantibodies were detected with either a peroxidase-conjugated murine polyclonal anti-human IgM (Zymed, South San Francisco, CA, USA) or an alkaline phosphatase-conjugated murine polyclonal anti-human IgG (Zymed). After 2 hours at RT and extensive washing, the plates were developed with either 100 µL of 0.4 mg/mL o-phenylenediamine (Kodak, Rochester, NY, USA) (IgM) or 100 µL of 1 mg/mL 4-nitrophenil phosphate (Sigma, St Louis, MO, USA) (IgG) and read at 492 or 405 nm respectively in a iEMS Reader (Labsystems, Helsinki, Finland).

To allow comparisons among plates, we chose a sample to be assayed in every plate (standard sample) which thus allowed us to introduce a correction factor. Arbitrary Units (AU) were defined as follows: for each patient sample (problem sample) the specific absorbance was calculated by subtracting the absorbance of blank wells and then multiplying by 1,000 and by a correction factor corresponding to the ratio between the absorbance of the standard sample assayed in a given plate (reference plate) and in the plate where the problem sample is assayed. Samples from patients were assayed together with those from their matched controls in the same plate to minimise differences due to the inter-assay variability. All samples were assayed twice. The intra- and inter-assay coefficients of variation were evaluated with five samples tested five and three times respectively and were always found to be lower than 5% and 10% respectively.

**Purification of anti-EPCR autoantibodies**

IgM fraction from serum or plasma samples containing high titres of IgM anti-EPCR was isolated using a HitTrap NHS-activated HP column (Amersham Biosciences) where a murine anti-human IgM Mo Ab had previously been immobilised. IgG fraction from serum or plasma samples containing IgG anti-EPCR was isolated using a
HiTrap Protein G HP column (Amersham Biosciences) according to the manufacturer’s instructions.

**APC generation on cultured endothelial cells**

50,000 EA.hy926 cells\(^{11}\) (endothelium-derived cell line expressing thrombomodulin and EPCR, kindly supplied by Dr. C.J. Edgell, University of North Carolina) were incubated with 0.02 U/mL (0.17 nM) thrombin (ERL, Swansea, UK) and increasing concentrations of PC (kindly supplied by Baxter, Deerfield, IL, USA) ranging between 50 and 1000 nM in a 20 mM Tris buffer, pH 7.4, supplemented with 150 mM NaCl, 5 mM CaCl\(_2\), 0.6 mM MgCl\(_2\), 1% BSA, 0.01% Tween-20 and 0.02% NaN\(_3\). After 45 minutes lepirudin (Schering AG, Berlin, Germany) was added at 0.2 µM to inhibit thrombin and 4 minutes afterwards the chromogenic substrate S-2366 (Chromogenix, Milan, Italy) was added at 0.4 mM and its proteolysis by APC kinetically monitored at 405 nm in the iEMS Reader. The data curve fitting the Michaelis-Menten equation was performed using Enzfitter software (Biosoft, Cambridge, UK) which calculated the Km and Vmax values of PC activation under these conditions. Where required, 20 µg/mL RCR-252 Mo Ab, which blocks PC-EPCR binding,\(^{21}\) or anti-EPCR autoantibodies from patients were added.

**Preparation of a sEPCR affinity column**

Two mg of recombinant sEPCR were coupled to a HiTrap NHS-activated HP column (Amersham Biosciences) following the manufacturer’s instructions. Since the sEPCR bound to the column retained its ability to bind PC, it probably retained the native conformation including the epitopes recognised by the autoantibodies. Thus, this column is suitable for anti-EPCR-depleting.

**Statistical methods**
In the APS case-control study the comparison between cases and controls for frequency of high levels of IgM and IgG anti-EPCR was performed with a chi-square test. In the foetal death matched case-control study, comparison between cases and controls for continuous and categorical variables was performed with a t-test for paired samples and McNemar test, respectively. Association between levels of IgM and IgG anti-EPCR with LAC and IgM aCL was assessed with correlation coefficients for continuous variables and Mann-Whitney test for categorical variables. In order to evaluate the risk of foetal death associated with high levels of IgM and IgG anti-EPCR, a conditional logistic regression analysis was used, with matched pairs of cases and controls. The main independent variables were levels of IgM and IgG anti-EPCR categorized, according to the distribution of these antibodies in controls. We used different cut-off points to determine the levels associated with a higher risk. Univariate and multivariate analysis were performed, adjusting for known risk factors of foetal death. The levels of anti-EPCR autoantibodies were also divided in quartiles to assess a relationship between this variable and risk of foetal death (dose-response). Tests for trend in foetal death across quartiles of anti-EPCR were assessed in conditional logistic models using a continuous variable with the median values for each quartile of the antibodies. Interaction (effect modification) was assessed introducing product-terms in the conditional logistic models. The Mann-Whitney U test was used to compare APC generation in the presence and absence of anti-EPCR autoantibodies.
RESULTS

More than 5 mg of sEPCR could be purified from a *Pichia* culture. By SDS-PAGE and Western blot, sEPCR appeared as a single band. The PC activation by thrombin on the EA.hy926 cells (Km = 69±18 nM) was hampered in the presence of 2 µM sEPCR (apparent Ki = 75±4 nM) suggesting that our recombinant sEPCR binds to PC with similar efficiency to native EPCR.²²,²³ Furthermore, the sEPCR was able to inhibit the anticoagulant activity of APC in a modified clotting assay, as expected²² (data not shown). This evidence strongly suggests that our recombinant sEPCR is folded in the correct conformation allowing us to use it to detect autoantibodies against EPCR.

**Anti-EPCR autoantibodies in APS patients**

The median values of both IgM and IgG anti-EPCR were higher among APS cases than among controls (57 AU vs 45 AU and 75 AU vs 72 AU, respectively) (figure 1). Even if the difference between mean levels was not statistically significant, 21% of APS patients had either IgM or IgG anti-EPCR levels exceeding the 97th percentile compared with 5% in control subjects (p = 0.024). Three patients displayed very high levels of IgM anti-EPCR (patient A = 407 AU, patient B = 301 AU, patient C = 293 AU) and two patients high IgG anti-EPCR levels (patient D = 230 AU, patient E = 220 AU). All these five patients were women with a previous history of thrombosis. Two women carrying IgM anti-EPCR (patients A and B) had suffered from multiple episodes of foetal death.

**Biochemical characterization of anti-EPCR autoantibodies**

IgM fractions from patients A, B and C and IgG fractions from patients D and E were purified from serum. IgM fraction of patient C remarkably inhibited the thrombin-induced APC generation by EA.hy926 cells in a dose-dependent manner (maximum effect about 80% reduction of PC activation, p = 0.02), an effect comparable to that obtained with RCR-252 (figure 2). The decrease in the APC generation was due to an
increase in the Km rather than Vmax, compatible with ablation of the EPCR-enhancing effect on activation (figure 3). To demonstrate that this effect was due to a specific IgM against EPCR, the patient C sample was anti-EPCR autoantibody-depleted by loading it onto the sEPCR affinity column. The sample lost the inhibitory capacity on APC generation indicating that an specific anti-EPCR autoantibody was responsible for this phenomenon (figure 2).

**Anti-EPCR autoantibodies in women with foetal death**

Since a relationship between anti-EPCR autoantibodies and foetal death could be hypothetised from the results presented above, we focused our analysis on the association between anti-EPCR and foetal death in a group of 87 women with a first episode of unexplained foetal death without previous history of venous and/or arterial thrombosis and 87 healthy age-matched controls. Table 1 shows the frequencies of risk factors classically related with foetal death together with the frequencies of IgM and IgG anti-EPCR found in patients and controls. The 95th percentile of the IgM anti-EPCR in the control group was 99 AU. 18% patients had values exceeding this cut-off as compared with only 3% controls. The crude relative risk, estimated by the matched odds ratio (OR), for foetal death in patients with IgM anti-EPCR above the 95th percentile was 14.0 [95% confidence interval (95% CI): 1.8-106.4] as compared with those who had a lower value. When the cut-off was set at the 90th percentile (83 AU), the OR was 5.2 (95% CI: 1.8 to 15.3).

The 95th percentile of the IgG anti-EPCR levels in the control group was 94 AU. 15% patients had values exceeding this cut-off as compared with 5% controls. The crude OR for foetal death in patients with IgG anti-EPCR above the 95th percentile was 4.3 (95% CI: 1.2-15.2). When the cut-off was set at the 90th percentile (80 AU), the OR was 2.3 (95% CI: 0.9-5.6).
In order to improve accuracy in the analysis of anti-EPCR autoantibodies as risk factors for foetal death, we performed a separate analysis adjusting for putative confounding variables (table 2). Due to the sample size, the data were sparse and we were not able to adjust simultaneously for factor V Leiden (FVL) and LAC in the same model. For this reason we considered two different models: Model 1, in which the adjustment was performed for APL (i.e. LAC and aCL), prothrombin G20210A and, in case of IgM anti-EPCR, for IgG anti-EPCR (vice versa in case of IgG anti-EPCR). Model 2, in which FVL was included instead of LAC. Using Model 1, the OR of foetal death associated with IgM anti-EPCR levels above the 95th and 90th percentiles were 23.0 (95% CI: 2.0-266.3) and 7.1 (95% CI: 1.9-25.6) respectively. The OR associated with IgG anti-EPCR exceeding the 95th and 90th percentiles were 6.8 (95% CI: 1.2-38.4) and 3.5 (95% CI: 1.9-11.8) respectively. In order to assess the risk associated with anti-EPCR autoantibodies whatever the isotype was, we created a composite variable which was either IgM or IgG exceeding the 95th or 90th cut-off. In this way, we obtained adjusted OR of 10.9 (95% CI: 2.2-54.7) and 6.7 (95% CI: 2.1-21.2) with cut-offs at 95th and 90th percentiles respectively. Using Model 2 the results were similar (table 2).

To determine whether the relationship between anti-EPCR autoantibodies levels and the risk of foetal death was dose-dependent, we stratified the subjects in quartiles according to the anti-EPCR levels, and OR for foetal death was calculated for each of the three upper quartiles using the lowest quartile as the reference. As table 3 shows, the adjusted OR increased monotonically with the IgM anti-EPCR levels, suggesting a dose-response effect. However, the adjusted OR did not increase linearly with the IgG anti-EPCR levels and thus a threshold response can be assumed.

Median (inter-quartile range) IgM and IgG anti-EPCR values were higher in patients with LAC than in patients without LAC [IgM: 54 (39-78) vs 82 (63-108) AU, p = 0.02;
IgG: 51 (37-64) vs 82 (56-117) AU, p = 0.01]. Likewise, levels of IgM aCL and anti-EPCR were positively correlated (IgM: r = 0.31, p < 0.0001; IgG: r = 0.24, p = 0.001).

The crude OR of foetal death associated with LAC (7.0) or IgM aCL (5.0) notably decreased and lost statistical significance when adjusting for anti-EPCR autoantibodies [3.1 (95% CI: 0.3-27.9) and 2.3 (95% CI: 0.4-12.8) respectively]. This finding indicates that the classical APL (LAC and aCL) were not independent predictors of foetal death, the apparent increased risk associated with them being at least partly due to anti-EPCR autoantibodies.

No interaction could be seen among the analysed variables (likelihood ratio test, p > 0.1). When we stratified these analyses by the number of pregnancies or by the period in which the foetal loss took place, the results did not substantially change.
DISCUSSION

We developed an ELISA to detect anti-EPCR autoantibodies and we provide evidence supporting a strong association between these autoantibodies and the risk of foetal death. Thus for the first time the presence of anti-EPCR autoantibodies in human pathology has been demonstrated. First, we detected high levels of these antibodies in five patients within a group of APS patients showing thrombosis and LAC. Then, we provided evidence that anti-EPCR autoantibodies are sometimes able to hamper the PC activation, which should compromise anticoagulant and antiinflammatory mechanisms. These PC activation-blocking antibodies may probably lead to a low level of APC in vivo, a risk condition for thrombosis in itself,24 which would help to explain the multiple venous thrombosis episodes experienced by the patient who carried PC activation-blocking antibodies in the APS group. Since we detected very high levels of IgM anti-EPCR in two APS women with previous history of multiple episodes of foetal death, we performed a matched case-control study to assess the risk of having a first episode of unexplained foetal death associated with the presence of anti-EPCR autoantibodies. This risk was dose-dependently higher as the level of IgM anti-EPCR increased. Very high levels of IgM anti-EPCR constituted a strong risk factor for the first episode of foetal death, with a 20-fold increase in relative risk. High levels of IgG anti-EPCR were also a strong risk factor although weaker than IgM and without evidence for a dose-response trend. The broad confidence intervals obtained in the estimates of the relative risks were probably due to the reduced number of women exposed to levels higher than the 95th percentile. Nevertheless, when a lower cut-off (90th percentile) was set, the estimation of the risk was still high and the confidence intervals were narrowed, the same occurring when IgM and IgG isotypes were considered together. This finding strongly suggests that high levels of either IgM or IgG anti-EPCR are risk factors for
foetal death. Furthermore, the associated risk might have been under-estimated since one of the criteria for including these patients in the study was to have neither a previous history of venous or arterial thrombosis nor previous obstetrical complications, entities which theoretically could be associated with a higher prevalence of elevated anti-EPCR autoantibodies. It is not surprising that IgM anti-EPCR was more predictive than IgG, since this is a common feature in obstetrical complications, although a likely plausible biological explanation for this phenomenon is not available. Known genetic risk factors, such as FVL or the prothrombin G20210A mutation, did not explain the increased risk of foetal death associated with anti-EPCR. Our findings are also not due to the effects of LAC or aCL. It is noteworthy that while LAC and IgM aCL were associated with an increased risk of foetal death in the univariate analysis, as previously reported, this association was markedly attenuated and lost its statistical significance in the multivariate model, where anti-EPCR autoantibodies were included in the analysis. Anti-EPCR autoantibodies could thus be at least partly responsible for the pathological effects observed in the patients showing LAC or aCL. This notion is coherent with the fact that there is no plausible explanation about how LAC or aCL might play a pathological role by themselves, while anti-EPCR autoantibodies could indeed exert a variety of pathological effects: the binding of autoantibodies to EPCR should be able to trigger the activation of the complement system. This will lead to a proinflammatory state with destruction of endothelium and trophoblast leading to a procoagulant state and undesiderable thrombosis and foetal loss as has recently been demonstrated in a mouse model of foetal death induced by APL. On the other hand, since the correct function of the PC system is essential for promoting placental cell growth, it is conceivable that the anti-EPCR autoantibodies able to block the PC activation will exert an additional effect by reducing the APC concentration in such a
manner that the placental development will be compromised. For these reasons, LAC and aCL could be just innocent bystanders or mere markers of a more complex autoimmune disorder.

In conclusion, we have demonstrated for the first time the presence of IgM and IgG anti-EPCR in humans. High levels of these autoantibodies are associated with a higher risk of a first episode of foetal death. Anti-EPCR autoantibodies are often found in patients with APS as well. It is conceivable that these antibodies may exert a causative role in the thrombotic and the foetal death episodes occurring in these patients.
REFERENCES


18. Gris JC, Quere I, Monpeyroux F, et al. Case-control study of the frequency of thrombophilic disorders in couples with late foetal loss and no thrombotic


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**Table 1.** Univariate OR and their 95% CI for foetal death associated with different variables studied. Anti-EPCR autoantibodies were categorised using as a cut-off point the 95th percentile for the variable in the control group. Levels of aCL higher than 15 G or M phospholipids units were considered positive.
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Table 2. Multivariate OR and their 95% CI for foetal death associated with anti-EPCR levels (categorised using as a cut-off points the 95th and 90th percentile for the variable in the control group). Model 1 was adjusted for G20210A prothrombin, LAC, aCL and, in case of IgM anti-EPCR, for IgG anti-EPCR (viceversa in case of IgG anti-EPCR). Model 2 is like Model 1 except that FVL is included for adjustment instead of LAC.
<table>
<thead>
<tr>
<th></th>
<th>N (cases/controls)</th>
<th>Matched OR</th>
<th>95% CI</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q1  (&lt;33 AU)</td>
<td>8/22</td>
<td>1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q2  (33-52 AU)</td>
<td>25/23</td>
<td>3.1</td>
<td>(1.0-9.6)</td>
<td></td>
</tr>
<tr>
<td>Q3  (53-70 AU)</td>
<td>19/21</td>
<td>2.6</td>
<td>(0.8-9.0)</td>
<td></td>
</tr>
<tr>
<td>Q4  (&gt;70 AU)</td>
<td>35/21</td>
<td>4.4</td>
<td>(1.4-14.3)</td>
<td>0.02</td>
</tr>
<tr>
<td>&gt;p95 (&gt;99 AU)</td>
<td>16/3</td>
<td>45.2</td>
<td>(2.9-662.4)</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q1  (&lt;35 AU)</td>
<td>18/22</td>
<td>1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q2  (35-49 AU)</td>
<td>18/23</td>
<td>1.3</td>
<td>(0.3-4.9)</td>
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</tr>
<tr>
<td>Q3  (50-57 AU)</td>
<td>14/20</td>
<td>0.8</td>
<td>(0.2-2.7)</td>
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<tr>
<td>Q4  (&gt;57 AU)</td>
<td>37/22</td>
<td>2.4</td>
<td>(0.7-8.4)</td>
<td>0.12</td>
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<td>&gt;p95 (&gt;94 AU)</td>
<td>13/4</td>
<td>10.2</td>
<td>(1.2-86.7)</td>
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</tr>
</tbody>
</table>

Table 3. OR for foetal death according to the anti-EPCR autoantibody levels. Patients and controls were stratified in quartiles according to the anti-EPCR level. OR for foetal death were calculated in the 2nd (Q2), 3rd (Q3), and 4th (Q4) quartiles, and 95th percentile (p95) as compared with those in the 1st (Q1) quartile. Adjustment for variables associated with foetal death included in Model 1 analysis was performed.
FIGURE LEGENDS

Figure 1. Anti-EPCR autoantibodies in APS patients and controls. Anti-EPCR levels are shown (median, interquartile range, and range). IgM anti-EPCR: controls, median = 45 AU; patients, median = 57 AU. Ig G antibodies: controls, median = 72 AU; patients, median = 75 AU.

Figure 2. Effect of anti-EPCR antibodies on APC generation by endothelial cells. Confluent EA.hy926 cells were incubated with buffer or the indicated Ab (RCR-252 at 20 μg/mL, the different patients fractions at 45 μg/ml except indicated) and the initial rate (Vo) of thrombin-dependent activation of 50 nM PC was determined. For each condition 2-4 independent experiments were performed. The mean ± SD of %Vo with respect to the Vo obtained in the absence of antibodies is represented. * p = 0.013, ** p = 0.02, with respect to the buffer condition.

Figure 3. Effect of patient C anti-EPCR autoantibody on kinetic parameters of APC generation by endothelial cells. Thrombin-catalysed protein C activation on EA.hy926 cells measured in the absence (solid circles) or presence (open circles) of patient C IgM fraction at 45 μg/mL. The Km for each condition is indicated. The Lineweaver-Burk plot in the inset shows that the Vmax in both conditions is essentially equal.
Figure 1
Figure 2
Figure 3

![Graph showing enzyme kinetics with Km values of 95 nM and 1,894 nM.](image)
Autoantibodies against EPCR are found in antiphospholipid syndrome and are a risk factor for foetal death

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