ARTICLE

Obstetric antiphospholipid syndrome: early variations of angiogenic factors are associated with adverse outcomes

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ABSTRACT

The prognostic value of angiogenic factors in newly pregnant women with obstetric antiphospholipid syndrome (oAPS) has not been documented. We observed 513 oAPS who experienced three consecutive spontaneous abortions before the 10th week of gestation or one fetal loss at or beyond the 10th week. We assessed the plasma concentrations of the proangiogenic factor placenta growth factor (PlGF) and of the antiangiogenic factor soluble fms-like tyrosine kinase-1 on the eve and on the 4th day of the low-molecular weight heparin-low-dose aspirin treatment. Placenta growth factor and fms-like tyrosine kinase-1 plasma concentrations showed marked increases. Treatment-associated variations of PlGF and of soluble fms-like tyrosine kinase-1 were antagonist risk factors for placenta-mediated complications (PMC) and for severe PMC, for fetal death, stillbirth and neonatal death. The ratio between PlGF increase and soluble fms-like tyrosine kinase-1 was a summary variable whose best cut-off values ($1.944.10^{-2}$) had high negative predictive values for PMC (0.918) and may be used to help rule out the development of PMC in evolutive pregnancies after 19 completed weeks. The early variations of PlGF and soluble fms-like tyrosine kinase-1 concentrations in newly pregnant oAPS may help to detect patients at low risk of PMC. (*clinicaltrials.gov identifier: 02855047*)

Introduction

Antiphospholipid antibody syndrome (APS) is defined by precise clinical symptoms associated with repeated positive results in laboratory tests for IgG or IgM antiphospholid antibodies (aPLAbs) such as lupus anticoagulant (LA), anticardiolipin antibody (aCL) and anti-β2 glycoprotein I antibody (aβ2GP1), present at moderate or high titers for diagnosis. Purely obstetric APS (oAPS) is an APS clinical presentation characterized by precisely defined morbidities occurring during pregnancy in women with no history of thrombosis.[1-5]

The use of low-dose aspirin (LDA) in association with heparin is generally recommended for the treatment of oAPS.[6] Among heparins, low-molecular weight heparins (LMWH) are favored for safety reasons and practical considerations. In oAPS defined on pregnancy loss criteria, the LDA-LMWH association, however, shows some limitations.[7] Available data support the hypothesis that alterations in placental trophoblast production of angiogenic proteins may help to explain the pathogenesis of preeclampsia,[8] placental abruption,[9] intrauterine growth restriction, and stillbirths.[10] The overall theory is that excessive release of antiangiogenic factors, such as soluble vascular endothelial growth factor (VEGF) receptor (sFlt1), by

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hypoxic trophoblasts, antagonize proangiogenic factors such as placenta growth factor (PlGF). This causes aberrant placental angiogenesis and vasculogenesis.[11]

In pregnant women receiving LMWH therapy, heparin increases circulating sFlt[12] and PlGF[13] immunoreactivity. Results of the PROMISSE study recently showed that circulating angiogenic factors, measured as early as 12-15 weeks in heterogeneous pregnant women with systemic lupus erythematous and/or aPLAbs, have a high negative predictive value in ruling out the development of severe adverse outcomes.[14]

The Nimes Obstetricians and Hematologists – AntiphosPholipid Syndrome (NOH-APS) observational cohort study[7] allowed us to evaluate the prognostic value on pregnancy outcomes of early sFlt1 and PlGF variations associated with the start of the LDA-LMWH treatment in oAPS women.

Methods

Patients

This study focuses on a subgroup of patients included in the NOH-APS cohort: those with an oAPS diagnosis who were treated for a new pregnancy during the 18 months individual observational period after diagnosis. The time window for recruitment was January 1st 1995 to January 1st 2005. Clinical follow up started on July 1st 1995 and ended on September 1st 2007. None of the observed treated pregnant oAPS women was lost to follow up.

Patient recruitment and definition of the patient groups included in the NOH-APS study have been described in detail elsewhere[7,15] (*Online Supplementary Figure S1*). Briefly, all women fulfilled one of the following inclusion criteria: 1) three unexplained consecutive spontaneous abortions before the 10th week of gestation that could not be accounted for by maternal anatomic or hormonal abnormalities, or paternal or maternal chromosomal causes (the recurrent embryo loss subgroup); or 2) one unexplained death of a morphologically normal fetus (fetal loss) at or after the 10th week of gestation, with the normal morphology of the fetus confirmed by ultrasound scan or direct examination of the fetus (fetal loss subgroup).

The exclusion criteria were: a history of thrombotic events (at least one clinical episode of venous, arterial, or small-vessel thrombosis in any tissue or organ other than the placenta confirmed by objective validated criteria, ie. unambiguous findings in appropriate imaging or histological studies), or any treatment given during previous pregnancies that might have modified the natural course of the condition, such as antithrombotic agents, or immunosuppressive or immunomodulatory drugs. We also excluded women whose pregnancy losses could be explained by infectious, metabolic, anatomic or hormonal factors. Women seropositive for HIV, or hepatitis B or C were also excluded. Patients were classified as primary aborters (no previous successful pregnancy) or secondary aborters.

Complete thrombophilia screening tests were systematically performed, leading to the definition of subgroups.[15]

Among the 4801 screened patients for thrombophilia[15] (*Online Supplementary Figure S1*), the present study focuses on the APS subgroup, conventionally defined as women being persistently positive for LA, and/or aCL, and/or aβ2GP1 (initially: n=517) assayed as described.[7,15] This subgroup was restricted to the women who initiated a new pregnancy during the 18 months observational period after oAPS diagnosis (n=513)[7] (*Online Supplementary Figure S1*). Pregnant oAPS women were regularly followed by obstetricians involved in the NOHA network, as needed, and were systematically evaluated once a month by internists and hematologists in our outpatient department of hematology, with no loss to follow up. Any initial missing values for clinical characteristics could be obtained during the subsequent consultations and with the help of the network obstetricians and general practitioners.

The study was approved by the University Hospital of Nîmes Institutional Review Board and ethics committee and by the local Comité de Protection des Personnes Soumises à la Recherche Biomédicale. This clinical investigation was conducted in accordance with the Declaration of Helsinki of 1975 as revised in 1996. All the women gave their informed consent to participate. Financial support was provided by Nîmes University hospital *via* an internal funding scheme; the funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Antithrombotics during new observed pregnancies

Low-molecular weight heparin [enoxaparin, 40 mg per day (4000 U/day)] was added to LDA (100 mg/day) from the day of positive pregnancy test result until delivery; these two treatments were administered concomitantly. Compliance to LMWH treatment was monitored by self-declaration of the patients and their partners, and systematic examination of the subcutaneous injection sites at each medical examination. Platelet counts were checked on the day before the first LMWH injection, twice a week during the first three weeks, and then once a month.

Outcomes

The primary end point was a composite outcome that included any of the following events occurring after 19 completed weeks of the observed pregnancy: preeclampsia, abruptio placenta, or small-gestational age newborn (< 10th percentile), summarized as the so-called placenta-mediated complications (PMCs).

The secondary outcome analysis included a composite outcome that included any of the severe PMCs: severe preeclampsia, severe small-gestational-age newborn less than 5th percentile, abruptio placenta leading to emergency delivery, pregnancy loss categorized as embryonic loss (before 10 week's gestation, WG), fetal death (before 20 WG), stillbirths (from 20 WG to delivery), and neonatal death defined before reaching 28 days of age.

The diagnosis of preeclampsia was the association of systolic blood pressure 140 mmHg or over or diastolic blood pressure 90 mmHg or over in a woman who was normotensive before 20 weeks' gestation and a significant proteinuria defined as the presence of 0.3 g or more of protein in a 24-hour urine specimen.[16] The diagnosis of severe preeclampsia was made according to American College of Obstetricians and Gynecologist criteria. Briefly, preeclampsia was considered severe if one or more of the following criteria was present: systolic blood pressure 160 mmHg or over or diastolic blood pressure 110 mmHg or over on two occasions at least six hours apart while the patient is on bed rest, proteinuria 5 g or over in a 24-hour urine specimen or 3+ or greater on two random urine samples collected at least four hours apart, oliguria less than 500 mL in 24 hours, eclamptic seizures, persistent headache or visual disturbances, abruptio placenta, pulmonary edema or cyanosis, epigastric or right upper-quadrant pain, impaired liver function (twice the normal range), thrombocytopenia (<100,000 cellsx10^{9}L), severe fetal growth restriction (< 5th percentile).[17]

Abruptio placenta was defined according to classical clinical prenatal signs and symptoms: vaginal bleeding accompanied by nonreassuring fetal status or uterine hypertonicity, or sonographic visualization of abruption, and evidence of retroplacental clots during examination of the delivered placenta. Cases were confirmed by histopathological diagnosis.

Birthweights were assessed by birthweight percentile charts

customized for maternal age, pre-pregnancy body mass index, parity, gestational age at delivery, and sex.[18] Small-gestational-age newborn was defined as birthweight under the 10th percentile, and severe when under the 5th percentile.

Chronic hypertension was defined as hypertension (blood pressure ≥140 mmHg systolic or 90 mmHg diastolic or more) that was present before pregnancy or that was diagnosed before the 20th week of gestation.[16] The diagnosis of superimposed preeclampsia in chronic hypertensive women needed one of the following findings: 1) in women with hypertension and no proteinuria early in pregnancy (<20 weeks' gestation), new-onset proteinuria as defined above; 2) in women with hypertension and proteinuria before 20 weeks' gestation; 3) sudden increase in proteinuria; 4) in women whose hypertension has previously been well controlled sudden increase in blood pressure; 5) thrombocytopenia (platelet count <100,000 cellsx10^{-9} L); or 6) increase in alanine aminotransferase or aspartate aminotransferase to abnormal levels.[16]

HELLP syndrome was defined by the presence of all 3 of the following criteria: hemolysis [characteristic peripheral blood smear and serum lactate dehydrogenase (LDH) ≥ 600 U/L or serum total bilirubin ≥ 20 μM/L-1], elevated liver enzymes [serum aspartate aminotransferase (AST) ≥ 70 U/L], and low platelet counts (< 100,000 cellsx10^{-9} L).[19]

Samples

We used blood samples collected for platelet monitoring under LMWH treatment. EDTA-anticoagulated blood samples were obtained by clean venipuncture: the first one the day before LMWH starting, the second one the 4th day of LMWH treatment, four hours after subcutaneous injection; both at 11.00 am (+ 30 minutes). After taking a platelet count, whole blood samples were centrifuged twice at 4000 g for 20 minutes, aliquots of platelet-poor plasma were then stored at -80°C until tested.

Assays

Stored plasma samples were subsequently used for sFlt1 and PlGF plasma level measurements, performed in a blind fashion for PMC outcome, and in duplicate using enzyme-linked immunosorbent assay kits (R&D Systems Europe, Lille, France). The calculated interassay coefficients of variation for sFlt-1 and PlGF were 4.0% and 7.5%, respectively; the calculated intraassay coefficients of variation were 2.5% and 4.5%, respectively. In order to minimize interassay variations, plasmas obtained from a given patient before and during LMWH treatment were assayed at the same time using the same standard curve (paired testing). We had no missing blood sample; 2.3% of the samples had to be assayed a second time due to a first accidental technical failure. Complete biological results were obtained for all study subjects.

Statistical analysis

Quantitative data are presented as the median, interquartile range (IQR) and minimum-maximum values. Qualitative data are presented as values and percentages. Mann-Whitney test, Kruskal-Wallis test, χ^2 tests and Fisher's exact tests were used, as appropriate, for comparisons between baseline characteristics.

All analyses were based on pregnancy outcomes that occurred during the first pregnancy after oAPS diagnosis, treated as described above.

Variations in the plasma concentrations of the tested angiogenic factors, defined as the difference "Δ" between plasma concentrations on day 4 of LMWH-LDA treatment and before treatment, were calculated for each patient and used for analysis (ΔPlGF; ΔsFlt1). The ΔPlGF/ΔsFlt1 ratio was analyzed as a continuous variable and after categorization into quartiles.

A priori selected factors of the various outcomes (primary outcome: PMCs; secondary outcomes: severe PMCs, pregnancy loss/neonatal death: abortion, fetal death, stillbirth, neonatal death) among the clinical predictors (age, body mass index, thrombotic familial antecedents, pregnancy loss familial antecedents, ethnicity, smoking history, pre-existing diabetes mellitus, pre-existing hypertension, pre-existing embryonic/fetal pregnancy loss, primary/secondary pregnancy loss and initial inflammatory disease), the metabolic markers at inclusion (hypercholesterolemia defined as a fasting cholesterol concentration above 5.2 mM/L-1 and hypertriglyceridemia as a fasting triglyceride concentration above 1.7 mM/L-1), the biological predictors at inclusion (positive LA, positive aCL-G, positive aCL-M, positive aβ2GP1-G, positive aβ2GP1-M, triple positivity and the *F5* rs6025 or F2 rs1799963 polymorphisms) and the variations of angiogenic factors associated with the LMWH-LDA treatment (ΔPlGF; ΔsFlt1; or the ΔPlGF / ΔsFlt1 ratio) were evaluated first by univariate then by multivariate logistic regression analysis.

For multivariate models, and because not all confounders were known for our study model, a stepwise backward elimination was performed after selecting all the variables identified by the univariate models as potential predictors at $P<0.20$, with adjustment being finally performed for all variables with $P<0.20$ in the multivariate models. The final model only included main effects with $P<0.05$. The goodness-of-fit of the model was assessed by the Hosmer-Lemeshow test.

Discrimination was assessed using computing receiver operating characteristic (ROC) curves and calculating the area under the curve (AUC). The best cut-off value was identified as the point maximizing the computed Youden index (sensivity Se + specificity Sp -1).

All tests were two-sided and assessed at the 5% significance level. The study design was based on our recruitment capacities assessed over a 10-year period, thus no sample size calculation was performed. However, hypothesizing a minimum clinically relevant relative risk of PMC of 10% and a 0.05 2-sided alpha level the study power to detect a 50% increase in the relative risk with the 513 observed patients is 0.935.

Statistical analyses were performed using StatView®-windows software v.5.0 (SAS Institute Inc., Cary, NC, USA) and XLSTAT® software v.2015.4.01.20116 (Addinsoft SARL, Paris, France).

Results

Patients' characteristics and new pregnancy outcomes

Patients' characteristics are available in the *Online Supplementary Table S1* and new pregnancy outcomes are shown in Table 1. Most of the patients were non-overweight Caucasian European women, under 35 years of age, with no positive family history of venous or arterial thrombotic disease or significant pregnancy loss (*Online Supplementary Table S1*). Primary oAPS and fetal death were the main clinical presentation and the main criterion, respectively. Pre-existing hypertension or/and hyperlipidemia were present in a minority of the patients and only one out of 10 women were current tobacco smokers.

ACL-M or LA were the most frequent aPlAb features. Seventy-five percent of the patients were positive for more than one marker and approximately 20% of the women were positive only for aCL-Ab (8.5% had only aCL-M antibodies). None had only aβ2GP1-Ab. Over 25% of the patients exhibited triple positivity, which was due in part to the high frequency of aCL-M-positive patients.

In the APS group, 3.3% of women were positive for the *F5* rs6025 or *F2* rs1799963 polymorphisms.

Despite the systematic prescription of the LMWH-LDA treatment during new pregnancies, only 75% of the women had an ongoing viable pregnancy after 19 completed weeks of gestation, with an 18.1% rate of abortions before 10 WG and a 7.2% rate of fetal deaths after 9 completed WG (Table 1). Fetal karyotype investigations on early loss before ten weeks through genomic hybridization on miscarriage tissue was only available in 78.5% of the cases and evidenced a significant rate of chromosomal abnormalities, mainly aneuploidies, in 59% of the investigated cases. The same test performed on fetal death was available for all cases and showed a 5.4% chromosomal abnormality rate, all in fetal loss cases during weeks 10 to 12.

An additional 5.7% of the women experienced stillbirth (normal karyotypes). Neonatal death before 28 days was reported in 3.5% of the women (normal karyotypes). PMCs were diagnosed in 17.7% of the women, the main feature being preeclampsia (11.1%; severe preeclampsia: 7.8%), followed by small-gestational-age newborn (14.2%; severe small-gestational-age newborn: 5.1%) and abruptio placenta (2.1%).

Safety outcomes outcomes for women taking antithrombotic treatment

No treatment had to be stopped or replaced for safety reasons. There were no cases of heparin-induced thrombocytopenia.

Angiogenic factors

The LMWH-LDA treatment was globally associated with a marked increase of PlGF plasma concentrations and a strong increase of sFlt1 concentrations, leading to a collapse of the PlGF/sFlt1 ratio values: in the whole oAPS group as well as in the two subgroups of women who later developed or did not develop any PMC (Table 2). The treatment-associated variations in PlGF and in sFlt1 concentrations were correlated (Spearman's rank-order correlation coefficient: 0.424; $P<0.0001$) (Figrue 1). Focusing on angiogenic factors on the 4th day of treatment, PlGF concentrations were lower, sFlt1 concentrations higher and the PlGF/sFlt1 values lower in women who finally developed PMCs than in those who did not (Table 2). Treatment-associated variations of PlGF and of sFlt1 concentrations were respectively lower and higher in women who developed PMCs than in those who did not (Table 2 and Figure 2). Consequently, the ratio of the treatment-associated variations of PlGF and of sFlt1 plasma concen-

Table 1. Pregnancy outcomes of the antiphospholipid syndromes (APS) women who initiated a new pregnancy during the 18 months individual observational period after obstetric APS diagnosis.

New pregnancy outcomes	N (%)
Abortion < 10 WG	93 (18.1%)
Fetal death ≥ 10 WG and < 20 WG	37 (7.2%)
Ongoing pregnancies at 20 WG	83 (74.7%)
Stillbirths ≥ 20 WG to delivery	29 (5.7%, 7.6%)
Any pregnancy loss	159 (31%)
Perinatal death > 22 WG and < 8 days	43 (8.4%, 11.2%)
Neonatal death < 28 days	18 (3.5%, 4.7%)
PE	57 (11.1%, 14.9%)
Severe PE	40 (7.8%, 10.4%)
Abruptio placenta	11 (2.1%, 2.9%)
SGA < p10	73 (14.2%, 19.1%)
SGA newborn < p5	26 (5.1%, 6.8%)
PMCs	91 (17.7%, 23.8%)

When two percentages are given, the first is calculated on the whole number of pregnancies, the second is restricted to ongoing pregnancies at 20 weeks of gestation (WG). PE: preeclampsia; SGA: small gestational age; p10: 10th percentile; p5: 5th percentile; PMC: placenta-mediate complications; PE and/or abruptio placenta and/or fetal growth restriction.

Table 2. Levels of angiogenic factor during early pregnancy in obstetric antiphospholipid syndromes (APS) women (oAPS) receiving the low-molecular weight heparins-low-dose aspirin (LMWH-LDA) treatment.

Angiogenic factors	oAPS Whole group (n=513)	PMC-negative (n=422)	PMC-positive (n=91)	*P*
PlGF, ng.L^{-1}				
Before treatment	9.3 [1.68] (5.6-12.6)	9.3 [1.71] (5.6-12.6)	9.3 [1.64] (5.9-12.3)	0.88
During treatment	31.8 [5.97] (16.8-49.5)	31.9 [5.57] (16.8-49.5)	29.8 [7.1] (16.8-44.8)	0.001
P	< 0.0001	< 0.0001	< 0.0001	
Δ	22.5 [6.21] (5.9-39.4)	22.6 [6.18] (5.9-39.4)	20.5 [7.1] (9.1-36.3)	0.0016
sFlt1, ng.L^{-1}				
Before treatment	32 [20] (1-81)	31 [20] (1-81)	34 [21] (2-62)	0.39
During treatment	1283 [728] (72-2992)	1247 [697] (72-2992)	1462 [782] (293-2560)	0.0006
P	< 0.0001	< 0.0001	< 0.0001	
Δ	1244 [718] (30-2928)	1216 [712] (30-2928)	1429 [756] (265-2511)	0.0007
PlGF:sFlT1 ratio				
Before treatment	0.296 [0.234] (0.069-11.3)	0.302 [0.227] (0.069-11.3)	0.265 [0.274] (0.099-5.42)	0.851
During treatment	0.025 [0.012] (0.012-2.26)	0.026 [0.013] (0.012-2.26)	0.021 [0.009] (0.012-0.089)	< 0.0001
P	< 0.0001	< 0.0001	< 0.0001	
ΔPlGF/ΔsFlt1	0.018 [0.009] (0.007-0.631)	0.018 [0.01] (0.008-0.631)	0.015 [0.007] (0.007-0.063)	<0.0001

Before treatment then on the 4th day of treatment (During treatment); in the whole group of patients and according to the subsequent development of placenta-mediated complications (PMC; PMC negative *vs.* PMC positive). Results are given as median, (interquartile range) and (minimum-maximum values). Δ: levels during treatment minus levels before treatment.

trations, namely the (ΔPlGF/ΔsFlt1) variable, was lower in women who developed PMCs (Table 2 and Figure 2).

We systematically looked for any associations between each of the five APS markers, the aPLAbs, and the angiogenic factors (basal concentrations, treatment-associated variations and ratio of the treatment-associated variations). Only basal sFlt1 concentrations and ab2Gp1-IgG titers were correlated (Spearmans' rank correlation coefficient r: 0.117; *P*=0.010) and higher values of sFlt1 were evidenced in ab2GP1-IgG positive women (*P*=0.0105).

Assuming that the fixed dose of 40 mg enoxaparin may not fit all women, we looked for any correlations between basal concentrations of angiogenic factors and their releases, and BMI values: no significant association could be evidenced (*data not shown*). No significant associations between BMI values and PMCs, severe PMCs, pregnancy loss before ten weeks, fetal death, stillbirth and neonatal death in patients receiving enoxaparin were observed (*data not shown*).

Risk factors for PMCs

Risk factors for PMCs are shown in Table 3. PMCs are pathologies arising after the 19th completed WG; therefore, risk factors for PMCs were investigated in the subgroup of 383 women with an ongoing pregnancy during the 20th gestational week. The treatment-associated variations of PlGF concentrations (ΔPlGF) were overall highly significant protecting factors against the development of PMCs whereas variations of sFlt1 (ΔsFlt1) were highly significant risk factors for PMCs. Both ΔPlGF and ΔsFlt1 remained independent indicators after adjustment for prior fetal death and triple positivity. The analysis of the summary variable (ΔPlGF/ΔsFlt1) ratio showed values belonging to the 2nd, 3rd and 4th quartiles to protect against the occurrence of PMCs even after adjustment for prior fetal death and for a triple positivity for aPLAbs, the risk values decreasing when moving to the highest quartile. The AUC related to the ROC computed from the (ΔPlGF/ΔsFlt1)*10^2 ratio values (Figure 2) was 0.745 (0.686-0.803; *P*<0.0001). Looking for any cut-off values, 1.944 maximized Youden index and was mainly associated with an interesting negative predictive value NPV for PMCs (0.918; negative likelihood ratio LR-: 0.286), thus ruling out PMCs, whereas its positive predictive value PPV was poor (0.363; positive likelihood ratio LR+: 1.830).

Risk factors for secondary outcomes

The study of severe PMCs also pointed out increasing ΔPlGF values to protect against severe PMCs and increasing ΔsFlt1 values to expose to severe PMCs (*Online Supplementary Table S2*). Indeed, the three highest quartiles of the (ΔPlGF/ΔsFlt1) ratio were also associated with a decreasing clinical risk, which was the lowest for values belonging to the upper quartile. These associations persisted after adjustment for prior fetal death and age. The AUC related to the ROC computed from the (ΔPlGF/ΔsFlt1)*10^2 ratio values was 0.737 (0.666-0.809; *P*<0.0001). The 1.589 value maximized Youden index and was mainly associated with an interesting NPV (0.927; LR-: 0.431) but with a poor PPV value (0.328; LR+: 2.679). Angiogenic factors best predicted severe preeclampsia [area under the ROC analyzing the (ΔPlGF / ΔsFlt1) *10^2 ratio in predicting severe preeclampsia: 0.769 (0.688-0.850)]. The 1.967 value maximized Youden index; its NPV was 0.982 (LR-: 0.118) with a poor PPV value (0.171; LR+: 1.763). The study of risk factors for difficult pregnancies within the whole cohort, ie. which were not concluded by a viable neonate at 28 days of age, led to final results which depended on the type of pregnancy failure. Angiogenic factors could not predict the occurrence of

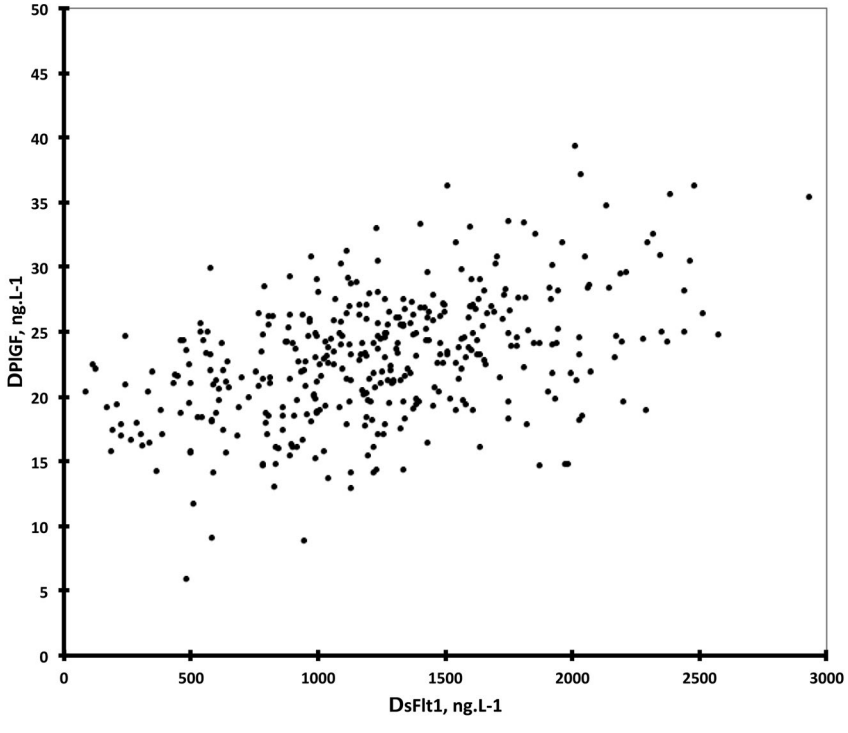

Figure 1. Correlation between the low-molecular weight heparins-low-dose aspirin (LMWH-LDA) treatment-associated early variations of the proangiogenic factor plancenta growth factor (PlGF) plasma concentrations (ΔPlGF) and of sFlt1 plasma concentrations (ΔsFlt1). Spearman coefficient of rank correlation 0.446, *P*<0.0001.

abortions/embryonic losses before 10 WG, which was the most frequent event; in this setting, an abnormal karyotype was a huge clinical predictor. An abnormal karyotype also strongly predicted fetal death. Deaths occurring after nine completed weeks, ie. fetal losses, stillbirths and neonatal deaths, were highly associated with the angiogenic factor variations during the onset of the LMWH-LDA treatment, increasing ΔPlGF values and increasing ΔsFlt1 values being negative and positive risk factors, respectively, with a significant progressive risk decrease from the second to the fourth quartiles of the (ΔPlGF / ΔsFlt1) $*10^2$ ratio values. Multivariate analysis also confirmed prior fetal death to predict for recurrence; familial thromboembolism and familial atherothrombosis to independently increase the risk of stillbirth and maternal tobacco smoking or triple positivity for aPLAbs to enhance the risk of neonatal death. We thus analyzed the discrimination power of the (ΔPlGF / ΔsFlt1)$*10^2$ ratio values on the occurrence of non-embryonic unhappy pregnancies in the 383 women with an ongoing pregnancy at 10 WG. The AUC was 0.725 (0.662-0.789; P<0.0001). The 1.545 value maximized Youden index; its NPV was 0.885 (LR-: 0.522) with a poor PPV value (0.381; LR+: 2.458).

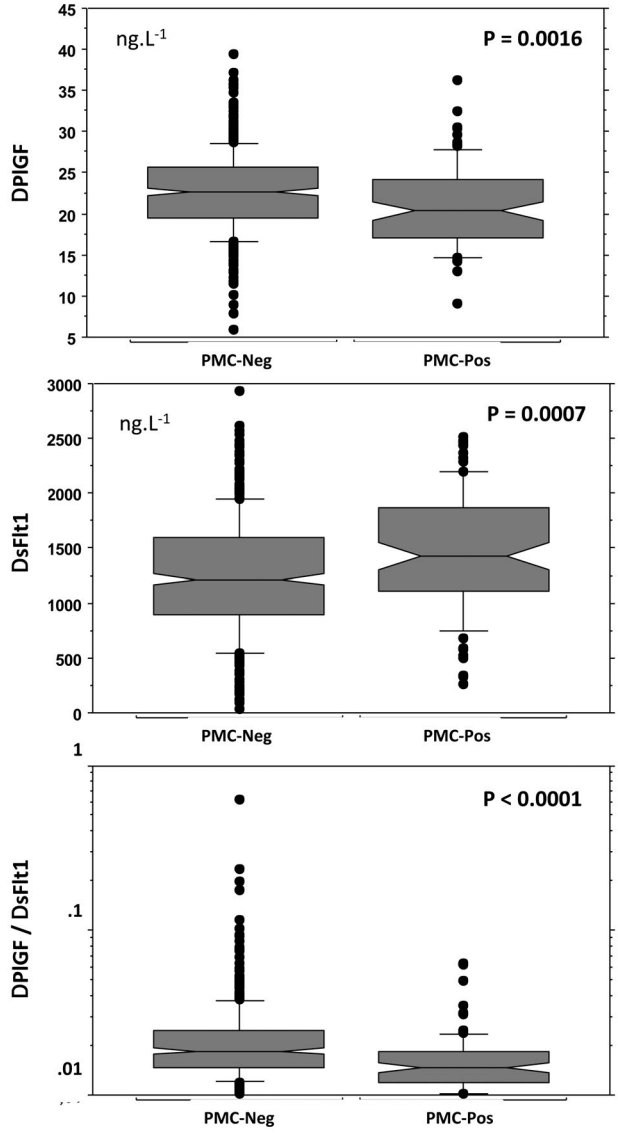

Figure 2. Variations of the proangiogenic factor placenta growth factor (PlGF) plasma concentrations (ΔPlGF), of sFlt1 plasma concentrations (ΔsFlt1) and of the ΔPlGF. Δ sFlt1 ratio (ΔPlGF/ΔsFlt1) associated with the beginning of the low molecular weight heparin–low dose aspirin treatment in obstetrical APS women who later developed (PMC-Pos) or did not develop (PMC-Neg) placenta-mediated complications.

Discussion

With this observational study we describe for the first time the early systemic blood variations of two angiogenic factors: PlGF, an agonist of placenta development, and sFlt1, a PlGF antagonist, in newly pregnant oAPS women clinically defined on the basis of previous pregnancy loss and receiving the LMWH-LDA treatment. Our observations highlighted significant PlGF and sFlt1 increases, which in turn have antagonistic effects on the risk of PMCs (PlGF: protecting, sFlt1: precipitating) of severe PMCs, and of fetal death / stillbirth / neonatal death. As a consequence, the categorization of the PlGF:sFlt1 ratio values into quartiles allowed a decrease of risks to be described as values jumped from the lower to the higher quartiles. Interestingly, a negative predictive value for the risk of PMCs and of severe PMCs were obtained, which may help to rule out the ulterior development of these still abnormally prevalent syndromes in conventionally-treated oAPS women.[7]

Table 3. Risk factors of placenta-mediated complications (PMCs) in antiphospholipid syndromes (APS) women.

Models	Variables	OR ($_{95\%}$CI)	*P*
Univariate			
	Fetal death	1.772 (1.072-2.89)	0.025
	aCL-M	1.818 (1.033-3.232)	0.041
	Triple positivity	1.637 (0.995-2.692)	0.052
	$\Delta PlGF.10^{-1}$	0.321 (0.188-0.548)	< 0.0001
	$\Delta sFlt1.10^{-3}$	2.852 (1.784-4.556)	< 0.0001
	$(\Delta PlGF/\Delta sFlt1).10^2$		
	Q1 (0.75-1.39)	1	
	Q2 (1.40-1.77)	0.348 (0.189-0.640)	0.0007
	Q3 (1.78-2.32)	0.163 (0.080-0.332)	< 0.0001
	Q4 (2.33-63.1)	0.083 (0.035-0.197)	< 0.0001
Multivariate 1*			
	$\Delta sFlt1.10^{-3}$	11.19 (5.688-22.0)	< 0.0001
	$\Delta PlGF.10^{-1}$	0.071 (0.034-0.150)	< 0.0001
	Fetal death	2.088 (1.166-3.739)	0.0133
Multivariate 2**			
	$(\Delta PlGF/\Delta sFlt1).10^2$		
	Q1 (0.75-1.39)	1	
	Q2 (1.40-1.77)	0.309 (0.163-0.586)	0.0003
	Q3 (1.78-2.32)	0.145 (0.070-0.302)	< 0.0001
	Q4 (2.33-63.1)	0.065 (0.026-0.159)	< 0.0001
	Fetal death	1.903 (1.097-3.3.3)	0.0220
	Triple positivity	1.893 (1.083-3.308)	0.0250

PMCs occurred in 91 of the 383 women with an ongoing pregnancy after the 19th completed gestational week. Univariate analysis: results are restricted to putative predictors with *P*-value <0.20. Multivariate analysis: final models include only main effects with P<0.05, adjusted for variables with P<0.20. aCL-M: positive for anticardiolipin IgM. Δ: levels during treatment minus levels before treatment. Q1: first quartile; Q2: second quartile; Q3: third quartile; Q4: fourth quartile. *Multivariate model 1 does not include the [$(\Delta PlGF/\Delta sFlt1).10^2$] variable; adjusted for triple positivity. **Multivariate model 2 does not include the [$\Delta PlGF.10^{-1}$] and the [$\Delta sFlt1 . 10^{-3}$] variables.

Administration of LMWH to preeclamptic women to enhance sFlt1 renal elimination has been described.[20] The proposed mechanism is a direct binding leading to mask sFlt1 positive charges.[20] The SFlt1 molecule is too big (≈100 kDa) to be filtered into urine in the absence of renal damage. The range of renal manifestations associated with APS has been broadened.[21] Renal function and the sFlt1 renal loss may thus be a strong modulating factor impacting on the negative prognostic value of circulating sFlt1. On the other hand, PlGF is a much smaller protein (≈30 kDa) which is readily filtered; however, decreased urinary PlGF at mid gestation is strongly associated with subsequent early development of preeclampsia.[22] Future studies must investigate the putative impact of LMWH-associated sFlt1 and PlGF renal clearances on APS pregnancies. Unfractionated heparin, 70 IU/kg body weight, intravenously administered over one minute has been described to increase plasma sFlt1 and PlGF in non-pregnant women with previous preeclampsia and uncomplicated pregnancy.[23] This resulted in a large vascular store, partly glycocalix-bound, of releasable sFlt1 and a smaller store of releasable PlGF.[23] VEGF/PlGF local regulation partly depends on the availability of their decoy receptor sFlt1, determined by sFlt1 local storage *versus* its systemic release. Heparin displaces natural sFlt1 stores from vascular smooth muscle cells and vessel walls *in vitro*, a phenomenon which is regulated by heparanase.[24] The same phenomenon is observed with human term placental villi explants.[24] As strong trophoblastic involvement is unlikely in our patients with an early new pregnancy, the treatment-associated PlGF and sFlt1 variations we could observe are presumably due to the heparin-mediated mobilization of vascular stores.

A recent study investigated the dysregulation of angiogenic factors in women with systemic lupus and/or antiphospholipid antibodies in relation to the development of pregnancy complications.[14] In this study, aPlAb positive patients were a minority (32%; 20% with only aPlAbs), treatments were non-uniformly applied, the first blood sample was taken at 6-11 weeks without any basal evaluation, the primary outcome was the development of adverse pregnancy outcomes (a composite of preeclampsia at any time, fetal death > 12 weeks, neonatal death prior to hospital discharge due to prematurity, preterm delivery < 36 weeks due to any placenta-mediated complications and small for gestational age < 5th percentile at birth). Despite these significant heterogeneities, a high NPV ruling out the development of severe adverse outcomes could be seen for PlGF and sFlt1 among the 12th-15th week measures, which is in line with the observations of our study.

Furthermore, our findings are consistent with available studies demonstrating a deleterious association between an angiogenic imbalance and pregnancy complications in which a dysfunctional placental organ is thought to play a key pathophysiological role. The similar risk profiles for preeclampsia, *abruptio placenta* and fetal growth restriction provide compelling evidence to suggest that these conditions may share common pathophysiological mechanisms.[25] Pro- and anti-angiogenic factors are dysregulated in patients with APS.[26-28] They are also dysregulated in pregnant women with PMCs.[29] *In vitro* studies showed that the modulation of 1st-trimester trophoblast angiogenic factor secretion by aPLAbs is insensitive to heparin.[30] Thus, the early variations of angiogenic factors may play a direct role in the outcome of APS pregnancies. Given this, heparin injections may serve as a truly dynamic sensitization test for assessing active mediators poorly accessible to plasma exploration in usual conditions.

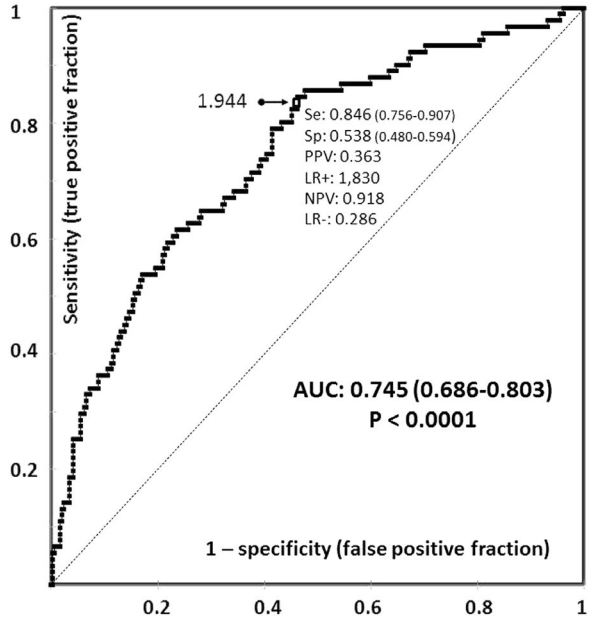

Figure 3. Receiver operating characteristic curve analyzing the discrimination power of the [(ΔPlGF / ΔsFlt1) * 10^2] ratio in predicting placenta-mediated complications.

Results showed non-uniform associations between pregnancy loss/death subtypes. Overall, prognostic values were only seen in non-embryonic losses, ie. the most severe phenotypes prone to the most devastating psychological consequences.[31] The high rate of chromosomal abnormalities in miscarriage tissues before ten weeks may at least partly explain the disappointing value of angiogenic factors in this clinical setting. Isolated recurrent early, embryonic loss defines a subgroup of oAPS patients which differ from other oAPS, with a better overall prognosis.[7] Studies suggest that the decidualized endometrium can select good quality embryos and rejects the incompetent embryos: the nature of the cellular and molecular dialogue between the maternal endometrium and the implanting embryo is central to early pregnancy failures. It has been proposed that patients experiencing recurrent implantation failure possess a selection mechanism that inappropriately rejects good quality embryos, recurrent embryo loss being caused by failure of natural embryo quality control.[32,33] No central role for angiogenic factors is currently considered in this setting.

The strength of this study includes a homogeneous and well characterized group of women receiving a single type of treatment during pregnancy, an accurate phenotyping of the various PMCs, and measurements performed in a single laboratory by personnel blinded to the outcomes. Limitations include the single-center design, the overall low number of PMCs, the retrospective study of frozen samples, the absence of any control group allowing analysis of the aPlAb-dependence and the LMWH-LDA treatment-dependence of the variations in angiogenic factors.

In summary, PlGF and sFlt1 plasma concentrations are

up-regulated in newly-pregnant obstetric APS beginning the traditional LMWH-LDA treatment. Variations of PlGF and of sFlt1 are risk factors for PMCs and for severe PMCs; and for fetal death, stillbirth and neonatal death. The ratio between PlGF increase and sFlt1 increase may help to rule out early on the development of PMCs or of severe PMCs in LMWH-LDA treated pregnancies: a prospective validation cohort is now mandatory to confirm the robustness of these data. Confirmation would lead to the non-selection of conventionally-treated patients with an overall good prognosis in the very first trials testing new therapeutic developments.

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