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Triple therapy with pravastatin, low molecular weight heparin and low dose aspirin improves placental haemodynamics and pregnancy outcomes in obstetric antiphospholipid syndrome in mice and women through a nitric oxide-dependent mechanism

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ABSTRACT

Objectives: A previous pilot study showed that pravastatin supplementation improved pregnancy outcomes in women with obstetric antiphospholipid syndrome (OAPS) that developed placental insufficiency despite standard of care treatment low molecular weight heparin plus low dose aspirin (LMWH + LDA). In this study we investigated the mechanism behind the beneficial effects of the triple therapy LMWH + LDA + pravastatin in improving uteroplacental vascular function and reducing pregnancy complications in OAPS. We hypothesized that nitric oxide (NO) is involved in the vasculoprotective effects of the triple therapy. A mouse model of OAPS that resembles the clinical scenario was used to test this hypothesis.

Methods: Eleven women with OAPS that developed preeclampsia (PE) and/or intrauterine growth restriction (IUGR) associated with uteroplacental vascular dysfunction despite treatment with LMWH + LDA participated in this study after given informed written consent. Seven women were supplemented with pravastatin at the time abnormal uterine artery Dopplers were detected and 4 remained on LMWH + LDA treatment only. Wire myography was used to identify the mechanisms underpinning the protective effects of the triple therapy in the mouse model of OAPS.

Results: The triple therapy increased serum NO levels, diminished uteroplacental vessels resistance improving placental function and prolonged pregnancies compared to conventional treatment LMWH + LDA, leading to live births in women with OAPS. Comparable to the observations in women, the triple therapy protected pregnancies in OAPS-mice, increasing placental perfusion and pregnancy outcomes. A synergistic vasculoprotective effect of the triple therapy on uterine arteries and aorta was demonstrated in OAPS-mice. LMWH + LDA showed a partial protection on endothelial function. Addition of pravastatin increase eNOS synthesis, expression and activity/signaling leading to a significant increment in nitric oxide (NO) generation, resulting in improved placental vascular function and total protection of pregnancies.

Conclusion: LMWH + LDA + PRAV increased serum NO levels and significantly improved placental haemodynamics and maternal and neonatal outcomes in women and mice with OAPS. A role for eNOS/NO in mediating the placental vasculoprotective effects in OAPS-mice was demonstrated, strengthening the concept that impaired NO production is a crucial mediator in the pathogenesis of OAPS and a potential target for pharmacological interventions. The efficacy of pravastatin supplementation should be confirmed in a larger clinical trial.

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1. Introduction

Placental insufficiency, characterized by abnormal uteroplacental vascular function, results in serious pregnancy complications, including preeclampsia (PE) and intrauterine growth restriction (IUGR), major causes of maternal and fetal mortality and morbidity [1].

Maternal antiphospholipid antibodies (APL) have been associated with the development of placental insufficiency. Between 20 and 30% of pregnancies in obstetric antiphospholipid syndrome (OAPS) patients result in an adverse pregnancy outcome related to abnormal placental vascular function such as PE and IUGR [2,5]. Animal studies highlighted the central role of the placenta in obstetric complications in APS. Animal studies using NanoSPECT/CT and MRI imaging studies demonstrated that the placenta is a highly susceptible target for APL and that binding of these antibodies results in robust complement activation, placental insufficiency and adverse pregnancy outcomes [6,7].

Standard obstetric care in women with OAPS is based on treatment with low molecular weight heparin and low dose aspirin (LMWH + LDA). Unfortunately, standard of care treatment fails in a significant number of pregnancies [8–10]. Efforts to develop effective therapeutic strategies to prevent/treat placental mal perfusion would be of significant clinical benefit for mothers and fetuses.

The last decade has seen the emergence of abundant experimental evidence supporting a favorable role for pravastatin in preventing placental insufficiency in mice. Studies performed in our laboratory and others, showed that pravastatin prevents adverse pregnancy outcomes in mouse models of OAPS and PE [11–14]. Interestingly, the mouse studies were successfully validated in studies in women with placental insufficiency, including OAPS [15–18].

One of the reasons of the longstanding shortage of drugs intended to treat pregnancy-related diseases is the concern regarding the potential adverse effects of drugs on the developing fetus. Importantly, numerous epidemiological studies demonstrated that pravastatin exposure during pregnancy is safe for the fetus [18–22]. Pravastatin being hydrosoluble has a limited transfer to the fetus [18,23].

In our previous pilot study supplementation with pravastatin improved placental hemodynamics and maternal and fetal outcomes significantly in OAPS women receiving standard of care LMHW + LDA [16]. In this study we investigated the effects of the triple therapy LMWH + LDA + pravastatin in improving uteroplacental vascular function and preventing pregnancy complications in OAPS. The hypothesis that nitric oxide (NO) plays a role in the mechanism/s underpinning the vasculoprotective effects of the triple therapy on the placenta was tested.

2. Material and methods

2.1. Study approval

Ethics committee approval was obtained for the human and mouse studies (PIL 60/13172, PPL 4305). The clinical study was performed at Narodni Front University Hospital (University of Belgrade Medical School, Serbia) in accordance with the Code of Ethics of the World Medical Association. All the women gave their informed consent to participate. All animal studies were performed at King's College London in accordance with the 1986 UK Home Office Animal Procedures Act.

2.2. Human studies

2.2.1. Patient information

Obstetric antiphospholipid syndrome (OAPS) is clinically characterized by pregnancy morbidity (recurrent unexplained abortions before the 10th week, unexplained fetal loss at or after the 10th week, or premature birth before the 34th week because of preeclampsia) [24–26]. The laboratory criteria for APS is based on repeated positive test results - persistently positive for APL Abs at 6 month - moderate to high titers, for APL anticardiolipin antibody (aCL), lupus anticoagulant (LA), and anti-β2 glycoprotein I antibody (aβ2GP1) of immunoglobulin isotype G (IgG) and/or IgM [25,27].

Eleven women that met criteria for OAPS and no systemic lupus erythematosus, developed preeclampsia (PE) and/or intrauterine growth restriction (IUGR) associated with abnormal placental Dopplers (Uterine artery mean PI > 95th centile) at 21 weeks (IQR[21–22]) despite treatment with low-dose aspirin (LDA) and low molecular weight heparin (LMWH) participated in this study. PE was defined according to the American College of Obstetricians and Gynecologists (ACOG) Task Force on Hypertension in Pregnancy guidelines [28]. IUGR was defined as being below the 10% percentile of the recommended gender-specific birth weight for gestational age reference curves [29]. Severe IUGR was defined as being below the 5% percentile. At the time abnormal Doppler flow values of the uteroplacental vessels were detected, women were counseled about the risks for the mothers and fetuses associated with placental dysfunction and supplementation with pravastatin (20 mg/daily) was offered. Seven women agreed to the triple therapy (LMWH + LDA + PRAV) and constituted the treated group and 4 women declined pravastatin supplementation and were included in the control group that continued to be treated with standard of care LMWH + LDA (enoxaparin, 40 mg subcutaneously, once daily) plus LDA (80 mg orally, once daily). This study prospectively recorded placental haemodynamics and pregnancy outcomes among standard of care-treated OAPS women who were either supplemented with pravastatin or not.

Some women presented additional risk factors for developing placental insufficiency such as advanced age (> 36 years) and conception after in vitro fertilization (IVF) (Table 1 and 2).

Women in the LMWH + LDA + PRAV group were older than the LMWH + LDA group (years, median 39 IQR [36–43]) vs 31.5 [26.5–35]). In the triple therapy group, six out of the seven women conceived after in vitro fertilization (IVF) (Table 2). In the control group only one woman conceived after IVF (Table 1).

Nitric oxide (NO) levels were measured at the time of placental insufficiency diagnosis/treatment and two weeks after. NO levels in serum were detected indirectly through the spectrophotometric measurement of its stable decomposition products nitrate and nitrite using a commercially available detection kit based on the Greiss reagent method (Cayman Chemical, Ann Arbor, MI, USA).

Table 1
Past and current obstetrical history and time of diagnosis of abnormal uterine artery Dopplers and preeclampsia (PE) / intrauterine growth restriction (IUGR) in OAPS patients (1–4) who were treated with standard LMW + LDA treatment.

Patient	Past obstetric history/ Risk factors	Currrent pregnancy / time of diagnosis of PE/IUR (weeks)
1	2 early miscarriages 1 late miscarriage	20 weeks: ↑UtA PI ↑ UmbA PI (aEDV) IUGR
2	1 stillbirth (24 weeks) 1 late miscarriage	29 weeks: ↑UtA PI Notching ↑ UmbA PI IUGR, PE (BP 180/100 mmHg, Prot = 690 mg/24 h)
3	3 early miscarriages 1 stillbirth (25 weeks)	21 weeks: ↑UtA PI Notching Severe IUGR
4	IVF 2 early miscarriages Vitamin D deficiency Age: 31.5 IQR[26.5–35] Adverse obstetric history:100%	22 weeks: ↑UtA PI IUGR 26 weeks: PE (PB175/105 mmHg, serum creatinine = 1.5 mg/dl) 21.5 weeks IQR [20.5–27.5]

IVF: in vitro fertilization, Prot: proteinuria, Ut art: uterine arteries, Umb art: umbilical artery, PI: pulsatility index, BP: blood pressure, bw: birth weight, NICU: neonatal intensive care unit, aEDV: absent end diastiolic velocity.

Table 2
Past obstetric history, risk factors, and characteristics of current pregnancy in APS patients (1–7) who were treated with triple therapy LMWH + LDA + PRAV.

Patient	Past obstetric history /Risk factors	Currrent pregnancy / time of diagnosis of PE/IUR (weeks)
1.	IVF, 2 early miscarriages, PE Age > 36	Conceived after IVF 21 weeks: ↑Ut art PI, bilateral notching, 22 weeks: PE (BP 160/100 mmHg, prot = 720 mg/24 h)
2.	IVF: 2 early miscarriages, 1PTB Age > 36 BMI > 35	Conceived after IVF: twins Cervical insufficiency – cerclage (18 week) 21 weeks: ↑Ut art PI, bilateral notching (one twin, boy) severe IUGR
3.	IVF, 1 early miscarriage 2 stillbirths (twins) Age > 36	Conceived after IVF: twins cerclage (17 weeks) 21 weeks: ↑Ut art PI, bilateral notching (both twins) PE (BP 167/105 mmHg, prot = 413 mg/24 h)
4.	IVF, 2 early miscarriages, PE Age > 36	Conceived after IVF: twins 21 weeks: ↑Ut art PI, bilateral notching (both twins) severe IUGR boy , IUGR girl 22 weeks: aEDV boy
5.	4 early miscarriages PE (30 weeks) 1 stillbirth (32 weeks)	7th pregnancy: normal conception 22 weeks: ↑Ut art PI, bilateral notching no hypertension or proteinuria IUGR
6.	IVF, 2 early miscarriages 1 placenta abruption (24 weeks) neonate died in NICU (14 days) age > 36	4th pregnancy: normal conception 19 weeks: ↑Ut art PI, IUGR, PE (BP 172/100 mmHg, thrombocytopenia: 93,000/μl serum creatinine: 1.4 mg/dl)
7.	PTB (25 weeks) 1 early miscarriage age > 36	3rd pregnancy: IVF (egg donor) 22 weeks: ↑Ut art PI, bilateral notching IUGR and PE (BP 165/105 mmHg, serum creatinine: 1.4 mg/dl)
	Age: 39 IQR [36–43] Adverse obstetric history:100%	Onset of PE/IUGR: 22 weeks IQR [21–22]

IVF: in vitro fertilization, PE: preeclampsia, Ut art: uterine arteries, PI: pulsatility index, PTB: preterm birth, BP: blood pressure, prot: proteinuria, IUGR: intrauterine growth restriction, NICU: neonatal intensive care unit, IQR: interquartile range. aEDV: absent end diastiolic velocity.

2.3. Mice

Adult mice (6–8 weeks) were used in all experiments. C57BL/6 mice were purchased from Charles Rivers (Margate, United Kingdom).

2.3.1. Mouse model of OAPS

Female mice were mated with previously isolated males, and the presence of a vaginal plug was defined as day 0 of pregnancy. From day 6 of pregnancy, mice were treated with daily IP injections of affinity-purified anti-β2 glycoprotein I ($a\beta_2$GPI) antibodies (100 μg) (OAPS-mice) until day 15 of pregnancy, when the dams were killed and pregnancy outcomes were evaluated. Among the different APL specificities, $a\beta_2$GPI antibodies are frequently found in OAPS women that develop adverse pregnancy outcomes. $a\beta_2$GPI antibodies were isolated using magnetic beads (Dynabeads, Thermo Fisher Scientific Waltham, MA, USA)[6] from serum from patients identified through the Registry of Connective tissue diseases (10/H0405/35) at St Thomas' Hospital. The NHS National Research Ethics Service approved the collection and utilization of samples for research purposes. The control group (control-mice) received normal human IgG (100 μg) purified using protein G sepharose chromatography (Zymed Laboratories, San Francisco, CA).

A group of OAPS-mice were treated with daily injections of enoxaparin (LMWH 250 μg, SC, Sigma-Aldrich, St Louis, MO, USA) and low dose aspirin (LDA, Acetylsalicylic acid 2.1 mg, IP, Sigma-Aldrich, St Louis, MO, USA) from day 6 of pregnancy until day 15 of pregnancy. Half of the mice that received LMWH + LDA as previously described were supplemented with pravastatin (PRAV) (10 μg, IP Sigma-Aldrich, St Louis, MO, USA). LMWH, PRAV and LDA doses were equivalent to the doses used in women with OAPS. Seven to eight mice were studies in each experimental group.

At day 15 of pregnancy, a group of control- and OAPS-mice (untreated and treated with LMWH + LDA or LMWH + LDA + PRAV) were subjected to placental perfusion evaluation. A second group of dams were killed at this time; uteri were dissected and fetal and placental weight were recorded. Thoracic aortas and uterine arteries were removed and placed into ice cold physiological salt solution for further wire myography studies to evaluate endothelial function. Placental tissue was harvested to measure NO content and eNOS synthesis, expression and signaling.

2.3.2. Placental perfusion studies

Placental perfusion was examined by injecting day 15 pregnant females in control and OAPS-mice (treated and untreated) with 100 μL of 25 mg/mL FITC-labeled dextran (MW 2 000 000; Sigma-Aldrich, St Louis, MO) intravenously as previously described [30]. Placentas were homogenized in 9 volumes of 0.1 M Tris (pH 7.4) and FITC-dextran content was measured with a Perkin-Elmer luminescence spectrometer (San Jose, CA, USA).

2.3.3. Wire myography

To examine vascular reactivity, wire myography using aortic and uterine arteries rings was performed [31]. The aorta and uterine arteries were dissected, cleaned of surrounding fat, cut into approximately 2.5 mm rings and mounted into a chamber unit in a multi-wire myograph system (610 M, Danish Nyo Technology, Denmark). Vessel tension data was recorded on a computer using Myodaq 2.02 analysis software (Danish Nyo Technology, Denmark).

At the start of each experiment, a passive circumference-tension curve was performed for each vessel to determine the optimum resting tension. Endothelium-intact aortic rings were then left to equilibrate for 30 min in physiological salt solution (PSS) followed by constriction with 125 mM potassium - substituted PSS (KPSS) as previously described [31]. Cumulative dose–response curves were initially performed for norepinephrine (NE, Sigma-Aldrich St Louis, MO, USA) (10^{-6} to 10^{-4} M). The concentration of NE required to produce an 80% response (EC_{80}) was used to preconstrict the vessels. Vessels were used for subsequent relaxation curves if they attained a minimum of 1.0 mN/mm of tension in response to NE. Following incubations with KPSS, the vessels were first contracted with 10^{-5} M NE for 5 min and then treated with cumulative concentrations of 10^{-9} to 10^{-5} M acetylcholine (ACh, Sigma-Aldrich St Louis, MO, USA, 3 min, respectively) to evaluate NO-dependent vasorelaxation. After the experiments, contractile ability of

Table 3
Maternal and fetal outcomes in women with OAPS treated with triple therapy LMWH + LDA + PRAV and conventional therapy (LMWH + LDA). Values are expressed as median and IQR: interquartile range. NICU = neonatal intensive care unit. PE = preeclampsia. * different from patients that received LMWH + LDA therapy, $p < 0.01$.

	IUGR/PE Time of diagnosis (weeks)	LMWH +LDA	PRAV	Survival	End of pregnancy(week)	Birth weight(g)	Neonatal outcomes
APS (n = 7)	21 IQR [21.5–23]	yes	yes	98*days IQR [84–119]	36* IQR[33–38]7 live births (100%)	Twins [3]: 1420 g [1287–1501], Singletons [4]: 3050 g [2850–3475]	Twins admitted to NICU: 37 days IQR [31–43] 1 necrotising enterocolitis all discharged with normal development survival: 100%*
APS (n = 4)	21.5 IQR [20.5–27.5]	yes	no	23 days [15.75–27.25]2 stillbirths1 neonatal death	24.5 IQR[23–30.6] 1 live birth (25%)	1 survived (1200 g, 32 weeks)	3 deaths 1 NICU: resuscitation, Hypoxic, acidotic93 days – normal development survival: 25%

the blood vessels was tested. Vessels which failed to contract to KPSS or NE were not included in the study.

2.3.4. Nitric oxide (NO) determination in placenta, aorta and uterine arteries

Placentas, endothelium-intact aortas and uterine arteries from day 15 control pregnant mice and OAPS-mice (untreated and treated with LMWH + LDA or LMWH + LDA + PRAV) were homogenized in isotonic solution of PBS containing 10 mM N-ethylmaleimide (NEM, Sigma-Aldrich St Louis, MO, USA) and 2.5 mM EDTA to prevent artefactual formation of NO products or metabolites during sample preparation. Determination of NO was performed indirectly through the spectrophotometric measurement of its stable decomposition products nitrate and nitrite. A commercially available nitric oxide metabolite detection kit was used (Cayman Chemical, Ann Arbor, MI, USA).

2.3.5. Endothelial nitric oxide synthase (eNOS) expression and signaling in aorta and uterine arteries

A previously described rapid isolation procedure was used to obtain endothelial-derived proteins without vascular smooth muscle contamination [32]. Briefly, portions of aortas and uterine arteries were opened longitudinally, and the endothelium/tunica intima was gently scraped (5 times) by using a round-end spatula and placed in lysis buffer [32,33]. Mouse eNOS expression was determined using a commercially available ELISA kit (Abcam, Cambridge, United Kingdom). cGMP levels, indicative of endogenous eNOS signaling activity, was measured by a direct immunoassay (Abcam, Cambridge, United Kingdom).

2.3.6. RNA extraction and quantification of gene expression by real-time qPCR

After cervical dislocation, thoracic aortas, uterine arteries and placentas were harvested. RNA was extracted using RNA extraction columns (Qiagen, Hilden, Germany) as per the manufacturer's instructions. RNA purity was verified using a NanoDrop spectrophotometer. 1 μg total RNA was reverse transcribed using a First Strand cDNA Synthesis kit (Fermentas Life Sciences, Waltham, Massachusetts, USA). Relative quantification of gene expression was performed by real-time PCR using iQ SYBR-Green Supermix on the iCycler iQ thermal cycler (BioRad, Hercules, CA, USA) following the manufacturer's protocols. Primer sequences are: GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used to normalize the quantitative experiments. Primer sequences were as follows: Mouse GAPDH sense, GGC CTT CCG TGT TCC TAC; antisense, TGT CAT CAT ATC TGG CAG GTT, mouse eNOS sense CCT GGA GGA ATA ATG CTG AAT; antisense, AAT GGT AAC GTG CAG GAC ATC. The relative quantities were expressed as the specific ratio between the gene of interest and the reference gene.

2.4. Statistics

All statistical analyses were performed using Prism 6.0 Software (GraphPad Software Inc.). Comparisons between groups were performed by one-way analysis of variance (ANOVA) followed by *t*-test or Mann-Whitney. Data are presented as mean ± standard deviation (SD). In all cases, $P \leq 0.05$ were deemed statistically significant. When > 2 groups were to be compared, ANOVA followed by a post-hoc Bonferroni test was used in order to avoid multiple-comparisons error.

Data from the human studies are presented as medians and interquartile range (IQR).

False discovery rate (FDR) was used for multiple comparisons of human NO-levels.

FDR adjusted p-values less than or equal to 5% were considered significant.

3. Results

3.1. Human studies

All OAPS patients were treated with LMWH + LDA since the beginning of pregnancy. Despite antithrombotic therapies all patients developed early placental insufficiency evidenced by abnormal Dopplers at 21 weeks (IQR[21–22] (Table 1 and 2). Patients on standard treatment LMWH + LDA (control group) showed no improvement in placental Dopplers and pregnancies survived 23 days IQR [15.75–27.25] after diagnosis (Table 3). Preeclampsia symptoms, observed in 2 patients in the control group, did not improve despite L-DOPA treatment. In the control group, 2 women suffered stillbirths and one gave birth preterm to a neonate with extremely low birth weight that died right after delivery (Table 3). The fourth neonate was born preterm, hypoxic and acidotic and required resuscitation. This neonate survived and now shows normal development after spending 3 months at NICU (Table 3).

LMWH + LDA + PRAV improved placental perfusion in all 7 patients as observed in our previous study [16]. Uterine arteries resistance dropped significantly and pulsatility index (PI) values reached normal values for gestational age (Fig. 1**A**) one week after the triple therapy (median:1 week IQR[1–1.5]). Uterine arteries PI in the control group not supplemented with PRAV remained elevated (> 95%centile) until the end of gestation (Fig. 1**A**).

Serum nitrite/nitrate levels increased significantly after pravastatin supplementation while no changes were observed in patients that remained on conventional therapy (Fig. 1**B**). Hypertension and proteinuria, observed in 3 OAPS patients, improved after LMWH + LDA + PRAV treatment (BP = 130/90 mmHg or less and proteinuria of 300 mg/dl). Pregnancies in patients treated with LMWH + LDA + PRAV survived 98 days, IQR[77–119] after diagnosis compared to 23 days IQR[15.75–27.25] in the control group (Table 3). Delivery in OAPS patients treated with the triple therapy occurred at 36 weeks (IQR[33–38]) diminishing neonatal complications associated with prematurity. In the control group that received only LMWH + LDA therapy deliveries occurred preterm (24.5 weeks IQR [23–30.6]) (Table 3). Only 1 fetus survived in the control group. In the group treated with LMWH + LDA + PRAV only twins were admitted at NICU (37 days IQR[31–43]) and no late sequelae were reported for these infants (Table 3).

3.2. Mouse studies

3.2.1. LMWH + LDA + PRAV increased placental blood perfusion and placental and fetal weight in OAPS-mice

Diminished placental perfusion was detected in OAPS-mice compared to control mice treated with NHIgG (Fig. 2**A,B),** mimicking the clinical disease in which increased placental vascular resistance was detected during Doppler scanning [16].

While conventional treatment LDA + LMWH induced a partial increase in placental blood flow (Fig. 2**A, B**) in OAPS-mice, LMWH + LDA + PRAV significantly increased placental perfusion to values comparable to control pregnancies that received NHIgG (Fig. 2**A, B**). In parallel to the improved placental perfusion, increased placental and fetal weights were observed in mice that received the triple therapy compared to untreated OAPS-mice and mice that received conventional treatment (Fig. 2**C**). LMWH + LDA treatment induced a mild increase in fetal weight but did not affect placental weight compared to untreated OAPS-mice (Fig. 2**C**). Triple therapy LMWH + LDA + PRAV completely prevented IUGR in OAPS-mice (Fig. 2**C**).

3.2.2. Endothelial and vascular function in aorta and uterine arteries from untreated and treated OAPS mice.

Aortic rings isolated from OAPS-mice showed a diminished vasorelaxant response to ACh compared to blood vessels from control mice treated with NHIgG-mice (Fig. 3 **A, B**). Increased relaxation to increasing doses of ACh was observed in aortic rings from LMWH + LDA-treated OAPS-mice. However, ACh-induced vasorelaxation in this group was still different from control mice, suggesting a partial protection on

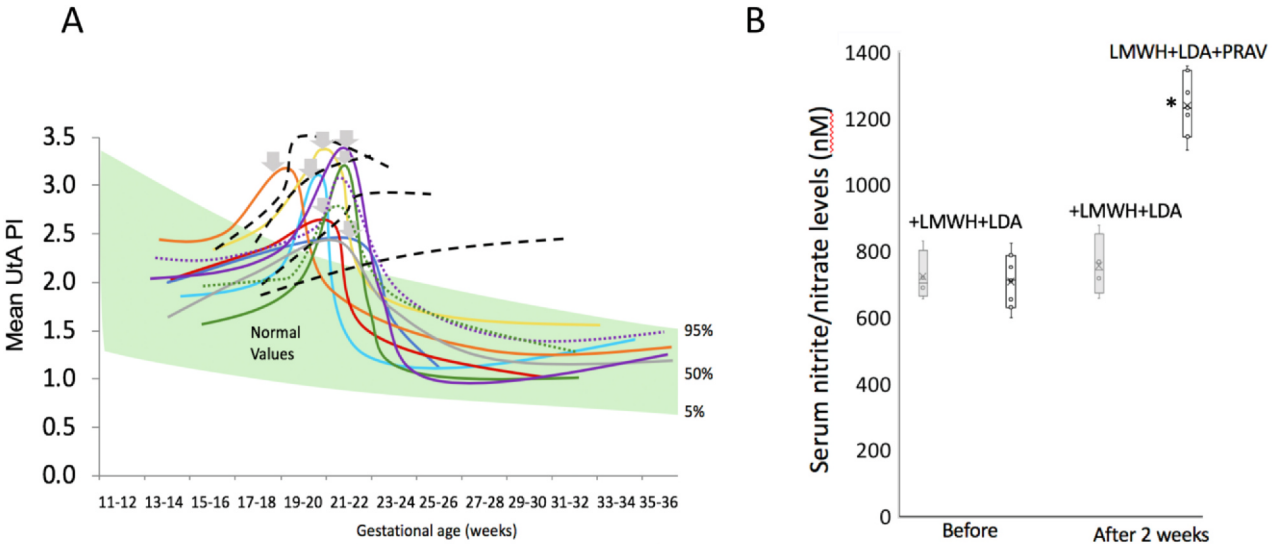

Fig. 1. Uteroplacental Doppler studies and serum nitric oxide levels in OAPS patients A- Uterine artery pulsatility (UtA PI) index during the course of pregnancy in OAPS patients that developed placental insufficiency despite antithrombotic therapy supplemented and non-supplemented with pravastatin. The gray arrows indicate the time at which pravastatin was added. The area in green represents the normal values (upper and lower lines correspond to 95 and 5 percentile respectively). UtA PI values diminished significantly in the patients supplemented with pravastatin (median time 8 days IQR [7–10]) and remained within normal values until delivery. UtA PI values remained high (> 95% centile) during the course of pregnancy in the 4 patients that did not receive pravastatin (dashed line). The dot line represent twin number 2. **B-** Serum nitrite/nitrate levels (indicative of nitric oxide content) in patients with OAPS receiving standard of care LMWH + LDA before and 2 weeks after supplementation with pravastatin or no supplementation. Pravastatin supplementation increased NO levels in OAPS-patients. Values are expressed as median, 1st quartile (25th percentile) and 3rd quartile (75th percentile). * different from values prior to pravastatin addition, $p < 0.05$, N = 7 in the LMWH + LDA supplement with pravastatin (PRAV), N = 4 in the group of women that did not receive PRAV. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

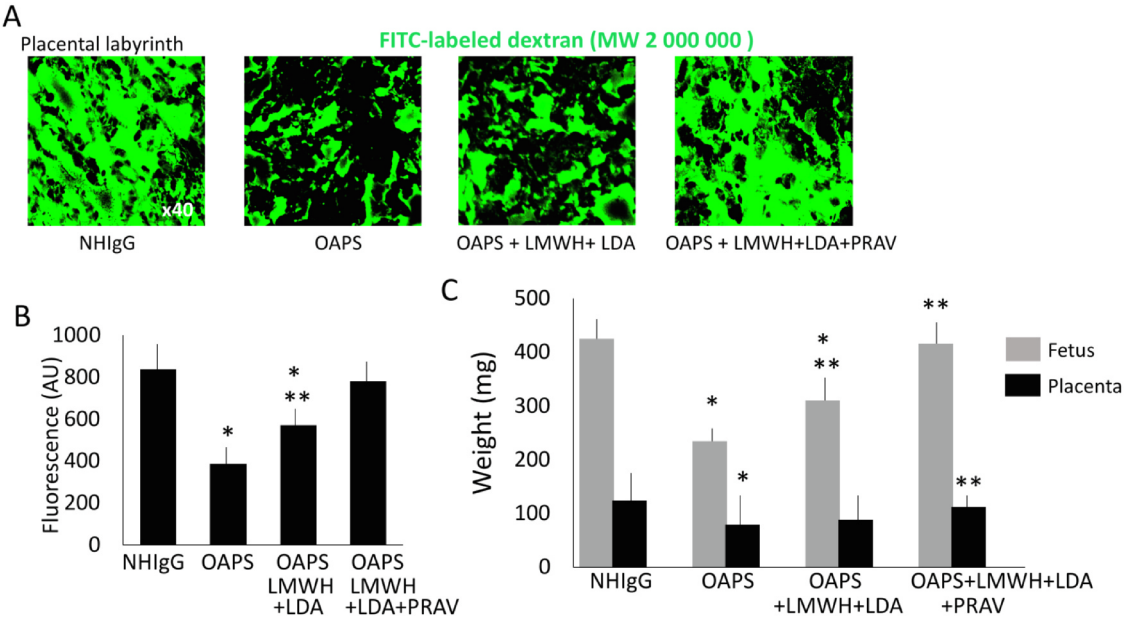

Fig. 2. Triple therapy LMWH + LDA + PRAV improved placental blood flow and prevented IUGR in OAPS-mice. **A**- Placental blood perfusion was measured in NHIgG- and OAPS-mice (treated and untreated) after fluorescein isothiocyanate (FITC)-dextran (molecular weight: 2 000 000) injection in the maternal circulation. Increased placental dextran-FITC is a measure of increased placental flow. In control NHIgG-mice, with normal pregnancies, the fluorescent tracer accumulated in the placental labyrinth. Less fluorescence, indicative of less blood perfusion, was observed in the labyrinth of OAPS-mice. A slight improvement in placental blood flow was observed in LMWH + LDA-treated OAPS-mice. LMWH + LDA + PRAV restored placental blood flow in OAPS-mice to control levels. N = 6 to 8 mice in each experimental group, 4 placentas/mouse. **B**- The bar graph shows the quantification of dextran-FITC in placental homogenates. *Different from control NHIgG, $P < 0.01$, ** Different from OAPS, $P < 0.01$. **C**- Placental and fetal weight measured at day 15 of pregnancy in NHIgG- and OAPS-mice (treated and untreated). *Different from control-mice, $P < 0.01$, ** Different from OAPS-mice, $P < 0.05$. N = 7 mice/group.

endothelial function (Fig. 3 **A, B**). Mice treated with triple therapy LMWH + LDA + PRAV showed increased vasorelaxant response to ACh and preserved endothelial function, comparable to control mice (Fig. 3 **A, B**).

The thoracic aorta, a compliance vessel continually subjected to different hemodynamic forces may adjust its vascular tone in a different way to smaller blood vessels such as uterine arteries affecting placental perfusion. To better understand the improvement in placental blood flow induced by the triple therapy, vascular function in uterine arteries rings was studied.

Interestingly, uterine arteries showed a vascular function similar to aortic rings (Fig. 3 **C, D**). Uterine artery rings isolated from OAPS-mice showed a significantly diminished vasorelaxant response to ACh compared to blood vessels from NHIgG-mice. LMWH + LDA treatment induced a partial protection in endothelial function in uterine arteries from OAPS-mice (Fig. 3 **C, D**). Mice treated with LMWH + LDA + PRAV showed a preserved uterine artery endothelial function comparable to control mice (Fig. 3 **C, D**).

3.2.3. eNOS expression and activity and NO generation

Diminished NO levels were detected in placentas and uterine arteries from OAPS-mice compared to NHIgG-mice (Fig. 4**A**). Interestingly, diminished levels of NO in OAPS-mice were not associated with a diminution in eNOS gene expression (Fig. 4**B**). In addition, eNOS protein expression was not diminished in the endothelium of uterine arteries from OAPS-mice compared to control mice (Fig. 4**C**), suggesting that the low levels of NO found in OAPS might be related to impaired eNOS activity. In this line, diminished cGMP generation was observed in the endothelium of uterine arteries from OAPS-mice (Fig. 4**D**) suggesting that reduced NO-cGMP signaling contributes to the vascular/endothelial dysfunction observed in APS. Reduced NO-cGMP signaling was also observed in the aortic endothelium of OAPS-mice (Fig. 4**B**).

Treatment with LMWH + LDA increased placental and uterine levels of NO in OAPS-mice, but NO levels were still lower than those measured in control mice (Fig. 4**A**). Despite the increase in NO levels in uterine arteries and placentas, RT-PCR experiments showed no changes in eNOS gene expression in OAPS-mice treated with LMWH + LDA compared to untreated OAPS-mice (Fig. 4**B**). A moderate increase in cGMP generation was observed in OAPS-mice treated with LMWH + LDA suggesting a mild modulatory effect of the antithrombotic therapy on eNOS signaling resulting in a small increase NO production (Fig. 4**D**).

Uterine arteries and placentas from mice that received LMWH + LDA + PRAV showed the highest levels of NO production and this increase was associated with increased eNOS gene expression (Fig. 4**B**) in parallel with increased eNOS protein expression and cGMP generation. The triple therapy induced the maximal increase in NO production by increasing eNOS gene and protein expression as well as activity (Fig. 4 **A, B, C, D**). Increased eNOS expression and signaling was also observed in the endothelial cells isolated from aortas from OAPS-mice that received the triple therapy (Fig. 4 **C, D**).

4. Discussion

A significant number of pregnant women with OAPS are refractory to conventional LMWH + LDA treatment and develop placenta insufficiency associated with adverse maternal and neonatal outcomes. In this study, 11 women showed abnormal uterine artery pulsatility index, and IUGR and/or preeclampsia, placental pathologies associated with uteroplacental vascular dysfunction, despite conventional therapy. In an attempt to improve placental perfusion/vascular function and prevent adverse pregnancy outcomes associated with placental insufficiency women were offered to receive pravastatin in addition to conventional therapy [15,16]. NO plays a major role in fetoplacental impedance during gestation, and it is known that pregnancies complicated by preeclampsia are associated with a lower level of nitric oxide synthetase (NOS) activity in the placenta; that leads to high-resistance fetoplacental circulation [35]. Pravastatin showed to increase NO levels

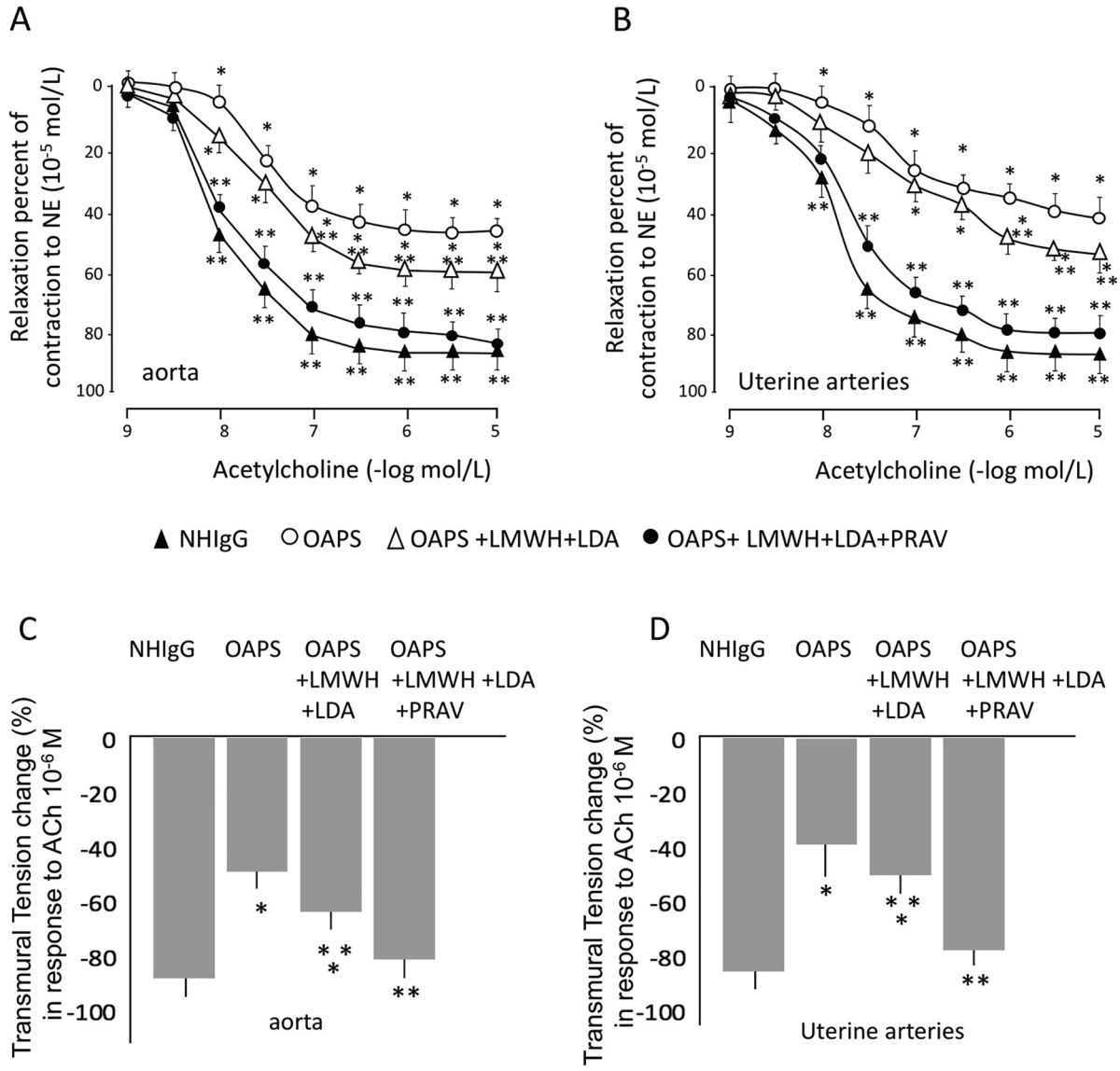

Fig. 3. Wire myography studies in aorta and uterine arteries in mice **A, C**- Concentration-response curves for acetylcholine (ACh) in aortas and uterine arteries with intact endothelium from control and OAPS-mice. Vasorelaxation induced by ACh (10^{-6} M) in precontracted (10^{-5} mol/L norepinephrine (NE)) endothelium-intact aortic rings (**A**) and uterine arteries rings (**C**) from day 15 pregnant NHIgG- and OAPS-mice (untreated and treated). N = 7–8 mice/experimental group. **B, D**- Response to different doses of ACh (10^{-9} to 10^{-5} M) in (**A**) aorta and (**B**) uterine arteries from day 15 pregnant NHIgG- and OAPS-mice untreated and treated. Each point represents the mean ± SEM (n = 5–7). Responses are expressed as percentages of the maximal relaxation induced by norepinephrine (NE, 10^{-5} mol/L) * Different from NHIgG-treated mice , $p < 0.01$ ** Different from OAPS-mice, $p < 0.05$.

and improve pregnancy outcomes in an animal model characterized by abnormal placental perfusion [34].Therefore, we hypothesized that pravastatin might improve placental perfusion in women with placental dysfunction through an NO-dependent mechanism. In agreement with our hypothesis, increased serum NO levels and improved uterine artery Dopplers were observed in all women that received LMWH + LDA + PRAV compared to the women that remained on conventional therapy. A significant increase in pregnancy length and favorable outcomes was associated with increased NO levels and improved Dopplers. Supplementation with pravastatin increased NO levels, improved placental haemodynamics, ameliorated preeclampsia symptoms and improved fetal growth leading to live birth very close to term. Therefore, preventing the neonatal health compromise associated with prematurity. On the other hand, no changes in serum NO levels and adverse pregnancy outcomes such as preterm birth, stillbirth and poor neonatal outcomes were observed in women that received LMWH + LDA without pravastatin. In the group supplemented with pravastatin no neonatal complications were observed and the infants show normal development and growth.

Mouse studies were performed to investigate the mechanism/s underpinning the protective effects of pravastatin on uteroplacental vessels function and the role of NO. Similar to the human studies, the triple therapy improved placental perfusion and improved placental and fetal weight to a greater extent than standard treatment LMWH + LDA in OAPS-mice. Pregnancy outcomes in OAPS-mice treated with the triple therapy were not different from control mice. Wire myography studies were performed to examine functional responses and vascular reactivity in mice treated with conventional therapy and conventional therapy supplemented with pravastatin. Endothelium-intact aortic and uterine artery rings from OAPS-mice showed an attenuated ACh-induced relaxation, suggesting an impaired production of NO. In this line, diminished levels of NO were measured in placenta and uterine arteries from OAPS-mice compared to control mice. A potential link between APS and diminished NO bioavailability has been reported in humans, as

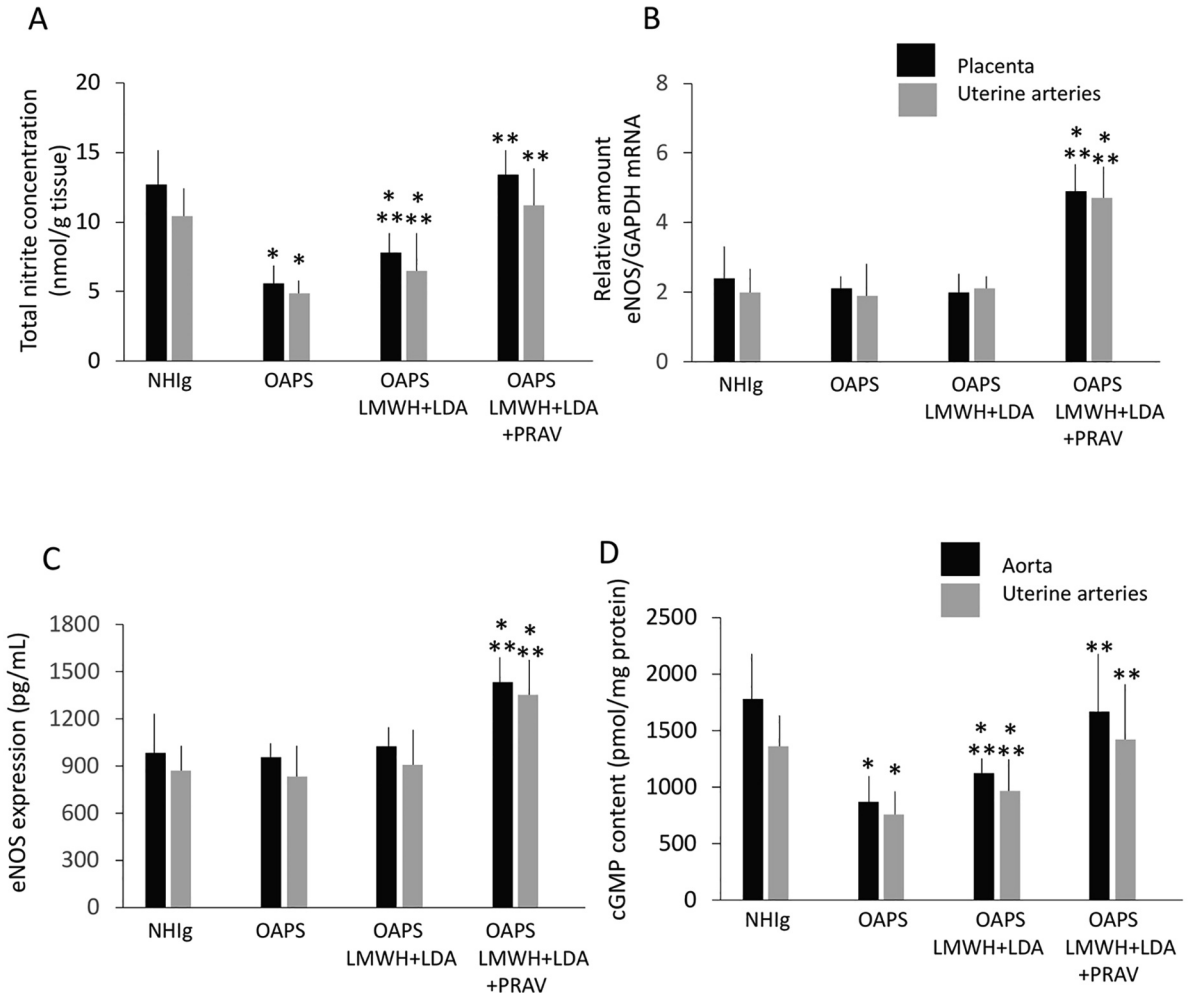

Fig. 4. Endothelial Nitric oxide synthase (eNOs) gene and protein expression and signaling and nitric oxide (NO) production. **A**- NO content in placentas and uterine arteries from NHIgG- and OAPS-mice (untreated and treated with LMWH + LDA with and without PRAV supplementation). Determination of NO was performed indirectly through the measurement of total nitrite content. Diminished levels of NO were measured in placenta and uterine arteries from OAPS-mice compared to control mice. A mild increase in NO levels was observed with LMWH + LDA treatment. NO levels in mice that received the triple therapy increased significantly, reaching values comparable to control mice (NHIgG). *Different from NHIgG-mice, $P < 0.01$, ** Different from OAPS-mice, $P < 0.01$. N = 7–8 mice/experimental group. **B**- *eNOS* gene expression quantification by real-time PCR in placentas and uterine arteries from NHIgG- and OAPS-mice (untreated and treated with LMWH + LDA with and without PRAV supplementation). The relative quantities are expressed as the specific ratio between the gene of interest *eNOS* and the reference gene (GAPDH). LMWH + LDA + PRAV treatment induced a significant increase in *eNOS* gene expression in placenta and uterine arteries. *Different from NHIgG, $P < 0.01$, ** Different from OAPS-mice, $P < 0.05$. N = 7–8 mice/experimental group. **C**- expression of eNOS protein in endothelium from aorta and uterine arteries from control (NHIgG) and untreated and treated OAPS-mice. The triple therapy LMWH + LDA + PRAV induced a significant increase in eNOS protein expression in both uterine arteries and aorta. *Different from NHIgG, $P < 0.01$, ** Different from OAPS-mice, $P < 0.05$. N = 6–7 mice/experimental group. **D**- Endogenous eNOS signaling/activity, measured by cGMP levels, in endothelium from aorta and uterine arteries from control (NHIgG) and OAPS-mice (untreated and treated). Reduced cGMP generation was observed in the aorta and uterine arteries endothelium from OAPS-mice, suggesting diminished eNOS signaling/activity. LMWH + LDA induced a small increase in cGMP levels while pravastatin supplementation restored eNOS activity to control values. *Different from NHIgG, $P < 0.01$, ** Different from OAPS-mice, $P < 0.05$. N = 6–7 mice/experimental group.

plasma APL levels have been shown to be inversely correlated with urinary NO metabolite excretion [36]. In agreement with this, APS patients have lower levels of plasma nitrites compared to control subjects [37].

Decreased availability of NO in OAPS-mice was not associated to changes in eNOS gene expression either in placentas or in the uterine arteries. This is in agreement with the studies by Ramesh et al, describing decreased NO bioavailability in the endothelium caused by a direct antagonism of eNOS activation by antibodies aβ_2GPI, antibodies with the same specificity to the ones used in this study [38]. Similarly to our studies in aortic and uterine artery rings, NO-dependent, ACh-induced vasorelaxation in the carotid artery was impaired in OAPS-mice that received aβ_2GPI antibodies [38].

Our original hypothesis envisaged triple treatment LMWH + LDA + PRAV to synergistically increase the production of NO in uterine arteries thus improving uteroplacental vascular function leading to improved pregnancy outcomes. In agreement with our hypothesis we identified a role for NO in the vasculoprotective effects of the triple combination therapy.

LMWH + LDA induced a moderate vasorelaxation in aortic and uterine artery rings associated with a partial increase in NO generation that was independent of eNOS synthesis. LMWH + LDA induced a mild increase in eNOS activity evidenced by a slight increase in cGMP generation. Activation of eNOS by heparin has been demonstrated through a mechanism involving inhibitory guanine nucleotide regulatory protein, independently from its anticoagulant effects [39]. Acetylation of eNOS protein by aspirin can lead to increased activity and NO generation in OAPS-mice treated with LMWH + LDA [40]. LDA might also contribute to placental blood improvement by relaxing the vascular smooth muscle [41]. In vivo, aspirin might also preserve the integrity of

the vascular wall through its free radical scavenging properties, thus preventing the consumption of NO through interaction with reactive oxygen species [42]. All these mechanisms can contribute to the partial protection of LMWH + LDA on placenta haemodynamics in OAPS.

Pravastatin supplementation in OAPS-mice treated with LMWH + LDA significantly restored endothelial function. Increased vasorelaxation induced by the triple therapy was associated with increases in eNOS gene and protein expression leading to maximal increase of NO. It has been reported that pravastatin increases eNOS mRNA stability, leading to increased eNOS synthesis and activity [43]. In this line, a significant increase in cGMP generation was observed in the endothelium of aortas and uterine arteries from OAPS-mice treated with triple therapy, suggesting an important modulatory effect of pravastatin in NO/cGMP signaling resulting in NO production. We also need to consider that pravastatin might contribute to the improvement of placental blood flow and function by reversing atherosclerotic lesions frequently found in the placenta of patients with preeclampsia and OAPS [44–46] and by modulating the activity of neutrophils and the release of reactive oxygen species that may affect endothelial function [11].

Along with identifying a role for NO in mediating the vasculoprotective effects of LMWH + LDA + PRAV therapy, these results strengthen the concept that impaired eNOS activity resulting in impaired NO production is a crucial mediator in the pathogenesis of OAPS. Therefore, therapies that can augment NO availability by increasing eNOS synthesis and activity, might be beneficial in the prevention of adverse pregnancy outcomes in APS.

In conclusion, this study demonstrates that the triple combination therapy LMWH + LDA + PRAV increases eNOS synthesis, expression and activity resulting in increased NO levels leading to improved placental perfusion and favorable pregnancy outcomes. This study in women involved a small group of patients, therefore a larger clinical trial testing the effects of pravastatin supplementation in APS women should be organized to confirm these observations.

CRediT authorship contribution statement

Eleftheria Lefkou: Conceptualization, Data curation, Investigation, Project administration, Writing - review & editing. **Katerina Varoudi:** Investigation. **Aleksandar Jurisic:** Conceptualization, Data curation, Investigation, Writing - review & editing. **Zaklina Jurisic:** Investigation. **Greg Contento:** Investigation. **Guillermina Girardi:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Writing - original draft, Writing - review & editing.

Source of funding

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Authors contribution

GG and EL conceived and designed the study. JP, GC and GG performed the animal studies. EL , KV, AJ and ZK were responsible for patient's care. GG and EL analyzed and interpreted data. GG wrote the manuscript.

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