King's Research Portal

Document Version
Peer reviewed version

[Link to publication record in King's Research Portal](#)

Accepted Manuscript

Pravastatin-l-arginine combination improves umbilical artery blood flow and neonatal outcomes in dichorionic twin pregnancies through an nitric oxide-dependent vasorelaxant effect

Aleksandar Jurisic, Zaklina Jurisic, Eleftheria Lefkou, Joaquim Pombo, Guillermina Girardi
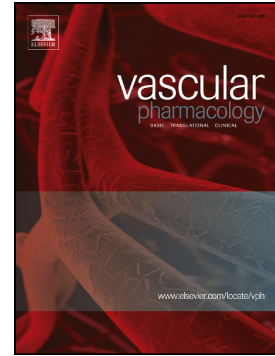
Please cite this article as: Aleksandar Jurisic, Zaklina Jurisic, Eleftheria Lefkou, Joaquim Pombo, Guillermina Girardi , Pravastatin-l-arginine combination improves umbilical artery blood flow and neonatal outcomes in dichorionic twin pregnancies through an nitric oxide-dependent vasorelaxant effect. Vph (2017), doi:10.1016/j.vph.2018.06.001

Pravastatin-L-arginine combination improves umbilical artery blood flow and neonatal outcomes in dichorionic twin pregnancies through an nitric oxide-dependent vasorelaxant effect

Aleksandar Jurisic[1], Zaklina Jurisic[2], Eleftheria Lefkou[3], Joaquim Pombo[4] and Guillermina Girardi[4]

[1]University of Belgrade Medical School, Narodni Front University Hospital, Belgrade, Serbia

[2]OB/GYN Polyclinic Jurisic, Belgrade, Serbia

[3]Institute of Thrombosis and Haemostasis, Athens, Greece

[4]Division of Women and Children's Health, King's College London, UK

Corresponding author
Prof Guillermina Girardi
Pregnancy Laboratory
Division of Women and Children's Health
The Rayne Institute - 4th Floor Lambeth Wing
St Thomas' Hospital - King's College London
London SE1 7EH - United Kingdom
Phone/ fax: +44(0) 207-188-1101
e-mail address: guillermina.girardi@kcl.ac.uk

Abstract

The increase in fetal and neonatal morbidity and mortality associated with twin pregnancies correlates with an increased risk of preterm delivery, low birth weight, and intrauterine growth restriction (IUGR). Although the pathogenesis of IUGR is unclear and thus management remains a major challenge, feto-placental blood vessels are compromised, and altered umbilical blood flow is observed. In this pilot observational study we investigated the effects of pravastatin plus L-arginine on umbilical artery (umb art) blood flow. Between 2013 and 2016, five women received daily doses L-arginine and pravastatin when an umb art pulsatility index above limits for gestational age was observed and concerns about selective growth restrictions arose. All patients showed selective absent or reversed end-diastolic umbilical artery Doppler flow (AREDV) associated with increased perinatal mortality. Pravastatin (PRAV) plus L-arginine (L-Arg) treatment diminished umb art resistance significantly and allowed pregnancy to continue. No signs of acidosis or hypoxia, normal cardiotocography tracing, normal fetal movement and fetal weight gain were observed in the twins that showed abnormal umb art Dopplers. All neonates were born around 33 weeks (median 33 weeks, IQR[31.4-33.0]), thus diminishing substantially the chances for any prematurity-associated adverse neonatal outcomes. The infants now show normal growth and development. In *in vitro* studies, pravastatin induced relaxation of aortic rings. Murine studies identified were performed to investigate the mechanism behind PRAV+L-Arg beneficial effects. A nitric oxide (NO)-dependent synergistic vasorelaxant effect of PRAV+L-Arg was demonstrated using aortic rings. Increased levels of placental NO and increased synthesis of eNOS in placental endothelial cells were observed in mice treated with PRAV+L-Arg compared to untreated mice and mice treated with PRAV- or L-Arg alone. This study suggests that PRAV plus L-Arg might be a good therapeutic option to improve blood flow in umbilical arteries prolonging pregnancy and improving pregnancy outcomes in twins. A RCT should be organized to confirm these results.

Introduction

The incidence of twin pregnancy is increasing, mainly due to delayed childbirth and advanced maternal age at conception and the extensive use of assisted reproduction techniques (1). Twin pregnancies are associated with a greater risk of preterm delivery, low birth weight, intrauterine growth restriction (IUGR) and intrauterine fetal death (IUFD). A fivefold increase in the rate of stillbirths is observed in dichorionic twins (DC) compared with singleton pregnancies. Preterm birth occurs in up to 60% of twin pregnancies, contributing to the increased risk of neonatal mortality (2). The recent global drive to prevent stillbirth has highlighted multiple pregnancy as a major risk factor (2).

IUGR, strongly associated with fetal demise, is commonly observed in twins, with an incidence of 25-35%, and can affect one or both fetuses. In the IUGR surviving fetuses, a positive correlation between low birth weight, abnormal neurodevelopment and increased risk of adult cardiovascular disease have been documented (3).

Although the pathogenesis of IUGR is unclear and thus management remains a major challenge, feto-placental blood vessels are compromised, resulting in altered umbilical artery (Umb art) blood flow in the severest cases.

Severe reduction in Umb art blood flow as reflected by absent or reverse end diastolic velocity (A/REDV) during pregnancy is highly associated with fetal morbidity and mortality and expeditious delivery should be contemplated to prevent IUFD.

L-arginine (L-Arg), a precursor for synthesis of vasodilator nitric oxide (NO), has been used to treat IUGR in humans and sheep (4, 5). While some studies have associated L-arginine supplementation to improved placental blood flow and increased birth weight compared to standard care, other studies including a well-designed double blinded trial of oral arginine versus placebo failed to show a benefit in fetal growth (6). Efforts to develop effective treatments to improve umbilical arteries blood flow and prevent IUGR and IUFD would be of important clinical significance.

In a recent study, pravastatin treatment improved uterine arteries blood flow and pregnancy outcomes in women with obstetric antiphospholipid syndrome and placental insufficiency-related pregnancy complications: preeclampsia and IUGR (7). Among the many pleiotropic effects attributed to statins, increased nitric oxide (NO) synthesis, vascular dilatation and anti-inflammatory and antithrombotic effects are described (8). Thus, there is a strong basis for using pravastatin to improve umbilical arteries (Umb Art) blood flow, ameliorate IUGR and protect twin pregnancies. Pravastatin showed not to be teratogenic in mice and women (9-11). Five women with twin pregnancies that presented umb art pulsatility index above limits for gestational age and thus at risk of fetal growth restriction and death were treated L-arginine and pravastatin. We hypothesized that this combined therapy might have a synergistic modulatory effect on nitric oxide synthesis leading to Umb art vasodilation, blood flow improvement and

prevention of growth restriction and IUFD in dichorionic twin pregnancies. In vitro studies, using aortic rings and isolated placental endothelial cells from mice were performed to test to test our hypothesis.

METHODS

Human studies

From 2013 to 2016, five women (median age 37, IQR [33.5-38.5]) with dichorionic twin pregnancies and selective abnormal Umb art Dopplers were treated with L-arginine and pravastatin to prevent IUGR and IUFD. None of them had previous live births. Four of them conceived after in vitro fertilization. Normal fetal development and normal placental haemodynamics were observed until the end of the second trimester (median: 23 weeks, IQR [21.3-23.7])), when selective Umb art pulsatility index (PI) above limits for gestational age was detected. At this time, all women were treated with daily oral doses of pravastatin (PRAV, 40 mg) and L-arginine (L-Arg, 1.5 g) until the end of the pregnancy. Patients were scanned every week to monitor changes in vascular resistance in Umb Art and fetal well-being and growth. Uterine arteries Doppler ultrasound parameters were normal throughout pregnancy in all patients. Approval for investigational drug use was obtained from the Narodni Front University Hospital Ethical Review Committee at the University of Belgrade Medical School, Serbia and written informed consent was obtained from all pregnant patients. All research was performed in accordance with relevant guidelines and regulations.

Ultrasonography of pregnancies

Doppler examinations were performed by two examiners (AJ and ZJ) using RM6C matrix 4D convex probe (Voluson E10, GE Healthcare) and V4-8 4D convex probe (Medison V20 Prestige, Korea) with the high-pass filter at 60 Hz. Spectral Doppler analysis of flow velocity waveforms in fetal blood vessels (middle cerebral artery, umbilical artery) was performed after obtaining a minimum of 10 heart rate cycles without fetal movement and fetal breathing. When AEDV was detected in umbilical artery, measurements were repeated 3 times on different free loops of umbilical cord.

The following variables were recorded: i) Umbilical artery pulsatility index(Umb Art PI) obtained from a free loop of the umbilical cord; ii) middle cerebral artery (MCA) pulsatility index (PI) measured in the straight portion of the artery avoiding head compression by the transducer and iii) the cerebroplacental ratio (CPR) was calculated as the ratio of the MCA PI divided by the Umb Art PI.

Animal studies

All animal studies were performed in accordance with the 1986 UK Home Office Animal Procedures Act. Approval was provided by the local ethics committee, Animal Welfare & Ethics Board (AWERB),

FWB & St Thomas' Hospital at King's College London. C57BL/6 mice were maintained at 20-22°C, with standard rodent chow available ad libitum and under 12:12 hours light dark schedule. Non pregnant and day 15 pregnant C57BL/6 females were used in this study. After mating, the presence of a vaginal plug was considered day 0 of pregnancy. Animals were killed by cervical dislocation and the thoracic aortas were removed and placed into ice cold physiological salt solution for further analysis of vascular reactivity using wire myography. A group of pregnant (15 dpc) females were treated with pravastatin (PRAV (0.6mg/mouse, intraperitoneal Sigma Aldrich), L-Arginine (L-Arg, 0.5 mg/mouse) or PRAV+L-Arg 4 hours prior to the isolation of the aortas and placentas for endothelial cell isolation and nitric oxide production measurements . These doses are equivalent to the doses of 40 mg of PRAV and 1.5 g L-Arg used in the human studies.

Wire myography

To examine the effects of L-Arg and PRAV on vascular reactivity, aortic rings and wire myography were used (13). The aorta was dissected, cleaned of surrounding fat, cut into approximately 2.5mm rings and mounted into a chamber unit in a multi-wire myograph system (610M, Danish Nyo Technology, Denmark). Vessel tension data was recorded and stored on a computer using Myodaq 2.02 analysis software (Danish Nyo Technology, Denmark). Vessels were then left to equilibrate for 30 minutes in physiological salt solution (PSS) and then subject to constriction with 125mM potassium - substituted PSS (KPSS) as previously described (13). Following incubations with KPSS, vessels were constricted with 50µL of 10^{-4}mol/L phenylephrine (Sigma Aldrich, UK) to induce a 80% maximum contraction in arteries. After vasoconstriction with phenylephrine, incubations with PRAV (10ng/ml, Sigma Aldrich) and L-Arg (350 µM, Sigma Aldrich), alone or in combination (L-Arg added 30 min before pravastatin), were performed to assess vasorelaxant effects. Pravastatin concentration was calculated based on the studies performed using the *ex vivo* technique of dual perfusion of placental lobule. The perfused placenta studies showed that 18± 4% of the perfusion concentration (50 ng/mL equivalent to an in vivo dose of 40 mg (14)) was transferred to the fetal circuit at the end of a 4-hour perfusion (15). L-Arg concentration was calculated based on the dose used in humans and a volume of distribution of 24L.

Incubation for 30 min with nitric oxide (NO) synthase inhibitor NG-nitro-L-arginine methyl ester (100µL 10^{-2} mol/L L-NAME; Sigma Aldrich, UK) prior to the addition of pravastatin and L-Arg was performed to evaluate the role of NO in the effects these drugs on the vascular tone. Aortic rings with intact endothelium from pregnant mice (untreated and treated with PRAV, L-Arg and PRAV+ L-Arg) were incubated with acetyl choline (Ach, 10^{-6}M, Sigma Aldrich) after preconstriction with phenylephrine to evaluate NO-dependent vasorelaxation.

After the experiments, contractile ability of the blood vessels was tested. Vessels which failed to contract to KPSS or phenylephrine were not included in the study.

NO production and synthesis in mouse placentas

The thoracic aorta, a compliance vessel continually subjected to different hemodynamic forces may adjust its vascular tone in a different way to smaller placenta blood vessels. Thus, we investigated NO production in placental blood vessels by measuring eNOS (endothelial NO synthase) synthesis by RT-PCR in endothelial cells isolated from placentas from control mice and mice treated with PRAV, L-Arg and PRAV+L-Arg. In addition, total content of NO was measured in placentas from all experimental groups.

Isolation of placental endothelial cells

Pregnant mice were killed by cervical dislocation. Placentas (4-5 per mouse) from control mice and mice treated with L-Arg, PRAV and PRAV+L-Arg (n=4 mice/experimental group) were dissected (free of umbilical cord and maternal decidua). A single-cell suspension from placental homogenates was obtained by enzymatic dissociation using the gentleMACS Dissociator (Miltenyi Biotec).

CD31/PECAM-1 is mainly expressed on endothelial cells. 2.8 μm superparamagnetic Dynabeads® M-270 Epoxy beads were conjugated with rat monoclonal anti mouse CD31 (ThermoFisher Scientific). The CD31+ cells in the single cell suspension from placentas were magnetically labeled with Dynabeads conjugated with anti CD31 antibodies after incubation with FcR blocking readent (Miltenyi Biotec). Next, the cell suspension was loaded onto a MACS® Column, which was placed in the magnetic field of a MACS Separator (Miltenyi Biotec). The magnetically labeled CD31+ cells were retained within the column. After removing the column from the magnetic field, the magnetically retained CD31+ cells were eluted as the positively selected cell fraction. The CD31+ placenta-derived cells were kept at -80°C for further RT-PCR studies. Because CD31 is also expressed on most leukocytes, these cells were depleted using microbeads conjugated with anti CD45 antibody (monoclonal [I3/2.3] antibody, Abcam) from dissociated primary placental tissue prior to CD31 positive selection. CD31 can also be found on platelets, however immunohistochemical detection of CD42 , marker of platelets, in whole placentas showed extremely low number of platelets in this tissue (data not shown).

Nitric oxide determination in placentas

Placentas were homogenized in isotonic solution of PBS (1:9 W/V) containing 10mM N-ethylmaleimide (NEM) 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. This method requires that nitrate

first be reduced to nitrite and then nitrite determined by the Griess reaction. Briefly, the Griess reaction is a two-step diazotization reaction in which the NO-derived nitrosating agent, dinitrogen trioxide (N_2O_3) generated from the acid-catalyzed formation of nitrous acid from nitrite (or autoxidation of NO) reacts with sulfanilamide to produce a diazonium ion which is then coupled to N-(1-napthyl) ethylenediamine to form a chromophoric azo product that absorbs strongly at 540 nm. A commercially available nitric oxide metabolite detection kit was used (Cayman Chemical, USA)

RNA extraction and quantification of eNOS expression by real-time qPCR

The expression of eNOS in isolated placental endothelial cells was analysed by RT-PCR. RNA from was extracted using Qiagen RNA extraction columns as per the manufacturer's instructions, including initial homogenisation in Trizol (Invitrogen). RNA purity was verified using a nanodrop. 1 µg total RNA was reverse transcribed using a First Strand cDNA Synthesis kit (Fermentas Life Sciences). Relative quantification of gene expression was performed by real-time PCR using iQ SYBR-Green Supermix on the iCycler iQ thermal cycler (BioRad) following the manufacturer's protocols. Primer sequences are: GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used to normalize the quantitative experiments based on prior reference-gene suitability testing and we verified for each experiment that the raw GAPDH values were not significantly different between groups. 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 are expressed as the specific ratio between the gene of interest and the reference gene.

Statistics

All statistical analyses were performed using Prism 6.0 Software (GraphPad Software Inc.). Comparisons between groups were performed by either one-way analysis of variance (ANOVA) with Dunnett's post hoc test within group and with Bonferroni's post hoc test between groups. In all cases, $P \leq 0.05$ were deemed statistically significant. Data are presented as mean ± standard deviation (SD).

AUTHORS CONTRIBUTIONS

AJ, EL and GG planned the studies. AJ and ZJ performed the human studies. GG and JP performed the mouse studies. GG wrote the manuscript text and prepared the figures. All authors reviewed the manuscript.

RESULTS

Human studies

Selective increased umbilical artery pulsatility indexes (Umb Art PI) above limits for gestational age were observed at 23 weeks (median: 23 weeks, IQR [21.3-23.7]) (Table 1, Fig 1 A and B) in all women. A/REDV were detected in all affected twins. Given the high perinatal mortality and morbidity rates associated with A/REDV, termination of the pregnancy to prevent intrauterine fetal death is recommended by the International Society of Ultrasound in Obstetrics and Gynecology guidelines (16). At this time, women received daily doses of pravastatin and L-arginine (PRAV+L-Arg). In the twins that presented increased Umb Art resistance, a significant improvement in the Umb Art blood flow was observed 2 weeks after PRAV+L-Arg treatment (median: 2 weeks IQR[2-2.4](Table 1, Fig 1A and 1B). The improvement in Umb Art blood flow was associated with an increase in fetal weight (Table 1, Fig 2A). While fetal weight stagnation is frequently observed in twins with diminished Umb Art blood flow, a significant weight gain was observed in the 5 fetuses after PRAV+L-Arg treatment (Table 1, Fig 2A) (weight gain median (% increase/week): twin with normal Umb Artery flow: 28.5 IQR[15.1-45.45], twin with abnormal Umb Art blood flow: 13.7% IQR [8.35-30.1]) (Table 1). At 27.5 [26.5-29.5] weeks Umb art blood flow started to increase again. However, PRAV+L-Arg treatment prevented IUFD in the twins with increased Umb Art PI and pregnancies survived 9 weeks (median: 9 IQR [7w2d – 12w]) after abnormal Umb art blood flow was initially detected.

A mild decrease in the cerebroplacental ratio (CPR) was observed in the fetuses with abnormal umbilical Dopplers, indicative of redistribution of the cardiac output to maximize the oxygen supply to brain (brain-sparing effect) (Figure 2B). No signs of acidosis or hypoxia, normal cardiotocography tracing, normal fetal movement and fetal weight gain were observed in the twins with compromised Umb Art blood flow until delivery. Cesarean delivery was performed due to concerns about fetal distress in 4 patients. One patient was delivered because of maternal health concerns after she developed late onset preeclampsia. All neonates were born around 33 weeks (median 33 weeks, IQR[31$^{4/7}$-33]), thus diminishing substantially the chances for prematurity-associated adverse neonatal outcomes. All neonates were born alive. Postnatal assessment showed Apgar scores at 1st and 5th minute higher or equal to 7. No signs of hypoglycemia, hypothermia or hyperbilirubinemia were observed in all neonates. None of the neonates required resuscitation and/or ventilation. Neonates were admitted at NICU because of prematurity and discharged without any developmental abnormalities. The infants are now 1-4 years old and show normal growth and development.

Animal studies

In an attempt to understand the mechanism behind the beneficial effects of PRAV+L-Arg in improving umbilical arteries blood flow observed in humans, animal studies were performed.

Synergistic vasorelaxant effect of PRAV+L-Arg

The effects of PRAV, L-Arg and the combined therapy on endothelial and vascular function were measured in aortic rings from pregnant mice using wire myography. Incubation with PRAV induced relaxation of aortic rings preconstricted with NE. PRAV-induced vasodilation was bigger than that elicited by Ach (Figure 3A). L-Arg showed a weaker vasorelaxant effect compared to Ach and PRAV (Figure 2A) . However, aortic rings incubated with PRAV+L-Arg showed enhanced relaxation compared to L-Arg, PRAV or ACh (Figure 3A). Removal of the endothelium from the aorta completely prevented relaxation responses to PRAV and L-Arg (data not shown). Relaxation in response to PRAV or L-Arg was abolished by inhibition of NO-synthase (nitro-L-arginine methyl ester L-NAME, 100 μM) (transmural tension change (%): PRAV+L-NAME= 5.5 ±3.2, L-Arg+L-NAME 6.8±2.9) indicating that NO plays a role in the vasorelaxant properties of PRAV and L-Arg. The ex-vivo studies also suggest that NO is an important mediator in PRAV and L-Arg induced vasodilation. Aortic rings from pregnant mice treated with PRAV+L-Arg showed an enhanced NO-dependent ACh induced vasorelaxation compared to aortic rings isolated from pregnant mice treated with PRAV or L-Arg alone (Figure 3B).

Increased synthesis of eNOS in placental endothelial cells from mice treated with PRAV+L-Arg

RT-PCR studies were used to determine the synthesis eNOS, enzyme that catalyzes the production of nitric oxide (NO) from L-Arg in isolated placental endothelial cells. A five fold increase in gene expression for eNOS was observed in endothelial cells from placentas isolated from L-Arg-treated mice compared to control placentas (Figure 4A). PRAV treatment induced an eight fold increase in the synthesis of eNOS compared to control. Provocatively, mice treated with the L-Arg +PRAV showed a twelve fold increase in eNOS synthesis compared to placentas from control animals (Figure 4B).

Increased production of NO in placentas from PRAV+L-Arg-treated mice

In agreement with the increased eNOs synthesis observed in placental endothelial cells, increased NO production was measured in whole homogenates (Figure 4B) from placentas isolated from PRAV, L-Arg and PRAV+L-Arg mice compared to control mice (Figure 4B). The biggest production of NO was observed in the placentas from mice that received the combined treatment PRAV+L-Arg. NO levels in placentas from PRAV+L-Arg-treated mice were higher than those observed in mice that received L-Arg or PRAV alone (Figure 4B).

DISCUSSION

Increased fetal and neonatal morbidity and mortality associated with twin pregnancies is associated with a greater risk of preterm delivery, low birth weight, and IUGR. IUGR can affect one or both twins. Twin pregnancies with discordant fetal growth are rare but highly associated with intrauterine death. Although the pathogenesis of IUGR is unclear, feto-placental blood vessels are compromised and diminished umbilical blood flow is observed in Doppler velocity waveform analysis. These abnormal waveforms are associated with adverse perinatal outcomes. The most severe waveform patterns observed, A/REDV, are associated with growth restriction, fetal death and prolonged stay in neonatal intensive care (17). An association between preoperative umbilical artery Doppler waveforms and umbilical vein pO2 and pH at elective cesarean section has been reported (18). A/REDV has a strong statistical association with hypoxia and acidosis thus being a clinically sensitive indicator of perinatal morbidity and mortality (18). A/REDV in the presence of IUGR is an ominous finding that requires immediate delivery to prevent intrauterine fetal death according to the International Society of Ultrasound in Obstetrics and Gynecology (16).

In this pilot study, 5 twin pregnancies with discordant abnormal Umb Art blood flow and discordant growth were treated successfully with a combination of PRAV+L-Arg. The IUGR-fetuses showed A/REDV that compromised fetal survival. PRAV+L-Arg treatment given at the time of abnormal umbilical artery Doppler detection prevented IUFD and prolonged pregnancies. The pregnancies continued for several weeks (median survival time: 9 weeks, IQR[72w2d-12w]). C-sections were indicated after signs of fetal distress were observed at a median time of 33 weeks IQR [$31^{4/7}$-33], thus diminishing the risks associated with prematurity. Some of the neonates spent time at neonatal intensive care but were discharged in good health with no developmental abnormalities. The infants are now between 1 and 4 years old, healthy and show normal development. No adverse effects were observed in the mothers during or after PRAV+L-Arg treatment. The beneficial effects of PRAV+L-Arg on Umb art blood flow were noticed as early as 2 week after treatment (median 14 days IQR[14-21]), suggesting that this combined therapy might modulate umbilical arteries resistance.

We hypothesised that vasorelaxant NO plays an important role in the effects of PRAV+L-Arg in restoring the Umb arteries blood flow and animal studies were performed to test this hypothesis. Indeed, an NO-dependent vasorelaxant effect of PRAV and L-Arg was detected in aortic rings from mice using wire myography. Aortic rings from control mice incubated with PRAV, L-Arg and PRAV+L-Arg showed a diminution in the vascular resistance. The vasorelaxant effect was blunted by inhibition of eNOS with L-NAME, demonstrating that an NO-dependent mechanism is involved. A direct vasodilator effect of PRAV on the endothelium in aortic rings was previously reported by Kaesemeyer et al in non pregnant rats (19). In this study, PRAV also induced NO production in cultured bovine aortic endothelial cells (19). Aortic rings

from pregnant mice treated with PRAV or L-Arg showed an enhanced Ach-induced vasorelaxation compared to aortic rings isolated from untreated pregnant mice. Dual therapy PRAV+L-Arg induced a much robust vasorelaxant response compared to L-Arg and PRAV alone.

While a vasorelaxant response to L-Arg and PRAV was observed in aortic rings, the aorta is a compliance vessel that may adjust its vascular tone in a different way to smaller placenta blood vessels. Therefore, synthesis of eNOS in endothelial placental cells was studied. It has been shown that eNOS augments fetoplacental blood flow, placental vascularization, and fetal growth in mice (20). In addition, it has been reported that PRAV protects the endothelium by activating eNOS independent of its cholesterol-lowering actions (21). Moreover, an increase in the synthesis and activity of eNOS was found in human placental cotyledons perfused with PRAV (22). Interestingly, it has also been described that L-Arg increases eNOS expression (23). L-Arg treated rabbits showed increased eNOS expression in aortas (23). In agreement with the bibliography and the NO-dependent vasorelaxant mechanism identified in aortic rings, increased synthesis of eNOS was detected in placental endothelial cells isolated from mice treated with L-Arg, PRAV and the combined therapy. PRAV+L-Arg induced a more significant increase in eNOS synthesis compared to PRAV and L-Arg alone.

Previous studies from our Laboratory that showed that PRAV restored plasma levels of NO in a mouse model of placental insufficiency (23). In addition, it has been reported that statins increase eNOS mRNA stability leading to increased eNOS activity (24). Increased eNOS synthesis and activity combined with the increased availability of eNOS substrate L-Arg might result in increased NO generation leading to vasorelaxation of placental vessels.

Pravastatin is a hydrophilic molecule with limited transfer across the placenta. However, the small amount of pravastatin that reachedthe placental fetal vessels (18% of the administered dose (15)) was able to induce relaxation in vitro in the aortic rings. L-Arg alone evoked only minor vasorelaxation in aortic rings with intact endothelium compared to ACh or PRAV. Small relaxations, or absence of relaxations, to L-Arg have also been observed in rat and rabbit aortas and in bovine pulmonary artery and vein (25, 26). While L-Arg –induced vasorelaxation in aortic rings was weaker compared to PRAV, the combined therapy induced significant vasorelaxation. The combination of PRAV+L-Arg induced a significant diminution in vessel resistance in the mouse in vitro studies and in the human Doppler studies. The amino acid L-Arg is converted to L-citrulline and NO by NOS and is a limiting factor in NO availability (27-29). Positive immunofluorescence for endothelial eNOS was described in human umbilical cord artery (30). By increasing intracellular endothelial L-Arg stores and enhancing eNOS synthesis and expression, PRAV+L-Arg might synergistically increase NO bioavailability, inducing vasorelaxation and improving umbilical arteries blood flow. We also need to consider that both the endothelial cells and the vascular smooth muscle

possess biochemical pathways converting L-Arg to NO, thus the relaxation of the smooth muscle might also contribute further to the vasorelaxation.

Among many other pleiotropic effects, PRAV showed to increase heme oxygenase/carbon monoxide (CO) production (8).   Interestingly, chronic inhalation of CO, potent vasodilator, throughout gestation showed to increase uterine blood flow in mice (31). Thus, PRAV-induced CO production might also contribute to the improvement of the blood flow in the umbilical arteries in the twin study.

The combination of PRAV+L-Arg improved Umb art blood flow, increased fetal weight and allowed progression of pregnancy with favorable outcomes in twin pregnancies with discordant umbilical artery resistance. This study provides evidence on the efficacy of PRAV+L-Arg in preventing adverse pregnancy outcomes associated with discordant abnormal Umb artery flow in twins.

Limitations of this study include the small number of patients which means that precise conclusions cannot be draw. Nevertheless, this clinical case report describes and analyzes the successful management of five pregnant patient with twin pregnancies with discordant fetal growth and may constitute the first line of evidence in health care to treat an unusual but serious pregnancy complication. Larger studies would be needed to more clearly demonstrate the beneficial effect of PRAV+L-Arg combination in preventing adverse pregnancy outcomes in twin pregnancies with discordant umbilical artery blood flow and intrauterine growth restriction.

Figure 1. Umbilical artery pulsatility index during the course of pregnancy in the five patients with twin pregnancy with discordant fetal growth.

The dash line corresponds to the fetuses with compromised umbilical artery flow. The colored arrows indicate the time at which pravastatin +L-arginine treatment was introduced for each patient. **B**- Doppler ultrasound imaging of umbilical arteries before and after pravastatin + L-arginine treatment (patient 5).



Case 5 –twin with abnormal Dopplers
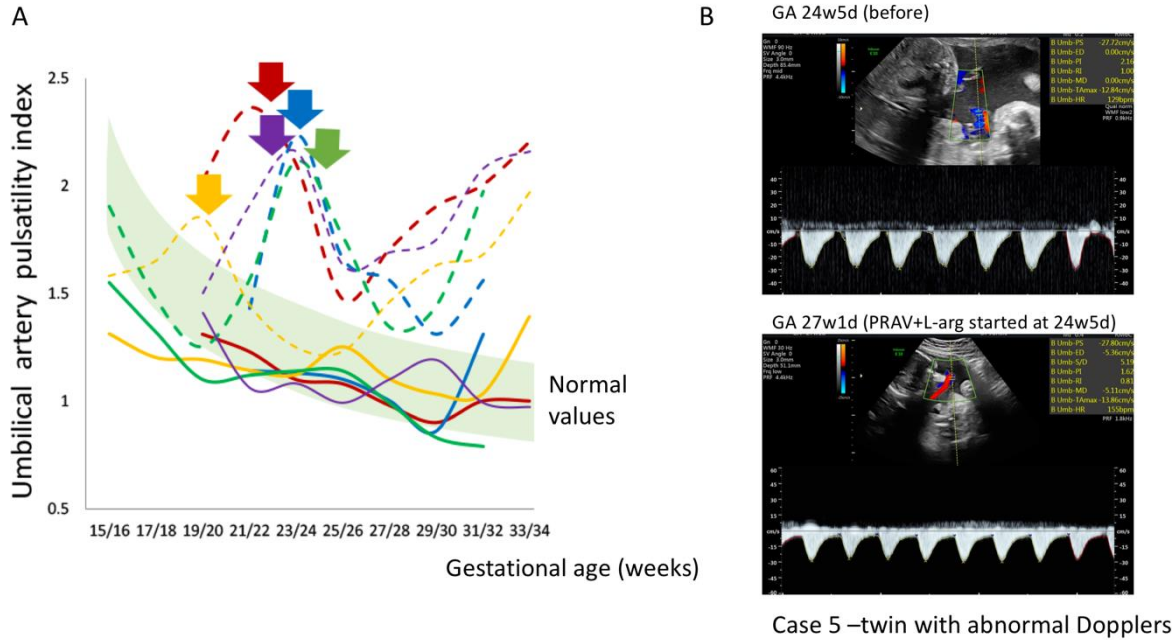
Figure 2. Expected fetal weight (EFW) and cerebroplacental ratio (CPR) in twin pregnancies with discordant growth.

A- EFW in grams during the course of pregnancy in the five patients with twin pregnancy with discordant fetal growth.

B- Mean values of CPR for the foetuses with normal and abnormal umb art blood flow. The area in green represents the normal values (upper and lower lines correspond to 95 and 5 percentile respectively). The dash line corresponds to the foetuses that showed compromised umbilical artery flow early in pregnancy. The coloured arrows indicate the time at which pravastatin +L-arginine treatment was introduced for each patient.
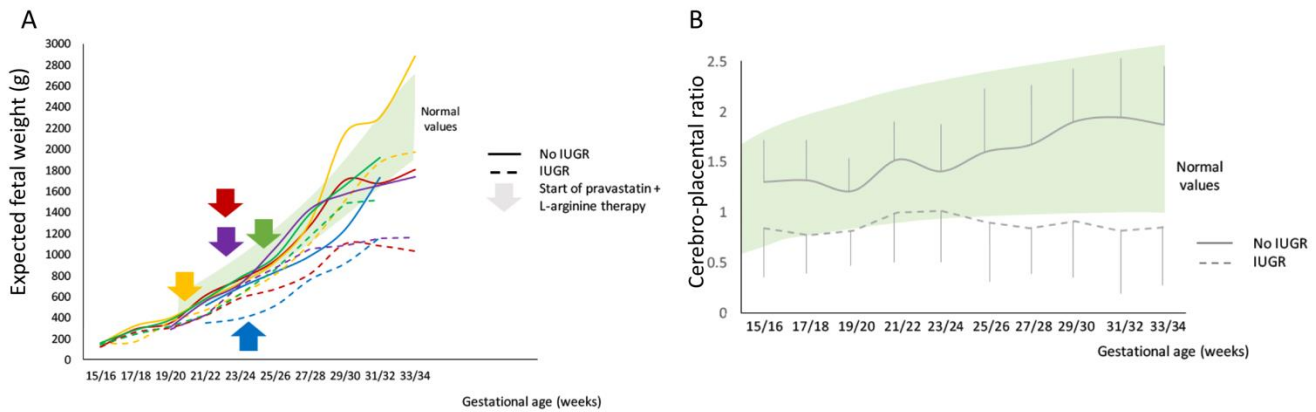
Figure 3. Wire myography studies A- Vasorelaxation induced by Ach (10^{-6} M), PRAV (10ng/ml, Sigma Aldrich) and L-Arg (350 μM, Sigma Aldrich), alone or in combination (L-Arg added 30 min before pravastatin) in precontracted (1 μM phenylephrine) endothelium-intact aortic rings from day 15 pregnant mice. *Different from ACh, $p < 0.05$, # Different from L-Arg, $p < 0.05$, ** Different from PRAV, $p < 0.05$.

B- Relaxation induced by 1 μM acetylcholine (ACh) in precontracted (1 μM phenylephrine) endothelium-intact aortic rings from pregnant mice (untreated (No TX) or treated with PRAV ((0.6mg/mouse, intraperitoneal Sigma Aldrich), L-Arg (0.5 mg/mouse) or PRAV+L-Arg.

 *Different from No TX, $p < 0.05$, # Different from L-Arg, $p < 0.05$, ** Different from PRAV, $p < 0.05$.
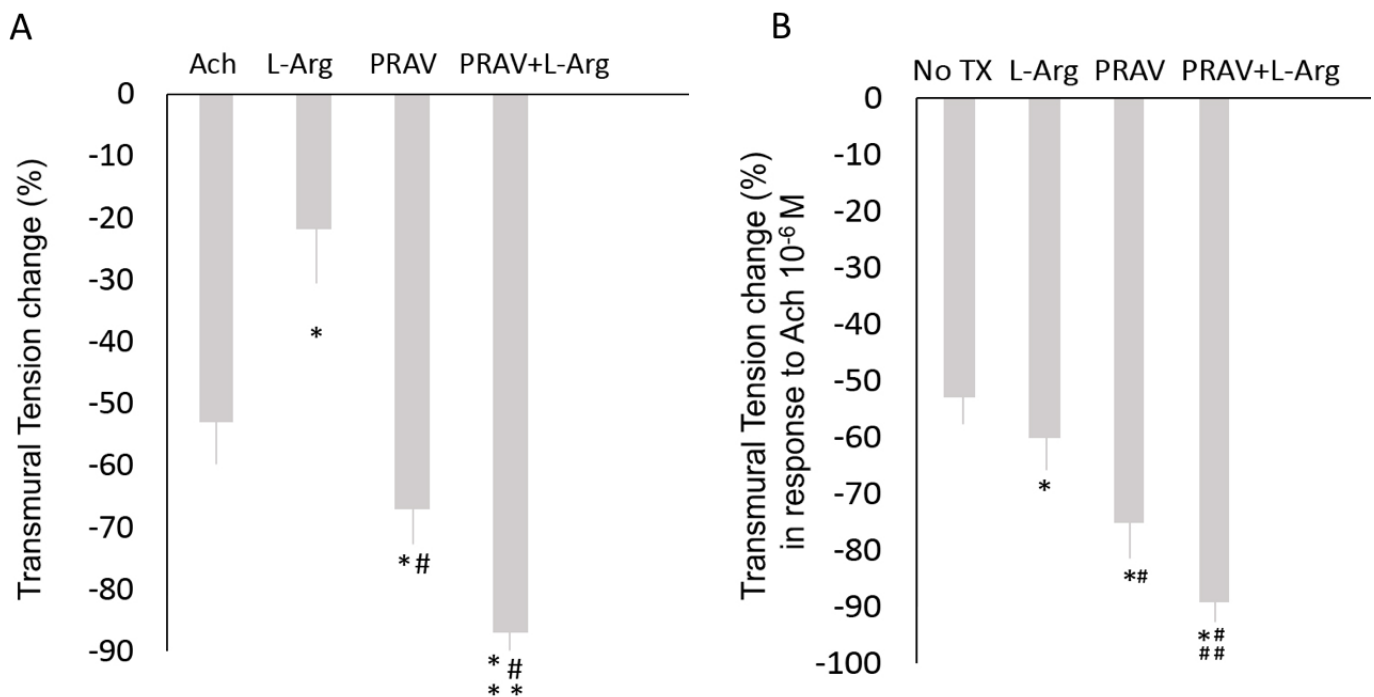
Figure 4. Pravastatin increases eNOS expression and NO synthesis in placenta

A- Nitric oxide (NO) production in placentas from untreated mice (No TX) and mice treated with L-Arg, PRAV and PRAV+L-Arg. NO was measured by its stable decomposition products nitrate and nitrite. * different from control, p<0.05

B- eNOS gene expression in endothelial cells isolated from placentas from untreated mice (No TX) and mice treated with L-Arg, PRAV and PRAV+L-Arg. Values are presented as the ratio between relative amount eNOS and GAPDH mRNA.

* different from control No TX , p<0.05, # different from L-Arg, p<0.05, ** different from PRAV, p<0.05
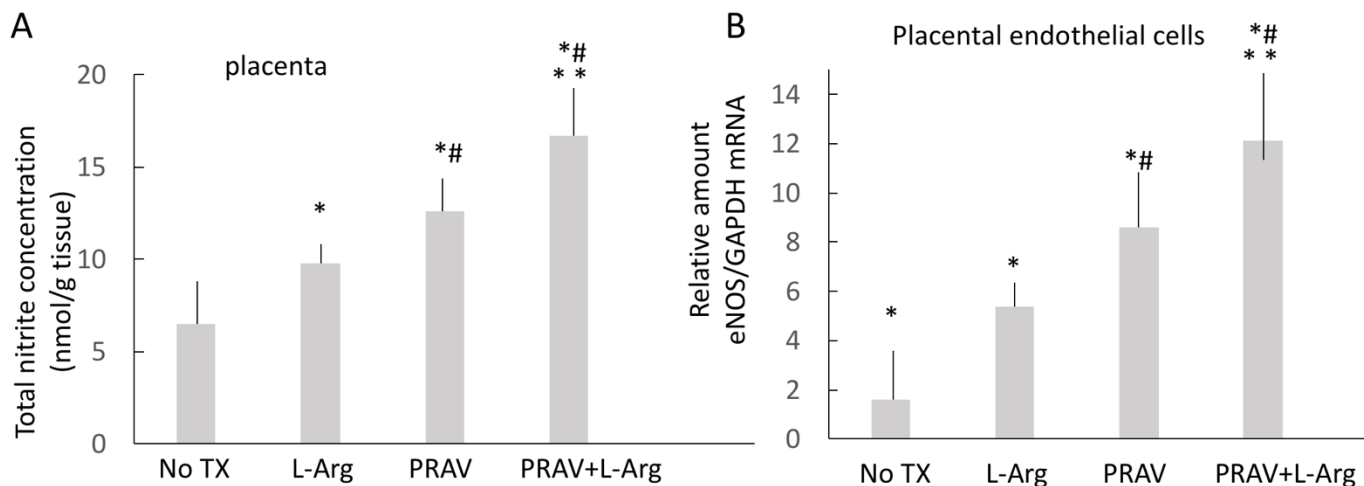
Table 1. Umb artery pulsatility index and expected fetal weight (before and after PRAV+L-Arg treatment), gestational age at delivery and fetal outcomes in 5 twin pregnancies. *Different from T1, p=0.0001, ** Different from T2 before pravastatin, p=0.0006, # not different from T1, p=0.395, *# not different from T1 birth weight, p=0.06, ## not different from T1, p=0.22.

Patient	PRAV+L-Arg given at:	Twins	EFW (g) at this time:	Umb art PI at this time	Improvement seen at	Min Umb art PI after PRAV+ L-Arg / week	Pregnancy Survival (weeks)	Delivery (week)	% Fetal weight increase after P/week	Birth weight (g)
1	23 weeks	T1	605	1.2	25 weeks	0.85 ($30^{5/7}$w)	11	34	16.4%	1700
		T2	458	**2.3**		1.63 ($27^{1/7}$ w)			13.7%	1150
2	23 weeks	T1	489	1.2	25 weeks	0.86 ($29^{4/7}$ w)	9	34	40.0%	2250
		T2	393	**2.3**		1.31 ($29^{4/7}$ w)			11.1%	1250
3	20 $^{1/7}$ weeks	T1	394	1.19	$23^{5/7}$ weeks	1.25 ($26^{6/7}$ w)	12w 5d	33	50.9%	2900
		T2	358	**1.78**		1.23 ($26^{6/7}$ w)			36.7%	2000
4	$24^{3/7}$ weeks	T1	557	1.14	$26^{3/7}$ weeks	0.99 ($28^{3/7}$w)	7w 2d	31.5	28.5%	1700
		T2	520	**2.11**		1.34 ($28^{3/7}$w)			23.5%	1400
5	$24^{5/7}$ weeks	T1	715	1.06	$27^{1/7}$ weeks	0.99 ($27^{1/7}$ w)	7w	31.2	13.8%	1550
		T2	679	**2.16**		1.63 ($27^{1/7}$ w)			5.6%	1000
Median IQR	23 [$21^{6/7}$-$24^{4/7}$]	T1	557 [441.5-660]	1.19[1.1-1.2]	25 [$24^{2/7}$-27]	0.99[0.85-1.12]	9 [7w2d-12w]	33	28.5 [15.1-45.45]	1700 [1625-2575]
		T2	458 [375-599] #	2.16[1.94-2.3] *		1.31[1.27-1.63] **		[$31^{4/7}$-33]	13.7 [8.35-30.1] ##	1250 [1075-1270] *#

Disclosure of interest

All the authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

REFERENCES

1- Ananth, C.V., Chauhan, S.P. Epidemiology of twinning in developed countries. Semin Perinatol. 36:156-161 (2012).

2- Cheong-See, F. et al. Prospective risk of stillbirth and neonatal complications in twin pregnancies: systematic review and meta-analysis. BMJ 354:i4353 (2016).

3- Padmanabhan, V., Cardoso, R.C. & Puttabyatappa, M. Developmental Programming, a Pathway to Disease. Endocrinology 157(4):1328-1340 (2016).

4- Krishna U, Bhalerao S. Placental Insufficiency and Fetal Growth Restriction. Journal of Obstetrics and Gynaecology of India 61(5):505-511. doi:10.1007/s13224-011-0092-x (2011).

5- Arantzatzu, L. et al. Parenteral Administration of L-Arginine Enhances Fetal Survival and Growth in Sheep Carrying Multiple Fetuses. J Nutr. 141(5):849-855 (2011).

6- Oyston C, Baker PN. Therapeutic strategies for the prevention and treatment of pre-eclampsia and intrauterine growth restriction. Obstet Gynecol & Reprod Med 23(12):375-380 (2013).

7- Lefkou, E. et al . Pravastatin improves pregnancy outcomes in obstetric antiphospholipid syndrome refractory to antithrombotic therapy. J Clin Invest. 126(8):2933-2940 (2016).

8- Girardi, G. Can statins prevent pregnancy complications? J. Reprod Immunol. 101–102:161–167 (2014).

9- Bateman, B.T. et al. Statins and congenital malformations: cohort study. BMJ. 350:h1035 (2015).

10- Zarek J, Koren G. The fetal safety of statins: a systematic review and meta-analysis.

J Obstet Gynaecol Can.36(6):506-509 (2014).

11- Costantine MM et al. Safety and pharmacokinetics of pravastatin used for the prevention of preeclampsia in high-risk pregnant women: a pilot randomized controlled trial. Am J Obstet Gynecol. ;214(6):720.e1-720.e17 (2016).

12- Hatanaka, T. Clinical pharmacokinetics of pravastatin: mechanisms of pharmacokinetic events. Review Clin Pharmacokinet. 39(6):397–412 (2000).

13- Mulvany, M.J., Halpern, W. Mechanical properties of vascular smooth muscle cells in situ. Nature 260:617–619 (1976).

14- Khalil, A. et al. ISUOG practice guidelines: role of ultrasound in twin pregnancy. Ultrasound Obstet. & Gynecol. 47(2):247-263 (2016).

15- Hatanaka, T. Clinical pharmacokinetics of pravastatin: mechanisms of pharmacokinetic events. Review Clin Pharmacokinet. 39(6):397–412 (2000).

16- Nanovskaya TN, Patrikeeva SL, Paul J, Costantine MM, Hankins GD, Ahmed MS. Transplacental transfer and distribution of pravastatin. Am J Obstet Gynecol. 209(4):373.e1-5 (2013).

17- Tannirandorn, Y., Phaosavasdi, S. Significance of an absent or reversed end-diastolic flow velocity in Doppler umbilical artery waveforms. J. Med Assoc Thai. 77(2):81-86 (1994).

18- Tyrrell, S., Obaid, A.H. & Lilford, R.J. Umbilical artery Doppler velocimetry as a predictor of fetal hypoxia and acidosis at birth. Obstet Gynecol. 74(3 Pt 1):332-337 (1989).

19- Kaesemeyer, W.H., Caldwell, R.B., Huang, J & Caldwell, R.W. Pravastatin sodium activates endothelial nitric oxide synthase independent of its cholesterol-lowering actions. J. Am Coll Cardiol. 33(1):234-241 (1999).

20- Kulandavelu, S., Whiteley, K.J., Bainbridge, S.A., Qu, D. & Adamson, L. Endothelial NO synthase augments fetoplacental blood flow, placental vascularization, and fetal growth in mice. Hypertension 61:259-266 (2013).

21- Balan A, Szaingurten-Solodkin I, Swissa SS, Feinshtein V, Huleihel et al . The effects of pravastatin on the normal human placenta: lessons from ex-vivo models. PLoS One 12(2):e0172174 (2017).

22- Javanmard SH, Nematbakhsh M, Mahmoodi F, Mohajeri MR. L-Arginine supplementation enhances eNOS expression in experimental model of hypercholesterolemic rabbits aorta. Pathophysiology 16(1):9-13 (2009).

23- Redecha, P, van Rooijen, N., Torry, D., Girardi, G. Pravastatin prevents miscarriages in mice: role of tissue factor in placental and fetal injury. Blood 113:4101-4109 (2009).

25- Laws PE, Spark JI, Cowled PW, Fitridge R. The Role of Statins in Vascular Disease, Eur J Vasc Endovasc Surgery 27(1):6-16 (2004).

26- Nelson, SH, Steinsland O, Chand YW. Increased Nitric Oxide Synthase Activity and Expression in the Human Uterine Artery During Pregnancy. Circ. Res. 87:406-411 (2000).

27- Thomas, G., Mostaghim, R.& Ramwell, P.W. Endothelium dependent vascular relaxation by arginine containing polypeptides. Biochem Biophys Res. Commun. 141:446-451 (1986).

28- Ignarro, L.J. et al. Basic polyamino acids rich in arginine, lysine, or ornithine cause both enhancement of and refractoriness to formation of endothelium-derived nitric oxide in pulmonary artery and vein. Circ Res. 64:315-329 (1989).

29- Palmer, R.M.J., Rees, D.D., Ashton, D.S.& Moncada, S. L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. Biochem. Biophys Res. Commun. 153:1251-1256 (1998).

30- Moncada, S., Higgs, E.A. The l-arginine—nitric oxide pathway. N Engl J. Med. 329:2002–2012 (1993).

31- Myatt, L., Brockman, D.E., Eis, A.L. & Pollock, J.S. Immunohistochemical localization of nitric oxide synthase in the human placenta. Placenta. 14(5):487-495 (1993).

32- Venditti, C.C. et al. Chronic carbon monoxide inhalation during pregnancy augments uterine artery blood flow and uteroplacental vascular growth in mice. Am J. Physiol. Regul. Integr Comp Physiol. 305(8):R939-948 (2013).

Graphical abstract
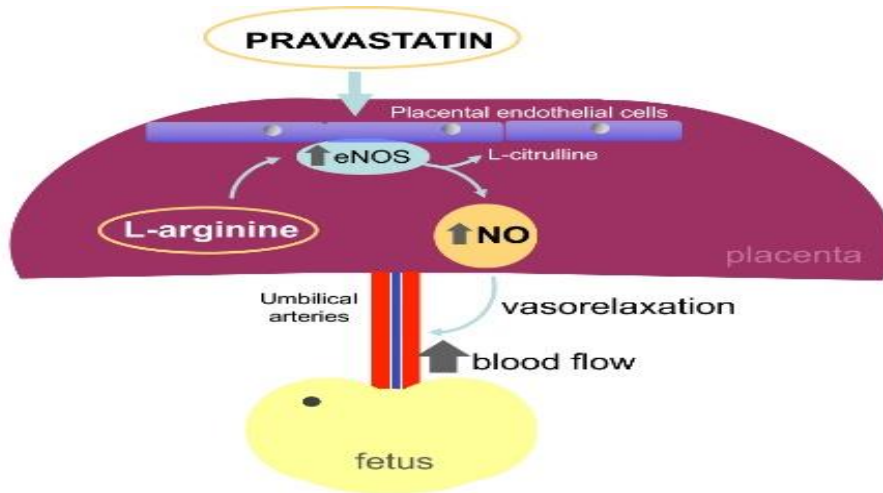
Pravastatin (PRAV) and L-arginine increase eNOS synthesis and NO production in placenta endothelial cells inducing vasorelaxation of umbilical arteries increasing blood flow to the fetus