Physiological and pharmacokinetic properties of Placental Protein 13 (PP13)

In vivo and *in vitro* studies in animals

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Thesis for the degree of Philosophiae Doctor

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Lífeðlisfræðilegir og lyfjahvarfafræðilegir eiginleikar fylgjupróteins 13 (PP13)

In vivo og in vitro rannsóknir í dýrum

Tijana Drobnjak

Ritgerð til doktorsgráðu

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Ágrip

Inngangur: Þegar kona verður þunguð, eiga sér stað miklar breytingar á æðakerfi hennar, til að mæta aukinni blóðþörf til fylgju og fósturs og tryggja eðlilegan vöxt fóstursins. Til að þetta sé hægt, þarf stækkun á æðum sem flytja blóð til fóstursins að eiga sér stað. Ef þessar breytingar á æðakerfi ná ekki að myndast, getur það leitt til ýmissa meðgöngutengdra sjúkdóma, eins og meðgöngueitrun. Tíðni meðgöngueitrunar er um 2-5% þungana í Evrópu og lýsir sér sem nýtilkominn háþrýstingur og því fylgir mælanlegt prótein í þvagi. Fylgjuprótein 13 (PP13, placental protein 13) er prótein sem er eingöngu seytt af fylgju og mælist í blóði mæðra frá fimmtu viku meðgöngu. Konur sem eru í áhættuhópi reynast vera með mun lægra magn af PP13 í sermi, en konur með heilbrigða meðgöngu. Því hefur verið lagt til að próteinið megi nýtast til greiningar á konum sem eru líklegri að þróa með sér meðgöngueitrun.

Markmið: 1) Að skoða skammtíma- og langtímaáhrif proteins á æðakerfið í kringum legið (Grein III). 2) Að skilgreina verkunarmáta próteinsins á einangraðar legæðar úr rottum (Grein I). 3) Að meta áhrif próteinsins á æðakerfið í kringum legið og fósturvöxt í rottum. 4) Að meta lyfjahvörf próteinsins í kanínum (Grein II).

Aðferðir og niðurstöður: Osmótískar dælur voru ígræddar í óþungaðar rottur sem losuðu um 10 µl/klst og tæmdu sig á sjö dögum. Sum dýranna (n=11) voru aflifuð eftir sjö daga meðferð til að meta skammtímaáhrifin, en önnur (n=16) voru aflifuð eftir 13 daga til að meta langtímaáhrif próteinsins á æðakerfið. Þvermál slag- og bláæða úr legi voru borin saman á milli hópa. Marktækur munur sást á öllum æðum í bæði langtíma- og skammtímahópnum. Til að skilgreina verkunarhátt fylgjupróteins 13, voru legslagæðar einangraðar og settar í sérhæfðan æðamæli (e. arteriograph). Próteinið reyndist hafa æðavíkkandi áhrif (38-50%) sem hægt var að hemja með því að hinda aðgengi nituroxíðs og arakídónsýru. Áhrifin hurfu ef æðaþelið var fjarlægt. EC_{50} mældist undir 1pM. Áhrif próteinsins í þunguðum rottum með ígræddar osmótískar dælur sem fengu nituroxíðhindra í drykkjarvatni leiddi í ljós marktækt minni unga samanborið við viðmiðunarhópinn. Þessi áhrif snertu einnig meginlegslagæð (MUA) og legbláæðar (RUV).

Lyfjahvörf fylgjupróteins 13, sem fóru fram í kanínum, voru skoðuð með því að gefa þrjá mismunandi styrki af próteininu (5 ng/mL, 10 ng/mL og 50 ng/mL) í æð (I.V.), og síðan einn styrkur (50 ng/mL) var gefinn undir húð (S.C.). Blóðþéttni var mæld með ELISA prófi og lyfjahvarfafræðileg heðgun próteinsins reiknuð út, sem fylgdi svokölluðu tveggja-hólfa kerfi. Helmingunartíminn var marktækt hærri hjá S.C. hópnum ($p<0.01$), en það kom í ljós að bæði dreifirúmmál og flatarmálið undir ferlinum (AUC) reyndust vera skammtaháð milli I.V. hópanna.

Ályktanir: Niðurstöðurnar sýndu að fylgjuprótein 13 hefur veruleg æðavíkkandi áhrif, sem jafnframt hefur jákvæð áhrif á vöxt fylgju og fósturs. Þetta prótein gæti verið lykilþáttur í undirbúningi æðakerfisins fyrir aukið blóðflæði síðar á meðgöngunni. Lyfjahvarfafræðilegar athuganir sýna að magn próteinsins sem er losað úr fylgju gæti verið mun hærri en áður var talið. Frekari rannsókna er þörf til að fá dýpri innsýn í áhrif þessa próteins á æðakerfi manna. Niðurstöður okkar er mikil hvatning fyrir áframhaldandi rannsóknum til að meta fylgjuprótein 13 sem mögulegan lyfjasprota til að fyrirbyggja meðgöngueitrun og aðra meðgöngutenda sjúkdóma sem tengjast ófullnægjandi blóðflæði til legs og fylgju.

Lykilorð:

Fylgjuprótein 13 (PP13), meðganga, meðgöngueitrun, verkunarháttur, lyfjahvörf.

Abstract

Background: During pregnancy extensive hemodynamic changes are required for optimal fetal growth and utero-placental circulation adaptation to increased blood volume. If those changes are altered or fail to occur they may lead to pathological pregnancies and preeclampsia. Preeclampsia is an obstetrical syndrome associated with high blood pressure and altered organ function that affects 2-5% of pregnant women. Placental protein 13 (PP13) is secreted solely by placenta and has been proposed as a potential biomarker whilst its serum levels in early pregnancy in women at risk are much lower than those in healthy pregnancies.

Aims: First, to study the long and short term effects (Paper III), and evaluate overall effect of PP13 exposure on uterine vasculature, and the fetal outcome in rats. Second aim is to define the mechanism of action in isolated rat arteries *in vitro* (Paper I). The final aim is to evaluate the pharmacokinetic profile of PP13 in rabbits (Paper II).

Methods and Main Results: Slow-release osmotic pums loaded with protein or saline were implanted in periscapular region in non-pregnt rats, releasing its content for seven days. Some animals (n=11) were sacrified after seven days with the pump (short-term), and some animals (n=16) were sacrified after thirteen days (long-term). The diameter of arteries and veins were compared between groups, resulting in significant vascular expansion of all vessels in rPP13 group both in short and long-term. Histidine tagged PP13 variant (his-PP13), caused significant vessel expansion in radial arteries, and only in short-term. Study conducted in pregnant rats, by inserting the osmotic pump at the time of placentation, in which some rats were treated with L-NAME via drinking water in order to inhibit the main vasodilation pathway (eNOS). The results showed that rPP13 treated animals had heavier pups and bigger placentas, in comparison to the control. All L-NAME treated groups resulted in significantly smaller pups and placentas in comparison to the control. Main uterine artery (MUA) and radial uterine veins (RUV) expansion in L-NAME treated animals were significatntly lower compared to the control. To study the mechanism of action uterine vessels were isolated and cannulated in arteriograph maintained at constant pressure of 50 mmHg. Vessels were then preconstricted and exposed to increasing concentrations of rPP13 (10^{-13} to 10^{-8} M). rPP13 elicited 38-50% arterial vasodilation

mediated via endothelial signaling pathways of nitric oxide and arachidonic acid, with half maximal response (EC_{50}) of approx. 1pM. In order to establish pharmacokinetic profile of PP13, three different concentrations of the protein (5 ng/mL, 10 ng/mL and 50 ng/mL) were administrated intravenously (I.V.) and one concentration (50 ng/mL) was administrated subcutaneously (S.C.) in New Zealand White rabbits. The serum levels of the protein were determined by enzyme-linked immunosorbent assay (ELISA). The pharmacokinetic profile is described as two-compartment model. The elimination half-life was found to be different between the groups ($p<0.01$), and volume of distribution and area under the curve were found to be dose dependent.

Conclusion: The results indicate that PP13 is a potent vasodilator, has a positive effect on fetal growth and may be a key factor in preconditioning the uterine vasculature in order to accommodate the blood flow increase during pregnancy. In addition pharmacokinetic studies indicate that the concentration of total PP13 released into maternal circulation may be much higher than previously estimated. Further studies are required in order to get an insight into the effect on human vasculature, however, these results encourage evaluation of PP13 as a potential therapeutic agent for obstetric syndromes characterized by insufficient uteroplacental blood flow.

Keywords:

Placental protein 13, pregnancy, preeclampsia, vasodilation, mechanism of action, pharmacokinetics.

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Contents

List of abbreviations

ACh	Acetylcholine
AUA	Arcuate uterine artery
AUC	Area under the curve
AUV	Arcuate uterine vein
BMI	Body mass index
CBD	Carbohydrate binding domain
Cl_{tot}	Total body clearance
CO	Cardiac output
COX	Cyclooxygenase
CV	Coefficient of variability
EC	Endothelial cell
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPE	Early onset preeclampsia
EVT	Extravillous trophoblasts
GFR	Glomerular filtration rate
H&E	Hematoxylin and eosin dye
HELLP	Hemolysis, elevated liver enzymes, and low platelet count syndrome
his-PP13	Histidene tagged placental protein 13
hPL	human Placental Lactogen
I.V.	Intravenous
IP	Prostacyclin
IUFD	Intra uterine fetal death
IUGR	Intrauterine growth restriction
L-NAME	Nω-nitroL-arginine-methyl-ester
L-NNA	Nω-nitro-L-arginine
LDL	Low-density lipoproteins
LPE	Late onset preeclampsia
MA	Mesenteric arteries
MP	Mid-pregnant
MUA	Main Uterine Artery
MUV	Main Uterine Vein

NP	Non-pregant
NZW	New Zealand White
PAPP-A	Pregnancy associated plasma protein – A
PBS	Phosphate buffered solution
PE	Preeclampsia
PG	Prostaglandin
Phe	Phenylephrine
PlGF	Placental growth factor
PP13	Placental Protein 13
PSS	Physiological salt solution
RUA	Radial uterine artery
RUV	Radial uterine vein
S.C.	Subcutaneous
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sFlt-1	fms-like tyrosine kinase 1
sPlGF	(soluble) Placental growth factor
STBM	Syncytiotrophoblast microparticles
UAA	Uterine arcuate arteries
V_d	Volume of distribution
VEGF	Vascular endothelial growth factor

List of figures

List of tables

List of original papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I. Drobnjak, T., Gizurarson, S., Gokina, N. I., Meiri, H., Mandala, M., Huppertz, B., Osol, G. Placental protein 13 (PP13)-induced vasodilation of resistance arteries from pregnant and nonpregnant rats occurs via endothelial-signaling pathways. Hypertens Pregnancy, 2017: 36(2):186-95.

II. Drobnjak T, Meiri H, Mandala M, Huppertz B, Gizurarson S. Pharmacokinetics of placental protein 13 after intravenous and subcutaneous administration in rabbits. Drug Des Dev Ther. 2018; 12:1977-83.

III. Drobnjak T, Jonsdottir A.M, Helgadottir Helga, Meiri H, Sammar M, Osol G, Mandala M, Huppertz B, Gizurarson S. Placental Protein 13 (PP13) causes vasodilation of rat uterine vessels after slow subcutaneous administration. Submitted for publication.

IV. Unpupblished data: Preliminary study – PP13 effect after in pregnant rats *in vivo* study.

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Declaration of contribution

The studies presented in this thesis were carried out under supervision and guidance of Prof. Sveinbjörn Gizurarson, Dr. Hamutal Meiri and Prof. George Osol. Tijana Drobnjak took part in performing all experiments presented in the thesis.

Paper I: All authors listed in the article contributed into design of the study. Prof. George Osol, Prof. Maurizio Mandála and Dr. Natalia I. Gokina supervised *in vitro* studies on isolated uterine arteries experimental work that was conducted by Tijana Drobnjak. Dr. Natalia I. Gokina developed and conducted the calcium measurement model and respective experiments, and designed the study along with Prof. George Osol, Prof. Sveinbjörn Gizurarson and Tijana Drobnjak. Tijana Drobnjak, Prof. Sveinbjörn Gizurarson, Prof. George Osol and Dr. Natalia I. Gokina managed the pharmacological aspects of the study. Dr. Hamutal Meiri and Prof. Berthold Huppertz were involved in the design of PP13 experiments and provision of the protein and its verification. All authors were involved in writing the manuscript, data analysis and disscussions.

Paper II: Tijana Drobnjak designed the study under the supervision of Prof. Sveinbjörn Gizurarson and Dr. Hamutal Meiri. Prof. Sveinbjörn Gizurarson and Tijana Drobnjak carried out the drug administration. Tijana Drobnjak performed blood collection, sample treatment and analysis of the samples. Dr. Hamutal Meiri provided PP13. All the authors contributed in writing the manuscript, data analysis and discussion.

Paper III: Insertation of the pumps, scarifying of the animals and dissecting the organs, as well as care of animals during the study was performed by staff of ArcticLAS. Tijana Drobnjak and Helga Helgadóttir contributed to the activities of animal care and during experiments. Anna Margrét Jónsdóttir, MD, and Tijana Drobnjak contributed to the fixation of the organs. Staff of the Univeristy Hospital, department of pathology, Reykjavik, Iceland, performed staining of the organs. Vessel size measurements were performed by Tijana Drobnjak. All authors contributed to data analysis, writing the manuscript and discussion.

1 Introduction

1.1 Physical and physiological changes during pregnancy

Pregnancy is a particularly important phase of a woman´s life, during which she experiences rapid changes in body contour, size and shape over a short time. During the course of a pregnancy significant anatomical and physiological changes take place in order to accommodate and nurture the growing fetus.

One of the major changes experienced is a change in the cardiovascular system where cardiac output (CO) increases until it gradually reaches its maximum at 20 weeks´ gestation (Hall, George, & Granger, 2011), resulting in a 50% increase in total blood volume by 34 weeks´ gestation (Konje, Kaufmann, Bell, & Taylor, 2001; Moore, McCullough, & Weil, 1987; Soma-Pillay, Nelson-Piercy, Tolppanen, & Mebazaa, 2016; Thornburg, Jacobson, Giraud, & Morton, 2000). As a secondary result of this increase in blood volume, renal plasma flow and glomerular filtration rate (GFR) increase by between 40-65% and 50-85% respectively (Soma-Pillay et al., 2016). In addition, systolic and diastolic arterial pressure – particularly the latter - drop slightly when trophoblast invasion into the uterine spiral arteries, peripheral vasodilation and pulse pressure reach their maximums in the middle of the second trimester of the pregnancy. The heart rate increases gradually at the same time until term, while stroke volume plateaus at 20 weeks´ gestation (Hall et al., 2011; Thornburg et al., 2000). Respiration changes as a result of the increased oxygen demands, that accompany a 15% increase in the maternal metabolic rate and a 20% increase in oxygen consumption (Soma-Pillay et al., 2016).

Endocrine changes occur as well. Steroids produced by the adrenal glands are reduced secondary to the reduction in vascular resistance and systolic and diastolic blood pressure (Soma-Pillay et al., 2016). Adaptations in glucose metabolism to promote fetal development are also necessary during pregnancy, while some researchers consider pregnancy a diabetogenic state (Soma-Pillay et al., 2016). Insulin-secreting pancreatic beta-cells undergo hyperplasia which leads to increased insulin secretion. Insulin-sensitivity in early pregnancy, largely as a result of the production of the glycoprotein hormone human Placental Lactogen (hPL), leads to progressive insulin resistance (Angueira et al., 2015; Butte, 2000). Total

serum cholesterol and triglycerides increase: associated with a 50% rise in low-density lipoproteins (LDL) at term. This is important for enabling placental steroidogenesis (Soma-Pillay et al., 2016). These major changes are summarized in Table 1.

Table 1: Hemodynamic changes in an uncomplicated pregnancy

Increased (%)*	**Decreased (%)***
Uterine blood flow (12%)	Platelet count (10%)
Plasma volume (50-60%)	Systemic vascular resistance (20%)
Red blood cell mass (30%)	Pulmonary vascular resistance
Diastolic dimension	Hematocrit
Stroke volume (30%)	Colloid osmotic pressure (10-15%)
Heart rate (10-20 bpm)**	Plasma albumin concentration
Arterial oxygen tension (5-10%)	Arterial carbon dioxide tension 15%)
Venous capacitance	Arterial hydrogen ion concentration
Renal plasma flow (40-65%)	Arterial blood pressure
GFR (50-85%)	

*If applicable. Values reflect the change at term. ** beats per minute. The table is based on (Hall et al., 2011; Soma-Pillay et al., 2016; Thaler et al., 1990; Thornburg et al., 2000).

Any impairment or maladaptation during pregnancy may therefore have a significant impact on fetal and placental development and could result in a number of complications for either the mother (preeclampsia (PE), hemolysis, elevated liver enzymes, low platelet count syndrome (HELLP) and gestational diabetes) or the fetus (intra uterine growth restriction (IUGR), intra uterine fetal death (IUFD) (Lyall, Robson, & Bulmer, 2013).

1.2 Maternal uterine circulation

The uteroplacental circulation undergoes great changes during the advancement of pregnancy, where by term vascular resistance is directing 20% of total cardiac output to the uterine vascular bed. This results in more than a ten-fold increase in blood flow over the level present in the non-pregnant state (Konje et al., 2001). Since in normal pregnancy blood pressure drops or changes very little overall (Hall et al., 2011), uterine hemodynamic changes are affected by a decrease in uterine vascular

resistance (Lyall et al., 2013; Thornburg et al., 2000). Uterine arteries arise from the iliac arteries and provide the major blood supply to the uterus. In humans, anastomoses are formed between the uterine arteries and the ovarian arteries (left and right branches), forming a bilateral arcade from which arcuate arteries branch from the uterine arteries in the mesometrium, passing over both the anterior and the posterior wall of the uterus and thus forming an arterial grid around the organ. Arcuate arteries that vary in size branch centripetally into the myometrium forming radial arteries. Radial arteries penetrate the myometrium and decidua, terminating in the spiral arteries, which ultimately supply the endometrium, decidua and placenta during pregnancy (Browne et al., 2015; Osol & Moore, 2014). In pregnancy, maternal and fetal circulations come into close contact, separated only by a single or double lining of trophoblast cells (cyto- and syncytiotrophoblastic layers). Trophoblasts invade the spiral arteries and their adjacent stroma to replace the vascular smooth muscle and endothelial layers, resulting in the formation of low-resistance vessels that can accommodate a highly increased blood flow. These altered vessels are almost independent of maternal vasoconstriction through a lack of both smooth muscle and receptors for circulating general vasoconstrictor substances (Lyall et al., 2013; Pijnenborg, Vercruysse, & Hanssens, 2006; Thornburg et al., 2000). The intravillous space is formed through these vessels and the placental villi surrounding them will have enough blood flow for nutritional exchange to occur. This type of placentation is called hemochorial and is present in mammals such as humans, higher order primates, rabbits, guinea pigs and rats (Moll, 2003; Pijnenborg et al., 2006). At term, around 200 spiral arteries open up into the intravillous space, while the blood flow in the uterine artery at the side of the placenta is greater than in the contralateral uterine artery (Browne et al., 2015; Konje et al., 2001). The anatomy of the blood vessels during the pregnant and non-pregnant states and in the case of preeclampsia (PE) is shown in Figure 1.

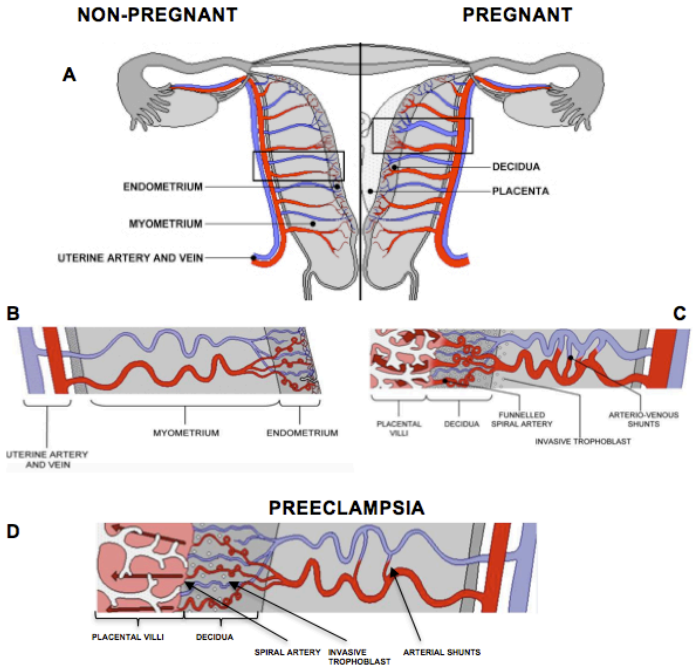

Figure 1: The anatomy of blood vessels of the human uterus during pregnant and non-pregnant states. The figure is based on (G. J. Burton, A. W. Woods, E. Jauniaux, & J. C. P. Kingdom, 2009b).

The figure abouve shows a diagramatic representation of uterine and placental vasculature (Figure 1 A) in non-pregnant (B) and pregnant states (C). In normal pregnancy, large arterio-venous shunts are present in the myometrium (Figure 1 C). In pregnancies with complications like PE (Figure 1 D), uterine arteries undergo a more limited trophoblastic invasion and transformation and are therefore narrower and minimal arterio-veonous shunts are present. In uncomplicated pregnancies, funneled spiral arteries are present while in preeclamptic pregnancies spiral arteries are much narrower (Burton et al., 2009b).

1.2.1 Placentation and trophoblast invasion

After the attachment of the zygote to the progesterone-dominated endometrium, the decidua, the differentiation process of the placental villi gives rise to extravillous trophoblasts (EVT). First of all, the cytotrophoblasts in the villi, which together with syncytiotrophoblasts (which again develop from the cytotrophoblasts) anchor the blastocyst to the maternal decidua and form the epithelial layer of the floating placental villi, start to differentiate into

a highly proliferative subtype cell column of trophoblasts. EVTs, during the first weeks of gestation, detach from the placental anchoring villi and migrate into decidual endometrium and inner myometrium by entering the trophoblast shell. As the interstitial EVTs migrate towards maternal vessels to induce vascular remodeling they differentiate into multinuclear giant cells (G. J. Burton, A. W. Woods, E. Jauniaux, & J. C. Kingdom, 2009a; Pijnenborg et al., 2006; Pollheimer & Knofler, 2012). When the trophoblast shell enters the spiral artery opening, trophoblast cells enter arterial lumen and form intraluminal plugs and invade the uterine spiral artery endothelial lining (Pollheimer & Knofler, 2012).

The EVT cells migrate along the endothelium of the spiral arteries and replace the endothelium by invading the arterial media, thereby destroying the elastic, muscular and neural tissue elements of the arteries (B. Sibai, Dekker, & Kupferminc, 2005). This is replaced by amorphous fibrinoid material in which trophoblast cells are embedded (Lyall et al., 2013). Finally, trophoblasts become incorporated into the arterial wall, by transforming the adhesion-receptor phenotype of the cells they replace, and an endothelial lining is reconstituted of endovascular extravillous trophoblasts (B. Sibai et al., 2005). With this remodeling change the steadily increasing placental blood flow can be accommodated and kept well above the needs of the fetoplacental unit without danger of vasoconstriction. Incomplete spiral artery remodeling leads, however, to reduced blood flow and a higher sensitivity to vasoconstrictor influences and thereby to damaging effects in the vascular endothelium of the mother and the complication of preeclampsia. The mechanisms involved in these structural changes are still not fully understood (Burton et al., 2009a; Pijnenborg et al., 2006). In pathologic pregnancies EVTs fail to transform spiral arteries in order to resemble the endothelial cells which they replace in healthy pregnancies (B. Sibai et al., 2005).

1.3 Preeclampsia

Preeclampsia is a human obstetrical condition affecting 2-5% of pregnancies worldwide, and is one of the major causes of maternal and perinatal death (Grimpel et al., 2011; Huppertz, 2008; Naljayan & Karumanchi, 2013; Roberts & Cooper, 2001; B. Sibai et al., 2005).

Preeclampsia is defined as the onset of hypertension and proteinuria in previously normotensive women, and mainly occurs after the 20^{th} week of gestation (B. Sibai et al., 2005). Preeclampsia is clinically divided into two main groups, i.e. early (EPE) (prior 34+0 weeks), and late (LPE) (after 34+0

weeks) (Akolekar, Syngelaki, Sarquis, Zvanca, & Nicolaides, 2011). The outcome of the disorder can vary in severity and frequency depending on different etiological risk factors, such as obesity, previous history of preeclampsia, gestational age at the time of onset, and its overall management (Madar-Shapiro et al., 2017; Naljayan & Karumanchi, 2013; Odibo et al., 2011; Roberge et al., 2012; B. Sibai et al., 2005). The disease can develop into a more serious clinical condition, eclampsia, which is characterized by convulsions accompanied by cerebral dysfunction and hemorrhages. The most severe and acute disease forms can be followed by multiple maternal organ failure that can lead to death. In general, the only cure for preeclampsia is delivery of the placenta and thereby also the fetus. Pulmonary edema and/or renal failure may develop, and hemolysis, elevated liver enzymes and a low platelet count constitute HELLP syndrome, a serious condition involving significant damage to the liver and severe hemorrhage (Naljayan & Karumanchi, 2013; B. M. Sibai & Stella, 2009). Since the presence of a hemochorial placenta is essential, for over 30 years research has had an increasing focus on placenta and placental bed pathologies, which are seen as central to the development of preeclampsia. Although full understanding of the pathogenesis of the disease has not yet been reached, our understanding of the disease has nonetheless advanced significantly over the last few decades (Phipps, Prasanna, Brima, & Jim, 2016).

1.3.1 Diagnosis of PE

Diagnosis of preeclampsia is made when hypertension, accompanied by proteinuria is present in previously normotensive women with no such symptoms (Brown, Lindheimer, de Swiet, Van Assche, & Moutquin, 2001; Macdonald-Wallis et al., 2015; Naljayan & Karumanchi, 2013; B. Sibai et al., 2005). Hypertension is defined as a blood pressure of at least 140 mmHg (systolic) and at least 90 mmHg (diastolic) on more than two occasions at a minimum of two measurements 4-6 hours apart (Macdonald-Wallis et al., 2015). Proteinuria is diagnosed when 1+ or more appears during urine dipstick testing on two occasions after 20 weeks of gestation (Brown et al., 2001; Macdonald-Wallis et al., 2015), and is better defined as protein excretion of ≥300 mg/l or ≥500 mg over 24 hours, or a protein/creatinine ratio of at least 0.3. In the absence of proteinuria, preeclampsia is diagnosed when hypertension is associated with new onset of systemic disorder such as cerebral symptoms, epigastric pain followed by nausea and vomiting, thrombocytopenia or abnormal liver enzyme measurements (ACOG, 2013; Brown et al., 2001; B. Sibai et al., 2005).

1.3.2 Short- and long-term complications

Maternal and fetal death is one outcome of the disease which has become rarer in high-resource countires but still represents real threats where resources are low (Alkema et al., 2016). Preeclampsia can have both short- and long-term effects on surviving babies and mothers. Children born with low a Apgar score may develop both short- and long-term consequences like intrapartum hypoxia, hypoxic brain injury (with possible also a long-term sequela), a need for neonatal respiratory support and other conditions due to prematurity chronic lung conditions and other complications associated with prematurity and prolonged stay in a neonatal intensive care unit (Jang, Jo, Lee, Kim, & Lee, 2011).

A number of studies have shown that mothers who have suffered PE are at 3-4 times greater risk of developing cardiovascular diseases in later life and , on average, with an up to 50% chance of hypertension on average 14 years later. This was first shown in Iceland (Arnadottir, Geirsson, Arngrimsson, Jonsdottir, & Olafsson, 2005; Jonsdottir, Arngrimsson, Geirsson, Sigvaldason, & Sigfusson, 1995), though the observation has been repeated in several other studies from different parts of the world (Behrens et al., 2017; Bellamy, Casas, Hingorani, & Williams, 2007; Bokslag, van Weissenbruch, Mol, & de Groot, 2016).

1.3.3 Treatment of PE

There is no known treatment to cure PE after the onset of clinical symptoms, except delivery of the placenta and fetus. However, treatment may be given to lower blood pressure and other symptoms.

To date, the most effective prophylactic treatment has been shown to be acetylsalicylic acid (Aspirin®, often colloquially called “aspirin”), 150 mg per day where trials involving over 30,000 women have shown that the incidence of early PE may be reduced by 62%, thought late PE is affected to a lesser extent (Rolnik et al., 2017). Many randomized trials of various sizes have investigated low-dose aspirin as a prophylactic treatment against PE (Atallah et al., 2017; Bujold et al., 2010; Haapsamo, Martikainen, & Rasanen, 2008; Roberge et al., 2012), and it is used as secondary prevention mainly in patients with history of the disorder and therefore based on individualized risk assessment.

1.3.4 Pathogenesis of PE

PE is generally considered as a disorder of two stages. Reduced placental

perfusion secondary to abnormal placentation and inadequate vascular remodeling in the placental bed mark the first stage of the disorder. This insufficient physiologic adaptation of the mother to her pregnancy is the necessary prerequisite to the disease syndrome. Immunological factors including damage to the placental villi (Redman & Sargent, 2010; Smarason, Sargent, Starkey, & Redman, 1993), inflammatory reactions (Hubel et al., 2008) and oxidative stress (Masse, Giguere, Kharfi, Girouard, & Forest, 2002) resulting from inadequate placental perfusion then develop gradually as the immediate precursors to a rise in blood pressure and other symptoms associated with the development of PE (Hod, Cerdeira, & Karumanchi, 2015; Roberts & Hubel, 2009). The second stage is marked by systemic complications, hypertension and multiple organ failure (Roberts & Cooper, 2001).

1.3.5 Prediction of PE

Considerable efforts have been directed at the identification of biophysical findings, demographic factors and biochemical analytes that could be used to predict the development of preeclampsia, both EPE and LPE. Early detection of women at risk would be beneficial, as it would enable better clinical management and sub-classification, which could facilitate identification of improved treatment regiments.

1.3.6 Demographic factors for prediction of PE

The demographic factors that have been reported as associated with increasing risk of preeclampsia are medical conditions such as chronic hypertension and diabetes, multiple gestation, ethnic origin, use of assisted reproductive techniques, history of the disorder in previous pregnancies, nulliparity and obesity (Akolekar, Syngelaki, Beta, Kocylowski, & Nicolaides, 2009; Breathett, Muhlestein, Foraker, & Gulati, 2014; Chafetz et al., 2007; Conde-Agudelo & Belizan, 2000; Odibo et al., 2011; Pare et al., 2014; Spencer, Cowans, Chefetz, Tal, & Meiri, 2007; Wang et al., 2002). From all these parameters, being overweight or obese (BMI > 25) carries the most prevalent risk factor (Pare et al., 2014).

None of these potential risk factors is a sure prediction marker, therefore a number of the studies have focused on measurement of the serum markers in order to establish a better monitoring of the possible development of the disorder.

1.3.7 Serum markers for prediction of PE

In general, the studies focus on early predictive markers, with the aim of allowing early detection of the hypertensive syndrome, the establishment of preventive measures such as the administration of acetylsalicylic acid, and for better management and reduction of possible complications once the disease is diagnosed, thus improving the overall outcome of the pregnancy for both mother and neonate. In the last decade a major focus of research has been the identification of potential serum biomarkers for such early preeclampsia prediction. Among the markers that have been investigated are pregnancy associated plasma protein-A (PAPP-A), soluble placental growth factor (PlGF), soluble VEGF receptor-1 also known as soluble fms-like tyrosine kinase 1 (sFlt-1) and placental protein 13 (PP13).

1.3.7.1 PAPP-A

In several studies a risk of pregnancy complications associated with low first-trimester maternal PAPP-A, including preeclampsia, have been reported (Cowans & Spencer, 2007; Spencer, Cowans, & Nicolaides, 2008; Spencer, Yu, Cowans, Otigbah, & Nicolaides, 2005). PAPP-A is produced by trophoblasts and maternal blood levels increase as pregnancy progresses. Early pregnancy PAPP-A concentrations are not a strong indicator for predicting preeclampsia by themselves, but a significant improvement in detection accuracy of the disorder is achieved when PAPP-A values are combined with Doppler velocimetry of the uterine artery in the second trimester (Spencer et al., 2008; Spencer et al., 2005). Although, it was proposed as a first trimester marker, decreased PAPP-A levels are seen in all trimesters in women who develop PE (Grill et al., 2009).

1.3.7.2 PlGF

Preeclampsia has also been associated with reduced production of the pro-angiogenic protein placental growth factor (PlGF), and a number of studies have reported reduction of maternal serum PlGF during the clinical phases of preeclampsia (Crispi et al., 2006; Reuvekamp, Velsing-Aarts, Poulina, Capello, & Duits, 1999; Teixeira et al., 2008; Torry, Wang, Wang, Caudle, & Torry, 1998). Reduced serum levels of PlGF are pronounced in both the first and second trimester of pregnancy (Crispi et al., 2006; Erez et al., 2008; Thadhani et al., 2004). In preeclamptic pregnancies, the maternal serum PlGF is reported to be lower than in controls, and an association between PlGF and severity of the disorder has also been suggested (Akolekar, Zaragoza, Poon, Pepes, & Nicolaides, 2008). The interrelation between

serum levels of PlGF and PAPP-A with uterine artery pulsativity index (PI) is compatible with postulated roles of these two markers in placental development and may be regarded as a reflection of the impaired placentation that is a hallmark of the first stage of preeclamptic disease (Akolekar et al., 2008; von Dadelszen, Magee, & Roberts, 2003).

1.3.7.3 sFlt

Flt-1 and soluble Flt-1 (sFlt-1) are the products of FLT-1 generated by different mRNA processing. Flt-1 is a membrane-spanning receptor of VEGF and PlGF. sFlt-1 is a shorter isoform that lacks cytoplasmic and transmembrane domains, but retains the ligand-binding one, and therefore is secreted into the circulation (Huckle & Roche, 2004; Kendall & Thomas, 1993; Thomas, Andrews, & Liu, 2007). sFlt-1 binds VEGF and PlGF in both the circulation and in tissues, acting as a scavenger and thus preventing them from interacting with their membrane receptors on the endothelium. This means that increased concentration of sFlt-1 in the circulation decreases free PlGF and VEGF (Hod et al., 2015; Sela et al., 2011). Although, the physiological function of sFlt-1 is not fully understood, it appears to be nonessential for normal placental development (Hiratsuka, Minowa, Kuno, Noda, & Shibuya, 1998). Therefore, the measurement of elevated sFlt or low VEGF levels in early pregnancy is not though useful in order to predict preeclampsia (Akolekar, de Cruz, Foidart, Munaut, & Nicolaides, 2010).

1.3.7.4 PP13

The biggest disadvantages of the aforedescribed serum markers is that they are not specific for the prediction of preeclampsia and can only be used a few weeks before the clinical onset of the disorder is evident. The disease processes will often have commenced by that time and a role for these substances in disease prevention is therefore not to be expected.

Several studies in humans have, however, concluded that placental protein 13 (PP13) might be a promising biomarker for prediction of preeclampsia. It can be detected in maternal serum as early as 6 weeks of gestation, and therefore it could potentially improve the risk assessment and overall pregnancy management and outcome (Akolekar et al., 2009; Gonen, Grimpel, et al., 2008; Gonen, Shahar, et al., 2008; Huppertz et al., 2008). It has in addition been reported that early PP13 serum concentration combined with PAPP-A can be useful to detect severe preeclampsia and HELLP cases (De Villiers et al., 2018; Moslemi Zadeh, Naghshvar, Peyvandi, Gheshlaghi,

& Ehetshami, 2012), and combining PP13 with Doppler (Nicolaides et al., 2006b; Odibo et al., 2011) can also improve early detection.

In normal pregnancy maternal PP13 increases in serum throughout pregnancy (approx. 200 pg/mL by weeks 5-8), reaching a peak late in the third trimester (400 pg/mL by weeks 36-40) with a total serum concentration increase of 1.4-2-fold. In preeclamptic pregnancies the PP13 serum levels are significantly lower compared to controls (< 50 ng/mL, weeks 5-8), reaching a peak of >600 pg/mL, or a 7-15-fold increase, compared to measurements in early pregnancy (Huppertz et al., 2008). Lower concentrations of PP13 in early pathological pregnancies have been reported by several studies (Chafetz et al., 2007; Romero et al., 2008; Spencer et al., 2007). In women at high risk of developing preeclampsia, PP13 was found to be an efficient marker with a detection rate for early onset preeclampsia of 71% for a false positive rate of 10% (Khalil et al., 2009). In a study by Nicolaides et al, patients with severe preeclampsia before week 34 were reported to have lower PP13 serum levels than normotensive women (Nicolaides et al., 2006a). Considering the severe short- and long-term consequences of preeclampsia the positive role of serum biomarkers such as PP13, in combination with other techniques, may add an accumulative value to the prediction of the disorder.

1.4 Structural and functional characterization of PP13

1.4.1 Homology of PP13

PP13 is one of 56 known placental proteins and was first isolated from human term placenta in 1983 and characterized by Bohn et al. (Bohn, Kraus, & Winckler, 1983). It has shown structural and functional homology to the ß-galactoside-binding lectins (Barondes et al., 1994), with DNA and amino-acid sequence homology, especially in their carbohydrate binding domain (CBD) (Sammar et al., 2014; N. G. Than et al., 2008; Than et al., 2009). There are several subgroups of galectines and PP13 (also referred to as galectin 13) belongs to the sub-groups that tend to form homodimers (Thijssen, Rabinovich, & Griffioen, 2013). Galectins appear not to have specific individual receptors, but bind to suitable oligosaccharide residues of glycoproteins in the cytoplasm, on cell surfaces in the extracellular matrix (Yang, Rabinovich, & Liu, 2008).

1.4.2 Structural properties and binding of PP13

PP13 is composed of 118 amino acids composing two identical monomers,

each of ~16 kDa and held together by disulfide bonds, through which it dimerizes resulting in 32 kDa homodimers with a carbohydrate content of 0.6% - the lowest among known placental proteins (Burger et al., 2004; Than et al., 2014; Than et al., 2004; Than, Sumegi, Than, Berente, & Bohn, 1999). The gene encoding for the protein, which is specific to primates, is called *LGALS13* and is positioned on chromosome 19 (Than et al., 2009; Than et al., 1999). PP13 is predominantly expressed by syncytiotrophoblasts, and is localized both in the cytoplasm and in the brush border membrane of the syncytiotrophoblast where it is found in syncytiotrophoblast microparticles (STBM) that are shed into the maternal circulation along with their PP13 content when the syncytiotrophoblast cells are damaged in the preeclamptic oxidative process which has been termed aponecrosis (N. Than et al., 2008). The specific binding site for PP13 has not yet been defined although, similar to most galectins, PP13 binds to sugars such as N-acetyl-lactosamine (Than et al., 2014) and to sugar residues of the B and AB antigen of the ABO blood groups (Than et al., 2011). These interactions are important regulators for the availability of free PP13 in the blood circulation and have been found to influence the risk assessment and prediction to develop preeclampsia (Watkins, 2001).

Affinity chromatography and mass spectroscopy identified high affinity binding of PP13 to annexin II, a member of Ca^{2+} and phospholipid binding protein families, and to beta/gamma actin (Than et al., 2004). As PP13 is a soluble protein lacking a transmembrane domain, it reaches the cell surface by binding to ß-galactoside residues and generates immune responses as well as influencing functions like apoptosis and molecular recognition (Than et al., 2004; Visegrady et al., 2001). Induction of apoptosis of various types of maternal white blood cells in the placenta is mediated through the CBD of PP13 (Kliman et al., 2012; Than et al., 2009).

1.4.3 PP13 and pathological pregnancies

As mentioned above, pregnancies destined to develop preeclampsia have a low concentration of PP13 in the first weeks of gestation (Huppertz et al., 2008; Sammar et al., 2009). In studies where term placentas were obtained after delivery, it was found that mRNA levels of PP13 were reduced 3.5-fold in women who developed PE and the related HELLP syndrome (Sammar et al., 2011; N. G. Than et al., 2008). Reduced expression of PP13 mRNA in villous trophoblast has been reported to be already lower in the first trimester in comparison with control samples. Lower mRNA expression is therefore associated with the pathogenesis of PE (Shimizu et al., 2009). There are a

few studies in which different ethnic groups have been examined for gene sequencing of the *LGALS13* in order to find the polymorphism relevant to reduced PP13 mRNA expression (Stolk et al., 2006). Three motives in the *LGALS13* gene lead to the polymorphism (Stolk et al., 2006), but the mutant proteins are degraded in the placenta almost instantly and not released into the circulation (Sammar et al., 2009).

In two longitudinal studies steep increases of PP13 in maternal serum around mid-gestation have been observed, i.e. at a time when severe early disorder starts to be active (Gonen, Grimpel, et al., 2008; Huppertz et al., 2008).

The majority of the studies referred to above have been done in vitro. The first in vivo study was conducted in pregnant rats, where the results showed a drop of blood pressure and an increase in pup and placental weights when compared to the control groups (Gizurarson et al., 2015).

1.5 Studying PE in animal models

Contributions from viable and reproducible animal models, mostly developed in rats, are important for understanding how preeclampsia progresses. Although many current models can be applied to preeclampsia research, they still poorly mirror the whole range of dysfunction in the human condition. PE is predominantly a human disease. Animal models focusing on mechanical interference with uterine blood flow have been developed mostly in mice and rats and vary from being used to show changes in blood pressure by drug infusion (Keiser et al., 2009; Orshal & Khalil, 2004) to do the same in a surgically modified circulation (LaMarca et al., 2008; Li, LaMarca, & Reckelhoff, 2012). Animal models have contributed to an increased understanding of the underlying etiology of preeclampsia. A brief comparison between human and rat maternal circulation will be presented below for clarification of the investigations presented in this thesis.

1.5.1 Uterine circulation in rats

Like humans, rats have a hemochorial type of placentation, in which intraplacental pressure created by the maternal blood within the intravillous space must be kept low to avoid compression of the intravillous fetal vessels, or intralabyrinthine fetal vessels in the case of rodents (Moll, 2003). In rats, changes in uteroplacental blood flow are greater than in humans, resulting in the blood flow at term being more than 70-fold higher than in the non-pregnant animal (Osol & Mandala, 2009; Page, Celia, Leddy, Taatjes, &

Osol, 2002). Uterine artery diameters in rodents increase most often with little or no thickening of the vascular wall (Mandala & Osol, 2012). Rats have a duplex V-shaped uterus (Figure 2), with main uterine arteries that lie parallel to each other out of each uterine horn. Arcuate arteries in the mesometrium at the uterine surface appear as loops branching form the main uterine artery. Tertiary mesometrial arteries (radial arteries) connect the arcuate loops with the uterine wall. The pre-myometral radial arteries enter the uterine wall between placentation sites and branch out into an intrauterine arterial plexus that supplies the myometrium (Osol, Barron, & Mandala, 2012; Osol & Mandala, 2009).

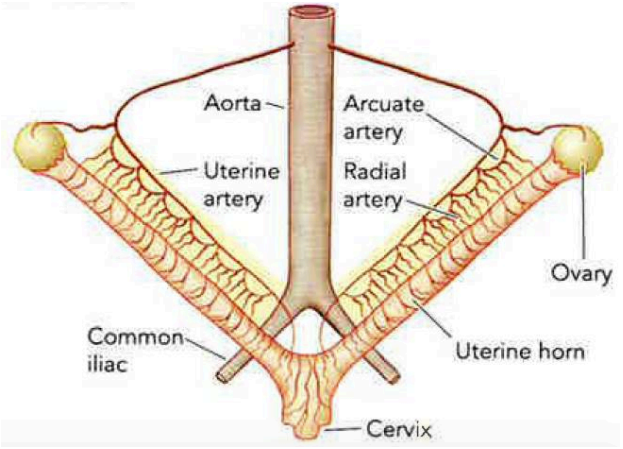

Figure 2: The anatomy of rodent uterus blood vessels. This figure is re-printed with the permission of the publisher and authors and is originally published by (Osol & Mandala, 2009). Rats have a duplex uterus with a main uterine artery and vein running parallel to the uterus and which are located in the mesometrium. Secondary arcuate,vessels form redundant loops with the main uterine artery and the tertiary vessels that in a radial fashion connect the arcuate loops with the uterine wall.

Current management of preeclampsia consists of monitoring and controlling maternal hypertension and organ function. Prediction and prophylaxis of the disorder in early pregnancy are of great interest in the field of fetal medicine, and a number of potential biomarkers have been identified, although these need to be externally validated in order to be used to facilitate screening in the patients identified at risk. The future of the therapy of preeclampsia lies in identifying physiologically relevant mechanisms that control the onset and development of the syndrome.

2 Aims

The general aim of the project was to gain insight into the pathways that are involved in mediated vasodilation by placental protein 13 (PP13) and on the effects PP13 has on the uterine vascular system.

The four specific aims, which are also reflected in three published papers, a manuscript submitted for publication and additional data included in this thesis are:

1) To evaluate the overall effect on vascular physiology uterine circulation after short- and long-term continuous exposure to PP13 in non-pregnant rats by histological staining. Manuscript (Paper III).

2) To explore the acute effects of exposure to PP13 on isolated non-pregnant rat model arteries by examining both the direct effect and the effect after pharmacologically based changes causing inhibition of various pathways that are mediating physiological changes in the arteries and anatomical changes leading in endothelial layer denudation. These studies are summarized by (Drobnjak et al., 2017), Paper I.

3) To follow up previous findings of the long-term effect of exposure to PP13 by slow delivery and additional treatment via drinking water. The overall effects on rats were evaluated on the vascular physiology, placental size and pup weights. Preliminary data.

4) To explore the pharmacokinetic profile and parameters of PP13 after intravenous (I.V.) and subcutaneous (S.C.) single-dose administration in a rabbit model, as described in (Drobnjak, Meiri, Mandala, Huppertz, & Gizurarson, 2018), Paper II.

3 Materials and methods

3.1 Placental protein 13 and his-PP13

PP13 used in this study was made in two variants: The wild type variant (rPP13) and the wild type variant that includes histidine Tag linked to its N terminal (his-PP13). Both were prepared by expression in *Escherichia coli*, and harvested from the soluble fraction as described by Sammar et al (Sammar et al., 2014). Proteins were harvested from the inclusion bodies, solubilized and refolded, than subsequently purified by ion exchange chromatography (Maymon et al., 2017). The his-PP13 variant was initially purified by NiNTA affinity purification followed by ion exchange chromatography as previously described (Sammar et al., 2014).

DNA sequences of both variants were determined from both strands by the dideoxy sequencing method, Sanger sequencing (Sanger, Nicklen, & Coulson, 1977; Than et al., 1999).

The molecular weight and the purity of the protein were verified by SDS-PAGE and immunoblots with PP13-specific monoclonal antibodies (Sammar et al., 2014). The molecular weight of rPP13 is 15.6 kDa and the his-tag PP13 is ~18 kDa (2kD heavier). In nature isolated PP13 forms stable dimer connected trough the disulphide (S-S) bridges that may exchange form with the monomer as was previosly described by (Than et al., 1999) and modeled by (Visegrady et al., 2001). His-PP13 has one of the S-H group bloked by the histidine tag and forms only monomers or poly-oligomer, which tends to sink down into soluble solution. Accordingly rPP13 appears to have a lower kinetic energy and is more stable and lasts longer in human fluid (Sammar et al., 2011).

3.2 Animals and ethical approvals

3.2.1 Spargue – Dawley Rats

The animal studies carried out at the University of Vermont were approved by the Institutional Animal Care and Use Committee at the University of Vermont, USA, and the studies carried out in Iceland were approved by the National Laboratory Animal Review Board of Iceland, (study no. 2015-02-01). All experiments were carried out in accordance with the US NIH guidelines for the care and use of laboratory animals (NAS, 2011). Fodder pellets and

water were provided *ad libidum*, and animals were kept under a 12/12 hours light/dark cycle. All efforts were undertaken according to the "3R principles" (www.nc3rs.org.uk) to reduce the number of animals used in this study and optimize experimental protocols for obtaining maximum data from each tested animal.

Pregnant and age-matched non-pregnant female Sprague-Dawley rats (12–14 weeks of age) were purchased from Charles River Laboratories International (St-Constant, Quebec, CA) and housed at the animal care facility at the University of Vermont, College of Medicine, Burlington, VT, USA. The animals were allowed to acclimatize for at least 72 hours prior to use, and pregnant animals were used during mid-pregnancy (day 15 of a 22-day gestation).

Sixteen female non-pregnant Sprague-Dawley rats (11 weeks of age) were purchased from Taconic Bioscience (Ejby, Denmark). Animals were acclimatized for two weeks prior to start of the experiments. All experiments were carried out at an authorized animal facility ArticLas, Reykjavik, Iceland.

3.2.2 New Zealand White rabbits

Twelve female non-pregnant New Zealand White (NZW) rabbits were purchased from Envigo, (Huntington, UK), and housed at the laboratory animal care facility at the University of Iceland (Reykjavik, Iceland). The animals, each weighing on average 3.9 ± 0.3 kg, were caged three together with access to fodder pellets and water *ad libidum* and maintained on a 12/12 hours light /dark cycle.

The study was approved by the Icelandic Animal Ethic Committee (study no. 2015-09-02) and carried out according to the US NIH guidelines for the care and use of laboratory animals. All efforts were undertaken according to the "3R principles" (www.nc3rs.org.uk) to reduce the number of animals used in the study and optimize experimental protocols for obtaining maximum data from each tested animal.

3.3 In vivo study in non-pregnant rats (Manuscript, Paper III)

3.3.1 Drug administration and osmotic pump implantation

All animals received slow-release osmotic mini-pumps (Alzet, Model 2 ML1), loaded with 2 mL of 127 ng non-his-tag PP13 (n=10), implanted subcutaneously in the periscapular region. The pumps released 10 µl/day of their content for a seven-day period. Control animals received similar osmotic

pumps releasing saline (n=9) or his-tag-PP13 (n=8), also loaded with 127 ng of the protein, released at the same rate.

Two groups of animals were used in this study. First part, active protein administration study, where animals were euthanized after seven days was performed at the University of Vermont, VT, USA. The second part, extended protein efficacy study, in which animals were euthanized thirteen days after the implantation of the pumps, was performed at ArctcLAS, Reykjavik, Iceland. The timing of the pump implantation was chosen in order to resemble the study performed in pregnant rats, where the osmotic pumps were implanted at day 8 of the pregnancy (at the time of placentation (Soares, Chakraborty, Rumi, Konno, & Renaud, 2012)) and scarified at day 21 (one day before the term delivery for rats).

3.3.2 Diameter evaluation of vessels

3.3.2.1 Active protein administration study

All uteri were pinned in Petri dishes filled with relaxing solution (10 mM HEPES saline at 4°C containing of 100μM papaverine / 1 μM diltiazem mixture). All vessels were photographed through a calibrated stereomicroscope (Zeiss, Germany) in order to estimate vascular structure and compare between groups.

3.3.2.2 Extended protein efficacy study

All rat uteri were dissected, one uterine horn was photographed on a Petri dish and the other placed into buffered formalin solution for 48 hours. Afterwards, they were transferred to phosphate buffered solution (PBS) with pH 7.4. Formalin-fixed sections of the uterine horns were paraffin-embedded and sliced to 4μm thick slices before staining. The slices were stained by hematoxylin and eosin dye (H&E), and visualized, and analyzed using Nanozoomer® digital pathology software. In order to determine vessel type, first vessels in the mesometrium were measured, and than perimetrial vessels (radial). All measurements were carried out by measuring the area of each vessel´s lumen, using a tool in the software. Vessel diameters were calculated using the following equation:

$$D = 2 * \sqrt{\frac{A}{\pi}}$$

Equation 1: Area of the circle (D-diameter; A: area; π =3.14)

The study groups were compared by non-parametric ANOVA test (for large samples) and Mann-Whitney test (for small samples) in order to evaluate the differences between rPP13, his-PP13 and saline control. All values are reported as means ± SD, with p-values <0.05 considered significant. All statistical analysis and plotting of all values were performed using Prism, Graph Pad Software, San Diego, CA, USA.

3.4 Mechanism of action in uterine and mesenteric vessels (Paper I)

3.4.1 Vessel preparation

Uterine arcuate arteries (UAA) and third-order mesenteric arteries (MA), both having similar resting diameters (150–250 μm), were dissected free of surrounding adipose and connective tissue. They were cannulated on to glass needles in a chamber of a small artery arteriograph (Instrumentation and Model Facility, University of Vermont, Burlington, VT, USA), and pressurized to 50 mmHg using a pressure-servo system (Living Systems Instrumentation, St. Albans City, VT, USA). Change in lumen diameters were measured under a microscope (Zeiss, Jena, Germany) using a video dimension analyzer (Living Systems Instrumentation, St Albans City, VT, USA) and recorded on WinDaq software, DATAQ Instruments, Inc, Akron, OH, USA.

Some vessels were treated by intraluminal air perfusion in order to remove the endothelium. The removal of the endothelium was accomplished and confirmed by a complete loss of acetylcholine (ACh 1 μM)-induced dilation in vessels pre-constricted with phenylephrine (Phe 0.1–0.3 mM).

3.4.2 Evaluation of vasodilator reactivity

Equilibration of each vessel took 40 min in oxygenated physiological salt solution (PSS) at 37°C and pH 7.4. All experiments were performed at an intraluminal pressure of 50 mmHg, a pressure that approximates in vivo conditions (Gokina, Kuzina, & Vance, 2010). Arteries were pre-constricted by the addition of Phe or a synthetic thromboxane analog (U46619) to the superfusate at concentrations sufficient to produce a 40–60% reduction in

lumen diameter, as described in earlier studies (Colton, Mandala, Morton, Davidge, & Osol, 2012). The preconstriction with Phe was allowed to stabilize for at least 15–20 min prior to continuing the experiment. Once constricted vessels have stabilized, PP13 was added to the superfusate in increasing concentrations (10^{-13} to 10^{-8} M). The resulting changes in lumen diameter were recorded, allowing sufficient time (typically 10–15 min) for the diameter to stabilize at each concentration. In cases when this stabilization was difficult to achieve, Phe was washed out and the synthetic thromboxane analog U46619 used instead. The level of preconstriction was 50% on the average for either agonist. Vascular reactivity was evaluated by determining both sensitivity and efficacy.

Sensitivity was defined as the concentration of PP13 required to produce half-maximal dilation (EC_{50}), and obtained by using sigmoid logistic curves. Efficacy was defined as the maximal extent of dilation induced by PP13 relative to complete dilation. The efficacy was determined at the end of each experiment in a solution containing a mixture of a phosphodiesterase inhibitor (papaverine, 100 μM) and an L-type Ca^{2+} channel blocker (diltiazem, 1 μM).

3.4.3 Nitric oxide and prostaglandin involvement in PP13-induced vasodilation

Some UAA were pretreated for 30 min with a combination of Nω-nitro-L-arginine (L-NNA) (100 μM) and Nω-nitroL-arginine-methyl-ester (L-NAME) (100 μM) in order to inhibit NO production. The combination of inhibitors had been shown to be more effective than either substance alone for generating NO inhibition (C. W. Jones, Mandala, Barron, Bernstein, & Osol, 2009). Inhibition of the production of prostaglandins (PGs) in arteries was accomplished by pretreatment with 10μM indomethacin (a cyclooxygenase [COX] 1 and 2 inhibitor) for 30 min. A prostacyclin (IP) receptor inhibitor, RO1138452, was used at a concentration of 10 μM to block the downstream effects of IP production (R. L. Jones, Wise, Clark, Whiting, & Bley, 2006). Vessels that had been pretreated with the blockers described above were than preconstricted with Phe and prior to when the increasing concentrations of PP13 were administrated. Differences in responses to PP13 in denuded vessels for multiple comparisons. *p* values ≤0.05 were considered significantly different.

3.4.4 Endothelial cell calcium measurements

The role of PP13 in calcium mobilization was previously described in BeWo cells and in cultured trophoblasts (Balogh et al., 2011; Burger et al., 2004;

Than et al., 2011). To examine the PP13 effect on endothelial cell (EC) cytosolic calcium levels, UAA ECs were loaded with the Ca^{2+} sensitive dye fura-2 by intraluminal perfusion with 5 μM fura-2-AM-containing solution for 5 min at room temperature, as previously described (Gokina & Goecks, 2006; Takahashi, Camacho, Lechleiter, & Herman, 1999). Excess fura-2 removal was carried out by the additional vessel perfusion with PSS for 10 min at 20mmHg intraluminal pressure. Measurement of EC fura-2 fluorescence was performed using a photomultiplier system (IonOptix Inc., Milton, MA, USA). Background fluorescence was defined for each vessel before loading with fura-2-AM. The background-corrected ratio of 510 nm emission was obtained at a sampling rate of 5 Hz from arteries alternately excited at 340 and 380 nm. After dye loading, the vessels were superfused for 10 min with PSS containing 10^{-8} M PP13 (maximally effective concentration for eliciting vasodilation). In these experiments, PP13 was tested without a pre-constriction step with Phe, and at an intraluminal pressure of 50 mmHg to eliminate movement artifact. Finally, ACh (10 μM) was applied as a positive control, due to its ability to elicit rapid, significant increases in calcium (16). Intracellular endothelial calcium ($[Ca^{2+}]_i$) of the EC was calculated using the following equation:

$$[Ca^{2+}]_i = K_d \beta \frac{R - R_{min}}{R_{max} - R}$$

Equation 2: Endothelial calcium concentration

In this formula R is the experimentally measured ratio (340/380 nm) of fluorescence intensities (Gokina & Goecks, 2006; Knot & Nelson, 1998), R_{min} is the ratio when $[Ca^{2+}]_i$ is not present, and R_{max} is the ratio at Ca^{2+} saturated fura-2 conditions. β is a ratio of the fluorescence intensities at 380 nm excitation wavelength at R_{min} and R_{max}, and β-values were determined by an in situ calibration procedure from the arteries treated with the ionophores ionomycin (10 μM) and nigericin (5 μM) (Gokina & Goecks, 2006).

3.4.5 Solutions and drugs

The PSS contained 119 mM NaCl, 4.7 mM KCl, 24.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.023 mM EDTA, and 11.0 mM glucose, and pH was adjusted to 7.4. For the fura-2 calibration procedure, we used a solution of the following composition: 140 mM KCl, 20 mM NaCl, 5 mM HEPES, 5 mM EGTA, 1 mM $MgCl_2$, 5 µM nigericin, and 10 µM ionomycin, pH = 7.1. Fura-2-AM and pluronic acid were purchased from Invitrogen (Carlsbad, CA, USA); ionomycin and nigericin were obtained from Calbiochem (La Jolla, CA, USA).

3.5 Preliminary study (Unpublished data)

All animals recieved 2 ml slow-release osmotic mini pumps (Alzet, Model 2 ML1) loaded with 2 mL of 127 ng of his-Tag PP13 (n=3), rPP13 (n=10) or saline solution (n=10). Pumps were subcutaneously implanted in the periscapular region at day 8 of the gestation, and released 10 µL/day of their content for a seven-day period. Animals from each group, his-PP13 (n=3), rPP13 (n=4) and saline (n=4), were treated with L-NAME (0.5 g/L) via drinking water. The treatment started on the day of pump implantation, and continued until mid-gestation, day 15, when the animals were euthanized.

3.5.1 Morphometric measurements

Morphometric measurements, i.e. length and width, were performed on the unstretched uterine vasculature of both horns. The uterus was kept in a relaxing solution for approx. 40 min before measurements were performed. Diameters of the both MUA and MUV were measured at three points: at ovarian end, in the middle and at cervical end. In addition radial veins of each animal were measured as well. All vessel diameter measurements were performed using a Stemi SR microscope, (Zeiss, Germany). Pups and placentas were dissected from the uterus and weighted separately. Individual measurements were compared using Mann-Whitney non-parametric test.

3.6 Pharmacokinetic study of PP13 in NZW Rabbits (Paper II)

3.6.1 Experimental procedure

The animals were divided into four experimental groups (n = 6/group). Each animal was used in one experimental group, then rested for a week and used again in a different experimental group, receiving different dose of the drug or being treated by different routes of administration. Three groups received single dose intravenous (I.V.) injection (1.3, 2.6 or 12.8 ng/kg) of PP13 that

was administered into the right marginal ear vein. The forth group received single dose subcutaneous (S.C.) injections (12.8 ng/kg), administered at the neck area on the back.

PP13 was diluted in 0.9% saline solution at concentrations of 5, 10 and 50 ng/mL, corresponding to amount of 1.3, 2.6 and 12.8 ng/kg, respectively.

A control blood samples of approx. 0.8 ml were collected from the marginal vein of the left ear (opposite of the administration side) of each animal and stored into serum vacutainer tubes (MiniCollect® Tube, GBO, Cat. No. 450472). The blood was collected at (T_0) prior to protein administration and also at a time points of 10, 30, 60 and 90 min, and 2, 4, 6 and 24 h, after PP13 administration.

Blood samples were centrifuged within 2h after the blood collection and separated serum was kept frozen at -80 °C until further analysis.

3.6.2 Analytical assay

Determination of PP13 concentration in collected serum samples was carried out by commercially available ELISA kit from Cusabio (Cusabio, Wuhan, China, Cat.No: CSB-E12733h). All samples were analyzed in duplicates. The working range of the assay is 2.5-1000 pg/ml. Calibration curves had a regression coefficient at R^2 >0.99. Intra-assay precision and inter-assay precision had a coefficient of variance (CV%) of 10% and 11%, respectively. Inter-assay precision was determined by measuring standard samples on three separate days. The calibration curves were fitted with four-parameter logistic equation. PP13 serum concentrations of each animal were analyzed over time, and in order to describe the pharmacokinetic profile of PP13 after both I.V. and S.C. administration, a two-compartment model with linear elimination was used. Data were collected and calculated in Microsoft Excel®, using standard pharmacokinetic equations and simulations. All statistical calculations were performed using GraphPad In Stat on Prism (GraphPad Software, San Diego, California, USA).

4 Results

4.1.1 Effect on vascular expansion after short-term slow-release administration of rPP13 and his-PP13 in non-pregnant rats

After seven day active exposure of rPP13, his-PP13 and saline control, animals were sacrified and vascular expansion examined. A significant difference was observed in main uterine arteries and veins (MUA and MUV) and in radial veins (RUV), in the rPP13 group when compare to the control. A significant effect between vessel diameters was also oberved in his-PP13 group, but only for radial veins when compared to the controls Table 2.

4.1.2 Effect on vascular expansion after long-term slow-release administration of rPP13 and his-PP13 in non-pregnant rats

After the PP13 exposure and histological staining of the uterine horns the diameter size of the veins measured resulted in significant expansion of all types of veins, main, arcuate and radial veins, compared to the saline control and his-PP13. The his-PP13 caused expansion of MUV, but to a lesser extent of the radial vein and arcuate veins as summarized in Table 3. The expansion differences with recombinant PP13 (rPP13) were significant for the MUV ($p<0.05$), the arcuate veins and radial veins ($p<0.01$, each) (Figure 3). The differences were not significant for his-PP13 compared to the saline treated group.

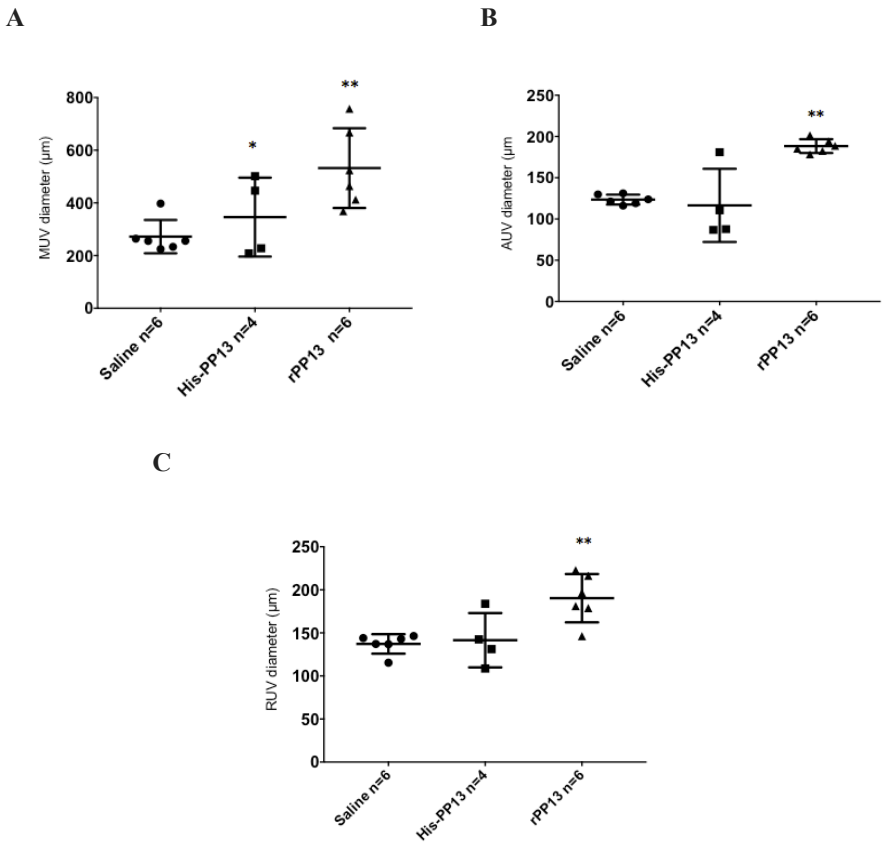

Figure 3: Diameter size comparison of veins by the size order.

Analysis of vessel expansion for the main uterine vein (MUV) (A), arcuate (B) and radial veins (C). The diameters were measured after in digital software after the H&E staining. The results are presented as means ± SD.

The diameter size comparison between the main uterine arteries (MUA), the arcuate arteries and the radial arteries are summarized in Figure 4, ABC, respectively. As summarized in the Table 3, there was no significant difference between groups in MUA diameter. However, a significant difference was observed between groups regarding both arcuate arteries and radial arteries ($p < 0.05$), when PP13 treated group was compared to the saline control and to the his-PP13.

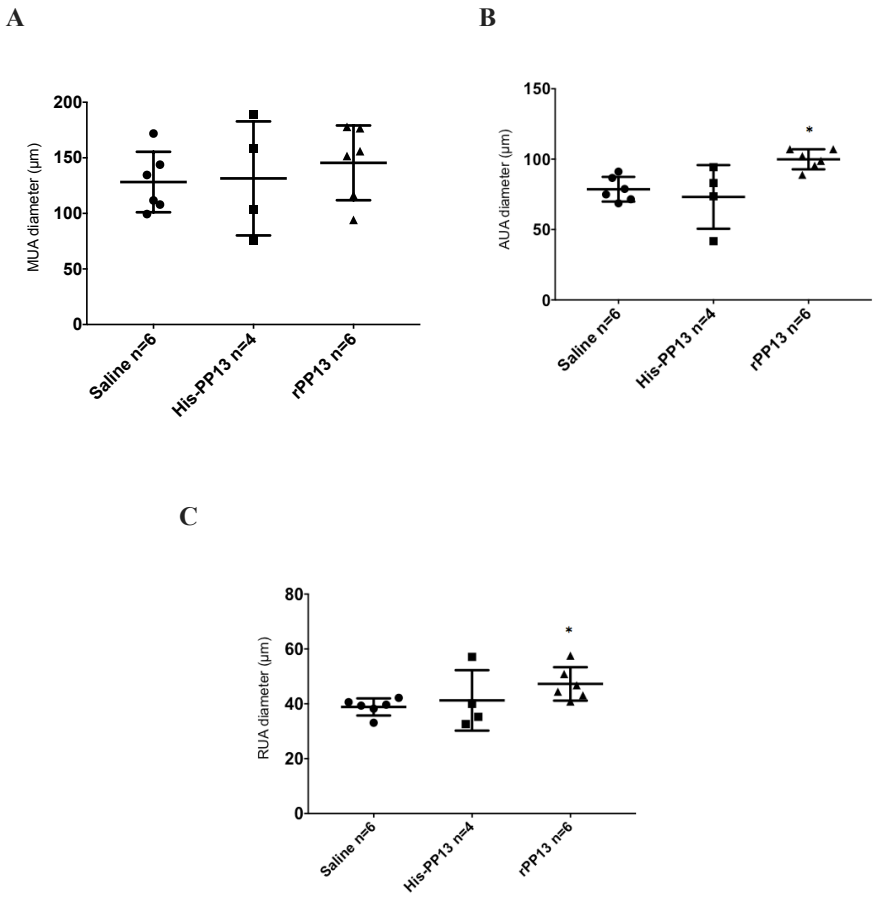

Figure 4: Diameter size comparison between arteries by size order.

Analysis of vessel expansion for the main uterine artery (MUA) (A), arcuate arteries (B) and radial arteries (C). The diameters were measured in digital software after the H&E staining. The results are presented as means ± SD.

Immediately after the animals were sacrified, the uterus of each animal was dissected, and one of the uterine horns was separated and photographed on a Petri dish while the other was transferred for histological examination. In Figure 5 the immediate visible effect of rPP13, his-PP13 and saline on vascular system configuration is presented.

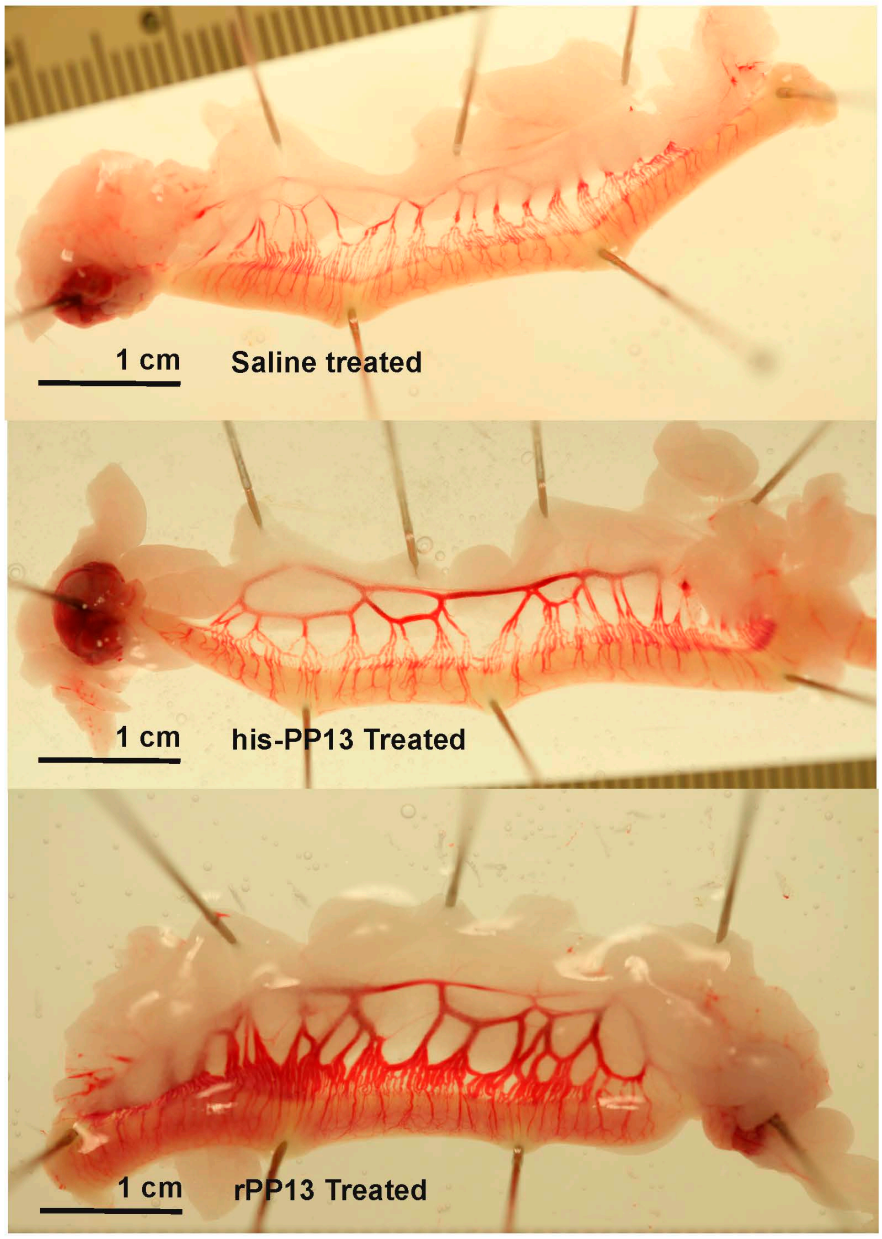

Figure 5: The post mortem uterine vasculature between rPP13, his-PP13 and saline.

The overview of the photographed uterine horns of three representative specimens showing the expanded uterine veins in the the saline control (top) compared to the his-PP13 (middle) and rPP13 (bottom).

Table 2 Vessels diameters measured seven days after exposure with PP13, his-PP13 and saline control.

Type of vessels	rPP13 (n=4)	His-PP13 (n=4)	Saline (n=3)	p
MUV (µm)	301.3±39.6* (31%)	302.0±97.0 (11%)	273.3±5.8	0.0172
MUA (µm)	151.9±15.5*** (16%)	128.8±23.2 (-2%)	141.5±32.0	<0.0001
RUV (µm)	184.2±18.8*** (39%)	173.7±65.3*** (27%)	137.1±14.9	<0.0001

Diameters of uterine veins and arteries from non-pregnant rats treated with rPP13, his-PP13 or saline released for seven days from sub-cutaneous pumps. The values in brackets show the size difference compared to saline control. Data are reported as means ± SD, n – number of animals. The significant difference in the p value column to the right is the Mann-Whitney value for vessels of the same type among the treatment groups. The asterisks represent p-values calculated for the differences between each group to saline with *p<0.05, **p<0.01, ***p<0.001. MUV, main uterine vein; MUA, main uterine artery; RUV, radial uterine vein.

Table 3 Vessels diameters measured thirteen days after exposure with PP13, his-PP13 and saline control.

	Saline / drink water	Saline / drink L-NAME	rPP13 / drink water	rPP13 / drink L-NAME	HisPP13 / drink L-NAME	p
n	24	21	36	24	17	
MUV (μm)	690^{c} [598-782]	949^{b} [774-1,123]	1,158^{a} [1075-1242]	993^{b} [906-1079]	971^{b} [855-1086]	<0.001
MUA (μm)	150^{a} [135-165]	121^{b} [105-136]	161^{a} [151-171]	128^{b} [113-143]	113^{b} [98-128]	<0.001
n	66	96	75	59	73	
Radial vein (μm)	340^{c} [303-376]	372^{bc} [355-389]	517^{a} [389-644]	388^{b} [368-409]	384^{b} [367-401]	<0.001
n	44	81	53	43	46	
Pup weight (gr)	3.6^{b} [3.3-3.9]	2.9^{c} [2.5-3.4]	4.6^{a} [4.3-4.8]	3.0^{c} [2.9-3.2]	2.9^{c} [2.8-3.1]	<0.001
Placenta weight (gr)	2.9^{ab} [2.7-3.2]	2.6^{b} [2.3-2.9]	3.2^{a} [3.0-3.3]	2.6^{b} [2.5-2.8]	2.5^{b} [2.3-2.7]	<0.001

Values are reported as Means $\pm$ 95% confidence interval; N- number of measurements; Statistical differences are by One-way ANOVA. A- Groups are significantly higher from others; B-Significantly higher from C but significantly lower from A; C-Significantly lower from A and from B; AB- significantly lower from A and higher from B; BC- significantly lower from B but higher from C.

4.2 rPP13-induced vasodilation on isolated uterine arteries in rat model

At a transmural pressure of 50 mmHg the lumen diameters of UAAs dissected from non-pregnant rats prior to treatment were 172 ± 21.7 µm (n = 5), compared with 230 ± 17.0 µm (n = 9) in mid-pregnant animals, showing that the UAAs had significantly enlarged (p = 0.002).

In mesenteric arteries, the lumen diameters were found to be similar in non-pregnant and mid-pregnant animals, averaging 213 ± 17.8 and 211 ± 22.6 µm, respectively, resulting in non significant difference (p = 0.26).

After pre-constriction with Phe or U46619, to an approximately half-maximal (40–60%) reduction in diameter was reached, the addition of PP13 in increasing concentrations (10^{-13}–10^{-8} M) followed. An induced progressive vasodilation in all intact vessels was observed. An example showing the diameter and time pattern of a UAA response to a single concentration (10^{-9} M) of PP13 is shown in Figure 6.

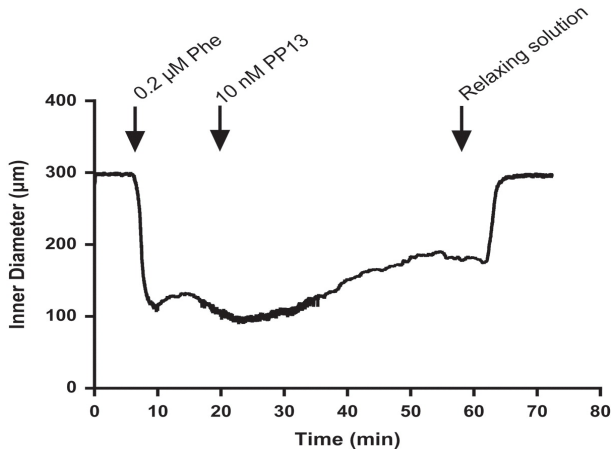

Figure 6: Concentration-response tracing of PP13. This figure is re-printed with the permission of the publisher and is originally published in (Drobnjak et al., 2017).

Tracing of a concentration–response to PP13 in a single uterine arcuate artery dissected from mid-pregnant rat. Artery was pre-constricted to approx. 60% of its original size with 0.2 µM Phe, and dilated with a single 10 nM concentration of PP13, prior to inducing complete vasodilation with a relaxing solution containing diltiazem and papaverine. The pressure was kept constant at 50 mmHg, and the recording has been made over 2 hours and 5 min (7412 s).

4.2.1 Dilation effect of PP13 on mesenteric and uterine arcuate arteries

After the administration of increasing concentrations of PP13, the concentration–response curves were plotted from data gained in UAAs from both non-pregnant and mid-pregnant animals, as depicted in Figure 7. Efficacies were 45 ± 4.8% in non-pregnant and 50 ± 5.9% in mid-pregnant. There were no significant differences in efficacy between groups (p = 0.25). Efficacies of PP13 in MAs were 51 ± 7.0% and 41 ± 5.9% from NP and MP respectively, and no significant difference was between the groups (p = 0.06). EC_{50} values were also not different between vessel types (p = 0.98), or treatment groups (p = 0.56), and ranged from 0.035 to 0.063 pM.

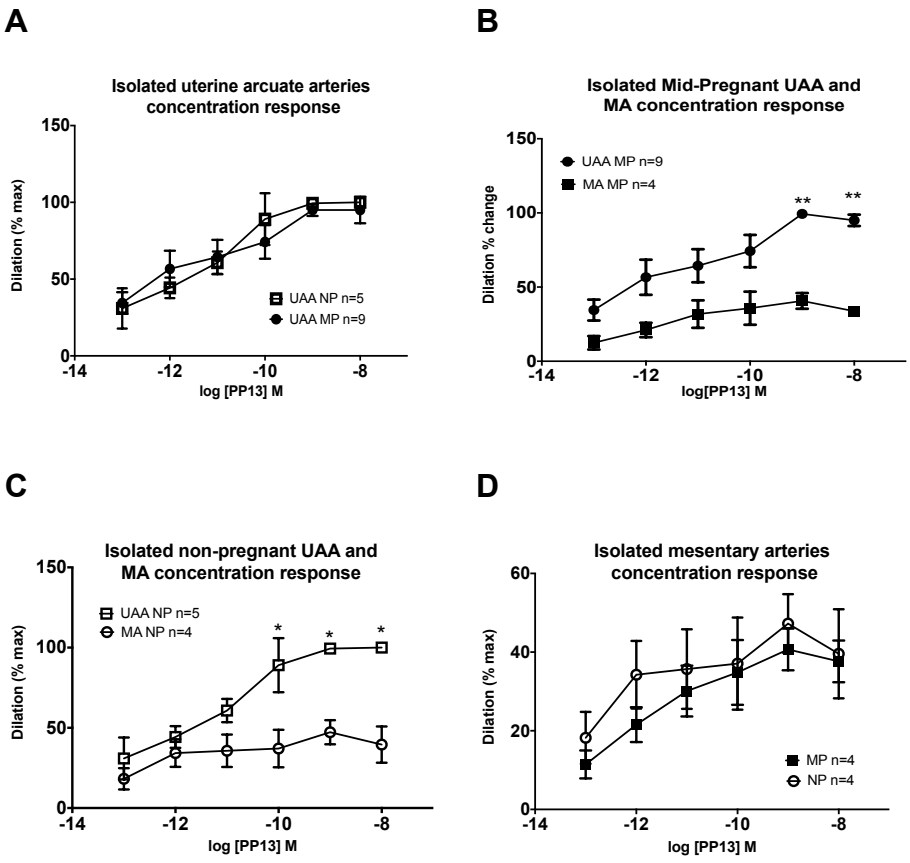

Figure 7: Concentration response of UAAs and MAs isolated from mid-pregnant (MP) and non-pregant (NP) rats.

Figure 7 shows concentration-response to rPP13 on isolated pressurized uterine arcuate arteries and third order mesenteric arteries from mid-pregnant (MP) and non-pregnant (NP) rats. Figure 7A shows responses of UAA to increasing concentrations of PP13. No significant difference (p=0.96) was observed between mid-pregnant and non-pregnant animals. In Figure 7B the mid-pregnant uterine arteries showed greater responses to PP13-induced dilation than the arteries dissected from the mesentery (also mid-pregnant animals), resulting in a significant difference at the highest concentration applied (p=0.08). Figure 7C shows the similar effect of PP13 was observed between artery types (uterine and mesenteric) in non-pregnant animals, resulting in a greater response of uterine arteries to PP13-induced vasodilation (p=0.02). In Figure 7D the effect of the PP13-induced

vasodilation in mesenteric arteries dissected from mid-pregnant and non-pregnant animals was not significant (p=0.34). Data are reported as mean ± SEM, n = number of experiments, *p< 0.05; **p<0.01.

4.2.2 The role of endothelium in uterine artery relaxation caused by PP13

After denuding the endothelium by air perfusion from some arteries dissected from mid-pregnant rats, the effect of PP13 was tested resulting in a complete abolishment of the dilation effect as shown in Figure 8.

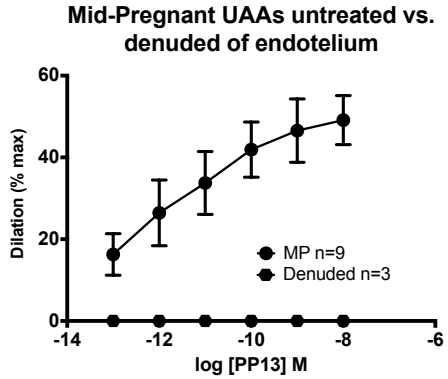

Figure 8: Tracing of endothelium denuded arteries and PP13 control

Arteries dissected from MP animals, denuded of the endothelium did not show any response to the PP13 in comparison to the control group (p<0.0001). Data are reported as mean ± SEM, n = number of experiments.

4.2.3 Mechanism of action

In order to define the mechanism of action within the endothelium, responsible for the PP13-induced vasodilation the effect of inhibiting NO and COX 1 and 2 receptors was examined by pretreating the arteries with L-NAME/LLNA (0.2 mM) and indomethacin (INDO) (10 µM), respectively.

Pretreatment of arteries where NOs and COX1/2 inhibitors were combined together resulted in almost complete abolishment of the PP13-dependent relaxation (p<0.001), while very little effect was observed in some arteries at highest tested concentrations of PP13, Figure 9.

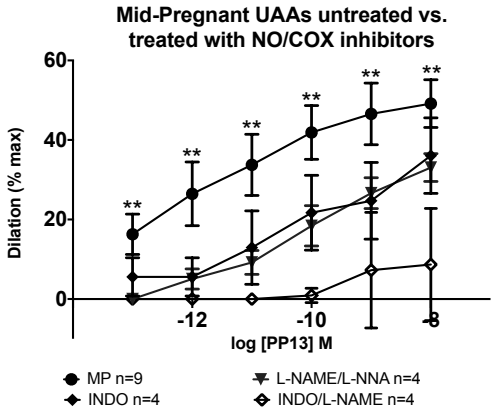

Figure 9: Concentration-response of PP13 after eNOS and COX 1/2 inhibition

Effects after NOS and/or COX 1 and 2 inhibition on PP13-induced vasodilation of uterine arcuate arteries from mid-pregnant rats. Vasodilation was measured relative to untreated vessels. The vasodilation was significantly reduced by pretreatment with INDO (10 μM) or L-NAME/L-NNA (2 × 100 μM), administered separately ($p<0.01$, in both cases) or combined together ($p<0.001$). Data are reported as mean ± SEM, n = number of experiments, $^{**}p < 0.01$.

Pretreatment of the arteries by separately inhibiting the receptors, either by treatment with indomethacine or L-NAME/LNNA, had intermediated effects, suppressing the maximal vasodilation by 28% and 34% respectively (Figure 9). Due to a significant inhibition effect of indomethacine we hypothesized that PP13 vasodilation was mediated by signaling trough prostacyclin (IP) receptor. The inhibition of the IP receptor was, therefore, evaluated by pretreatment of the arteries with 10μM solution of the specific IP receptor antagonist RO1136452.

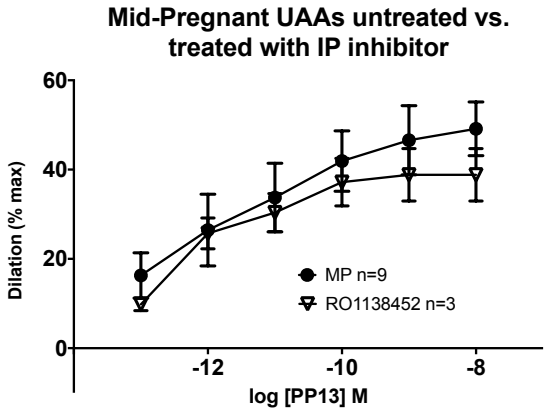

Figure 10: Concentration-response of PP13 after IP receptor inhibition.

PP13-induced vasodilation after pretreatment of arteries with 10μM IP receptor inhibitor RO1138452 is shown in Figure 10. IP receptor inhibition did not affect PP13 vasodilation on uterine arcuate arteries from mid-pregnant rats (p=0.44) (data are reported as mean ± SEM, n = number of experiments).

4.2.4 Endothelial cell Ca^{2+} measurement

As presented in Figure 11, there were no detectable changes in endothelial Ca^{2+} in response to application of 10 mM PP13 (n=3). However, a significant rise of endothelial Ca^{2+} was observed after subsequent administration of ACh that was used as a positive control.

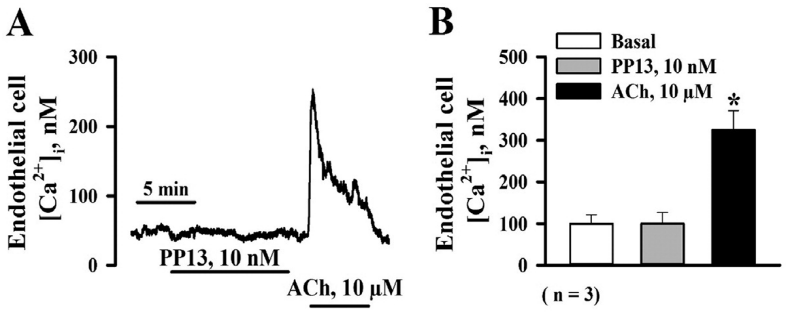

Figure 11: Endothelial calcium tracing. This figure is re-printed with the permission of the publisher and is originally published in (Drobnjak et al., 2017).

Figure 11 A shows the tracing of endothelial calcium (EC $[Ca^{2+}]_i$) in an isolated, pressurized uterine arcuate artery from a mid-pregnant rat showing that a high concentration of PP13 (10 nM) did not alter basal calcium levels, while subsequent addition of ACh (10 µM) induced rapid, significant $[Ca^{2+}]_i$ elevation. In Figure 11 B a summary graph demonstrating lack of changes in EC $[Ca^{2+}]_i$ to application of PP13 is shown. In the same arteries, ACh induced a marked increase in EC $[Ca^{2+}]_i$ (Data are reported as mean ± SEM, n = number of experiments. *$p < 0.05$).

4.4 Preliminary study

4.4.1 The *in vivo* effect of rPP13 and his-PP13 on diameters of MUA and MUV in mid-pregnant rats after inhibition of eNOS via drinking water

eNOs inhibition was carried out by adding L-NAME into the drinking water. After slow release osmotic pumps were inserted (day 8 of gestation) they released their content over seven days, prior to euthanizing the animals on day 15 of gestation. In order to observe the effect of this treatment, MUV and MUA were measured after the specimens had been left in relaxing solution (diltiazem / papaverine) for approx. 40 minutes. All the results are summarized in Table 4.

The group receiving rPP13 without L-NAME in drinking water had signicantly larger MUVs in comparison to all other groups, $p<0.001$. His-PP13 and L-NAME treated group was observed to have significantly larger MUVs compared to saline treated group without L-NAME. Significant difference was observed between MUA diameters when compared between rPP13 group and all other groups, $p<0.001$. His-PP13 and L-NAME treated group and was found to be significantly lower than saline treated groups, both with and without L-NAME addition in drinking water. Finally, significant difference was observed between radial vein diameters where the rPP13 group developed the largest radial veins by far ($p<0.001$). His-PP13 effect was observed to be significantly higher than the saline treatment without L-NAME, but not significantly different from saline group treated with L-NAME in drinking water.

It was also observed that in L-NAME treated groups, both venous and arterial diameters, pup and placental size did not differ regardless if the rPP13 treatment was implemented or not.

4.4.2 The *in vivo* effect of rPP13 and his-PP13 on pup and placenta weights in mid-pregnant rats after inhibition of eNOS via drinking water

After seven-day administration of rPP13, his-PP13 or saline solution via osmotic pumps animals were sacrified and pups and placentas were dissected separately and each weighted and recorded. Animals that received rPP13 without L-NAME in drinking water had heaviest pups and placentas. L-NAME treatment had a biggest effect on fetal growth resulting in all L-NAME treated groups were significantly lower from the saline group without L-NAME treatment. The results are presented in Table 4.

Table 4 Vessel diameters, pups and placental size after *in vivo* treatment with L-NAME.

	Saline / drink water	Saline / drink L-NAME	rPP13 / drink water	rPP13 / drink L-NAME	HisPP13 / drink L-NAME	p
n	24	21	36	24	17	
MUV (µm)	690^{c} [598-782]	949^{b} [774-1,123]	$1{,}158^{a}$ [1075-1242]	993^{b} [906-1079]	971^{b} [855-1086]	<0.001
MUA (µm)	150^{a} [135-165]	121^{b} [105-136]	161^{a} [151-171]	128^{b} [113-143]	113^{b} [98-128]	<0.001
n	66	96	75	59	73	
Radial vein (µm)	340^{c} [303-376]	372^{bc} [355-389]	517^{a} [389-644]	388^{b} [368-409]	384^{b} [367-401]	<0.001
n	44	81	53	43	46	
Pup weight (gr)	3.6^{b} [3.3-3.9]	2.9^{c} [2.5-3.4]	4.6^{a} [4.3-4.8]	3.0^{c} [2.9-3.2]	2.9^{c} [2.8-3.1]	<0.001
Placenta weight (gr)	2.9^{ab} [2.7-3.2]	2.6^{b} [2.3-2.9]	3.2^{a} [3.0-3.3]	2.6^{b} [2.5-2.8]	2.5^{b} [2.3-2.7]	<0.001

Values are reported as Means $\pm$ 95% confidence interval; N- number of measurements; Statistical differences are by One-way ANOVA. A- Groups are significantly higher from others; B-Significantly higher from C but significantly lower from A; C-Significantly lower from A and from B; AB- significantly lower from A and higher from B; BC- significantly lower from B but higher from C.

4.5 Pharmacokinetics of PP13 in NZW rabbits

4.5.1 Intravenous administration

Serum concentration-time profiles of PP13, following single dose I.V. administration of 1.3, 2.6 and 12.8 ng/kg, are shown in Figure 12. The two-compartment model was found to fit best for the majority of animals, and used to calculate pharmacokinetic parameters. The calculated values for AUC were 315 ± 47 pg*h/ml, 495 ± 28 pg*h/ml, and 1360 ± 82 pg*h/ml, for 1.3, 2.6 and 12.8 ng/kg, respectively. When corrected for the dose given, AUC was equivalent to 246, 193 and 106 pg*h/ml, showing that there was a drastic significant reduction in AUC with increased dose ($p=0.004$). Likewise, when volume of distribution was compared, there was a significant difference in the value between the lowest and the highest dose ($p<0.001$). These findings suggest that PP13 binding reached saturation, and thus it appears that the protein might follow non-linear pharmacokinetics.

The half-life following I.V. injection was calculated to be 5.16 ± 0.97 h, 4.42 ± 0.77 h and 4.60 ± 0.09 h for these three doses, respectively. The clearance for these three doses was found to be 14.0 ± 1.9 ml/kg/h, 20.3 ± 3.5 ml/kg/h and, 33.5 ± 2.6 ml/kg/h, respectively.

4.5.2 Subcutaneous administration

Mean serum concentration-time profiles of PP13 following a single dose (12.8 ng/kg) at S.C administration, in comparison to I.V. administration, is presented in Figure 13. The corresponding pharmacokinetic parameters were obtained by using two-compartmental model analysis. Following S.C. administration at single dose of 12.8 ng/kg the $t_{1/2}$, $t_{1/2abs}$, C_{max}, AUC, and Cl were 11.4 ± 1.0 h, 4.5 ± 0.4 h, 130 ± 11 pg/ml, 805 ± 62 pg*h/ml, 60.4 ± 12.5 ml/kg/h, respectively. The bioavailability (F) was calculated based on equivalent I.V. dose to be 57.1 ± 3.2%.

When the study was performed all rabbits were clinically healthy and no side effects were observed after the administration of PP13 in any concentration, neither after I.V. (1.3 ng/kg; 2.6 ng/kg; 12.8 ng/kg), nor after S.C. (12.8 ng/kg) injections. S.C. administration was less invasive and easier to manage.

4.5.3 Comparison of parameters between the routes of administration

The half-life ($t_{1/2}$) was significantly different between the I.V. and S.C groups receiving the equivalent dose (p =0.002). AUC and Cl_{tot} were also found to be significantly different (p<0.001). After the S.C. administration, PP13 reached the C_{max} at 90 min, and the bioavailability was calculated to be about 57%, compared with the same dose administered I.V. The protein was almost completely eliminated from the blood during the next 24 hours in all groups.

The comparison between the different doses (1.3 and 12.8 ng/kg) for I.V. administration revealed a significant difference between AUC/dose (p=0.004) and Vd (p<0.001).

For all pharmacokinetic parameters of PP13 for both I.V. and S.C. administration routes see Table 5.

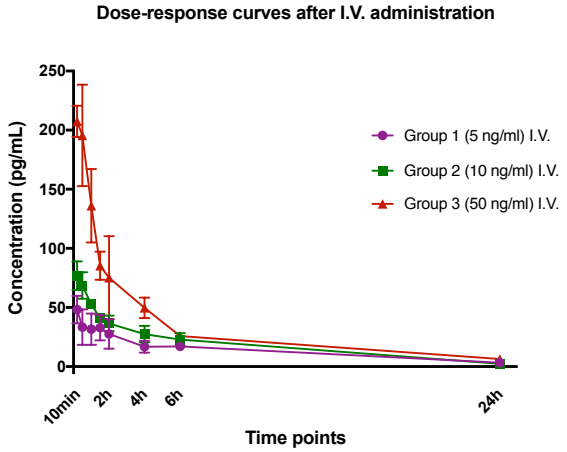

Figure 12 Serum concentration-time curves after using single I.V. administration. This figure is re-printed with the permission of the publisher and is originally published in (Drobnjak et al., 2018).

Group 1: received 5 ng/ml (1.3 ng/kg), n=6; Group 2: received 10 ng/ml (2.6 ng/kg), n=6; Group 3: received 50 ng/ml (12.8 ng/kg), n=6. Data are reported as mean ± SD.

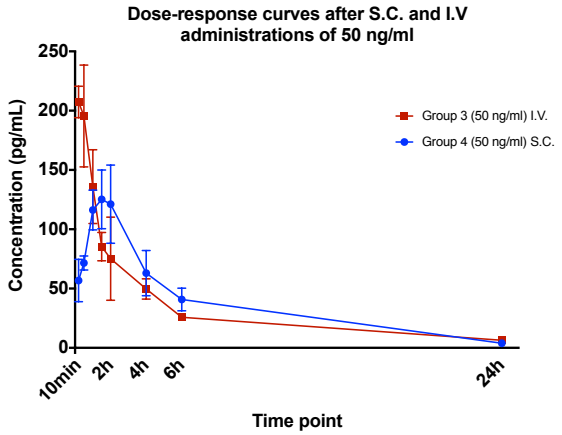

Figure 13: Serum concentration-time curves after single I.V. and S.C. This figure is reprinted with the permission of the publisher and is originally published in (Drobnjak et al., 2018).

The dose-response curve after administration of 50 ng/mL (12.8 ng/kg), n=6. Group 3 received I.V. administration and Group 4 recieved S.C. administration. Data are reported as mean ± SD.

Table 5 Pharmacokinetic parameters for both administration routes based on individual data. This table is re-printed with the permission of the publisher and is originally published in Drobnjak, et al, 2018.

Parameters	Group 1 (1.3 ng/kg I.V.)	Group 2 (2.6 ng/kg I.V)	Group 3 (12.8 ng/kg I.V.)	Group 4 (12.8 ng/kg S.C.)	p (Group 3 vs. group 4)	p (I.V. groups)
$t_{½}$ (half-life) (h)[a]	5.16 ± 0.97	4.42 ± 0.77	4.60 ± 0.09	11.40 ± 1.03	0.002**	0.29
$t_{1/2\ abs}$ (h)	N/A	N/A	N/A	4.50	N/A	N/A
k_e (h^{-1})	0.15 ± 0.07	0.16 ± 0.03	0.15 ± 0.01	0.06 ± 0.02	N/A	N/A
k_{abs} (h^{-1})	N/A	N/A	N/A	1.78 ± 0.34	N/A	N/A
α (h^{-1}) [b]	0.36 ± 0.14	0.50 ± 0.13	0.60 ± 0.23	0.24 ± 0.09	N/A	N/A
β (h^{-1})[b]	0.09 ± 0.03	0.13 ± 0.04	0.04 ± 0.03	0.14 ± 0.04	N/A	N/A
MRT (h)	1.84 ± 0.19	1.23 ± 0.17	0.62 ± 0.18	N/A	N/A	N/A
Vd_{ss} (mL/kg)	25.8 ± 0.0	25.7 ± 0.0	14.3 ± 0.7	N/A	N/A	N/A
$AUC_{0\text{-}24h}$ (pg*h/mL)[a]	315 ± 47	495 ± 28	1360 ± 82	805 ± 62	0.001**[b]	N/A
Cl_{tot} (ml/kg/h)	14.0 ± 1.9	20.3 ± 3.5	33.5 ± 2.6	60.4 ± 12.5	0.001**	0.001**
C_{max} (pg/mL)	58.4 ± 10.9	78.9 ± 9.6	217.0 ± 19.3	130.0 ± 11.4	N/A	N/A
t_{max}(min)	N/A	N/A	N/A	90	N/A	N/A
Vd (L)	110 ± 13	132 ± 20	228 ± 33	1126 ± 498	N/A	0.0001**
Vd_{AUC} (L)	3.05 ± 0.74	3.89 ± 0.93	6.40 ± 1.94	N/A	N/A	N/A
AUC/dose (h/mL)	0.07 ± 0.02	0.05 ± 0.01	0.03 ± 0.004	0.02± 0.003	N/A	0.004*
F%	N/A	N/A	N/A	57.1 ± 3.2	N/A	

$t_{1/2.}$: half-life; $t_{1/2\ abs}$: absorption half-life; ke: elimination constant, k_{abs} absorption rate constant; β: elimination rate constant, α: distribution rate constant, MRT: mean resistance time; Vd_{ss}: Volume of distribution at steady state; AUC: Area under the concentration time curve; Cl_{tot}: Total body clearance, C_{max}: maximal plasma concentration; t_{max}: Time to maximum concentration; V_d : volume of distribution; Vd_{AUC}: Vd corrected for AUC; AUC/dose: AUC corrected for dose; F%: Bioavailability; N/A: not applicable. [a] Harmonic mean. ** p≤0.001 [b] P-value was calculated between two groups where the same concentration of the drug was given.

5 Discussion

This study was designed to evaluate the effect of placental protein 13 (PP13) on uterine vasculature and study its pharmacokinetics. PP13 is a specific placental protein that has been identified as a potential biomarker for prediction of preeclampsia early in gestation. Preeclampsia is a potentially deadly pregnancy-specific complication, for mother and fetus, whose cause is still unknown. The unique expression of PP13 in the human placenta, along with its plausible role as a biomarker for preeclampsia, triggered the research for the evaluation of whether there are grounds for this short polypeptide to be developed as a therapeutic agent for fighting the syndrome. Previous studies performed by our research group at the University of Iceland, which showed the blood pressure reduction caused by PP13 and hypertension to be a main target when dealing with preeclampsia, provided the foundation to proceed with this line of research.

Accordingly, the research was focused on three tasks:

Evaluation of the structural effects of the protein on the uterine vascular system *in vivo.*

Investigation of the *in vitro* mechanism of action of PP13 on the uterine arteries, using a pharmacological approach based on direct inhibition of various pathways.

Determination of the pharmacokinetic profile of PP13 and establishment of its pharmacokinetic parameters.

An attempt was also made to use an *in vivo* pharmacologically based model to test PP13 variants.

5.1 *In vivo* PP13 effect in non-pregnant rats

To evaluate the overall effect on the uterine vasculature in the non-pregnant rat model caused by PP13 during long-term exposure, the design of the study was based on administration of the recombinant polypeptide (rPP13) and its polyhistidine-tag variant (his-PP13) from peristaltic pumps that slowly released their content over a period of seven days. The animals were later sacrificed either seven or thirteen days after surgical implantation of the pumps.

It was found that both uterine arteries and veins were structurally expanded in the group receiving rPP13 compared to the control. Tested at 13 days, 6 days after active release of the protein had stopped, the expansion found by rPP13 group was maintained and even more pronounced for the main uterine vein (MUV). After short-term exposure of 7 days, his-PP13 had expanded the MUV and the radial veins but the effect on the main uterine artery was marginal. After 13 days the effect of his-PP13 was insignificant and only had an effect on MUV.

An increase in the blood flow to the uterus was observed in the long-term administration of rPP13. The measured expansion factor of veins was 1.39-1.84, which was higher compared to the expansion factor of the corresponding size order of the arteries (1.19-1.46). During pregnancy both veins and arteries undergo remodeling to meet the greatly increased demand for supply of oxygen and nutrients to the placenta and the growing fetus (Moser & Huppertz, 2017; Moser et al., 2017). With regard to the rPP13-related expansion and the effect it caused, we found that rPP13 was present in both veins and arteries, although more pronounced in larger veins, and therefore appears to be relevant for preparing the vascular system to accommodate the demand for increased blood flow during pregnancy. The schematic description of the process is presented in Figure 14.

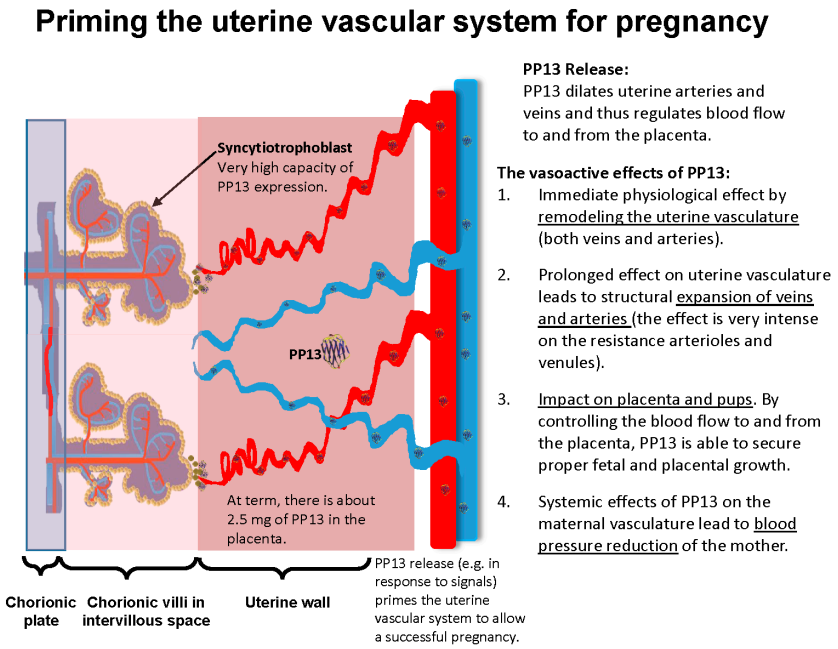

Figure 14 A schematic description integrating the in vivo impact of PP13. The in vivo impact of PP13 on the vascular system shows how PP13 could influence the vascular system and prepare it for its increased role in pregnancy.

These effects need to be evaluated in relation to the manner by which rPP13 reaches the circulation from the installed pumps. By this type of administration it is more likely that the released rPP13 is first absorbed by venules, taking it up to the major veins. Therefore, the greatest observed effect was in the largest veins. rPP13, if absorbed as we estimate, is subsequently transported on to the lungs and to the heart prior to being delivered to the uterus. In the uterus it probably accumulates in the terminal narrow arteries, where it binds to the mediators/receptors and yields the greater effect noted in the experiments. The postulated pathway described above might be associated with the blood flow direction and/ or with the receptor distribution embedded on the endothelial walls of the vessels. Observed differences in the effect may lead to the conclusion that there are many receptors or mediators PP13 is able to bind to that are probably widely distributed in large veins and arterioles. Alternatively, the different effect of PP13 on arteries and veins could also be attributed to a different distribution of the receptors or mediators of PP13 along the different blood vessels.

Further studies are required to assess whether the differential level of PP13 effects on the expansion of veins and arteries is derived by the mode of the polypeptide absorption or if it is a result of the diverse receptor distribution, or maybe both. Regardless of the underlying cause for this differential expansion, the effects discovered here had to be taken into future consideration when designing rPP13 delivery to influence blood flow in various pregnancy models.

Interestingly, the effect of his-PP13 on both arteries and veins was much smaller and in some cases appears to be negligible compared to rPP13. This is likely to be derived from the difference in stability of the two in blood (Sammar et al., 2014). In nature, the native PP13 form of the homo-dimer that can be also formed by rPP13, and this dimer, which is in equilibrium with the monomer, was identified to be more stable in saline solution. In his-PP13 the presence of the his-tag on the N-terminal prohibits the formation of the dimer through S-S bridges. Thus, his-PP13 can only be present as a monomer or in the form tail to head poly oligomer that was found to precipitate and became biologically inactive (Sammar et al., 2014; Than et al., 2004). This analysis corresponds to the findings evaluated six days after the active compound is no longer released, when no obvious structural effect of his-PP13 could be determined. Therefore the his-PP13 effects disappear more rapidly, resulting in minor or even insufficient vessel expansion six days after the active drug release is completed. This interpretation accounts for the greater long-term effect of rPP13 compared to his-PP13, although additional monitoring of the clearance from blood after continuous infusion is required to cast light on this matter.

5.2 PP13-induced vasodilation and mechanism of action

Here, the *in vitro* study of rPP13 was conducted using a pharmacological approach to explore the underlying pathways that are involved in the polypeptide effect on blood vessels. The effect was measured on isolated arteries using various agents that act as antagonists of various pathways that are known to be involved in arterial expansion. The main pathways involved are the eNOS and AA pathways and were blocked by specific antagonists on isolated rat arteries prior to rPP13 exposure. The experiments were done on uterine and mesenteric arteries that were isolated from both pregnant and non-pregnant rat models. To study the tissue location of the mediators involved in the rPP13 effect, experiments in endothelium-denuded arteries were performed on uterine arteries from pregnant animals.

In the work presented in the thesis we observed that rPP13 is a potent vasodilator in rats. The *in vitro* studies with isolated arteries have demonstrated a dose-response related expansion with rPP13 compared to the saline controls, in both uterine and systemic (mesenteric) arteries. The vasodilation induced by rPP13 decreased significantly when arteries were pre-treated in solutions containing inhibitors of physiologically active vasodilators, such as L-NAME/L-NNA (eNOS, specific inhibitors) and indomethacin (COX 1 and 2 inhibitor). It is known that eNOS and COX 1 and 2 pathways are present in the endothelial wall of the vessels. The present study proved that when both pathways are blocked at the same time, the effect of rPP13 becomes marginal. On the other hand, complete loss of the response was observed after some arteries underwent endothelial removal by air perfusion. The PP13-induced vasodilation did not include the prostacyclin receptor (IP), which is also present in the endothelium, and was not associated with an increase in endothelial flux of calcium (EC).

During the normal course of pregnancy, there is a process of first trimester vasodilation that occurs parallel to the expansion and remodeling of uterine arteries. The lack of such expansion or its partial or non-homogeneous development was identified as one of the origins for subsequent development of preeclampsia, especially of the sub-type of the disorders that develop before term delivery. This impaired remodeling can be evaluated by a larger mean arterial blood pressure (MAP) (approximately 10 mmHg) that can be determined during gestational weeks 11-13, which is 20-30 weeks prior to the development of the clinical symptoms of preeclampsia (L. C. Poon & Nicolaides, 2014). Although the higher MAP is way below values that have clinical significance, it is used as one of the strongest markers for early prediction of the later development of hypertension disorders in pregnancy (L. C. Poon & Nicolaides, 2014; L. C. Y. Poon, Kametas, Maiz, Akolekar, & Nicolaides, 2009). Therefore vasodilation is considered to be one of the earliest hallmarks of successful pregnancy and its impaired development is a main indication of a suboptimal pregnancy progression. Several studies have shown that first trimester artery vasodilation is associated with increased levels of prostacyclins, prostaglandins and eNOS substances that are important in the regulation of both the systemic and the uteroplacental blood flow (Conrad et al., 1993; Sladek, Magness, & Conrad, 1997). The results of this study imply that rPP13 activates these pathways and is therefore a trigger for the main process involved in the adjustment of the vascular system to the demand of blood flow increase to the uterus and fetus.

The mechanism of action of PP13 through both eNOS and AA pathways in the endothelium, independent of the EC, is presented in Figure 15.

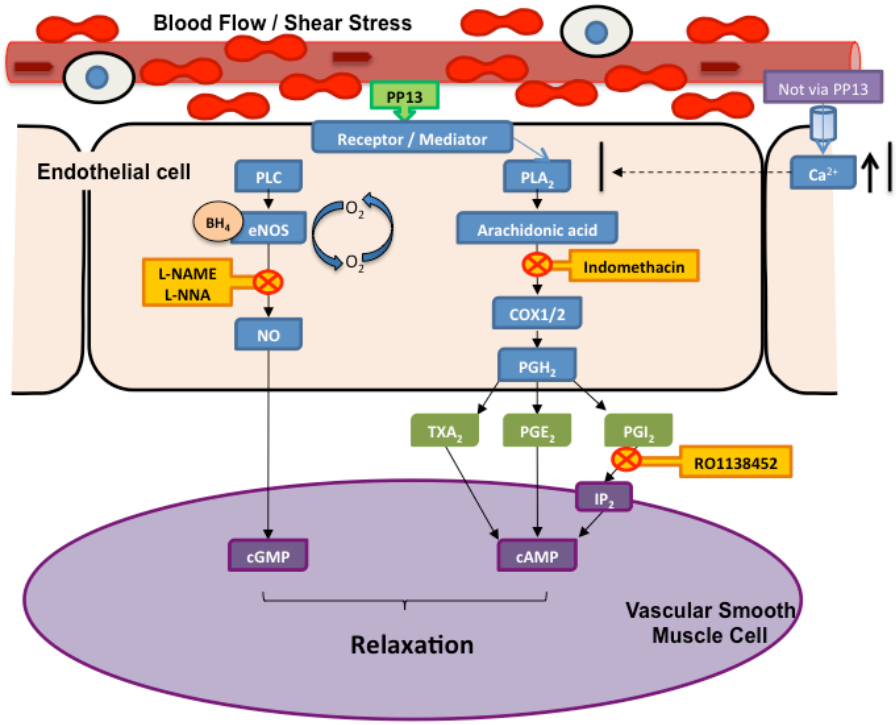

Figure 15: Signaling pathways of PP13-induced vasodilation. Figure based on (Drobnjak et al., 2017)

Endothelium-dependent vascular relaxation in response to placental protein 13 (PP13) occurs via undefined mediator(s) whose activation results in stimulation of nitric oxide (NO) production through eNOS, as well as the metabolism of arachidonic acid (AA) to prostaglandins (PG) through the COX 1 and 2 enzymes.

In blood vessels NO and PG normally elicit relaxation of vascular smooth muscle cells via cGMP and cAMP respectively. The finding that PP13 does not alter endothelial systolic Ca^{2+} levels, and that the IP receptor may not be involved in the mechanism of action, suggests that another prostaglandin, such as prostaglandin E2 (PGE2), might be responsible. In previous studies, PP13 has been shown to have high affinity for sugar residues on the annexin IIA receptor, the beta- and gamma actin of the syncytiotrophoblasts and decidual and erythrocyte B group antigens (Than et al., 2014; Than et al.,

1999). PP13 also interacts with phospholipase A as well as releasing PGs from cultured trophoblasts (Than et al., 2014; Than et al., 2004; Than et al., 1999). The potent vasodilatory effect of rPP13 and its activation on both NO and PG-signaling pathways supports the previously reported effects on reducing blood pressure (Gizurarson et al., 2015). The limitation of this study is that the evolution of the LGALS13, the gene encoding for PP13, starts from primates and thus the polypeptide is not expressed by the rat model used in the study. Therefore, further studies on isolated human uterine arteries are required in order to establish the relevance to human pregnancy. However, the mechanisms involved in blood pressure regulation and vasodilation between rats and humans are relatively similar when endothelial function is involved. Therefore, the results from this study provide the mechanistic basis for PP13-induced vasodilation of resistance arteries that is specific to endothelial pathways.

5.3 *In vivo* effect on pregnant rats after inhibition of eNOS production via drinking water

To establish the *in vivo* effect of PP13 on uterine vasculature and the sizeof pups and placentas after inhibition of one of the main vasodilation pathways the study was based on adding L-NAME into the drinking water of three study groups. On day 8 of gestation, the pregnant animals received peristaltic osmotic pumps that contained 127 ng of rPP13, his-PP13 or saline. After seven-day active release of the protein or saline solution, all the animals were sacrificed (day 15 of gestation).

It was found that rPP13 wihtout additional treatment with L-NAME had significant effect in respect to vessel diameters of the main uterine vein (MUV) and artery (MUA), as well as in radial uterine veins (RUV). Weights of pups and placentas were also significantly higher than in other groups measured. The effect decreased significantly in the groups receiving L-NAME. These findings confirm the previously published data on rPP13 that showed that it causes an increase in the placenta and pup weight and expands venous size (Gizurarson et al., 2015). The effect of his-PP13 was only measured in animals that received L-NAME in drinking water.

The effect observed between all three groups receiving L-NAME in drinking water was insignificant in all measured parameters. The size of pups and placentas as well as the MUA diameters were significantly lower when compared to the saline control group. However, MUV and radial vein diameters in all three L-NAME treated groups were significantly wider when

compared to the saline control group. The majority of studies mediate their focus on arterial remodeling in pregnancy and little is known about the venous role in the remodeling process during pregnancy. eNOs activity in human IUGR placentas has been shown to be lower that in normal pregnancies (Krause et al., 2013), and also that eNOS is the key mediator in the uterine remodeling process during pregnancy (Ko, Mandala, John, Gelinne, & Osol, 2018; Nelson et al., 2000; Tronc et al., 2000; Tuttle et al., 2001; van der Heijden et al., 2005). The effect we observed might be explained, as some studies have hypothesized, by eNOS impairment triggering the activation of the arachidonic acid pathway (Habib et al., 1993; Hajjar, Jacovina, & Chacko, 1994; Koller, Sun, Messina, & Kaley, 1993), and therefore a rise in cyclooxygenase. As we have shown in our previous study, PP13 can mediate its effect via both eNOS and the arachidonic acid pathway (Drobnjak et al., 2017), however, further inverstigation needs to be carried out in order to find out if inhibition of one pathway triggers the other as a back-up mechanism.

After inhibitionl eNOS endothelial production, remodeling of veins still occurred. This can be explained by possible venoarterial cross communication that needs to be further studied (Celia & Osol, 2002, 2005; Ko et al., 2018).

The findings in this preliminary study have confirmed that the eNOS pathway is important in mediating the PP13 effect or uterine vessel expansive remodeling. To our knowledge no studies have been carried out on venous response to either short or long-term eNOS inhibition.

5.4 Pharmacokinetics of PP13 in rabbits

In order to establish the potential clinical use of the PP13, an understanding of its pharmacokinetic behavior is important, therefore the study was designed to investigate the pharmacokinetic profile of rPP13 in rabbits via two administration routes. In this study the half-life of rPP13 was found to be relatively short, or 6-12 hours regardless of the route of administration. The dose-corrected pharmacokinetic parameters varied between 1.3 and 12.8 ng/kg as regards I.V. administration for both AUC and Vd. This was unusual and might be explained by blood-pressure-lowering and vasodilatory effect of the protein that binds to the endothelial receptors related to eNOs and prostaglandins (Drobnjak et al., 2017; Gizurarson et al., 2013). As PP13 is a potent vasodilator (EC_{50} of approx. 1pM) the effect seems to be reversible effect after single dose administration, as the *in vitro* studies showed.

However, continuous supplementation of rPP13 prolongs the effect of the drug. This leads to another factor that might be involved, which is that the level of free PP13 determined in serum from rabbits may vary due to its high affinity to red blood cells (Than et al., 2011) or its binding to tissue mediators (Than et al., 2014). PP13 might then be continuously released in very low amounts from tissues and mediators that continue to be present in the system such slow release supports the long-term effect of the protein.

The study was conducted on rabbits that, like rats, lack the LGLAS13 gene. Therefore evaluation of the pharmacokinetic profile in additional species that are more similar to humans is required. The overall antigen binding may differ between species and the pharmacokinetic profile described here may vary between animal models used. In addition, testing was performed using non-pregnant rabbits and evaluation in pregnant animals has to be made as well to verify the duration and total amount required. The protein absorption of the lymphatic vessels was not evaluated and that might have affected the results. In order to establish the clinical use of PP13 it is important to understand its pharmacokinetic behavior, and when it comes to administration to pregnant women, if PP13 is going to be developed and tried as a drug candidate the duration of administration and total amount of the protein need to be tested in greater detail.

5.5 Could PP13 serve as a drug to prevent preeclampsia?

In this thesis it was demonstrated that PP13 expands arteries and veins of both pregnant and non-pregnant rats. Thus it can be considered as an agent that prepares the vascular system for the physiological changes associated with pregnancy. As a galectin, PP13 generates its effect by binding to sugar residues on glycoproteins and glycolipids through specific oligosaccharide structures. This binding of PP13 has already been demonstrated to certain oligosaccharides on white blood cell membranes, and to the ABO blood group glycoproteins (Than et al., 2009). Galectin binding to sugar residues is highly dependent on the 3D structure of other glycoproteins and their respective sugar residue composition (Hernandez & Baum, 2002; Lie et al., 1998; Than et al., 2014; Than et al., 2009). PP13 has the highest affinity to oligosaccharides like N-acetyl-lactosamine, mannose and N-acetyl-galactosamine, which are quite common in many proteins (Than et al., 2004). Accordingly, PP13 has no receptor entity in the classical sense but it interacts with a large variety of sugar residues, and thus the search for a specific receptor appears counter-productive. On the other hand, unlike other galectins, PP13 is solely expressed by the placenta of humans and of some

anthropoid apes (Than et al., 2009), and is found in the cytoplasm and brush border membrane of the syncytiotrophoblasts. Our findings in the rat model indicate that the pregnancy-related vascular system reacts to the protein, although the animal genome does not express PP13.

The amount of PP13 present in the blood of pregnant women in the first trimester, as determined in previous studies (Akolekar et al., 2009; Chafetz et al., 2007; Huppertz et al., 2008; Odibo et al., 2011; Spencer et al., 2007) appears to be relevant to the measured dose-response curve measured in (Drobnjak et al., 2017) (Paper I), and to the amount required for vessel expansion. Studies revealed that the determined level is related to disorder severity, genetics and ethnicity (Akolekar et al., 2009; Chafetz et al., 2007; Odibo et al., 2011; Smarason, Sargent, & Redman, 1996; Spencer et al., 2007).

During the active stage of the disorder, when the clinical symptoms emerge, the level of PP13 is higher (Huppertz et al., 2008). Increased PP13 release was determined in pathological conditions and in hypoxia that is associated with shedding of necrotic bodies off the syncytiotrophoblast, bringing a very large amount of PP13 to the maternal circulation (N. G. Than et al., 2008), (Smarason et al., 1996). The question is therefore whether the increase of PP13 during the onset of the disorder is part of the pathological process or whether it is a mechanism developed by the body to fight against preeclampsia, by providing more PP13 in order to enable the vessel expansion as a feedback process to fight hypertension an effect that might help in some way in the containment of the disorder progression.

In this thesis, evidence was collected to show that the protein has a substantial impact on the uteroplacental vascular system during pregnancy and that its effect is related to vasodilation of arteries and veins. Therefore the protein could be linked to the remodeling of rat uterine vessels remodeling during pregnancy as shown in a series of studies (Drobnjak et al., 2017; Gizurarson et al., 2015; Huppertz, Meiri, Gizurarson, Osol, & Sammar, 2013; Sammar et al., 2014). The stronger effect was observed in veins, with the PP13-induced effect being partly mediated by the eNOS pathway in the endothelium. This was supported by studies showing that the vasodilative responsiveness of veins in pregnant rat models is primarily mediated by an NO-dependent mechanism (C. W. Jones et al., 2009).

Thus PP13 may favor hypotension during pregnancy by causing vasodilation, which is followed by an increase in uteroplacental blood flow. PP13-induced relaxation is abolished completely in the absence of the

endothelium, supporting an entirely endothelium-dependent mechanism of action. This indicates that a therapeutic I.V. administration route would allow PP13 to access its target tissue directly. In comparison, when PP13 is given by S.C. application its bioavailability is 57%. Thus, a higher amount of the protein would need to be given. However, the absorption kinetics of proteins following S.C. administration and the distribution to tissues involves a complex interaction of multiple processes that include lymphatic vessels, interstitial fluid transport, and both specific and non-specific binding to receptors and cell membranes, while the structure of the skin and subcutaneous space might have an impact on the absorption as well (Kagan et al., 2007; Richter, Bhansali, & Morris, 2012). The half-life of the protein observed in pharmacokinetic study needs to be examined further in the pregnant model and additional species, but is consistent with previous studies that have shown the protein to be stable in human serum samples for seven hours or for four days if kept at 4°C (Burger et al., 2004).

If potential benefits of PP13 were to be further explored by *in vitro* and *in vivo* studies, then this would be done based on the hypothesis that replenishment of PP13 could be used to treat or prevent preeclampsia. Treatment with L-NAME / L-NNA to inhibit NO production is only one of the ways to demonstrate hypertension in pregnancy in animal models. One of the widely used preeclampsia models in pregnant rats is the reduced perfusion pressure model (RUPP), made by my clipping the ovarian arteries and abdominal aorta with silver clips. As a result, the RUPP model mimics the physiological symptoms of human disorder, including hypertension, proteinuria and fetal growth restriction (Fushima et al., 2016). Another preeclampsia model is the transgenic mouse model that overexpresses the STOX1 gene, the susceptibility gene found in a Dutch population in daughters of mothers who suffered preeclampsia (van Dijk et al., 2005). This animal model mimics preeclamptic physiological factors such as hypertension, proteinuria, increased sEng levels, and sFlt1 levels. In placentas of the transgenic STOX1 mice model, alterations of the mitochondrial function are seen that could trigger the disorder (Doridot et al., 2014). Therefore, the effect of PP13 on the aforementioned animal models might give invaluable information on the regulation of physiological aspects caused by the disorder. However, these models vary in their reproducibility and characterization and none of them mirror the exact pathophysiology of human disorder, in particular not the impairment in trophoblast invasion.

Therefore, even though animal models have contributed to our understanding of preeclampsia and facilitated the testing of PP13, no ideal

animal model exists. Thus the extrapolation of these data for the human condition must be done with caution. In order to enter clinical trials, the potential benefits of PP13 replenishment can only be verified if evidence of a positive effect is satisfactory. Preferably, women would first be screened for the risk of development of PE, possibly by measuring insufficient serum concentrations of PP13 or in combination with other screening technologies and biomarkers discussed above. This could also involve heritability and genetic markers (van Dijk et al., 2005). If PP13 is to be accepted as a therapeutic agent, the major challenge is to verify that the protein has no negative effects on the growing fetus. This might be achieved by studying PP13 in the placenta in different species and how its development has evolved from animals to humans. Many challenges lie ahead if PP13 is goingto be developed as a treatment for preeclampsia.

Studies performed in the past have shown that PP13 hardly reaches the fetal arteries, which seem to be protected from PP13 penetration (Sammar et al, 2011). Many challenges can be anticipated in the systemic examination of the therapeutic potential of PP13 in order to evaluate the drug as a treatment for preeclampsia – a disorder that, in spite of many decades of study, has still not been identified with an effective treatment.

6 Conclusions

This study provides a significant contribution to the hypothesis that PP13 replenishment may be used for fighting preeclampsia in a prophylactic manner. It is shown that PP13 is a potent vasodilator, mediating its effect calcium-independently through the eNOS and AA pathways in the vessel endothelium. The effects on mediating a uterine blood flow increase were observed after slow release of the protein over a period of seven days, which in some vessels lasted for an additional six days, suggesting possible effects of PP13 as a preconditioning agent to the uterine vascular system in early pregnancy. Pharmacokinetic profiles of the protein suggest that the circulating levels of free PP13 are only a fraction of the amount secreted by the placenta, and that a single dose of the protein is present in serum for several hours. The rest remains bound to red blood cells, the endothelial layer and possibly other maternal organs. The mechanism and the target of action of PP13´s stimulating effect to uterine artery and vein diameters might explain heavier rat pups. It is important to gain better understanding of uterine blood flow regulation in order to deliver better and more effective treatment of preeclampsia.

If benefits of PP13 are further validated, then the replenishment of PP13 could be used to prevent preeclampsia. This study has some limitations since there is no production of PP13 in rats as it is only expressed in higher primates (Than et al., 2009). Yet it appears that the underlying pathway may already exist in lower mammals and thus its potential therapeutic use seems reasonable, if additional preclinical evidence is collected.

Safety studies still need to be undertaken, as PP13 was found not to reach the fetal compartment and only small residues could be detected in the umbilical arteries (Sammar et al., 2011). Therefore, additional toxicological studies are required to rule out such effects. The results obtained in this study provide a foundation for continuing with research on PP13 as the potential preventive therapeutic agent of preeclampsia in those at risk of developing the disease. Many challenges lie ahead on this path as the research is still in an early phase of development and many questions remain unanswered.

7 Future Perspectives

Despite extensive research in the field of obstetrical hypertensive disorders and preeclampsia, the pathogenesis has not been fully elucidated. Although identification of useful biomarkers and preventive strategies has been the focus of the last decade, no fully effective treatment has yet been implemented concerning therapeutic use. In particular, vascular remodeling and uteroplacental circulation in early pregnancy are believed to be involved in many pregnancy-related complications.

Our findings suggest that PP13 plays an important role through endothelial function in maternal uterine blood flow adaptaion. Based on the results presented in this thesis, further research ought to be conducted in order to explore the effects of PP13 in human preeclampsia. This includes 1) a study on veins, both direct mechanism of action and venoarterial communication; 2) different animal models (e.g. STOX1 transgenic mice, RUPP rat model); 3) different species (e.g. sheep and primates) and 4) human arteries (isolated uterine arteries). If positive and promising results are obtained from these studies, PP13 might enter Phase I clinical trials in humans.

While preeclampsia is associated not only with inadequate implantation of the placenta but also with systemic maternal vascular dysfunction, resulting in increased risk of cardiovascular diseases later in life, additional studies on the long-term effect of PP13 are needed.

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