The effects of placental protein 13 and its mutant *in vivo* in gravid rats

Thesis for the degree of Master of Science
University of Iceland
Faculty of Pharmaceutical Sciences
School of Health Sciences

HÁSKÓLI ÍSLANDS

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Thesis for the degree of Master of Pharmaceutical Science

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October 2013

Áhrif fylgjupróteins 13 og stökkbreyttu fylgjupróteins 13 *in vivo* í þunguðum rottum

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Meistararitgerð í lyfjavísindum

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Lyfjafræðideild

Heilbrigðisvísindasvið Háskóla Íslands

Október 2013

Printed by Háskólaprent
Reykjavík, Iceland 2013

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ABSTRACT

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Pre-eclampsia is the second most common cause for maternal mortality in the world and causes the death of nearly 46,000 women yearly. It has been connected to maternal morbidity, as well as numerous cardiovascular diseases later in life for the mother. Although much time and resources have been spent on research of the cause and therapy for pre-eclampsia, the pathology of pre-eclampsia still remains a mystery. The only current treatment of pre-eclampsia is premature delivery of the baby and the placenta.

PP13 is a recently discovered placental protein that shows promise as a predictive biomarker for pre-eclampsia. PP13 levels are lower in the serum during the first trimester in women that develop pre-eclampsia later in pregnancy. The effects of this protein during pregnancy are unknown, but the serum levels of the protein increase at the same time that the symptoms of pre-eclampsia emerge.

Our theory is that PP13 has effects on the blood pressure in pregnant women and that it plays a vital part for women with pre-eclampsia. As an attempt to understand the behaviour of the protein, *in vivo* effects of PP13 and a mutated form of it on blood pressure were researched on foetuses and uterine arteries in 21 gravid rats.

Our results show that PP13 has a lowering effect on blood pressure, increases placental- and pup weight and increases uterine artery width in comparison with a group that was treated with saline. The results also show that PP13 can cause dilation in an artery *in vitro.* In contrast the PP13 mutant caused limited effect on blood pressure but decreased the pup and placental weights and had only a little effect on dilation in the uterine arteries.

This research gives an idea about the effects of PP13 during pregnancy even though there are many questions to be addressed.

ÁGRIP

Áhrif fylgjupróteins 13 og stökkbreyttu fylgjupróteini 13 *in vivo* í þunguðum rottum

Meðgöngueitrun en önnur algengasta orsök fyrir andláti á meðgöngu og veldur dauða um 46 þúsund kvenna árlega og hefur verið tengd við hjarta og æðasjúkdóma seinna í lífi fyrir móður. Þrátt fyrir að sjúkdómurinn hafi verið töluvert rannsakaður þá er orsök meðgöngueitrunar enn óþekkt og engin meðferð hefur komið í veg fyrir eða hindrað framgöngu sjúkdómsins. Eina meðferðin í dag er að fæða barn og fylgju fyrir tímann.

Fylgjuprótein 13 (PP13) er nýlega greint meðgönguprótein sem lofar góðu sem greiningarmerki fyrir konur sem eru í hættu á að fá meðgöngueitrun. Í fyrsta þriðjungi meðgöngu er mikil lækkun á próteininu í blóði kvenna sem fá meðgöngueitrun seinna á meðgöngunni.. Áhrif PP13 á meðan meðgöngu stendur eru óþekkt en magn próteinsins hækkar langt yfir eðlilegt mörk á síðasta þriðjungi meðgöngu, á sama tíma og helstu einkenni meðgöngueitrunar koma fram.

Kenning okkar er sú að PP13 hafi áhrif á blóðþrýsting í óléttum konum og spili mikilvægt hlutverk í meðgöngueitrun. Hlutverk þessa verkefnis er að reyna að skilja hegðun próteinsins og stökkbreytts afbrigði þess betur með því að skoða áhrif þeirra í 21 þungaðri rottu.

Niðurstöður okkar sýna annars vegar að PP13 lækkaði blóðþrýsting, jók þyngd hvolpa og fylgju ásamt því að legæðar voru marktækt breiðari í meðferðarhóp. Einnig sáum við að próteinið hafði æðaslakandi áhrif *in vitro*. Hinsvegar hafði stökkbreytta próteinið ekki mikil áhrif á blóðþrýsting, lækkaði fæðingarþyngd hvolpa svo um munaði ásamt því að hafa aðeins lítil áhrif á útvíkkun legæða.

Þessi rannsókn gefur hugmynd um verkun PP13 á meðgöngu þó svo að mörgum spurningum sé enn ósvarað um verkun þess.

LIST OF ABBREVIATIONS

Haemolysis, elevated liver enzymes and low palate count syndrome	HELLP
Disseminated intravascular coagulation	DIC
Accreditation of Laboratory Animal Care	AAALAC
Monoclonal anti-body	mAb
Mean arterial pressure	MAP
Placental protein 13	PP13
Analysis of variance	ANOVA
Area under curve	AUC
Prostaglandin F2 alpha	$PGF_{2\alpha}$
Prostacyclin	PGI_2
Thromboxane	TXA_2

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1. INTRODUCTION

1.1 Pre-eclampsia

Pre-eclampsia and subsequent complications are the second most common cause for maternal mortality in the world, surpassed only by HIV. Causing 16% of maternal deaths yearly, pre-eclampsia affects 2-8% pregnancies. There are differences between areas with the most devastating effects in third world countries where access to health-care is limited (Duley, 2009; Lipstein, Lee, & Crupi, 2003; Vatten & Skjaerven, 2004).

The outcome of pre-eclampsia has been improving with time, and the prevalence of pre-eclampsia has been rather steady for the last decades, with a slight increase in the past decades according to a study where 626,272 births were evaluated over a period of 25 years in Norway. By using meta-analysis, this study showed an increase in numbers of mothers that develop pre-eclampsia during pregnancy and the overall risk of pre-eclampsia rise from 2,6% relative risk in 1967-1974 to 3,6% in 2008. This increase might be explained by change in the definition of pre-eclampsia, increased follow up during pregnancy, advancements in data collection and lifestyle changes (Irgens, Reisaeter, Irgens, & Lie, 2001b). Prevalence of pre-eclampsia differs between populations, and is not only connected to the maternal ethnicity but paternal factors as well. Prevalence of pre-eclampsia is highest in black maternity but lowest in Asian paternity (Caughey, Stotland, Washington, & Escobar, 2005; MacKay, Berg, & Atrash, 2001). Further analysis has shown that there are differences between subgroups of ethnicities as well, women in the Asian subgroup from the Philippines had higher prevalence of pre-eclampsia compared to women from China and Japan (Rao, Cheng, & Caughey, 2006). Analyses of other ethnical differences have not been published. As mentioned paternal genetics have an impact factor as well, males that have fathered a pre-eclamptic pregnancy are more likely to father another one (Lie et al., 1998) and mothers that were the product of a pre-eclamptic pregnancy are more likely to develop pre-eclampsia during their own pregnancies (Mogren, Hogberg, Winkvist, & Stenlund, 1999). This gives ground to assume that the cause of pre-eclampsia is somewhat connected to genetic traits.

1.1.1 Definition of Pre-eclampsia

Diagnosis of pre-eclampsia is confirmed by two factors, an elevated blood pressure and proteinuria. Elevated blood pressure is defined as diastolic blood pressure of 90 mmHg or higher, two measurements in a row and at least 4 hours apart. There is a great importance in measuring blood pressure correctly since incorrect measurements can lead to misdiagnosis. Proteinuria is defined as 0.3 g in a 24 hour urine collection or 2+ on a dipstick method (Milne et al., 2005b; Steegers, von Dadelszen, Duvekot, & Pijnenborg, 2010). Serum protein loss by proteinuria can affect a number of organs, and usually the most vital ones such as the liver, brain, kidneys and the lungs. The seriousness of the symptoms connected to proteinuria can differ between individuals and is connected to the amount of protein loss. Pre-eclampsia can develop into haemolysis, elevated liver enzymes and low palate count syndrome (HELLP) if the protein loss through the kidneys continues, becomes greater or if the liver of the mother fails to supplement this loss. Other forms of the disease include severe pre-eclampsia and eclampsia (Walker, 2000).

Pre-eclampsia develops into severe pre-eclampsia in 2.5% of pre-eclamptic pregnancies and is characterized by systolic blood pressure of 160 mmHg or higher and diastolic blood pressure of 110 mmHg or higher, 3+ on a protein dipstick method in two urine samples taken at a random time intervals. Other symptoms include oliguria of less than 500mL in 24 hours, cerebral or visual disturbances, pulmonary oedema or cyanosis, epigastric or right upper-quadrant pain, impaired liver function, thrombocytopenia and/or foetal growth restriction ("Diagnosis and management of preeclampsia and eclampsia," 2002; Stamilio, Sehdev, Morgan, Propert, & Macones, 2000).

Eclampsia is another form of the disease and is sometimes preceded by HELLP, and is mainly characterized by eclamptic convulsions. The diagnostic criteria for eclampsia are convulsions during pregnancy or within 10 days postpartum and two of the following syndromes: hypertension, proteinuria, thrombocytopenia and/or raised liver enzymes (Zwart et al., 2008).

1.1.2 Causes of pre-eclampsia

The direct cause of pre-eclampsia is yet to be discovered and there are still many hypotheses about the origins and pathways of the disease. Common theories include genetic, immunogenic and environmental causes (Redman, Sacks, & Sargent, 1999; Roberts & Cooper, 2001; B. M. Sibai et al., 1997). Poor placentation is thought to be one of the key factors in pre-eclampsia. In normal pregnancy the placental bed is invaded by syncytotrophoblasts that penetrate the myometrium, along with spiral arteries. The spiral arteries are later transformed to large arteries that can support the increased blood flow that the placenta requires, especially during the third trimester. In pre-eclamptic pregnancies this syncytotrophoblast invasion seems to be insufficient and the remodelling of the spiral arteries is not extensive enough, leading to a decrease in blood flow through the placenta in the third trimester (Zhou, Damsky, & Fisher, 1997). What causes this insufficiency of invasion of the syncytotrophoblasts is under debate but common theories are that the immune response from the mother hinders the syncytotrophoblast invasion due to too much inhibition of natural killer cells, or that lack or over expression of placental proteins causes hypoxia of the placenta which in turn causes reduced spiral artery invasion (Hiby et al., 2004).

The factors most commonly linked to the increased risk of developing pre-eclampsia include diabetes (type 1 and gestational diabetes), multiple gestation, high maternal weight (BMI >29), null parity, previous chronic hypertension, kidney disorders and previous history of pre-eclampsia. Smoking, however, has on more than one occasion been connected to lowered risk of pre-eclampsia (Conde-Agudelo & Belizan, 2000; Odegard, Vatten, Nilsen, Salvesen, & Austgulen, 2000; Ros, Cnattingius, & Lipworth, 1998). There are theories that vitamin D deficiency might be the cause for pre-eclampsia due to its effects on the immune system, which might explain racial differences in pre-eclampsia since black women are more prone to be vitamin D deficient and are more likely to suffer from pre-eclampsia (Bodnar et al., 2007). Currently no published research is available that connects vitamin D supplementation to decreased prevalence of pre-eclampsia.

1.1.3 Effects of pre-eclampsia

Although pre-eclampsia has always been labelled as a pregnancy disease there is emerging evidence that pre-eclampsia and eclampsia are connected to a variety of diseases later in life, for both mother and child. In women that are affected by pre-eclampsia it has been shown that the disease can increase prevalence of end stage renal disease (Vikse, Irgens, Leivestad, Skjaerven, & Iversen, 2008), venous thromboembolism disease later in life (van Walraven, 2003), cause chronic hypertension after birth (B. M. Sibai, Elnazer, & Gonzalezruiz, 1986) and lead to increased mortality due to ischemic heart diseases later in life (Jonsdottir, Arngrimsson, Geirsson, Sigvaldason, & Sigfusson, 1995). It has also been connected to an elevated risk of ischemic heart disease later in life in individuals that are the product of a pre-eclamptic pregnancy. Pre-eclampsia can cause preterm birth in 15-67% of affected pregnancies and for comparison, hypertensive disorders are the cause of at least 12% of preterm deliveries worldwide (B. Sibai, Dekker, & Kupferminc, 2005; Slattery & Morrison, 2002; Smith, Pell, & Walsh, 2001; Vatten & Skjaerven, 2004; Wilson et al., 2003). Multiple diseases and effects later in life have been connected to being the product of preterm birth such as insulin resistance, cardiovascular diseases and in general higher mortality due to cardiovascular disease in later years. (Eriksson et al., 1999; Hofman et al., 2004; Irgens, Reisaeter, Irgens, & Lie, 2001a).

1.1.4 Serious conditions following pre-eclampsia

Even though pre-eclampsia is neither acute nor life threatening, women with the disease are recommended to be under routine check-ups, because if the disease deteriorates into HELLP syndrome or eclampsia the state of the mother must be assessed and treated as soon as possible. If pre-eclampsia is not treated properly it can result in death of the mother, the child or both (B. M. Sibai et al., 1986). When pre-eclampsia develops into severe pre-eclampsia or eclampsia it's often followed by HELLP syndrome, nausea, pain in the upper gastric tract and/or general malaise (Martin et al., 1999). HELLP occurs in about 20% of cases of severe pre-eclampsia and is considered a life threatening complication of pre-eclampsia (Geary, 1997; Vinnars et al., 2008). A mother that is suffering from HELLP can show signs of heartburn, nausea and/or vomiting, these symptoms might last for a few days before a more acute phase of the

disease presents itself (Martin et al., 1999). Research has shown that due to these vague symptoms a delay of a correct diagnosis is in average 8 days (B. M. Sibai et al., 1986). The delay can be serious for the health of the mother since about 5-38% of women who suffer from HELLP get disseminated intravascular coagulation (DIC) and other life threatening symptoms such as abruption placenta, renal failure, pulmonary oedema and/or sub capsular liver hematoma that can be treated properly if diagnosed in time (Celik et al., 2003; Haram, Svendsen, & Abildgaard, 2009; Kirkpatrick, 2010; B. M. Sibai et al., 1993).

1.1.5 Morbidity and mortality

Maternal deaths connected to hypertensive disorders, where pre-eclampsia and subsequent conditions are the most common, are 9.1% of overall maternal deaths in Africa and Asia, 25.7% in Latin America and 16.1% in developed countries. It's worth noting that a large part of African countries and a few Asian are excluded from these numbers since there is no data readily available, the percentage might therefore be higher in those areas (Khan, Wojdyla, Say, Gulmezoglu, & Van Look, 2006). Maternal death due to eclampsia in developed countries is 1.4 - 2.4% (Douglas & Redman, 1994; Zwart et al., 2008) while in Columbia it's 10% and in Nigeria 8.5% of mothers that suffer from eclampsia (Agida, Adeka, & Jibril, 2010; Conde-Agudelo & Kafury-Goeta, 1998). The trend for the last decades in western countries has shown a decrease in mortality in both mothers and babies due to pre-eclampsia, most likely because of progress in prenatal care, increased follow up and improved treatment options for eclamptic convulsion and minimizing the effects of HELLP syndrome (Basso et al., 2006; Odendaal et al., 1995).

1.1.6 Treatment

No treatment currently exists to prevent or treat pre-eclampsia, all therapies aim at minimizing symptoms. At this time the only cure is to give premature birth to the baby and deliver the placenta (B. Sibai et al., 2005). If the placenta is not delivered it can continue to cause the mother symptoms of pre-eclampsia, and even after delivery there is a small risk in the first 48 hours post-partum of complications, such as pulmonary oedema, renal failure, HELLP syndrome, postpartum eclampsia, and/or stroke (B. M. Sibai, 2003, 2004). Prenatal care seems to be the best possible way of helping women

that suffer from pre-eclampsia according to research conducted by MacKay et al. The research showed that women that received no prenatal care had more than 7-fold mortality risk in comparison to women that received adequate care; it can be assumed that prenatal care increases the chance that abnormalities will be found and treated, therefore preventing more serious complications (MacKay et al., 2001). It has also been shown that the outcome for the new born improves greatly if the gestational age can be increased from 34 weeks to 36 weeks. By this increment the chance of jaundice and admissions to intensive care following birth decreases, so there is much to gain for the health of the baby from delaying delivery as long as possible, without risking the health of the mother (Barton et al., 2011).

Therapy to prevent pre-eclampsia aims at keeping the blood pressure down but no direct action is made unless the condition worsens and threatens the mother. Increased follow up is the recommended action after a diagnosis of pre-eclampsia. Even though it is discovered during prenatal care, no therapeutic action is taken unless the syndrome progresses towards serious pre-eclampsia or HELLP syndrome is confirmed (Milne et al., 2005a). When the disease progresses into the more serious levels such as HELLP syndrome or eclampsia more aggressive therapy is available and should the mother's condition deteriorate, labour is induced (Leeman & Fontaine, 2008).

1.2 Current state of research

So far, no research has provided convincing results in preventing or stopping the outcome of pre-eclampsia, despite many different vitamins and supplements being suggested and tested. Notable trials include calcium, zinc, fish oils, vitamin C, vitamin E, low-dose aspirin and variety of different antihypertensive drugs (Briceno-Perez, Briceno-Sanabria, & Vigil-De Gracia, 2009; Poston et al., 2006; B. M. Sibai, 1998; Xu et al., 2010). Research has shown that there is no gain by administering these supplements during pregnancy. In fact, research have even shown that some of these supplements can have adverse effects on the pregnancy, such as lowering birth weight (Poston et al., 2006). Low to medium dose aspirin treatment that's initiated before week 16 of pregnancy has been connected to a decrease in cases of serious pre-eclampsia, but the treatment showed no reduction in preeclampsia and gestational hypertension. It's recommended for high risk pregnancies to start a medium dose aspirin therapy as soon

as pregnancy is confirmed since that is the only therapy that decreases the morbidity of the disease although not preventing it (Roberge et al., 2012).

1.2.1 Searching for biomarkers

It's feasible to develop a simple test to screen women in their first trimester to assess the risk of pre-eclampsia as they progress towards full term. An early screening makes it easier for healthcare professionals to increase follow up during prenatal care for high risk pregnancies as well as shorten reaction time, which can drastically improve the outcome if the mother develops serious pre-eclampsia or HELLP syndrome. Symptoms for HELLP are quite typical and might be discarded as general malaise if there is not previous suspicion of pre-eclampsia. This early identification could prepare the mothers for what's ahead as well and make them more aware of serious implications that might occur during the pregnancy.

Currently there are no effective screening methods to identify women who may be susceptible to develop pre-eclampsia during pregnancy. The most promising biomarkers include endoglin, PlGF, and sFlt-1. None of these show a significant increase during the first trimester of pregnancy so their shift is more likely to be caused by pre-eclampsia rather than to be the cause of the disease. The use of these proteins as a marker is therefore not optimal since it's too late to start aspirin therapy after the first trimester (Levine et al., 2006).

1.3 Placental protein 13

Placental protein 13 (PP13) was first characterized in 1983 from tissue taken from a delivered full term placenta. PP13 is a 16 kDa homodimer that is connected *via* disulphide bonds and its gene expression is located on chromosome 19 (19q13.1) close to five other members of the galectin family (Bohn, Kraus, & Winckler, 1983; Than et al., 2009a). Due to its high homology with galectin 10 (69% homology) as well as shared characteristics that are attributed to galectins, such as homodimerization, reactive SH groups, intra cell solubility and sugar binding abilities, PP13 was added to the galectin family which currently contains 16 proteins (Visegrady et al., 2001). The main expression of PP13 is in the syncytiotrophoblast cytosol, which covers the placental villi, and is transported to the intervillous space via non-classical export through Ca^{2+} influx.

PP13 enters the bloodstream of the mother from the intervillous space (Balogh et al., 2011; Than et al., 2004).

Expression of PP13 is also present in the foetal spleen and liver in small quantities and extremely low concentrations can be found in the liver, bladder and tumorous tissues of adults. PP13 is non-existent in healthy non pregnant women, males and is not found in foetal blood unless the mother develops HELLP syndrome (Sammar et al., 2011).

A mutant variation of PP13 has been diagnosed. The mutation causes a deletion of a single thymine base at nucleotide position 221 which results in a premature stop codon and shortens the protein by 38 amino acids. This deletion has been connected to adverse pregnancy outcomes even though the mechanism behind this protein is unknown, and all the women that were diagnosed with the mutation were heterozygous for the mutation. These women were in overall more prone to preterm labour but no direct connection can be made to the mutant gene and hypertension (Gebhardt, Bruiners, & Hillermann, 2009).

1.3.1 PP13 as a biomarker

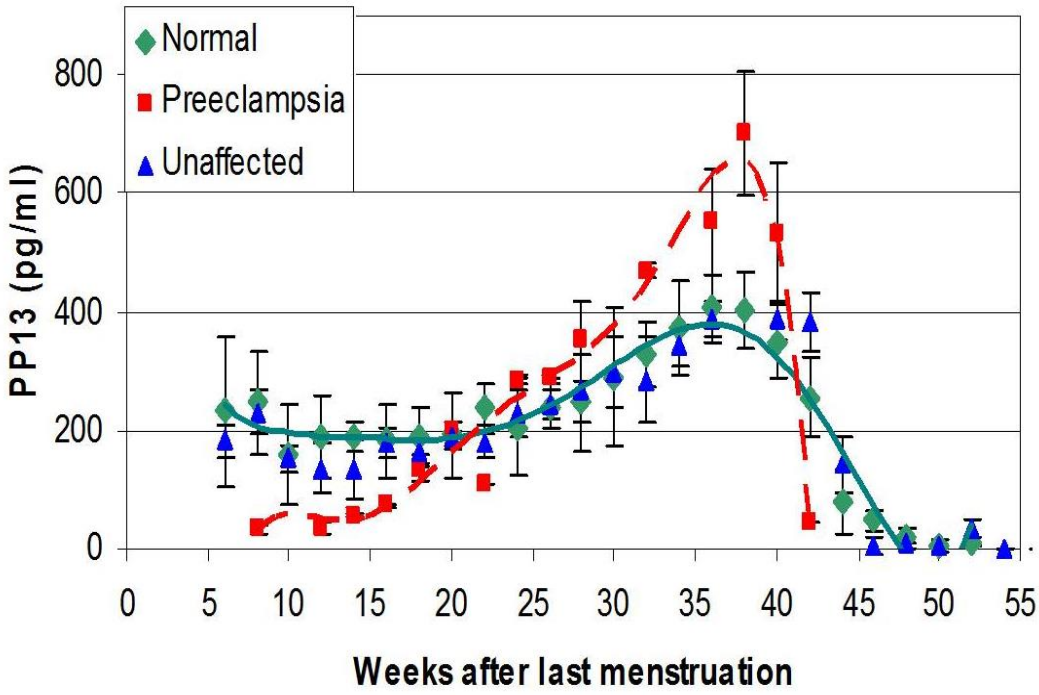

Figure 1: The change of biological levels of PP13 during pregnancy. Here the difference between pre-eclamptic women and unaffected women is quite visible (Huppertz et al., 2008).

Research for the use of PP13 as a diagnostic marker was initiated in 2003 when the level of PP13 in 50 pregnant women was measured. In the initial research it was reported that lower PP13 levels in the first trimester were associated with abnormal pregnancies (Spencer, Cowans, Chefetz, Tal, & Meiri, 2007). Further research has shown that low value of PP13 in the first trimester is strong indication for pre-eclampsia later in pregnancy. These results have been confirmed by multiple trials (Akolekar, Syngelaki, Beta, Kocylowski, & Nicolaides, 2009; Burger et al., 2004; Chafetz et al., 2007; Khalil et al., 2009; Nicolaides et al., 2006; Odibo et al., 2011; Spencer et al., 2007).

Figure 1 compares serum levels of PP13 between normal pregnancy (uneventful pregnancy that resulted in birth after 37 weeks), unaffected pregnancy (birth before week 37 or cervix shorter than 25mm) and pre-eclamptic pregnancies. In this graph,

which has been supported by other research, shows that the amount of PP13 is reduced in the first trimester. In the second trimester there is a sharp increase of the protein and it stays above normal levels until after birth in comparison to normal and unaffected pregnancies (Huppertz et al., 2008).

Some published articles claim that PP13 is ineffective as a predictive biomarker since there is little to no significant differences between pre-eclamptic groups and control groups. It's worth noting that all articles that make this claim are based on trials that used the AutoDELFIA automated immunoassay from PerkinElmer Inc. Trials that have shown PP13 to be an effective predictive biomarker used a non-automated ELISA assay from Hy-Labs (Huppertz, Meiri, Gizurarson, Osol, & Sammar, 2013). A neutral comparison study showed that the ELISA method was marginally better than the AutoDELFIA method (Cowans, Stamatopoulou, Khalil, & Spencer, 2011). This difference can be explained by differences in the construction of the tests. The automated assay from PerkinElmer is unfortunately unavailable in June 2013 and therefore replication of these results is impossible.

1.3.2 Role of PP13 during pregnancy

Galectins are known to have diverse and highly regulated effects during the first weeks of pregnancy along with other roles such as in cell proliferation, migration, tissue differentiation, inflammation, immunodeficiency, and malignant conditions (Burger et al., 2004; Visegrady et al., 2001). Exact effects of PP13 in of pregnant women are not yet known but deductions have been made that it serves immunological functions due to its homologues and that it has some haemostatic effects on the foetal maternal interface (Than et al., 2004). PP13 has a binding affinity to β-galactoside residues, which are present on the cell surface, cytoskeleton, and extracellular matrix. These residues partly regulate immune responses, influence apoptosis and molecular recognition, which are all pathways that are part of the placentation process. This suggests that PP13 might be a part of a regulatory mechanism for placentation (Spencer et al., 2007; Visegrady et al., 2001). Effects of PP13 on macrophage cell cultures showed an increase in cell apoptosis and decreased cell viability which might serve as a part in placentation in pregnant women (Boronkai et al., 2009). The binding of PP13 seems to shift from the cytoplasm of syncytiotrophoblasts in normal pregnancies to their membrane if the pregnancy is

affected by pre-eclampsia or HELLP syndrome which might cause abnormal transportation (Balogh et al., 2011). These results support the hypothesis that PP13 plays an important part in placentation and that deficiency of the protein can have dire effects later in pregnancy.

In pre-eclamptic pregnancies there is a reduction of PP13 available in the venous blood but higher accumulation of the protein in other tissues such as the placenta and umbilical cord blood, which might be explained by either a foetal source of the protein or a transfer from the placenta to the foetus. It's been shown that there is a reduction of PP13 mRNA in the pre-eclamptic placenta but an elevated protein level in various organs and in blood, especially during the third trimester (Sekizawa et al., 2009). Although not a common occurrence it's not unheard of that there is an increase in the protein levels despite a reduction of mRNA levels. The sharp rise in PP13 levels in the third trimester in pre-eclamptic pregnancies is probably due to increased shedding from the syncytiothrophoblasts that contain PP13 as a response to the low levels of PP13 in the first trimester (Than et al., 2008) or to counteract the high blood pressure that occurs when the toxic sFlt-1 appears in the systemic circulation and causes severe changes to the endothelia and induces toxic effects to the kidneys.

There are currently multiple theories about the biological functions of PP13 during pregnancy. They include that PP13 reduces immunological function at the brush border membrane of the placenta, that it affects the blood pressure of the mother due to lysophospholipase A function, induces spiral artery invasion and encourages the production of vital factors in placentation with its sugar-binding abilities (Kliman et al., 2012; Than et al., 2009a)

2. AIMS OF STUDY

The aim of this study is to evaluate the physiological effects of PP13 in gravid *Rattus norvegicus*. A pilot study has shown that PP13 has effects on the blood pressure in rats, but further research is required to confirm these findings and to characterize and understand the role of PP13 in pregnancy. As well as looking at the effects of PP13, a replica of the mutant PP13 (PP13M) has been made. We used that replica to do the first *in vivo* observations of this mutant.

2.1 Specific aims

1. Evaluate the effects of PP13 and mutated PP13 on blood pressure, pup and placenta weight in gravid rats following continuous treatment over several days.
2. Evaluate the effects of PP13 and mutated PP13 on the uterine vasculature following continuous treatment over several days in gravid rats.
3. Evaluate the pharmacological effects of PP13 on an isolated artery.
4. Measure PP13 levels in blood.
5. Estimate the basic pharmacokinetics of PP13 in gravid rats.

3. MATERIALS AND METHODS

3.1 Approvals

The animal studies were approved by the National Laboratory Animal Review Board in Iceland (approval number 0180-2902) and by IACUC (under protocol 12-023) and carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in conformance with the US NIH guidelines for the care and use of laboratory animals. The animals were housed in the Small Animal Facility at the University of Vermont which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Feed and water were provided *ad libitum*. All efforts were undertaken according to the '3R principles' of the directive to reduce the number of animals used in this study, and optimize experimental protocols to obtain maximum data from each tested animal.

3.2 Animals

The animals used in this research were female Sprague-Dawleys that arrived 12 weeks old. The animals were given a 72-hour acclimation period after arrival before any contact was made and were impregnated within 4 weeks of arrival by male Sprague-Dawley that were certified breeders by the provider. The animals were kept at 20-26°C and humidity at 30-70% in accordance to AAALACs guide to care of laboratory animals (Worlein, Baker, Bloomsmith, Coleman, & Koban, 2011). Prior to initial blood pressure measurements the animals were acclimatised to the procedure at least once so the base point blood pressure measurement would not be affected by stress that can be caused by new procedures.

3.2.1 Determination of fertility

To see if a female was likely to reproduce, a vaginal smear was sampled by injecting a small dose of H_2O vaginally and removed with the same dripper. The smear was examined with 20x magnification and estimated in accordance to Figure 2. If a rat is in diestrus 2 or proestrus phase she was determined likely to get pregnant and was thereafter placed in a metabolic chamber overnight with a male that had not been in contact with a female for at least 24 hours. Following morning the animal and the cage was examined for a vaginal plug which is a confirmation of a successful impregnation. If a vaginal plug was found, the female rat was moved to another (smaller) room, located in the animal facility dedicated for gravid rats only (Weyrauch, Torres, Baird, & Dunnett, 2009).

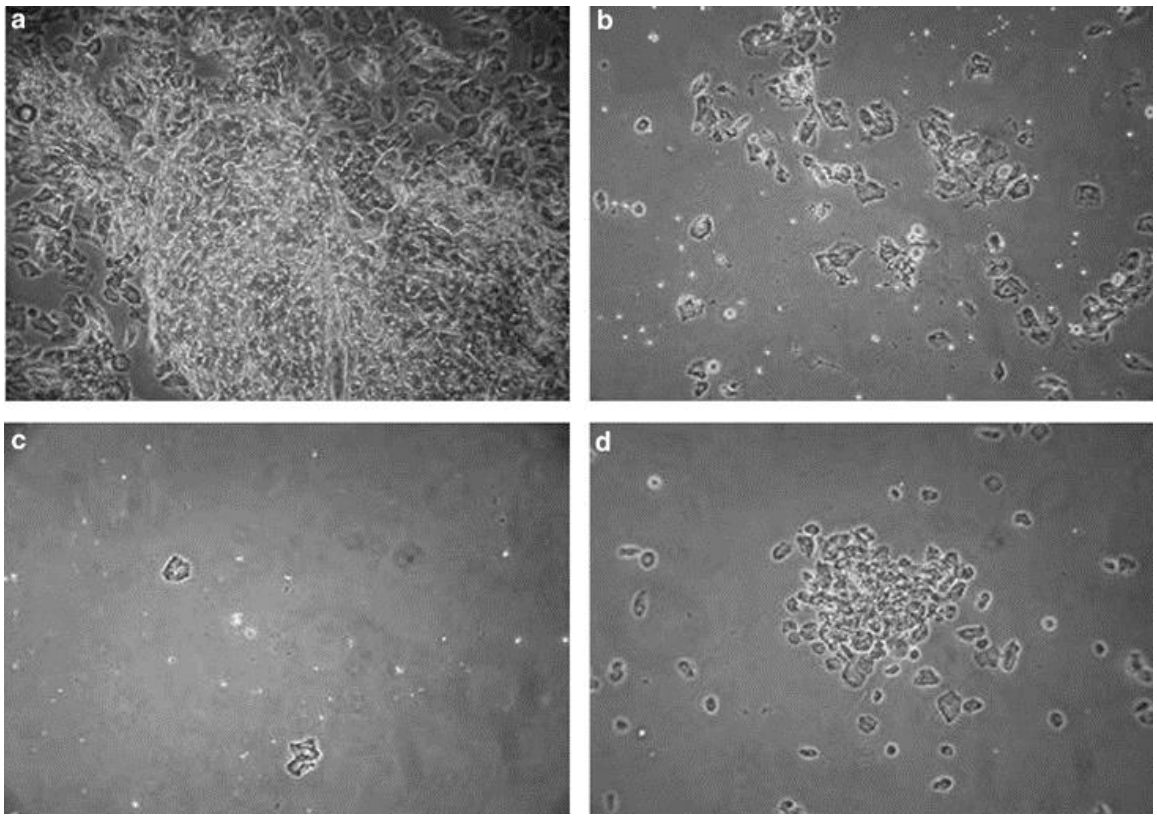

Figure 2: Different phases of estrous cycle. Estrus (a), diestrus 1 (b), diestrus 2 (c), and proestrus (d) are each characterized by the number and morphology of the cells in vaginal fluid. Magnification ×100 (Dazzi et al., 2007)

3.3 Materials

3.3.1 Chemicals

Chemicals, drugs and their usage are listed in Table 1. HEPES solution was prepared fresh each morning. Papaverine and PP13 were stored on ice.

Table 1: Overview of drugs and chemicals and their manufacturer that were used in this project.

Brand name	Generic name	Concentration	Molecular weight (g/mol)	Usage	Producer
Isosol	Isoflurane	2-4%	184.5	Anaesthesia	Vedico
Papaverine	Papaverine hydrochloride	100 µM	375.85	Artery dilation *in vitro*	Sigma chemical co.
-	Phenylephrine hydrochloride	0.1-0.7 µM	203.67	Artery constriction *in vitro*	Sigma chemical co.
Nembutal	Pentobarbital sodium	50 mg/kg	226.27	Euthanasia	Ovation Pharma-ceuticals
Buprenex	Buprenorphine	0.05 mg/kg	467.64	Pain killer	Hospira inc.
-	HEPES	10mM	238.30	Physiological buffer	Sigma chemical co.
-	Sodium Chloride (NaCl)	0.9% (w/v)	58.44	Physiological saline	Merck

Biological samples that were photographed and cannulised were stored in HEPES on ice to ensure physical effects on the tissue. HEPES is one of Goods buffers and consists of: 141.8 mM NaCl, 4.7 mM KCl, 1.7 mM $MgSO_4$, 0.5 mM EDTA, 2.8 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 5 mM glucose.

3.3.2 PP13 and PP13M

PP13 was prepared and provided for this project by Hy-Laboratories Ltd. (Rehovot, Israel). The PP13-R/pQE30 expression vector was transformed into M15 (pREP4) Escherichia coli host strain, and the bacteria were induced with IPTG. The expressed protein was subsequently purified with Ni-nitrilotriacetic acid columns to affinity purify PP13 by its His-tag residues. Mutant PP13 (PP13M) was constructed to include thymidine deletion at position 221 to simulate a real life mutation. It was inserted into the bacteria, expressed, induced, harvested and purified in a similar manner to the wild type. Both proteins were verified by molecular weight verification on SDS-PAGE, immunoblots and ELISA. A PP13 stock of 31 μg/ml with 30% glycerol and 3 mM diethylthiol was prepared for this study and stored at –80°C. To simulate clinically relevant human blood levels of PP13, Alzet osmotic pumps were selected to provide constant continuous administration of PP13 and PP13M. The pumps were inserted via surgery on the eight day of pregnancy. The eight day was chosen to minimize risk of abortion due to the surgery, but still early enough to ensure that PP13 would have maximal effects on placentation.

3.4 Animal Experiments

3.4.1 Dosage of PP13 in Rats

Normal concentrations of PP13 in pregnant women are about 100–300 pg/ml. While PP13 DNA is specific to primates, galectins that are similar to PP13 in structure have been identified in lower mammals (Than et al., 2009b). Thus, a protein with similar function as PP13 could be present in other animal species such as rats. Pharmacokinetic parameters in animals are unknown such as volume of distribution (Vd) nor interspecies variation of these parameters. The dosage of PP13 given was estimated based on an empirical Vd of 125 ml/kg, to 67 ng/kg or 17 ng/rat (Mahmood, 2007; Svavarsdóttir, 2008). This estimate was used when appropriate dosage was calculated for the pumps. The pumps released about 10.1 μl/hr so we estimated that a release of 0.625 ng/hr over seven days would give a blood concentration that reflects a normal blood concentration of PP13.

3.4.2 Pump Implants

The rats were anesthetized using isoflurane (4% for induction and 2% for maintenance during surgery) and the Alzet osmotic pumps (model 2ML1) were surgically inserted subcutaneously into the back of rats (ALZET, 2013). Immediately after surgery the rats received injections with the analgesic buprenorphine (0.05 mg/kg) and maintenance dose 12-hours post operation. The dose of PP13 was prepared immediately before loading into the pumps to minimize possible degradation of the protein. The aliquots that were inserted in the pumps were made by diluting the former mentioned 31 mg/mL stock solution of PP13 or PP13M with 0.9% saline. The control group received pumps filled with 0.9% saline. The pumps were loaded with 126.9 ng of PP13 in 9 animals, 126.9 ng of PP13M in 6 animals and with saline in 6 animals that served as a control group.

3.4.3 Blood Pressure and heart rate

Blood pressure and heart rate were measured in all animals non-invasively by determining the tail blood volume with a volume pressure recording sensor and an occlusion tail cuff (Kent Scientific Corp.). The animals were given half an hour to acclimate to the room temperature in which the measurements were made, and another 20 minutes to acclimate to the cones they were placed in and to warm up before blood pressure measurements started. Tail heat was measured and by aimed at keeping it at 34°C by recommendation from machine provider. Blood pressure and heart rate measurements were taken late morning (between 10 Am. and 12 Pm.) and measured for at least 25 measurements that were averaged to minimize standard deviation.

3.4.4 Blood Sampling

Blood samples were collected in BD serum vacutainers on day 11 via either saphenous or tail vein, spun down with 2000g force for 15 minutes at 20°C. After spinning the samples, the serum was collected into Eppendorf tubes and frozen at -80°C. Trunk blood was collected after sacrifice and treated the same way as described above.

3.4.5 Sacrificing

Each animal was euthanized with an intra-peritoneal injection of Nembutal and once a full anaesthesia was attained (toe pinch test), the rat was decapitated in a small animal guillotine. The abdomen was opened and the uterus and its contents were removed en bloc and pinned in a Petri dish with a gel bottom. The dish was filled with ice cold regular 10mM HEPES. The pH was adjusted to 7.4 with either HCl or NaOH.

Care was taken to not to force any abnormal stretch or compression on the tissue. The uterus was photographed through a stereomicroscope (Zeiss, Germany) to estimate and compare vascularization between groups. The pups and placentas were weighted on a precision scale and the weight was recorded.

3.4.6 Artery Cannulation

After taking the photographs an artery was excised from the uterus using micro-point scissors and forceps under a microscope. Segments (1–2 mm long) of uterine arteries were dissected free from connective and adipose tissue and transferred to a dual chamber arteriograph (Living Systems Instrumentation, Burlington, VT). The arteriograph consists of a chamber containing two glass cannulas and has its own independent super fusion system for drug delivery. The arteries were cannulated, and to simulate *in vivo* condition an intraluminal pressure was set to 50 mmHg prior to a 45-60 min equilibration at 37°C using a servo-controlled pump (PS-200) and in-line pressure transducer (Living Systems Instrumentation).

Following cannulation, the arteriograph was set on a mobile stage attached to an inverted microscope (Zeiss SR, Carl Zeiss, Thornwood, NY) with an attached monochromatic video camera (TV-C-Mono) that was connected to a video dimension analyser (VDA-10) and a television monitor. After the equilibration, phenylephrine (0.1 µM) was added in a stepwise manner until the artery reached 60-80% constriction from its original diameter. After the constriction, the artery was rested for 20 minutes to ensure stability of constriction. Once constriction was achieved, PP13 was added into the chamber in a stepwise manner and the reaction was recorded.

3.4.7 PP13 serum analysis

PP13 blood levels were determined in serum collected from the rat tail or saphenous vein three days after the insertion of the Alzet pump, using ELISA microtiter plates coated with PP13-specific monoclonal anti-body (mAb) from Hy-Laboratories (LOT:12081549B). The analysis was completed with a PP13-specific second mAb conjugated to biotin and further reacted with streptavidin conjugated to horseradish peroxidase. The reaction was developed with 3, 3‘,5,5‘-tetramethylbenzidine and stopped with 1M HCl. Optical density at 450 versus 630 nm was converted to PP13 levels using standards processed in parallel.

3.4.8 Analysis of vascular diameter

Photos were taken of each uterus and uterine vascularization, both without and with 32x magnification. These pictures were later analysed to determine resorptions and the width of various uterine arteries. Each branch of the arteries was measured from a photo containing a ruler with magnification. The petri dish containing the uterus was placed under the microscope and the focus was fixed in one position to maintain homology of each measurement. Pictures were taken of the uteruses containing enough arteries for analysis as a representative for each uterus. The number of pixels that spanned one mm was measured and used to translate number of pixels into artery width to mm.

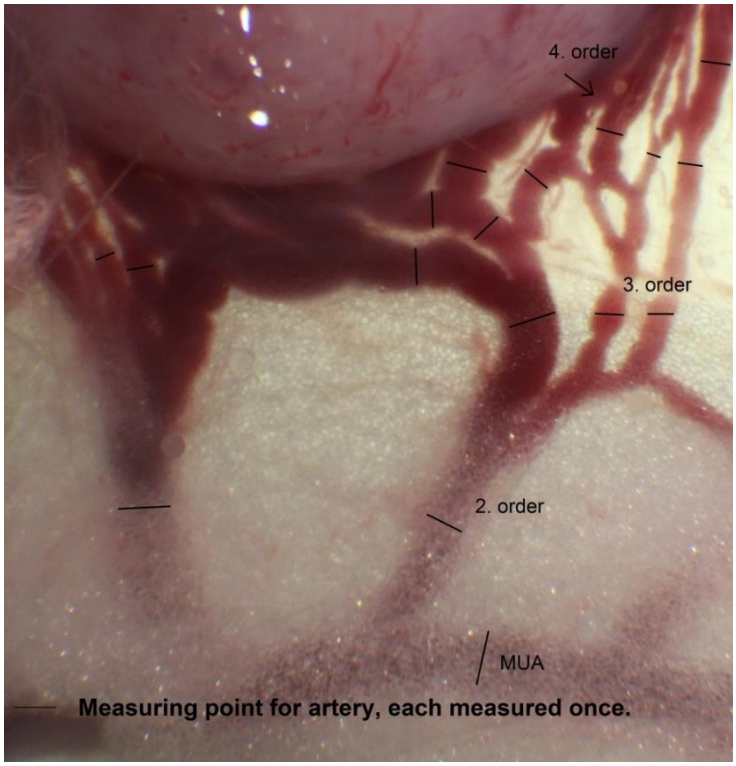

Figure 3: Example of artery measurements.

When an artery splits into two or more branches it was considered as a new order. In 32x magnification 4th order arteries were the smallest arteries that were measureable with confidence. An example of measurements can be seen in Figure 3.

3.5 Statistical analysis

CODA software was used to record blood pressure. The results were exported to Excel for further analysis such as mean blood pressure, mean heart rate and standard deviations. Statistical package in Excel was used to carry out paired *t*-test and analysis of variance (ANOVA). Significant difference was determined when $p<0.05$. Matlab was used to calculate polynomials that area under curve (AUC) was derived from.

4. RESULTS

This study contained 21 gravid rats treated for five days and sacrificed at day 15 and day 20. These two different lengths of pregnancy were made to allow the second group to recover after being treated with the test compounds. All rats received pump implants on the eight day of pregnancy, six of them carried out pregnancy near full term (until 20^{th} day of pregnancy) and the remaining 15 rats were sacrificed on day 15 of pregnancy.

The difference in the length of pregnancy was to make it possible to evaluate if PP13 and PP13M had any delayed effects on the pups and the placenta, even though the main interest was on the effects observed at day 15 (after seven day therapy). In some animals it was not possible to extract data from different days in pregnancy due to too much stress during blood pressure measurements. All data possible was used but note that on some days there might be a measurement lacking from a single animal. Full photographic data was extracted from all animals except one that was sacrificed on day 20 after PP13 therapy. When that particular rat was sacrificed the camera was unavailable, so it was not possible to take magnified pictures the uterus. All data collected in this research is available in appendix A.

4.1 Physiological effects of PP13

4.1.1 Effects of PP13 on blood pressure in gravid rats

To determine change in diastolic and systolic pressure simultaneously, mean arterial pressure (MAP) is calculated. Equation (4.1) is the general formula that is used to calculate MAP and gives an overall view of change in blood pressure.

$$MAP = \frac{(2 \cdot Diastolic\ pressure) + systolic\ pressure}{3} \tag{4.1}$$

MAP was calculated from average diastolic and systolic pressure from each group. An ANOVA test was made to compare the blood pressure between rats that received PP13 pumps, PP13M pumps, and saline pumps.

It's interesting to look at MAP in the context of the therapeutic days. In Figure 4 the average MAP is plotted against day of pregnancy when the blood pressure measurement was made.

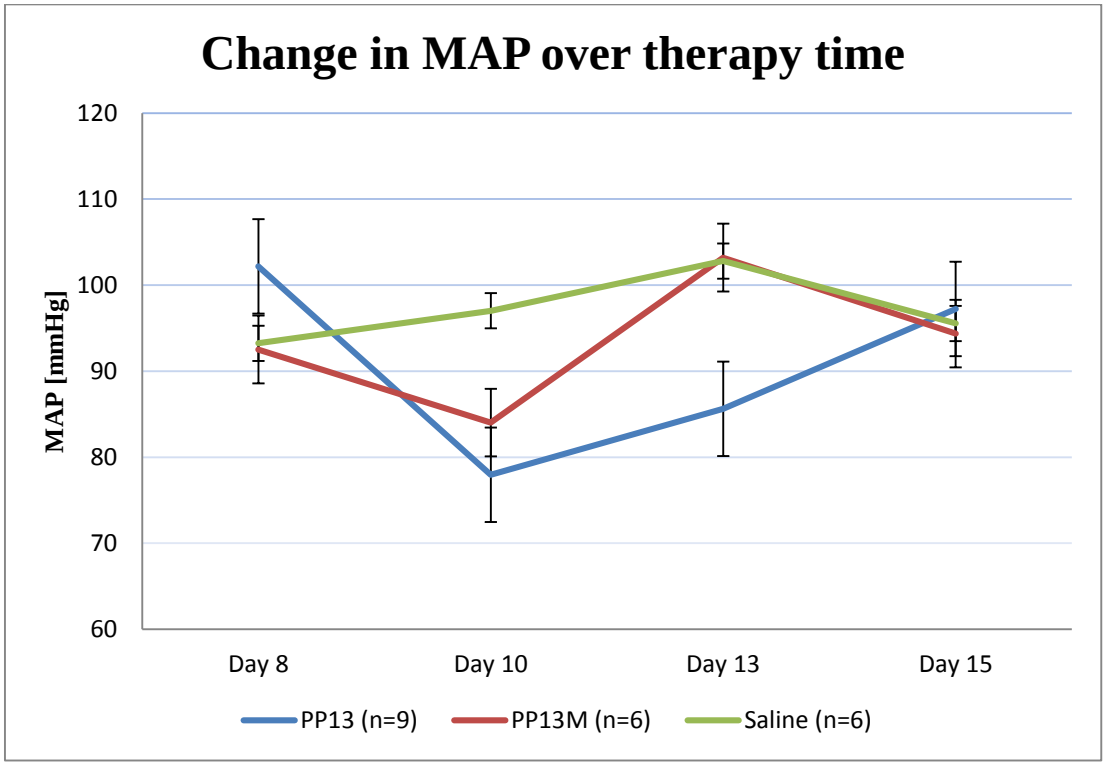

Figure 4: Changes of average MAP between therapy groups.

The error bars show standard deviation from the average. From this graph there is a visible nadir in the MAP in both PP13 and PP13M groups two days after therapy is initiated. This nadir fades out over time until all groups have returned to similar MAP values.

A two factor ANOVA without repetition was made on the slope between therapy groups to see if there was a significant change in the MAP between groups. The results of the tests are presented in Table 2.

Table 2: Results from a two factor ANOVA test made between therapy groups that were sacrificed on day 15.

Source of Variation	F	F crit	P-value
Variance within group	0,4910	3,3541	0,6173
Variance between days	3,5087	3,3541	0,0442
Variance between groups	6,5690	2,7277	0,0007

The ANOVA comparison in Table 2 shows that the intra variance in each group is not significant. There is a variance between days in the groups as well as there is a variance between all the groups. This shows that there is a significant change in the MAP between each group. A paired t-test between the groups shows that the PP13 therapy group has the lowest MAP on day 10 of pregnancy ($p<0.05$).

Baseline blood pressure differs between individuals and rats are no exception of that. To verify this drop three days after therapy the blood pressure was plotted out in context to initial blood pressure and percentage change is calculated Figure 5

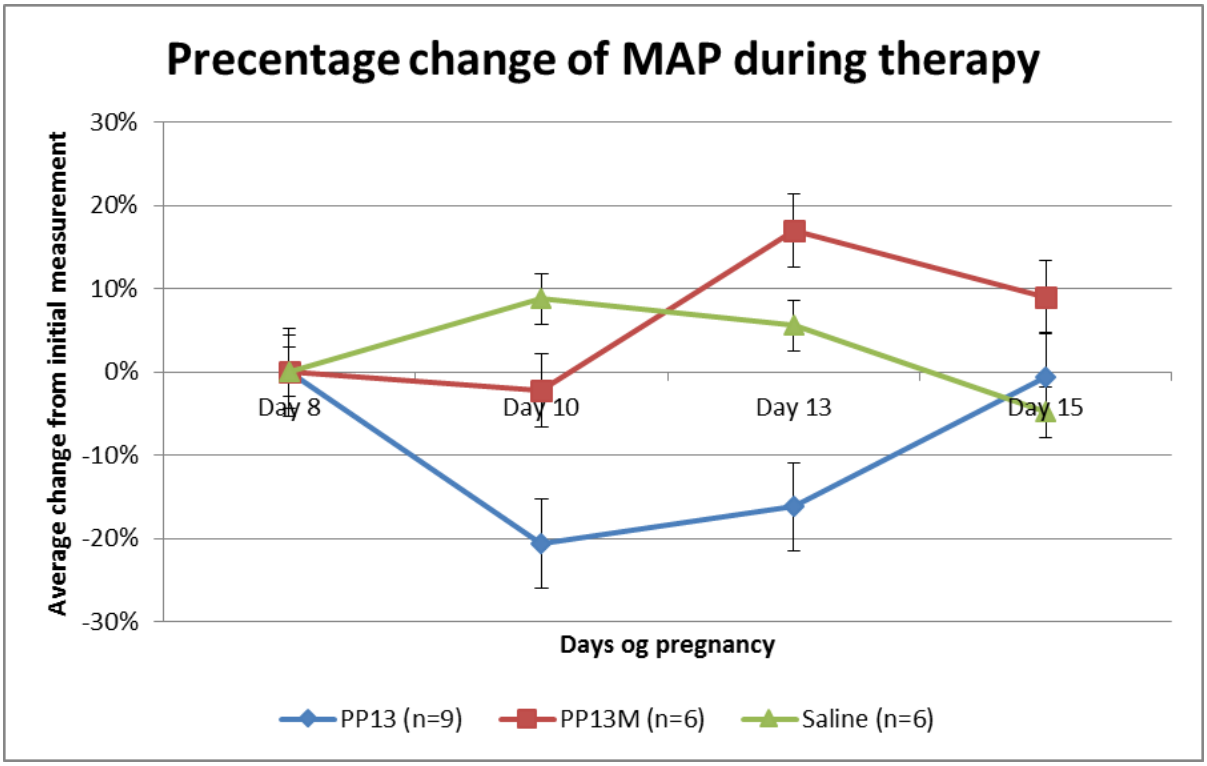

Figure 5: The percentage of change in blood pressure between groups where initial measurement is a reference point and their standard error

In the figure the drop is much more prominent for the PP13 group while the data is not as convincing for PP13M. By performing a *t*-test between all the groups we see that there is a statistical difference between the PP13 group and the saline group both on days 10 and 13 of pregnancy and between PP13M and PP13 on day 13.

Due to the noticeable drop in MAP on the tenth day of pregnancy (second day of therapy), regression analysis was made from the data from that day to examine if the blood pressure followed linearity or if some abnormalities would be visible due to therapy. In Figure 6 a regression analysis is plotted out.

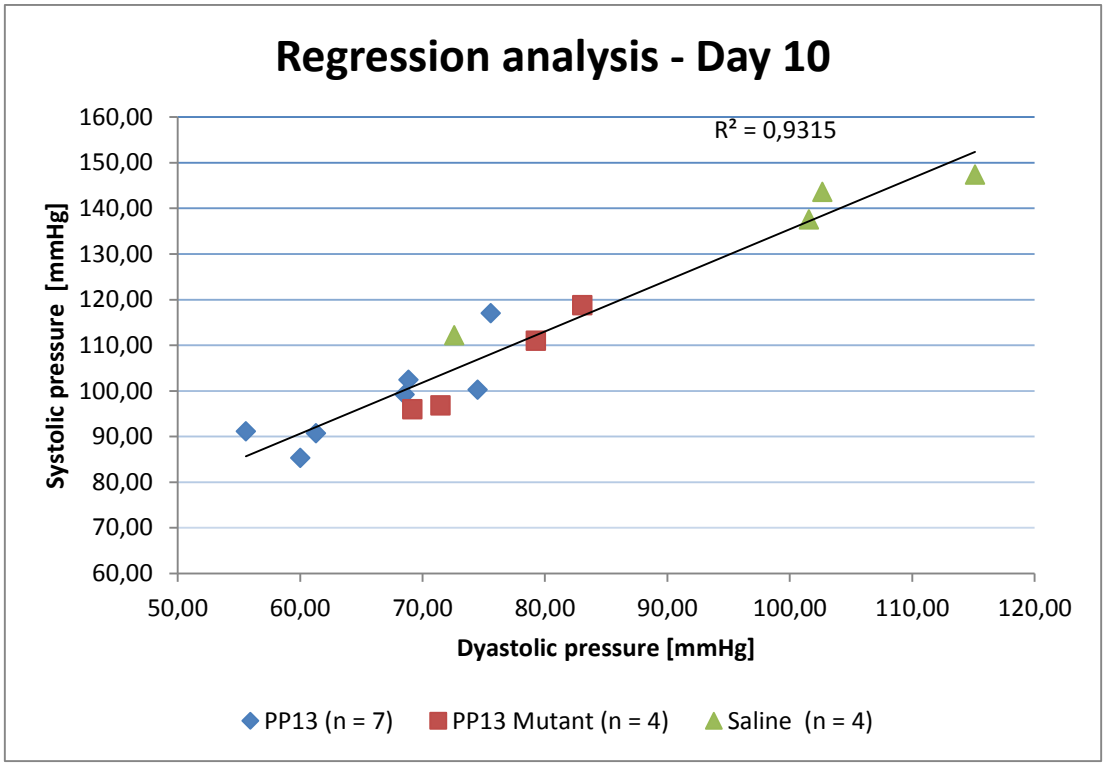

Figure 6: Regression analysis of blood pressure on day 10 of pregnancy.

Adjusted R^2 is 0.93 which determines that there is a good correlation between the data. The *p*-value is $6.06 \cdot 10^{-9}$ which indicates a statistical difference between the therapy groups where the PP13 group has the lowest diastolic and systolic pressures. AUC was calculated as well for each therapy group. The result from the AUC calculations agreed with the other data, AUC in PP13 treated groups was significantly lower than in saline treated groups. There was no statistical difference between PP13M groups and other groups.

Measurements that were made on rats that carried through pregnancy until day 20 are included in these graphs. It‘s not desirable to plot their data since the therapy groups are so small and high in variance. It‘s worth noting that the blood pressure stayed close to normal values in all groups after therapy should have ended, except in the PP13M therapy group. In that group the MAP was higher than the saline and PP13 groups.

4.1.2 Effects of PP13 on heart rate in gravid rats

As a compensation for the lowering of blood pressure there could be an increase of heart rate. Therefore the heart rate was collected and analysed. A graph depicting the average heart rate measurements for each rat is in Figure 7 as a scatter plot. Heart rate was measured simultaneously as blood pressure. All values over 700 beats per minute (BPM) were excluded as abnormalities that might be caused by stress in rat or responses to outside stimulus. The data does not represent all the animals as some animals had incomplete datasets.

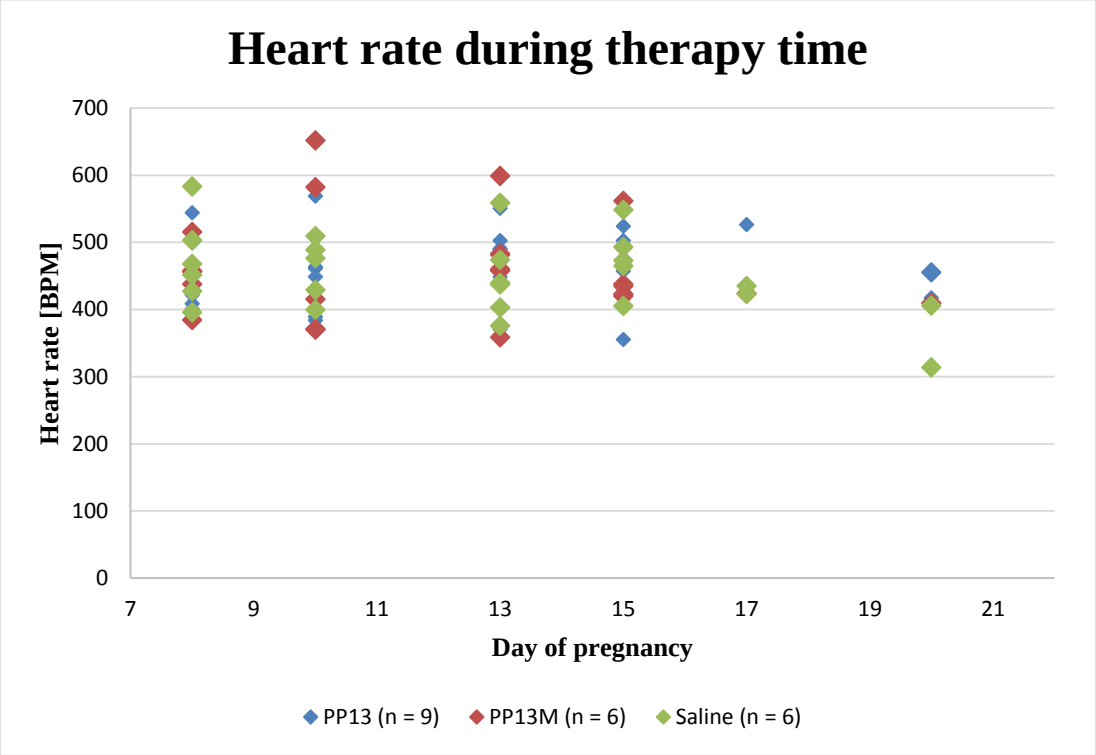

Figure 7: Distribution of heart rate during therapy. Heart rate over 700 BPM was omitted as abnormal.

The scatter plot in Figure 7 shows that the dispersion of the heart rate is rather consistent during therapy. An ANOVA test between the therapy groups reveals that there is no statistical difference in BPM between the groups on any day of the therapy ($p = 0.453 – 0.956$).

4.1.3 Effects of PP13 on foetal weight

One of the main concerns in drug therapy during pregnancy is the effects that a drug might have on the placenta, the foetus and the pregnancy. One of the major concerns are the risk of teratogenic effects. The behaviour of the mothers was recorded and assed subjectively. No obvious difference in behaviour between the groups was noted during therapy time. Rather than aborting a foetus that is not viable, the rat resorbs it, which leads to a visible plaque in the uterus (Telford, Woodruff, & Linford, 1962). In this research project a resorption was defined as a visible plaque inside the uterus where a foetus had previously been, as demonstrated in figure 8

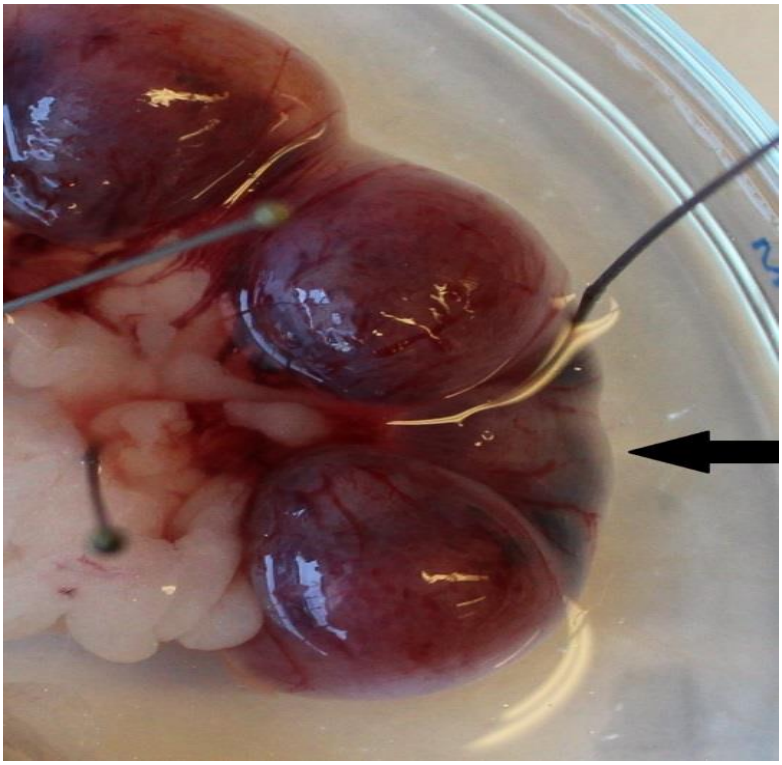

Figure 8: A site of resorption in an otherwise healthy uterus. The arrow points to a site of resorption.

To be able to assess the changes between therapy groups, foetal weight and placental size was measured on a precision scale and the weights averaged between groups and compared in Table 3.

Table 3: Differences in number of pups and resorptions, and size of pups and placentas between therapy groups. To obtain *p*-values, ANOVA were made. Resorptions are represented as % of total pups born to each group. Foetal and placental weight are represented as means ± standard deviation

	Litter size	Resorptions	Foetal weight	Placental weight
PP13 - 15 days (n = 7)	13.6	9.5%	0.182 ± 0.042	0.174 ± 0.033
PP13M - 15 days (n = 4)	14.3	14%	0.164 ± 0.033	0.155 ± 0.034
Saline - 15 days (n = 4)	15.5	0%	0.150 ± 0.027	0.179 ± 0.036
p-values	0.4832	0.1182	$3.4 \cdot 10^{-5}$	0.001102
PP13 - 20 days (n = 2)	14.0	0%	2.474 ± 0.28	0.639 ± 0.099
PP13M - 20 days (n = 2)	13.5	0%	0.927 ± 0.11	0.364 ± 0.073
Saline - 20 days (n = 2)	12.5	4%	2.213 ± 0.34	0.453 ± 0.069
p-values	0.8831	0.5629	$4.1 \cdot 10^{-33}$	$1.9 \cdot 10^{-17}$

ANOVAs were performed between all the different characteristics on both pregnancy lengths. The percentage of resorptions was calculated as the number of resorptions divided by the total number of pups viable to each group. Foetal and placental weights are represented as means ± standard deviation. From the *p*-values in Table 3 we see that there were no notable differences between the number of viable pups and number of resorptions. There is a great difference between foetal and placental weight between groups which was confirmed to between all groups with a *t*-test. There is a statistical difference between each group and the foetuses that received PP13 therapy were significantly larger than foetuses that received saline. In groups that were sacrificed on day 20 in pregnancy the placental weight is significantly higher in the PP13 groups ($p<0.05$). In rats that were sacrificed on day 15 of pregnancy the placentas of the control group are slightly heavier than in the PP13 therapy group, but no significant difference was recorded between those groups. Placentas in rats that were sacrificed on day 15 were statistically lighter than in the other two groups.

4.1.4 Effects on placental arteries

One of the theories on effects of PP13 during pregnancy is induction of placentation including angiogenesis. It was therefore interesting to observe the effect PP13 might have on vasculature in the uterus and where this difference might be most prominent.

Table 4: Comparison between the width and number of arteries taken from a 1 cm^2 piece of the excised uterus. Numbers are presented as an average ± standard deviation. P-values were obtained by ANOVA except noted by *, there *t*-test was used between saline and PP13M.

		Main uterine artery [mm]	2 order arteries [mm]	3 order arteries [mm]	4 order arteries [mm]	Total number of arteries
15 day	PP13 (n = 7)	0.522 ± 0.074	0.419 ± 0.099	0.409 ± 0.126	0.387 ± 0.196	33.4± 4.6
	PP13M (n = 4)	0.454 ± 0.147	0.353 ± 0.106	0.344 ± 0.119	0.197 ± 0.044	25.5± 5.9
	Saline (n = 4)	0.367 ± 0.051	0.317 ± 0.104	0.333 ± 0.117	0.206 ± 0.084	38.3± 7.0
	p-values	0.0992	0.0241	0.0198	0.0000001	0.03933
20 day	PP13 (n = 2)	1.224 ± 0.000	0.908 ± 0.223	0.610 ± 0.117	0.410 ± 0.099	29.0±0.0
	PP13M (n = 2)	0.874 ± 0.139	0.518 ± 0.152	0.331 ± 0.152	0.244 ± 0.091	39.5± 5.5
	Saline (n = 2)	0.796 ± 0.140	0.752 ± 0.295	0.546 ± 0.329	0.283 ± 0.701	35 ± 8.0
	p-values	0.3882*	0.1577	0.4952	0.0015	0.7162

From the data presented in Table 4 there is a statistical difference between all lower orders of arteries in rats that were sacrificed on day 15 of pregnancy. The results from the ANOVA test were confirmed by a paired *t*-test and showed that there was a statistical difference between the width of the arteries in all orders that were treated with PP13 and the other therapy groups. There is a reduction in these differences between rats that were sacrificed on day 20 but still a significant difference in 4^{th} order arteries where PP13 treated rats had the widest ones and the 3^{rd} order arteries in rats treated with

PP13 are narrower than the saline group. There is a statistical difference between the total numbers of arteries per cm^2 in rats that were sacrificed on day 15 where rats treated with saline had most arteries and rats treated with PP13M had the fewest. This difference is non-existent between therapy groups that were sacrificed on day 20.

By plotting out the percentage of the total number of arteries of all the groups in 0.1mm intervals the distribution of artery width between therapy groups becomes visible in Figure 9.

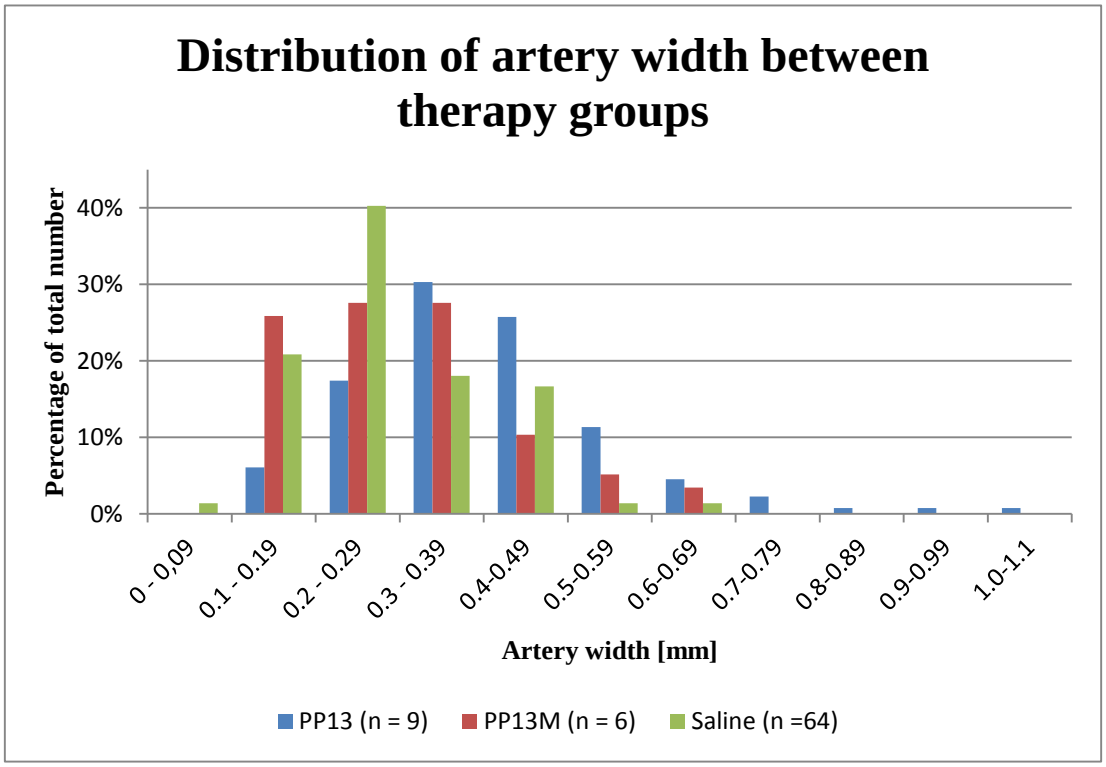

Figure 9: The percentage of total number of arteries plotted in 0.1mm intervals.

There is a noticeable shift to the right in the PP13 group which indicates that the arteries are in general wider than in the other groups. Similar shift is obvious in PP13M group where the shift is to the left which in turn indicated in general smaller arteries than in the other groups. No artery in PP13M and saline groups exceed 0.7 mm width, while the widest artery of PP13 therapy group is 1.1 mm.

4.2 Effects of PP13 on arteries *in vitro*

A method to assess artery response *in vitro* was used to estimate if PP13 has any effects on blood pressure by constriction or vasodilation. This gives an interesting view of how PP13 works, but so far it has only been shown that PP13 has a potential function to lower blood pressure, but by which pathways is still unknown. By using artery cannulation we can see if PP13 has any effects on contraction or dilation on arteries *via* intra artery receptors. Example of data from such cannulation is in figure 10.

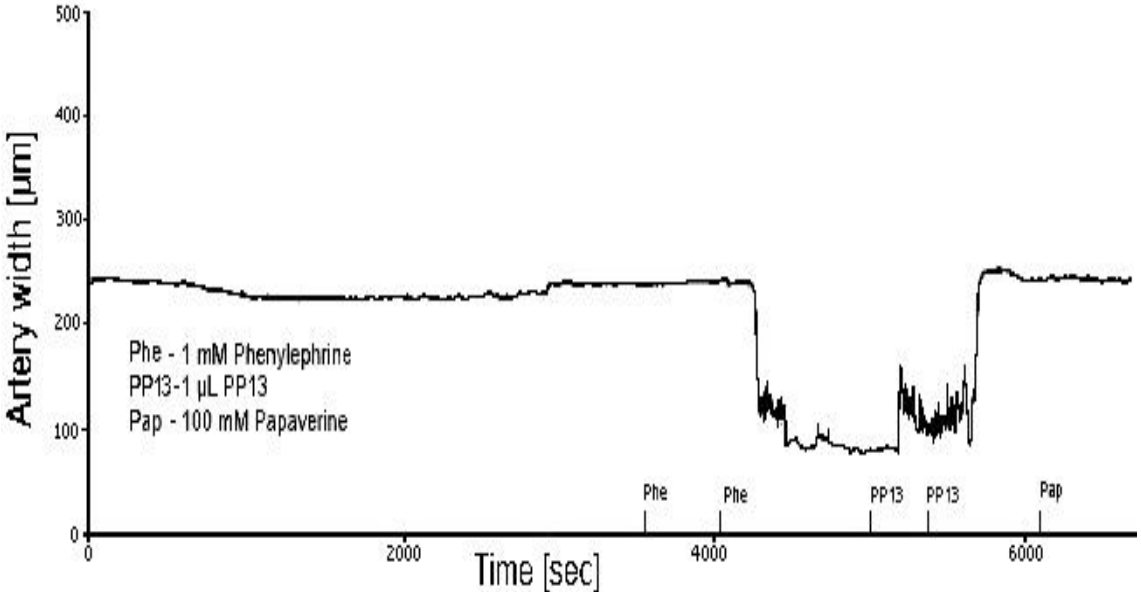

Figure 10: Example of artery constriction in vitro. The short lines on timescale indicate drug addition to the artery. After 4000 sec. PP13 was given and relaxation followed shortly after.

The artery was given a dose of 310 mM PP13, the whole volume of the system was 50 mL so the total concentration of PP13 was 6.2 pg/mL. It can be seen in this picture that there was a spike shortly after the first addition of PP13. The artery dilated and seemed constrict with time. After the second dose there was a short time lag but shortly after PP13 reaches the artery there is a full dilation and the artery is in completely relaxed state. This is confirmed by dosing the artery with a high dose of papaverine which is a strong vasodilator. These results were repeated but in two cases of four there was no response from the artery and in one case the artery fluctuated too much to give reliable results. Unfortunately there was not enough time to repeat these measurements. All pictures can be viewed in appendix A.

4.3 Measurements of PP13 in blood

An ELISA has been developed to detect PP13 concentrations in human serum. Blood was collected on day three of therapy when PP13 levels in the rat sera should be maximal. In Figure 11 the standard curve for the ELISA is shown.

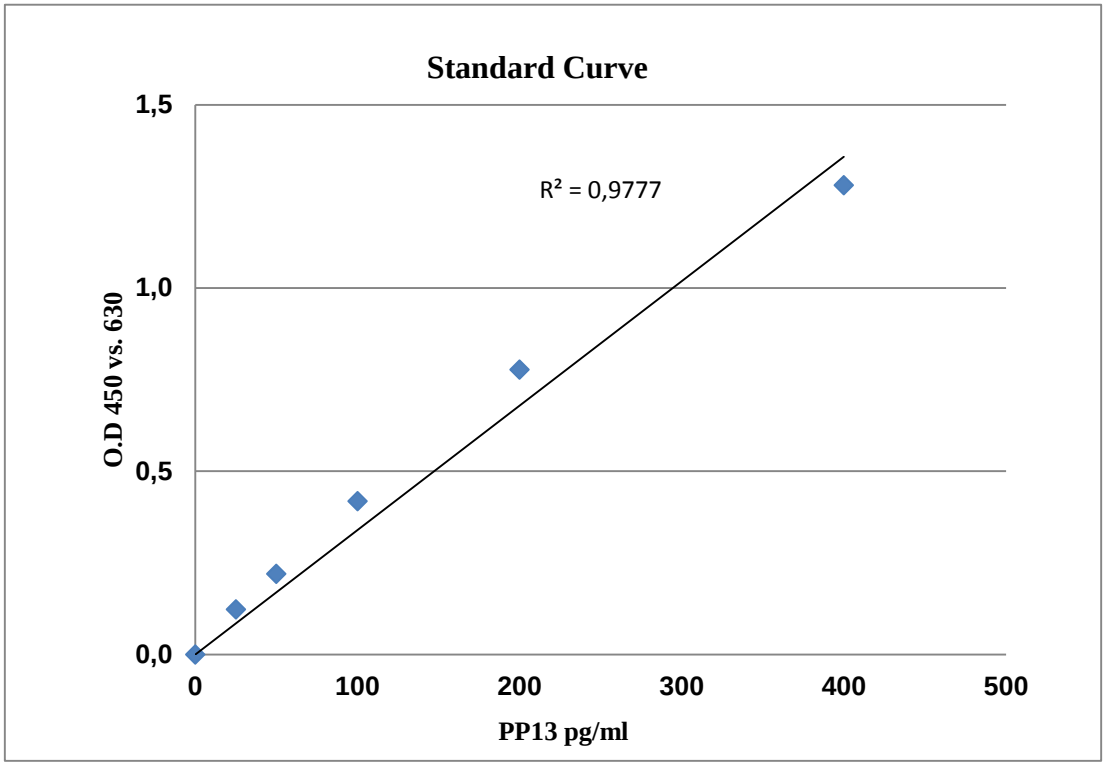

Figure 11: Standard curve from ELISA depicting an accurate measurement of PP13

The high R^2 in the trend line in Figure 11 shows that the standards are accurately mixed and the ELISA should show levels that are true to the serum values.

The results from the ELISA measurements of the rat sera are in Figure 12.

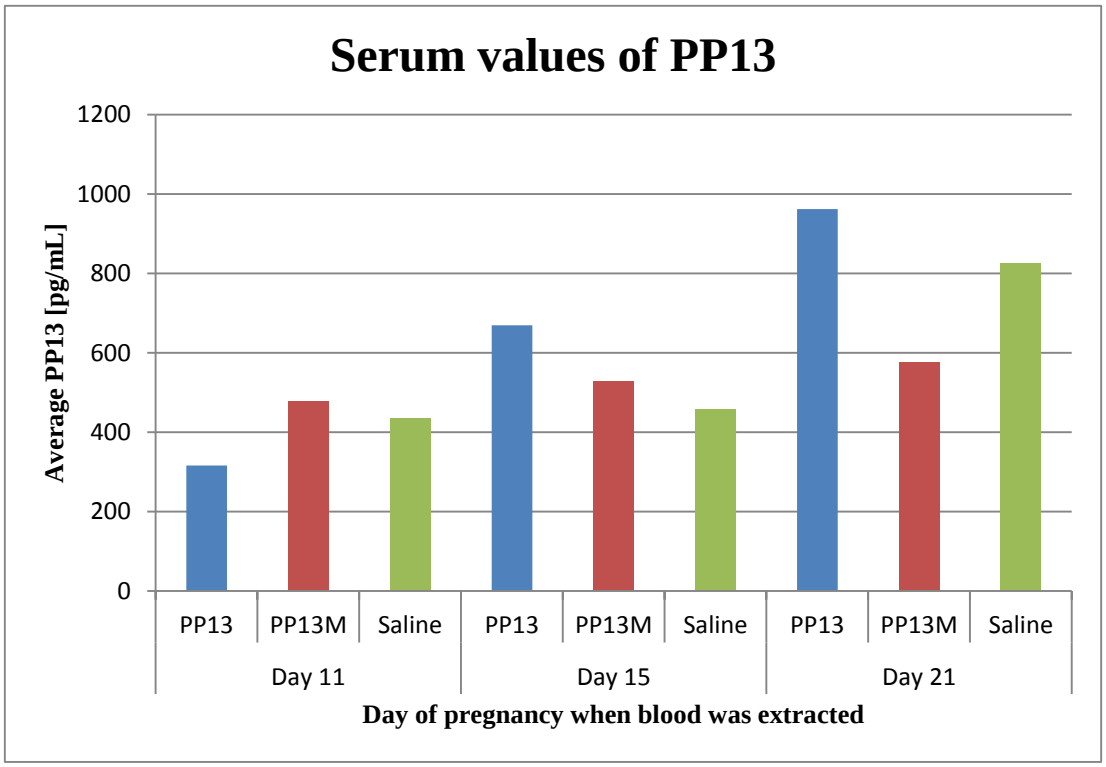

Figure 12: Results from ELISA on rat serum. Measurements were made from blood samples taken on day 11 of pregnancy and trunk blood acquired after sacrifice. Values are represented as average of therapy group

From the result Figure 12 it is evident that the measurements of PP13 in blood are inconclusive. An improved analytical method has been developed and should be used to reanalyse all samples. The data from this ELISA is therefore unusable to measure PP13 in the rat sera and the calculation of pharmacokinetics of PP13.

5. DISCUSSIONS

One of the main aims of this research project was to understand the effects of PP13 in gravid rats. We saw significant decrease in the blood pressure in rats that received PP13 treatment shortly after the therapy was initiated. Some change was visible as well in the rats that received PP13M but not as drastic as in the PP13 group. In addition to these effects, we saw a significant change in both vascularisation of the uterine arteries as well as in the pup and placental weights.

The PP13M was used in this project to compare the effects of human PP13 and mutated PP13. By using the mutant protein we aimed at gaining a better knowledge of the physiological effects of PP13 by comparison to the PP13M, and additional understanding of its mechanism and possible "pharmacological" effects.

5.1 Change in physiological characteristics

The blood pressure was measured in each group and expressed as MAP as well as mean percentage change in each group. Both methods showed a significant lowering of blood pressure in the rats that received PP13 treatment. How does PP13 affect the blood pressure in these rats? Several hypotheses can be suggested. It has been shown *in vitro* that PP13 has effects on prostaglandin formation inducing liberation of linoleic and arachidonic acid and therefore elevate the levels of prostacyclin (PGI_2) and thromboxane (TXA_2) (Burger et al., 2004). Different prostaglandins are known to have different effects on the blood pressure and although PGI_2 and TXA_2 are antagonists there is not necessarily equal synthesis of each prostaglandin. In the previous mentioned study it was noted that there was a higher biosynthesis of TXA_2, which may lead to a higher blood pressure in return. There are other less known prostaglandins that could cause an increase in the blood pressure such as prostaglandin F2 alpha ($PGF_{2\alpha}$). Activity from $PGF_{2\alpha}$ might contribute to the symptoms of pre-eclampsia and be a factor in the eclamptic convulsions since it's one of the essential factors in inducing labour. $PGF_{2\alpha}$ has not been as thoroughly researched as other prostaglandins, outside of inducing labour and as a therapy against glaucoma, so there is still a lot of unanswered questions about its effects in the human body (Goodman, Gilman, Hardman, Limbird, & Gilman, 2001). In a recently published article connection was made between $PGF_{2\alpha}$ and

cardiovascular diseases, $PGF_{2\alpha}$ was shown to increase arterial plaques by promoting fibrosis in coronary arteries and inducing formation of reactive oxygen species (Yu, 2010). Even though further research is needed on the subject an interesting theory is that PP13 induces release of $PGF_{2\alpha}$ during a pre-eclamptic pregnancy and might be the cause for the increased prevalence of cardiovascular diseases in mothers that have suffered from pre-eclampsia.

By analysing the blood pressure in context to the initial blood pressure measurement the data is represented quite conclusively. On the third day of therapy there is a drop in blood pressure in PP13 group but in the PP13M group the relevant blood pressure does not show the same drop and there is also an increase in the blood pressure in the saline group, which might be connected to the shock following the pump implantation. Still it's interesting to see that there is a rise in the blood pressure on the seventh day of therapy in the PP13 group even though there should have been enough protein to last for seven days. This might be explained by a number of reasons, such as that the protein is highly unstable at room temperature and breaks down easily. The protein could be degraded by some enzyme native to the rat, too much of the protein might have been released prematurely or there might be a saturation of receptors. By the end of the pregnancy the PP13 group blood pressure returned to a normal value which shows that the effect of PP13 is reversible and does not have a delayed effect on the blood pressure, as rats that where sacrificed on day 20 of pregnancy all had normal blood pressure levels as well. The study showed a clear decrease in the blood pressure, but this work was too short to discover the mechanism behind this effect. Does it occur via endothelial receptors, intra-arterial or intravascular receptors, release of some hormones or prostaglandin or maybe some other mechanisms? That remains to be studied further.

We expected to see an increase in the heart rate complimenting the lowering of blood pressure to maintain normal perfusion. This increase was not observed. Previous pilot studies showed an increase in heart rate, but in that study the PP13 was administered very late in pregnancy (day 15) (Gizurarson et al.). This difference between studies might be because of number of reasons since there is a difference in the protein delivery and the method behind the blood pressure measurements between these researches. No adverse effects were observed to the health of neither the rats nor any effects on organs

visible to the eye *post mortem* and therefore we might assume that the perfusion is high enough for both mother and pups.

5.2 Change of foetal characteristics

There was a significant difference in the foetal and placental weight between groups. There were neither statistical differences between the numbers of viable pups between the groups nor difference in pup resorption, which happened in all groups. Both PP13 and PP13M had significant effects on the pup and placental weights, but these effects differed greatly.

There is a significantly increased placental weight in the PP13 group which could be explained by a higher volume of blood reaching the placenta, resulting in an increase in growth. The large placenta is then able to increase the transport of nutrients to the foetus. This is in accordance with the fact that in human pregnancies the placental weight is directly proportional to foetal weight (Kay, Nelson, & Wang, 2011) and in pre-eclamptic pregnancies it's common the child suffers from some growth restriction (Duley, 2009). However, in the PP13M treated group, there was a significant reduction in the placental weight both after 15 and 20 days of pregnancy.

In the PP13 was, as former mentioned, a statistically significant increase in foetal size, both after 15 and 20 days of pregnancy. In the PP13M group the foetal weigh is close to normal values but after 20 day pregnancy there is a great statistical reduction in foetal weight.

Additionally, there was a decreased width of placental arteries in the PP13M group which may have led to hypoxia for the pups. This calls to mind a similarity to pre-eclampsia in humans, where hypoxia caused by poor placentation can be linked to intrauterine growth restriction, which does not occur with women that suffer from pre-existing hypertension (Wollmann, 1998).

As well as changes in the placental size there was a change in the vascular structure in the placentas that received PP13 therapy and mainly in the width of the arteries. This increased width of the arteries gives grounds that PP13 must play an important role in the placentation and influences the placentation process. By increasing the width of the arteries might be an increased blood flow and flow of nutrient to the pups which could

explain the increased size of the pups. An interesting follow up to these results would be a similar analysis where the spiral artery invasion is assessed and compared to PP13M and saline groups. That additional data would give us a realistic idea of the change in resistance arteries in the placenta and if the appearance for increased blood flow that we see is real.

In comparison to PP13 the effects of PP13M on the rat and the foetus seem to be delayed since there is no statistical difference between foetal and placental weight in PP13 and the PP13M group on day 15, but in day 20 there is a significant difference between those groups. We expected that the protein was ineffective so the changes we witnessed in those therapy groups came as a surprise. An interesting follow up to these results would be to increase the duration of the PP13M therapy and looking at the viability of the pups after birth.

5.3 Effects of PP13 *in vitro*

In order to assess if PP13 has any effects *via* intra-arterial receptors the arterial cannulation gives a good idea if the protein has a constrictive or relaxing effects. This method gives us the opportunity to review responses from arteries without any hormonal or other biochemical influences interfering with the protein, since the artery is under constant circulation with fresh HEPES and is given time to reach equilibrium and flush out any biological agents. By subjecting the pre-constricted artery to PP13 we were able to see a vasodilatation in the artery on two occasions. The dose used was rather low and in both cases two doses were needed to reach fully relaxed state. To confirm that the artery had reached fully relaxed state a dose of papaverine was given and in both cases no further reaction was noted. It must be taken into consideration that we were using human PP13, and it is not known if the rat has a protein that is equivalent to human PP13.

Since there was a relaxing response from these arteries it gives grounds to theorize further about effects of PP13 on blood pressure. These results suggest that PP13 affects some receptors that augment the dilation of arteries e.g. by binding of PP13 to intra-arterial or endothelial receptors. This might explain the vast difference in the width of placental arteries in rats that were treated with PP13. This also gives grounds for theories of the role of PP13 during human pregnancies. Does this protein cause the

elevation in blood pressure that is one of the main factors in pre-eclampsia or a biological response as an attempt to lower the blood pressure but happens too late? It's important to repeat these experiments in a bigger group to verify this response. It would be interesting as well to see if any similar responses occur if PP13M is used instead of PP13.

5.4 Pharmacokinetics of PP13

Originally it was our intention to measure the pharmacokinetics and blood values of PP13 following administration. Unfortunately, that was not possible due to lack of material and also because the pharmacological effects were the focus of this project rather than method development.

6. CONCLUSIONS

My aim with this study was to evaluate the effects of PP13 and PP13M on gravid rats. From the data that we collected we saw that there were definite effects on blood pressure, pup and placental weights and effects on uterine artery width, in rats that received the PP13 therapy for 7 days. While seeing these effects of PP13, I saw that the PP13M had rather adverse effects on both rats and pups. Rats that received PP13M therapy showed a greatly reduced pup and placenta weight after 20 days pregnancy.

I also saw a significant change in the vasculature of the rats that received the PP13 therapy. The arteries were significantly wider in the group that received the PP13 therapy compared to both saline and PP13M groups which suggests an increased blood flow through the placenta.

Some response was recorded in a cannulation of an isolated uterine artery. Due to a lack of time and limited number of animals we were not able to get results from enough number of animals to be able to draw a definite conclusion of the pharmacological effects of PP13 within the artery but by the definite response that we got on two occasions we conclude that there is some effect by PP13 *via* intra-artery receptors.

We were unable to measure the amount of PP13 in rat sera and therefore unable to estimate the pharmacokinetics of PP13.

Over all, I observed numerous effects visible in rats that received PP13 therapy over placentation period in pregnancy. These results are quite positive and open up further research questions about the effects and behaviour of this protein in pregnant women.

7. ACKNOWLEDGEMENTS

I would like to thank my advisor Sveinbjörn Gizurarson for the opportunity to do this research. It's been a great experience, challenging and has made me a better scientist.

Lilja Guðrún Steinsdóttir deserves special thanks for assistance and teaching in animal handling as well as Pétur Henry and his students in the RTL for access to his lab and guidance in western blotting.

George Osol at the University of Vermont for his immense hospitality and giving me the opportunity to experience a new culture and welcoming me into his lab. Shannon, Nicole, Darren and Dee in the Osol lab for immense patience, help, discussions and their friendship. Luke Ruddick for assistance in language, grammar and other complex English terms.

I’d like to express special gratitude to my boyfriend Siggi Smári, for his endless patience, support and love through this project.

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9. Appendix A

A table containing the treatment that each rat was subjected to

Table A1: Rat labels and their treatment

Rats number	Treatment	Day of pregnancy when sacrificed
1-2	PP13	20
3-4	PP13M	20
5-6	Saline	20
6-12, 17	PP13	15
13-16	PP13M	15
17-21	Saline	15

Following are tables that contain the values obtained through different measurements. Numbers that are represented as means contain too many measurement to be represented.

Table A2: Average from blood pressure measurements. Rat number is in bold and beneath are day of pregnancy when measurement was conducted

Row Labels	Average of Diastolic [mmHg]	Average of Systolic [mmHg]	StdDev of Diastolic [mmHg]	StdDev of Systolic [mmHg]
1				
8	103,00	134,20	8,37	11,55
10	75,00	106,47	12,89	16,08
15	60,00	84,22	13,42	14,76
17	86,58	123,50	14,71	12,92
20	75,74	119,00	18,26	16,58
2				
8	82,50	113,44	9,30	8,26
13	76,69	120,08	14,80	15,76
15	99,50	124,25	4,93	8,12
18	79,11	113,47	9,27	9,82
20	61,60	89,40	10,49	9,12
3				

Row Labels	Average of Diastolic [mmHg]	Average of Systolic [mmHg]	StdDev of Diastolic [mmHg]	StdDev of Systolic [mmHg]
8	95,50	128,75	5,21	4,41
12	101,43	136,14	8,56	11,32
18	102,50	135,19	11,71	12,88
20	107,40	147,00	23,24	22,32
4				
8	74,18	105,91	11,12	15,18
10	64,33	103,22	14,44	15,50
13	86,35	119,85	5,61	10,94
15	74,52	103,74	9,99	15,13
17	63,29	94,14	3,64	3,85
5				
8	58,79	82,50	5,98	6,64
10	65,15	91,46	10,55	10,73
12	91,43	121,29	5,24	5,08
15	84,71	127,86	16,52	13,06
17	82,67	123,67	6,71	23,10
20	68,40	95,70	9,51	13,33
6				
8	78,35	113,41	11,74	12,27
10	61,00	78,00	8,15	3,58
12	77,70	115,25	13,27	16,30
17	67,64	109,50	11,16	12,00
20	84,50	117,20	15,49	13,68
7				
8	69,00	102,40	10,56	8,71
10	61,29	90,71	11,32	12,49
12	63,50	113,75	11,96	24,02
14	65,13	103,63	7,66	2,97
15	96,53	136,11	4,94	3,80
8				
8	107,78	147,56	12,33	9,54
10	74,50	100,22	9,78	11,49
12	91,00	120,62	12,09	14,28
15	103,36	137,82	4,11	5,44
9				
8	96,05	129,55	15,47	16,30
10	68,54	99,23	7,17	15,19
13	83,85	120,23	2,67	7,35
15	98,30	139,70	15,51	10,66
10				

Row Labels	Average of Diastolic [mmHg]	Average of Systolic [mmHg]	StdDev of Diastolic [mmHg]	StdDev of Systolic [mmHg]
8	97,73	126,86	3,82	4,23
10	55,56	91,11	9,42	9,73
13	60,75	93,25	11,07	9,91
15	69,21	109,68	12,19	11,68
11				
8	80,43	109,38	8,40	11,99
10	60,00	85,33	2,00	6,66
13	66,00	91,67	11,31	13,25
15	58,40	91,50	7,85	10,37
12				
8	81,36	119,00	7,33	9,40
10	75,57	117,00	5,03	3,61
13	66,60	95,60	12,47	12,06
15	93,25	130,88	11,52	11,90
13				
8	88,77	112,62	4,17	6,42
10	69,15	96,00	11,10	9,57
13	70,55	99,09	5,61	6,07
15	67,00	109,25	9,62	14,05
14				
8	61,33	79,33	3,01	7,63
10	79,25	111,00	11,83	8,62
13	99,00	150,00	#DIV/0!	#DIV/0!
15	96,80	141,90	15,32	13,27
15				
8	89,20	122,60	11,58	15,14
10	83,04	118,75	7,33	8,88
13	101,71	135,54	6,12	8,19
15	89,83	129,83	9,45	7,31
16				
8	89,65	118,83	7,02	7,39
10	71,46	96,83	4,94	6,88
13	87,69	123,54	8,36	12,93
15	81,25	111,92	11,66	7,27
17				
8	101,68	137,32	4,71	5,48
10	68,86	102,43	6,87	12,15
13	83,00	116,92	5,55	6,89
15	90,08	133,62	6,99	9,03
18				

Row Labels	Average of Diastolic [mmHg]	Average of Systolic [mmHg]	StdDev of Diastolic [mmHg]	StdDev of Systolic [mmHg]
8	98,70	136,91	8,82	7,05
10	115,14	147,43	3,53	6,32
13	111,83	147,83	9,86	13,76
15	86,58	123,83	6,56	10,21
19				
8	70,69	103,85	13,54	15,37
10	101,56	137,56	6,97	12,62
13	81,88	113,25	11,18	13,12
15	63,31	93,23	7,90	7,52
20				
8	89,39	124,94	6,90	18,47
10	72,58	112,17	7,27	12,41
13	68,43	102,43	8,96	12,97
15	88,13	127,80	10,14	9,75
21				
8	97,00	130,86	8,87	8,17
10	102,67	143,61	15,29	15,61
13	116,92	153,92	6,42	4,85
15	90,38	134,25	8,78	9,92

Table A3: Average measurements for heart rate and their standard deviations

Row Labels	Average of Rate [BPM]	StdDev of Rate [BPM]	Row Labels	Average of Rate [BPM]	StdDev of Rate [BPM]
1			**11**		
8	465,4285714	82,5628188	8	438,6470588	79,63505916
10	448,6	115,004541	10	652	#DIV/0!
15	502,8	116,7334571	13	502,8	116,7334571
17	526,25	63,97590692	15	496,4615385	99,05774022
20	455,2	125,0469626	**12**		
2			8	421,75	70,92008947
8	400,7777778	34,59098073	10	384,1	15,23482852
13	488,875	151,0812813	13	490,75	96,8559313
15	524	132,0757358	15	490	95,48298278
18	432,3125	86,5145219	**13**		
20	417,2727273	109,6121261	8	457,2222222	40,25784948
3			10	651,75	40,63147384
8	384,4166667	26,9695395	13	481,6363636	99,42662896
12	460,047619	98,40705066	15	437,6666667	161,3887646

Row Labels	Average of Rate [BPM]	StdDev of Rate [BPM]	Row Labels	Average of Rate [BPM]	StdDev of Rate [BPM]
18	424,0588235	78,91488341	**14**		
20	409,8	80,23216313	10	371,0909091	79,86169864
4			13	599	#DIV/0!
8	456,4285714	99,68091951	15	434,6666667	49,95998399
10	582,3333333	86,52359986	**15**		
13	483,1176471	77,02019407	8	437,9375	59,08013626
15	561,6666667	93,08093578	10	370,173913	87,2689949
5			13	358,6666667	22,71308121
8	583	67,88225099	15	423,1666667	102,6360885
10	489	129,2284798	**16**		
12	375,9285714	82,7279734	8	515,5238095	72,40277553
15	473,0714286	129,1894639	10	415,4166667	24,93803916
17	434,6666667	130,5782013	13	457,5714286	94,52367574
20	405,6666667	79,95206897	15	420,1	53,51105805
6			**17**		
8	451,75	122,9828676	8	435	66,81036596
12	439,5625	105,6541331	10	568,6666667	72,59017381
17	422,8571429	133,0476243	13	448,2307692	73,40203658
20	313,8888889	10,01803928	15	457,25	123,1850972
7			**18**		
10	389	#DIV/0!	8	427,1304348	51,36792804
12	370,5	10,72380529	10	429,1428571	38,62395013
15	355,2222222	14,19840735	13	437,05	65,48079268
8			15	405,5	40,48085967
8	544	82,61557561	**19**		
10	461,0555556	57,46965667	8	395,75	37,67814827
12	471,3846154	93,62917856	10	399,75	46,51093061
15	434	84,3836477	13	402,8333333	111,5928612
9			15	464,8333333	130,3494022
8	499,2142857	100,3395609	**20**		
10	463,2727273	51,27005151	8	467,9545455	81,55423394
13	438,1666667	68,51982681	10	509,3333333	117,3839853
15	416,7272727	43,99566094	13	558,75	123,7534511
10			15	492,9230769	122,3182063
8	408,8181818	36,24340513	**21**		
10	418,4	65,42018037	8	503,2857143	102,592583
13	551	#DIV/0!	10	476,5	77,14056434
15	496,1	129,5182956	13	473,9166667	84,55709027
			15	548,25	124,5428307

Table A4: Average measurements for pup and placental weights and their standard deviations

Row Labels	Average of Pup [gr]	Average of Placenta [gr]	StdDev of Pup [gr]	StdDev of Placenta [gr]	Count of Pup	Sum of Resorptions
Rat 1	2,677838462	0,6838	0,397356422	0,115769088	13	0
Rat 2	2,2697	0,594166667	0,15198616	0,081930063	15	0
Rat 3	0,9123	0,407264286	0,117106644	0,084956085	14	0
Rat 4	0,941946154	0,319730769	0,093655758	0,060794454	13	0
Rat 5	2,123811111	0,473444444	0,415372226	0,074399179	9	1
Rat 6	2,30160625	0,4316125	0,261177088	0,064596314	16	0
Rat 7	0,1716875	0,1705375	0,033172697	0,037618008	16	1
Rat 8	0,18455	0,1379	0,067069089	0,032745768	16	1
Rat 9	0,144415385	0,169092308	0,036910903	0,035953708	13	2
Rat 10	0,1895	0,192028571	0,051519362	0,047821486	14	1
Rat11	0,193528571	0,199771429	0,045858586	0,029396845	14	1
Rat 12	0,206866667	0,156916667	0,033139604	0,019303454	12	0
Rat 13	0,186055556	0,166811111	0,033150215	0,025976956	18	0
Rat 14	0,17272	0,1491	0,031209824	0,027453259	15	1
Rat 15	0,143944444	0,160955556	0,030801384	0,054628292	9	5
Rat16	0,153326667	0,144616	0,036776554	0,02624093	15	2
Rat 17	0,18077	0,19345	0,025141248	0,030609485	10	3
Rat18	0,17174375	0,18248125	0,028481291	0,046573844	16	0
Rat 19	0,1501875	0,195	0,032974796	0,03342171	16	0
Rat20	0,129335714	0,168042857	0,010670348	0,025367809	14	0
Rat21	0,14908125	0,1742625	0,034125733	0,037428757	16	0

Table A5: All measurements for artery width

Rat No.	MUA Width	2 order	3 order	4 order
2	1,223880597	0,685074627	0,565671642	0,574627
		1,131343284	0,770149254	0,461194

Rat No.	MUA Width	2 order	3 order	4 order
			0,494029851	0,391045
				0,343284
				0,253731
				0,437313
3	1,012631579	0,537894737	0,723684211	0,268947
		0,481842105	0,284210526	0,243421
		0,525789474	0,325789474	0,222895
		0,799210526	0,342368421	0,337368
			0,6	0,229737
			0,318157895	0,332368
			0,553684211	0,378947
			0,263684211	0,247105
				0,386316
4	0,734506503	0,374394287	0,252486611	0,255037
		0,39020658	0,239224688	0,092068
			0,26013772	0,256567
			0,23820454	0,099464
			0,306044376	0,299923
			0,200714104	0,123693
			0,137975006	0,17317
			0,256567202	0,113746
				0,335374
5	0,602777778	0,773737374	0,356060606	0,363636
		1,040151515	0,362626263	0,393939
		0,604545455	0,515151515	0,274495
			0,44040404	0,318434
			0,660606061	0,386364
6	0,989769821	0,41713555	0,192327366	0,178005
		0,439130435	0,315601023	0,240921
		0,700255754	0,432736573	0,184143
		1,291048593	0,420204604	0,230179
			0,852941176	0,289514
			1,496930946	0,299233
			0,508951407	0,230179
				0,352941
				0,215345
7	0,545205479	0,407945205	0,453972603	0,466575
		0,63260274	0,261643836	0,494247
			0,358356164	0,345479
			0,424383562	0,320548

Rat No.	MUA Width	2 order	3 order	4 order
			0,427671233	0,343288
			0,488219178	0,566575
				1,110137
				0,663014
8	0,580152672	0,374045802	0,243002545	0,403053
		0,402290076	0,375318066	0,418321
		0,360559796	0,313231552	0,391094
			0,387022901	0,291349
			0,303816794	0,248092
			0,346310433	0,224173
				0,315522
				0,266667
				0,167939
9	0,597159091	0,486363636	0,398863636	0,31392
		0,573863636	0,377840909	0,430682
			0,577272727	0,442045
			0,791761364	0,666477
			0,688068182	0,982102
			0,475852273	0,507386
			0,477272727	0,502841
			0,795454545	0,381818
			0,523011364	0,398864
				0,527557
				0,89375
				0,71108
10	0,542929293	0,482323232	0,425	0,309091
		0,293181818	0,28510101	0,142929
		0,351262626	0,46010101	0,483586
			0,474494949	0,618434
				0,380556
				0,335606
				0,308586
				0,254545
				0,402778
				0,464646
				0,32096
11	0,567692308	0,452051282	0,474871795	0,663077
		0,501538462	0,326410256	0,324615
		0,423589744	0,579230769	0,212821
			0,424871795	0,354615
			0,361282051	0,392564

Rat No.	MUA Width	2 order	3 order	4 order
			0,273846154	0,344103
			0,36025641	0,416923
			0,461538462	
12	0,388601036	0,419430052	0,380051813	0,232124
		0,294818653	0,373056995	0,181347
		0,329274611	0,297668394	0,243523
			0,378497409	0,207254
			0,233678756	0,186528
			0,327979275	0,233161
				0,33342
17	0,432835821	0,433084577	0,394278607	0,203731
		0,44079602	0,3039801	0,120647
		0,514925373	0,447263682	0,231343
		0,17238806	0,413432836	0,202985
		0,447014925	0,253731343	0,196269
			0,243532338	0,178109
				0,314925
13	0,427777778	0,282575758	0,173989899	0,247475
		0,298484848	0,146717172	0,229293
		0,222222222	0,56489899	0,192172
			0,325505051	
			0,277777778	
			0,217171717	
			0,303030303	
			0,355050505	
14	0,336650485	0,353398058	0,291990291	0,308981
		0,328640777	0,180582524	0,23665
			0,185436893	0,208738
			0,327184466	0,176699
			0,374514563	0,170631
			0,248786408	
15	0,696052632	0,629210526	0,32	0,186053
		0,438684211	0,3	0,144737
			0,502894737	0,112632
			0,559473684	
16	0,355526316	0,344736842	0,489736842	0,211842
		0,341578947	0,49	0,187368
		0,292368421	0,381842105	0,205789
			0,342631579	0,231579
			0,438947368	0,223684
			0,452105263	0,111842

Rat No.	MUA Width	2 order	3 order	4 order
				0,194737
				0,181842
				0,17
18	0,408977556	0,165835411	0,208229426	0,141147
		0,214962594	0,257356608	0,181297
		0,271321696	0,225436409	0,237905
		0,278054863	0,202992519	0,176309
		0,234912718	0,304488778	0,102743
			0,359600998	0,306234
			0,377306733	0,150125
			0,377057357	
			0,2319202	
19	0,346928747	0,263390663	0,44963145	0,162162
		0,249140049	0,431203931	0,226044
		0,368796069	0,35036855	0,459459
			0,235135135	0,259214
			0,41990172	0,24398
			0,535626536	0,134152
				0,195577
				0,203931
20	0,419764706	0,408235294	0,448705882	0,113882
		0,492705882	0,669411765	0,112235
		0,487529412	0,235764706	0,324235
		0,471294118	0,367058824	0,266118
			0,490823529	
			0,368235294	
			0,371294118	
21	0,292628993	0,277395577	0,28992629	0,221622
		0,256265356	0,288452088	0,108108
			0,245454545	0,093612
			0,13980344	0,176904
			0,224570025	0,162162
			0,223095823	0,271499
				0,313514

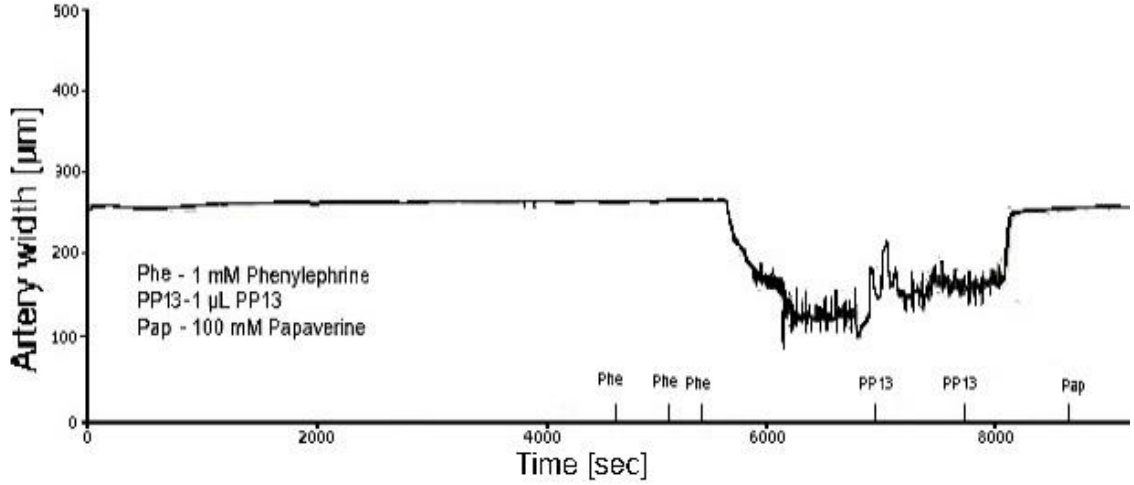

Figure A1: Cannulation performed 2. April. A reaction to PP13 doses

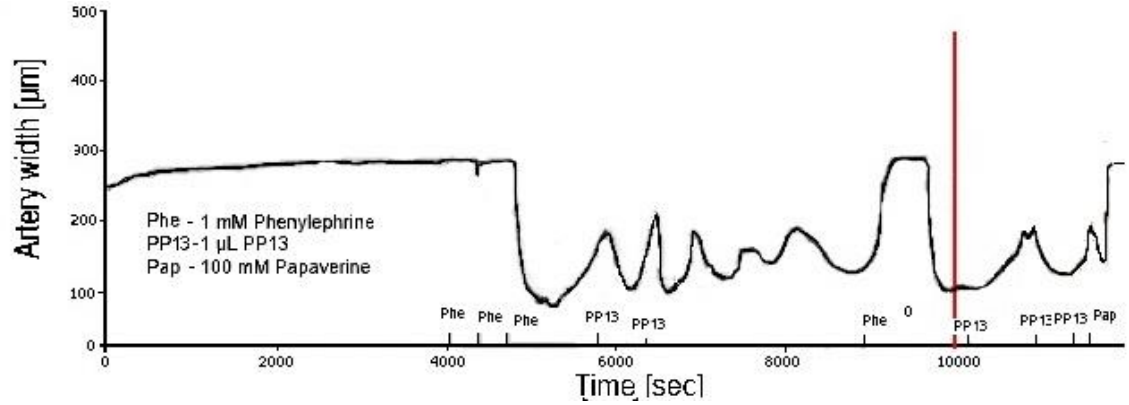

Figure A2: Cannulation performed 3. April. Too much fluctuation to make a conclusive assumption.

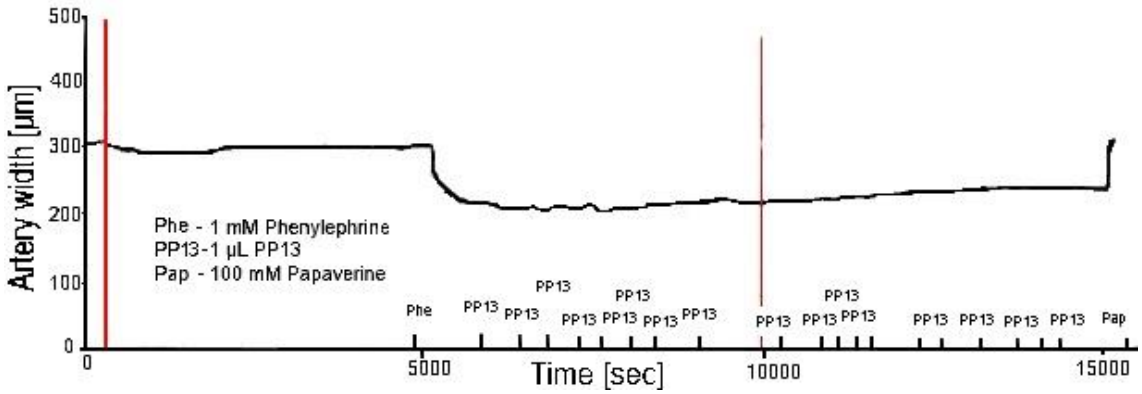

Figure A2: Cannulation performed 4. April. No response to PP13 doses

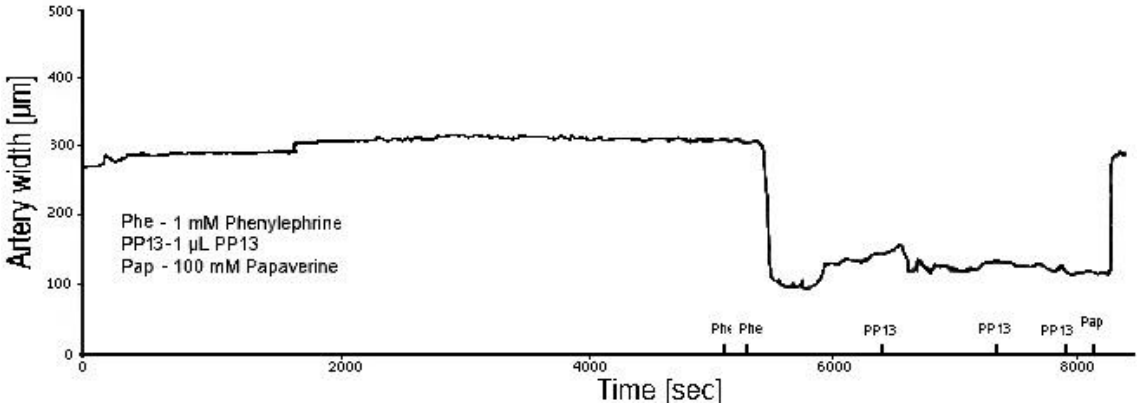

Figure A4: Cannulation performed 5. April. No reaction to PP13 doses

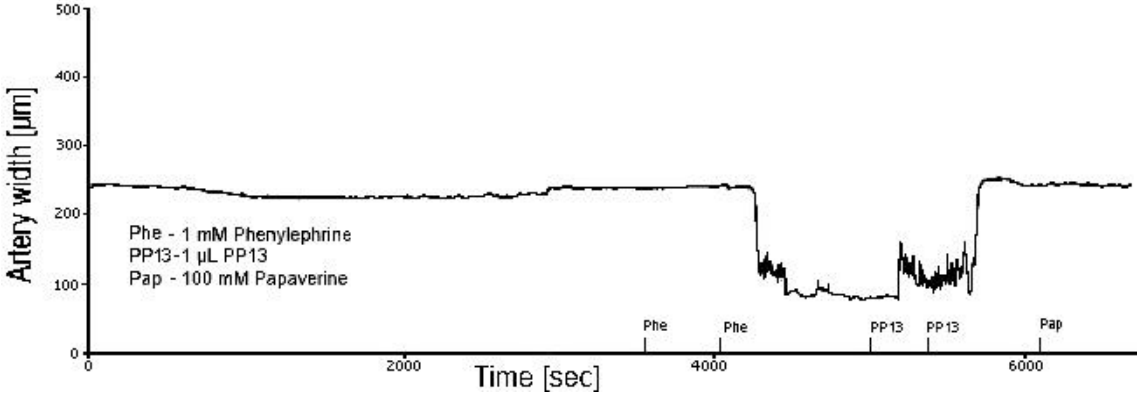

Figure A5: Cannulation performed 8. April. A reaction to PP13 doses