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Ph.D. thesis

ROLE OF HTRA1 IN PREGNANCY: A POSSIBLE EARLY MARKER OF PREECLAMPSIA

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Chapter 1: INTRODUCTION

1.1 *The Human Placenta*

The Placenta is a transient organ which represents the interface between the mother and the fetus. It allows the fetus growth ensuring nutrients and oxygen, it furthermore acts as a barrier against maternal and environmental stressors. Normal placental development is essential for the intrauterine phase because a perturbation of the fetal environment may can affect life-long health of the offspring [1].

Everything starts when the fertilized oocyte attaches to the maternal endometrium leading to the implantation process, an important mechanism tightly regulated during pregnancy. In fact, the invasion of extravillous trophoblasts into the maternal decidua (implantation) lead to the spiral arteries remodeling allowing a low resistance circulation in the intervillous space (placental circulation). An improper extravillous trophoblast invasion lead to a shallow placentation causing pregnancy complications that can impair pregnancy outcome [2].

In addition to the placenta, a key role in fetal development is due to the fetal annexes (chorion, amnion, yolk sac and allantoid), a system of membranes that surrounding the fetus to which it is connected by umbilical cord (***Figure 1***). All these structures are able to separate the mother from the fetus maintaining fetal nutrition and protection [3].
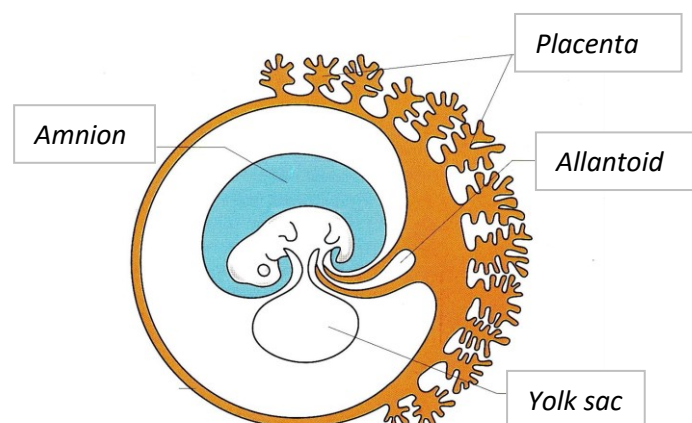
***Figure 1****: Placenta and fetal annexes*

1.2 Structure of the Human Placenta

After three days post fertilization the embryo enters in the uterine cavity and undergoes a compaction process forming the morula which will develop into blastocyst [4].

Subsequently, the villi that previously covered the entire surface of the blastocyst continue to progress only at the embryonic pole giving rise to the *Chorion frondosum*, from which will develop the **placenta** that develops from the same blastocyst that forms the fetus. The villi of the abembryonic pole degenerate from the third month of pregnancy give rise at the *Chorion leave.*

At this point, the endometrium is divided in:

- *Decidua basalis*, that represents the **basal plate,** localized against the Chorion frondosum
- *Decidua capsularis,* the portion of decidua attached to the fetal membranes surrounding the fetus
- *Decidua parietalis,* the uterine wall located on the opposite side of the embryonic pole, which, after capsularis decidua degeneration, come into contact with the *chorion leave* (**Figure 2**).
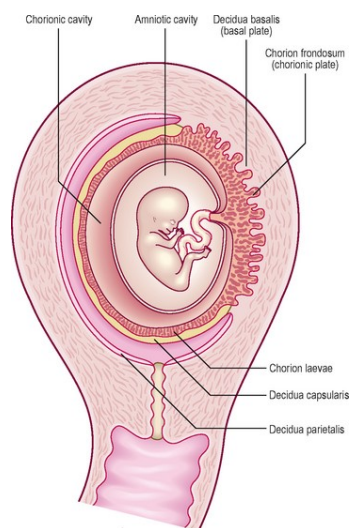
Figure 2: *Placenta and Decidua structures*

The mature human placenta appears as a disk-like structure approximately of 22 cm in diameter, 2,5 cm in thickness and 500 gr of weight. It is composed by two surfaces: the fetal surface called chorionic plate, where there are the fetal chorionic blood vessels, which are branching radials from the umbilical vessels, and the maternal surface called basal plate. The functional units of the placenta are the chorionic villi, highly vascularized structures playing a key role in maternal—fetal exchanges. In placental villi, fetal blood is separated by only three cell layers from maternal blood presents in the surrounding intervillous space (***Figure 3***) [5].
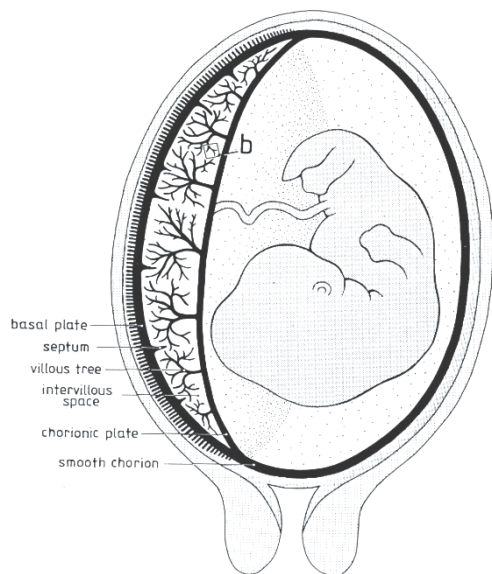
Figure 3: Human Placenta

1.3 The Placentation

At 4-5 days after fertilization, the human blastocyst is surrounded by the trophectoderm (TE), the precursor of all trophoblast cells, which is segregated from the inner cell mass (ICM) from which develops the embryo. The adhesion of the blastocyst to the uterine epithelium give rise at the process called implantation (6-7 days post-conception), the first step of placenta development. At day 8 post-conception TE stem cells generate the early mononuclear cytotrophoblasts (CTBs) and the multinuclear primitive syncytium (PS), the latter represents the invasive placenta. At 9 days p.c. in the PS appear some vacuoles that upon fusion create

networks of lacunar spaces (*lacunae*) bordered by septa of syncytiotrophoblast called *trabeculae* [6, 7] (***Figure 4***).
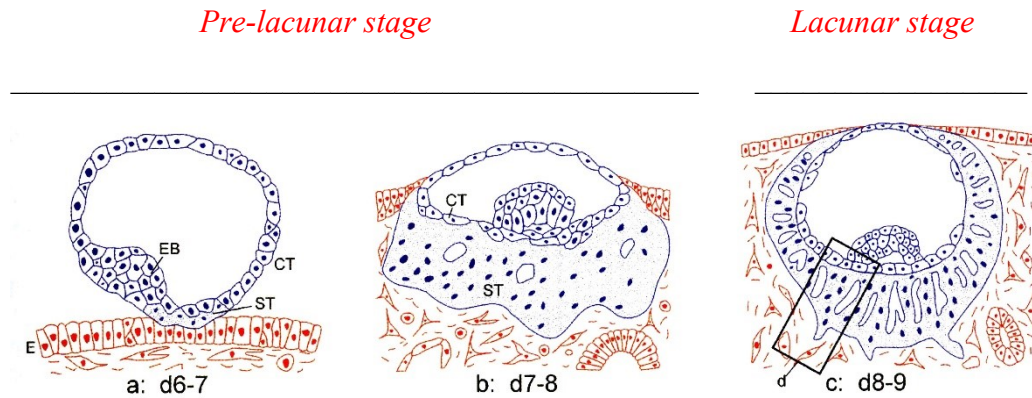
Figure 4: Early stages of placenta development (days 6-9 of gestation). a) and b) pre-lacunar stage, c) lacunar stage. Note lacunae (d) formation during days 8-9 of pregnancy.

The syncytium continues to penetrate the interstitium of the endometrium reaching the maternal spiral arteries of the uterus, the erosion of the maternal vessels causes the spill of maternal blood in the lacunae of syncytiotrophoblast. At 12 days p.c. the cytotrophoblast begins to penetrate into the syncytiotrophoblast trabeculae reaching the maternal side of the placenta. Furthermore, a part of the cytotrophoblast leaves the placenta and differentiate in extravillous cytotrophoblast (EVT) invading the endometrium. A group of this cells called endovascular trophoblast penetrate maternal spiral arteries replacing the arterial muscular wall [3].

The last stage of placentation is the villous stage that occur starting from 12-18 days p.c. The trophoblastic trabeculae composed by syncytiotrophblast externally and cytotrophoblast internally begin to protrude in the intervillous space forming the primary villi (***Figure 5***). Subsequently the embryonic mesoderm invades these primary villi forming the secondary villi. Starting at 20 days p.c. in the mesenchymal stroma of the secondary villi appear haematopoietic progenitor cells which develop in foetal capillaries transforming the secondary villi in tertiary villi. The first generation of tertiary villi are called mesenchymal villi from which will generate new villi [8].
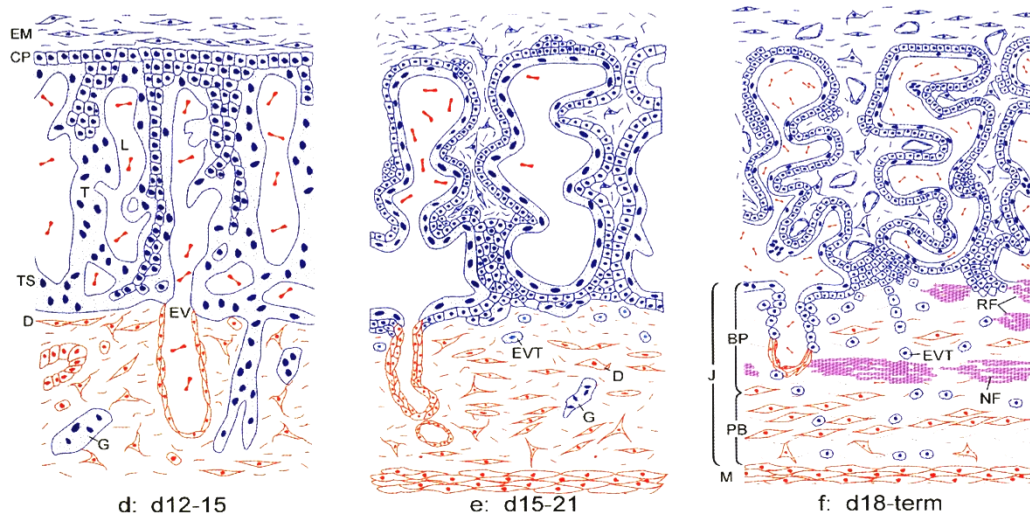
Figure 5: Late stages of placenta formation (days 12-term of gestation). Note the formation of the mature villi (tertiary villi) (f) starting from primary villi (d) and secondary villi (e).

In the human placenta it has been identified five types of different placental villi (***Figure 6***):

1. Mesenchymal villi
2. Stem villi
3. Immature intermediate villi
4. Mature intermediate villi
5. Terminal villi.

The **mesenchymal villi** are the most primitive villi mainly represented in the first stage of pregnancy from which will develop the immature intermediate villi. In this latter the connective tissue core of the mesenchymal villi becomes reticular and surrounds the large stromal channels where there are the fetal macrophages called Hofbauer cells. The **immature intermediate villi** are poorly vascularised and are present until term although they are less numerous.

At 14th week of pregnancy, the collagen fibres begin to fill the stromal channels of the proximal immature intermediate villi, generating a highly compact fibrous stroma characteristic of the stem villus. The main **stem villous** represents the connection between the villous tree with the chorionic plate. Moreover, some stem villi connect the villous tree to the basal plate by a cell column, this kind of stem villi are called anchoring villi.

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The immature intermediate villi begin to branch generating the **mature intermediate villi** that differ from the first in the tight disposition of the connective tissue fibres which fill the space between stromal channels and foetal vessels.

Around the 30th week of pregnancy, from the surface of the mature intermediate villi begin to develop the **terminal villi** characterized by a stroma composed by collagen fibres and sinusoidally dilated capillaries placed in proximity to the trophoblast. The terminal villi are the final ramifications of the villi and represent the main sites of feto-maternal exchange [9, 10].

Villo terminale

Villo stem o staminale

Villo mesenchimale

Villo intermedio immaturo

Villo intermedio maturo
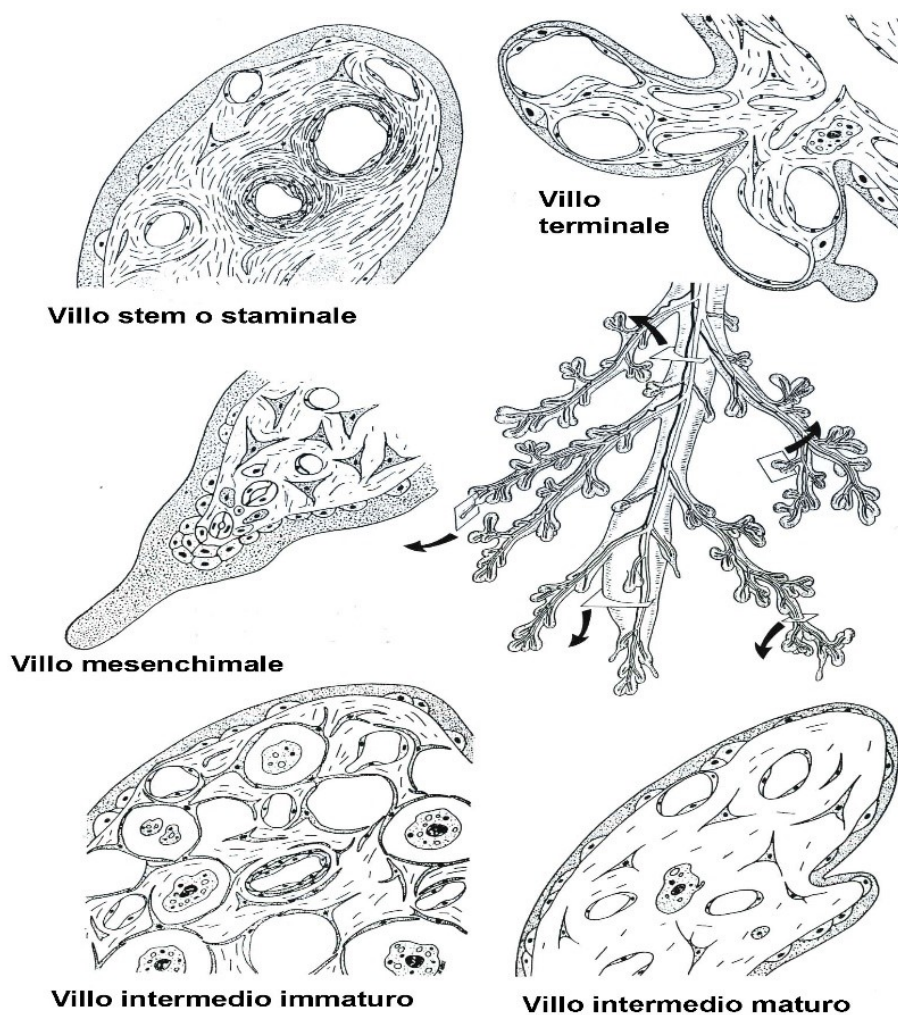
Figure 6: schematic view of different villous types normally found during human placenta development.

1.4 Preeclampsia (PE)

Preeclampsia (PE) is a multisystem hypertensive disorder that affect the 3-5% of the pregnancy worldwide [11]. Preeclampsia is characterized by new onset of hypertension (systolic pressure≥140 mmHg; diastolic pressure >90 mmHg) in normotensive woman after 20 weeks of gestation associated to new onset proteinuria (>300 mg/day). In addition, complications of this disorder may lead to maternal renal insufficiency, liver impairment (Hemolysis, ELevated Liver enzymes and Low Platelet count) and uteroplacental dysfunction [12].

Depending to the time of the disease onset, preeclampsia can be distinguished in two types: early onset PE, occurring before 34 weeks of gestation, and late onset PE, occurring after 34 weeks of gestation. Late onset PE is more prevalent than early onset of PE, but the latter has higher risk of maternal and fetal morbidity and mortality [13].

During the first stage of gestation, the nutrition of the fetus is ensured by blood plasma and secretion products of uterine glands. Only at the beginning of the second trimester of gestation, the maternal blood reaches the placenta and the continuous flow is due by the erosion of spiral arteries and the replacement of the vessel walls reducing the velocity of maternal blood flowing [14].

Preeclampsia, is characterized by a swallow extravillous trophoblast invasion which does not allow the uterine spiral arteries transformation in the placental bed. This condition causes a massively increased blood flow velocities, and reduction of the blood flow from the mother to the placental intervillous space. The reduction of blood flow velocity is necessary for the maintenance of the villous tree and for the adhesion of anchoring villi to the basal plate, which can be damaged. The pathological hypoxia due to placental hypoperfusion, induces the damaged villous trophoblast to release placental-specific factors into the maternal circulation. This factors generate maternal inflammation developing the clinical symptoms [15]. Moreover, the cytotrophoblast that doesn't invade the spiral arteries, doesn't express endothelial adhesion markers like VE-cadherin and $\alpha 1\beta 1$ and $\alpha V\beta 3$ integrin differently to normal one [16].

The whole process of trophoblast invasion trough the maternal decidua is tightly regulated and requires a strict interplay between extravillous trophoblast and maternal cells. In order to proper remodel the spiral arteries, the invasive capacity of trophoblast requires the expression and activity of different extracellular proteases including matrix metalloproteases (MMPs) and cathepsins. Moreover, invasive trophoblast and decidua cells express protease inhibitors such as tissue inhibitors of metalloproteinases (TIMPs) and cystatins (cathepsin inhibitors) in order to regulate and control the invasion [17].

Preeclampsia can be complicated by fetal growth restriction (FGR), that is due to a poor maternal nutrition or an insufficient oxygen and nutrition exchange at the placenta level [18]. When fetal growth restriction occurs, the placenta appears smaller than the normal one and intermediate and terminal villi are poor vascularized compromising maternal-fetal exchange. This is due to an excessive placenta remodeling that is not balanced by a subsequent villous growth [19]. Recent studies speculated that placental insufficiency contributes to a large proportion of spontaneous preterm birth (SPTB).

Spontaneous preterm births occur before the 37th week of gestation causing perinatal mortality in 75% of cases or long-term morbidity [20]. In fact, a lot of preterm babies have a high risk of developing diseases such as neurodevelopmental impairments, respiratory and gastrointestinal complications [21].

1.5 HtrA family proteins

The high temperature requirement A proteins (HtrAs) are a family of serine proteases, highly conserved in prokaryotes and eukaryotes organisms, having a role in protein quality control. In bacteria, they are involved in response to protein-folding stress and degradation of misfolded and mis-localized cell envelope proteins. In eukaryotes, HtrAs are able to remove misfolded proteins in different cellular organelles and in extracellular space. In particular, human HtrAs are involved in regulating cell development and aging process. Moreover, they play pivotal roles in pathological conditions such as arthritis, cancer, Parkinson's and Alzheimer's disease [22].

The HtrA family was firstly identified in *E. coli* and then four human homologues were identified: HtrA1 (L56 or PRSS11), HtrA2/Omi, HtrA3 (PRSP) and HtrA4 (***Figure 7)***[23].

This family proteins share a very well conserved architecture domain composed by:

- N- terminal region, whit regulatory function,
- Proteolytic domain (PD),
- PDZ domains, which mediates the specific protein-protein interactions.

In particular, the HtrAs belong to the trypsin clan SA that is characterized by the presence, in the catalytic site, of the triad His-Asp-Ser [23]. The PDZ domain is composed by: PSD-95 (mammalian postsynaptic density protein of 95 kDa), DLG (Discs large homolog protein) and ZO-1 (zonula occludens 1 protein) [24]. The N-terminus domain of HtrA proteins can be variable depending on its cellular localization and functionality [25]. In particular, the N-terminal regions of HtrA1, 3 and 4 are similar and characterized by the presence of a predicted signal peptide (SP); an Insulin Like Growth Factor – Binding Domain (IGF-BD) and a protease inhibitor domain (Kazal-type serine protease inhibitor domain). Instead, only HtrA2/Omi has a transmembrane protein domain. Initially, HtrA2/Omi is expressed as a precursor protein containing a mitochondrial localization signal (MTS) at N-terminus that are removed after the proteolytic maturation [26] [27]. Moreover, the HtrA3, due to an alternative splicing, can be expressed in two isoforms: long form

of 49 kDa (Htra3-L) and short form of 39 kDa (HtrA3-S), which differ in the lack of PDZ domain [28].

Figure 7: Domain organization of human serine protease HtrA family. SP= signal peptide. IGF-BD= domain with homology to insuline-like growth factor binding proteins. KM =Kazal type serine protease inhibitor motif. PROTEOLYTIC= proteolytic domain. PDZ= PDZ domain. MTS= mitochondrial localization signal TM= Trasnsmembrane sequence.

HtrAs, differently to the other heat-shock proteins, act in an ATP-independent way as chaperones or proteases [29].

All of them form homotrimers, through interaction among their protease domains, and homotrimers can form higher-order oligomers whose assembly and disassembly allowing the regulation of the protease activity [29]. The HtrAs activation mechanism is characterized by a pre-protein cleavage induced by the binding of regulatory peptide ligand on the PDZ domain of HTRA protein that leads to a change of conformation in the active site of the protein itself. [24].

1.6 HtrA1 and Pregnancy

HtrA1 is a member of the HtrA family proteins involved in different physiological processes such as extracellular matrix remodeling, mitochondrial homeostasis, apoptosis and cell signaling. It is a secreted proteins with serine-protease activity highly conserved and expressed in normal tissue of various species. An imbalance in HtrA1 expression is found in some pathologies such as osteoarthritis, age-related macular degeneration and cancer highlighting the importance of this protease in maintenance of normal cellular processes [30]. Previous study showed an higher HtrA1 expression in healthy tissue than in tumoral tissue identifying this protein as a possible prognostic factor in cancer [31]. In fact, HtrA1 expression was downregulated in ovarian cancer and in metastatic melanoma, whereas an elevated expression of this protein could lead to cell death acting as a proapoptotic protein [32].

HtrA1 is a ubiquitous protein involved in different cellular processes and it is highly expressed in human placenta suggesting a possible role of this serine protease in human placenta homeostasis. Moreover, it was demonstrated that HtrA1 was up-regulated in both endometrial glands and decidual cells during the uterine secretory phase, when blastocyst implantation can take place. During the first stage of pregnancy, HtrA1 protein was highly expressed in decidua and its expression further increased with the progression of pregnancy [33].

In normal human placenta of first trimester of gestation, HtrA1 is localized in the villous cytotrophoblast and villous stroma; in the second trimester it is present also in the syncytiotrophoblast and in third trimester it is mainly expressed in the latter structure [34]. Moreover, it has been shown that HtrA1 was expressed in key compartments for placental developement, i.e. i) the stroma of mesenchymal villi, that are forerunners of other villus types, and ii) the villous stroma adjacent to cell columns and cell islands that are two placental highly proliferative compartments [35]. This suggests that HtrA1 can interact with the Extra Cellular Matrix (ECM) molecules in villous remodeling during placental development. Hasan and colleagues demonstrated that $HtrA1^{-/-}$ mice had a smaller placenta than a control one, showing the junctional zone and labyrinth reduced. In addtion, the labyrinth had an insufficient vasculogenesis confirming the importance of the role of HtrA1 in placenta development [36].

HtrA1 has two isoforms, a native form (50 kDa) and a short form (30 kDa), which originates by an autocatalytic cleavage of the native protein, causing a triple increase of its protease activity. In addition, it was found that short form was increased and the native form was decreased in placenta affected by PE, whereas a significant decrease of both forms were found in PE placenta affected by IUGR, suggesting a different involvement of HTRA1 isoforms in these two pathologies. Recently, PE can be considered a pathology of maternal origin in contrast to PE-IUGR, that is considered of fetal origin [37].

Since HtrA1 is secreted by placenta, many studies have been focused in evaluating its levels in maternal blood of pregnant women. Interestingly, HtrA1 serum levels increase from first to third trimester of gestation showing an inverse trend compared to the HtrA1 expression in placental tissues [38]. HtrA1 serum levels change according to the different functions of trophoblast cells, in first trimester placenta, the trophoblast cells proliferate and differentiate actively leading to increased HtrA1 plasma levels, while in third trimester placenta, trophoblast cells proliferation is reduced leading to decreased HtrA1 plasma levels [39].

Levels of HTA1 secreted in the maternal blood between PE and normal gestation at third trimester, do not vary while they are significantly higher in PE pregnancy complicated by IUGR [38].

A further study analyzed HtrA1 maternal plasma levels during 28-32 weeks of gestation founding a significant increase of HtrA1 levels in PE and PE-IUGR pregnancies compared with the control group [40].

Moreover, in early-onset PE, HtrA1 plasma levels were significantly elevated compared to gestation age matched controls, whereas HtrA1 plasma levels in late-onset PE were lower than the age matched controls [41].

In an *in vitro* study, using HTR-8/SVneo cells as a model of trophoblast cells, it has been demonstrated that HtrA1 attenuated cellular chemotactic migration and invasion suggesting that an aberrant expression of HtrA1 during placentation could potentially affect trophoblast migration and invasion, resulting in shallow invasion typically found in preeclampsia [42]. These data suggest that endogenous HtrA1 may regulate migratory phenotype affecting trophoblast cells invasion and migration.

Chapter 2: AIM

The aim of this study is to evaluate whether HtrA1 could be considered a useful early marker of PE onset and how HtrA1 expression can be modified with the use of common and innovative compounds for treatment of PE. To this end, we will confirm HtrA1 expression in human placentas, from first to third trimester of normal gestation, and in placentas complicated by PE. In addition, we will evaluate whether HtrA1expression could be modified by low molecular weight heparin treatments, which is the compound of excellence for treatment of PE. In addition, due to their antioxidant and anti-inflammatory proprieties, curcumin and acetylsalicylic acid will be also tested. A multidisciplinary approach will be used to achieve this aim. Immunohistochemistry will be used to verify in whose placental structures HtrA1 is expressed; in vitro cellular models will be used to evaluate the effect of low molecular weight heparin, curcumin and acetylsalicylic acid treatment in modulating HtrA1 protein expression. Finally, HtrA1 plasma levels will be evaluated, by ELISA assay, in maternal plasma of first trimester of gestation in women that will develop PE later.

Chapter 3: Material and Methods

3.1 Tissue Collection

We analysed a total of 30 human placentas to study the HtrA1 expression: 10 from first (Obstetrics and Gynaecology of San Severino Hospital, MC, Italy) and 10 from third trimester of gestation (Department of Woman and Child Health, A. Gemelli Hospital, Università Cattolica Del Sacro Cuore Roma; Obstetrics and Gynecology, Department of Clinical Sciences, Polytechnic University of Marche, Ancona, Italy) and 10 from pathological placentas from pregnancies complicated by PE (Obstetrics and Gynecology, Department of Clinical Sciences, Polytechnic University of Marche, Ancona, Italy).

PE was defined as high blood pressure (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥ 90 mmHg on 2 occasions, at least 4 hours apart), developed after 20 weeks of gestation, with proteinuria (≥ 300 mg/24 hours or protein/creatinine ratio ≥ 0.3).

Specific exclusion criteria for the control group included a history of hypertension, diabetes mellitus, cardiac disease, renal disease, thyroid and immunological disease and congenital or acquired thrombophilia disorders, and the presence of chromosomal and other foetal anomalies.

All the procedure for this research project complies with the World Medical Association Declaration of Helsinki and were performed according to relevant national regulations and institutional guidelines.

Samples for immunohistochemistry were fixed in 4% neutral buffered formalin for 24 hours at 4°C. The tissues were rapidly and routinely processed for paraffin embedding at 56°C. Paraffin sections were cut and stretched at 45°C, allowed to dry.

3.2 Immunohistochemistry

Placental section were deparaffinized with xylene and rehydrated with graded series of ethyl alcohol. In order to inhibit endogenous peroxidase activity, sections were incubated for 50 min with 3% hydrogen peroxide in deionized water. Antigen retrieval was performed by with 0.1% trypsin (Sigma Chemical Co, St Louis, MO, USA) in phosphate buffer saline (PBS) for 5 min at 37°C. In order to block non-specific background, sections were incubated for 1 h at room temperature with PBS-6% non-fat dry milk (BioRad; Hercules CA). Sections were then incubated overnight at 4° C with anti-HtrA1 rabbit polyclonal antiserum raised against a purified bacterially expressed glutathione-S-transferase (GST)- HtrA1 (aa 363-480) human fusion protein [43] diluted 1:50 in PBS-3% non-fat dry milk. After washing in PBS, they were incubated with biotinylated secondary antibody 1:200 (Vector Laboratories, Burlingame, CA, USA) for 30 minutes at room temperature (RT). We used peroxidase ABC method (Vector laboratories, Burlingame, CA) for 1 h at room temperature and 3',3'- diaminobenzidine hydrochloride (Sigma- Aldrich, St. Louis, MO, USA) was used as the chromogen. Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted with Eukitt solution (Kindler GmbH and Co., Freiburg, Germany). Negative controls were performed by omitting the first or secondary antibody and using an isotype control antibody at the same dilution of primary anti-HtrA1 antibody for all the immunohistochemical reactions performed in this study.

3.3 Patients

This study involved healthy pregnant women at 11 weeks + 0 days‑13 weeks + 6 days of gestation. 158 healthy women and 14 of whom had a diagnosis of PE at delivery were recruited. Women attending a routine antenatal care visit at Department of Obstetrics and Gynecology of Hospital ʿS. Maria della Misericordiaʾ (Perugia, Italy), were recruited   prospectively followed until delivery.

Exclusion criteria were: multiple gestation; abortion or fetal demise or new born life < 7 days; preexisting diabetes or hypertension; women with renal failure, chronic disease or a history of consumption of drugs. We also excluded pregnancies with imprecise dates, fetal deaths, medical terminations of pregnancy and cases of induced preterm delivery before 37 weeks.

At the first visit, pregnancy dating was calculated from the date of the last period in a patient with regular menses and confirmed by ultrasound crown-rump-length measurement and the following data were collected: baseline demographics, medical history including obstetric history, current and before pregnancy habits (smoking, eating, physical activity) and anthropometric characteristics (Body Mass Index – BMI). Anthropometric and clinical features were monitored during gestation.

After birth, data concerning fetal gender, birth weight and new born health were collected.

3.4 Plasma collection and HTRA1 ELISA

Blood samples were obtained by Vacutainer venipuncture of the median cubital vein after overnight fasting. Plasma samples were prepared from a fresh EDTA venous blood centrifuged at 1500 g for 15 min at 4 °C.

Plasma were then aliquoted and stored at -80 °C until use. We have previously tested a good stability of HtrA1 protein at this temperature in plasma samples. Plasma HtrA1 concentrations were measured using commercial ELISA kit (USCN Life Sciences Inc, Wuhan, P. R. China). The measurements were conducted in duplicate, according to the manufacturer's recommended protocol. The minimum detectable dose of HtrA1 is less than 13.5 pg/ml as described in the instruction manual of over-mentioned ELISA kit. One under ml of plasma samples was used for each well. Internal negative and positive quality controls were supplied with the kit. Total proteins were used to normalize the expression data.

3.5 Statistical Analysis

A non-parametric approach was used since variables had a no-normal distribution, when evaluated with the Shapiro test. Quantitative variables were summarized using median and interquartile range (IR, 1st-3rd quartiles), respectively as measure of centrality and variability; qualitative variables were expressed as absolute and percent frequencies.

Comparisons between groups were evaluated using Wilcoxon rank sum test and Fisher exact test. Htra1 levels were analyzed after log transformation. A multiple logistic regression analysis was used to estimate the independent effect of women' characteristics on the probability of developing PE.

All estimates were obtained calculating 95% Confidence Intervals (95%CI). Likelihood ratio (LR) test and Hosmer-Lemeshow test were used to select the most parsimonious model and to evaluate the model's goodness of fit. The accuracy of the model in predicting preeclampsia was analyzed using the ROC curve, the Area Under Curve and 95% CIs. ROC curve was estimated including the variables having a p-value lower than 0.1 at the logistic regression analysis.

The R statistical program was used for the analyses and a probability of 0.05 was set as the threshold for statistical significance.

For western blotting analysis, data represent the mean ± SD, and were analyzed for statistical significance ($p < 0.05$) using Student's t-test. All experiments were performed in duplicate and were repeated at least three times. Prof. Rosaria Gesuita performed statistical analysis (Centre of Epidemiology and Biostatistics, Università Politecnica delle Marche, Ancona, Italy)

3.6 Cell culture

Human first trimester villous trophoblast cell line, HTR8/SVneo, were routinely cultured in RPMI 1640 medium (Life technologies, CA, USA). The human choriocarcinoma cell line JEG3 were routinely cultured in MEM with Earle's Salts (Euroclone). BeWo (human choriocarcinoma cell lines, used as syncytiotrophoblast model) were routinely cultured in DMEM/F12 medium (Gibco; Thermo Fisher

Scientific, MA, USA). All these media were supplemented with 10% foetal bovine serum (FBS; Gibco) and 100 U/ml penicillin and streptomycin (Gibco).

The Human Umbilical Vein Endothelial Cells (HUVEC, used as foetal vessel model) were cultured in EGM-2 Endothelial Cell Growth Medium-2 Bullet Kit (Lonza).

All cell lines were cultured in a humidified incubator with 5% CO_2 at 37°C.

3.7 Immunofluorescence of cell lines

HTR-8/SVneo, JEG3, BeWo and HUVEC cell lines were cultured on Lab-Tek II Chamber Slides (Nalge Nunc International, Naperville, IL, USA). The Chamber Slides of every cell line, were washed in Dulbecco's PBS (Lifetechnology, Monza, Italy), fixed in 4% paraformaldehyde in PBS for 10 min and permeabilized in 0,2% Triton X-100 (Sigma, Milano, Italy) in PBS for 5 min. After washing in PBS at RT, cells were blocked with 3% Normal Donkey Serum (Jackson ImmunoResearch, Pennsylvania, USA) in PBS and incubated overnight at 4° C with anti-HtrA1 (ab199529, Abcam). Cells were then washed three times in PBS and incubated with the Alexa Fluor 488-conjugated donkey anti-rabbit IgG secondary antibody (Thermo Fisher Scientific, Waltham, US) diluted 1:400 in PBS for 1 hour at RT. TOTO3 probe was used for nuclear staining. Finally, the slides were cover-slipped with propyl gallate and evaluated under a Nikon Eclipse E600 fluorescence microscope (Nikon, Düsseldorf, Germany).

3.8 MTT Assay

HTR8/SVneo and HUVEC cell lines were selected for the following treatments because they represent the cellular compartment expressing HtrA1 during first trimester of gestation. BeWo and JEG cell lines, considered syncytiotrophoblast models, were excluded because syncytiotrophoblast was negative in first trimester placental tissue. HTR8/SVneo and HUVEC cell lines were seeded at a concentration of 5×10^4 cells/well in 96-well plates. After reaching a confluence of

70%, the cells were treated with crescent concentration of Curcumin, ASA and Heparin:

- 2,5µM, 5µM, 10 µM, 20 µM of Curcumin
- 1µg/ml, 2,5µg/ml, 5 µg/ml, 10 µg/ml of ASA
- 1UI/ml, 10 UI/ml, 20 UI/ml, 30 UI/ml of Heparin.

After removing the media and washing with PBS, in each well it was added 100 µl of Thiazolyl Blue Tetrazolium Bromide solution (MTT) (Merk Life Science, Milano, Italy) and incubated for 3h in a humidified incubator with 5% CO_2 at 37°C. After the incubation period, MTT solution was removed and it was added 200 µl of Dimethyl sulfoxide (DMSO) to each well for solubilize the purple formazan crystals.

The absorbance of the samples, was measured using a microplate reader at a wavelength of 570 nm.

3.8 Cell treatments

HTR8/SVneo and HUVEC cell lines were treated for 24 and 48 hours with 10 µM curcumin, 10 µg/ml Acetylsalicylic acid (ASA, A5376 Sigma-Aldrich), 30 UI/ml Heparin (Inhixa 4000 UI/0,4 ml, Techdow). These concentrations have been chosen after performing MTT assay to evaluate a possible cytotoxicity of these compounds.

3.9 Protein extraction and Western Blotting analysis

The cells were lysed by using lysis buffer, containing 0,1 M PBS, 0.1% (w/v) SDS, 1% (w/w) NONIDET-P40, 1 mM (w/v) Na orthovanadate, 1 mM (w/w) PMSF (Phenyl Methane Sulfonyl Fluoride), 12 mM (w/v) Na deoxycholate, 1.7 µg/ml Aprotinin, pH 7.5. The samples were centrifuged at 15000 g for 10 min at 4°C and the supernatants were aliquoted and stored at -80°C until use.

The protein concentrations were determined by Bradford protein assay (Biorad Laboratories, Milan,Italy).

For western blotting assay, 20µg of protein for each sample were fractionated through electrophoresis in 10% SDS- polyacrylamide gels and transferred to

nitrocellulose membranes (Biorad Laboratories, Milan, Italy). In order to avoid non-specific protein binding, membranes were incubated with EveryBlot Blocking Buffer (Biorad Laboratories, Milan, Italy) for 5 minutes. Membranes were incubated with the primary antibodies rabbit polyclonal HtrA1 antibody (ab199529, Abcam) diluted 1:300 in TBS/0,05% Tween 20 (TBS-T), overnight at 4°C. After washing in TBS-T, membranes were incubated with secondary anti-rabbit antibody conjugated with horseradish peroxidase (715-036-150, Jackson ImmunoResearch) diluted 1:5000 in TBS-T for 1,5 h at RT. Detection of antibody binding was performed with the Clarity Western ECL Substrate (Biorad Laboratories, Milan, Italy) and the densitometry analysis of the obtained bands using Chemidoc (Biorad Laboratories, Milan, Italy) and ImageJ program (Ver. 1.52).

HepG2 human cell line was used as positive control as shown in manufacture's instruction. The relative quantities were expressed as the ratio of densitometry reading for analysed proteins normalized for β-actin expression.

Chapter 4: Results

4.1 HtrA1 expression in placental tissues

In first trimester placentas, HtrA1 was expressed in the cytotrophoblast layer (***Figure 8 b*** arrows) and in fetal vessels (***Figure 8 a,b*** arrowhead). In third trimester placenta there was a shift in HtrA1 expression, in fact the cytotrophoblast appeared negative and syncytiotrophoblast highly positive for HtrA1 (***Figure 8 c,d*** arrows). Moreover, the fetal vessels lost their positivity in third trimester placentas (***Figure 8 c,d***). In the trophoblast from pregnancy complicated by PE, there was no difference with the controls at third trimester gestation (***Figure 8 e,f***). On the contrary, fetal vessels from PE showed a positive immunostaining for HtrA1 (***Figure f***).
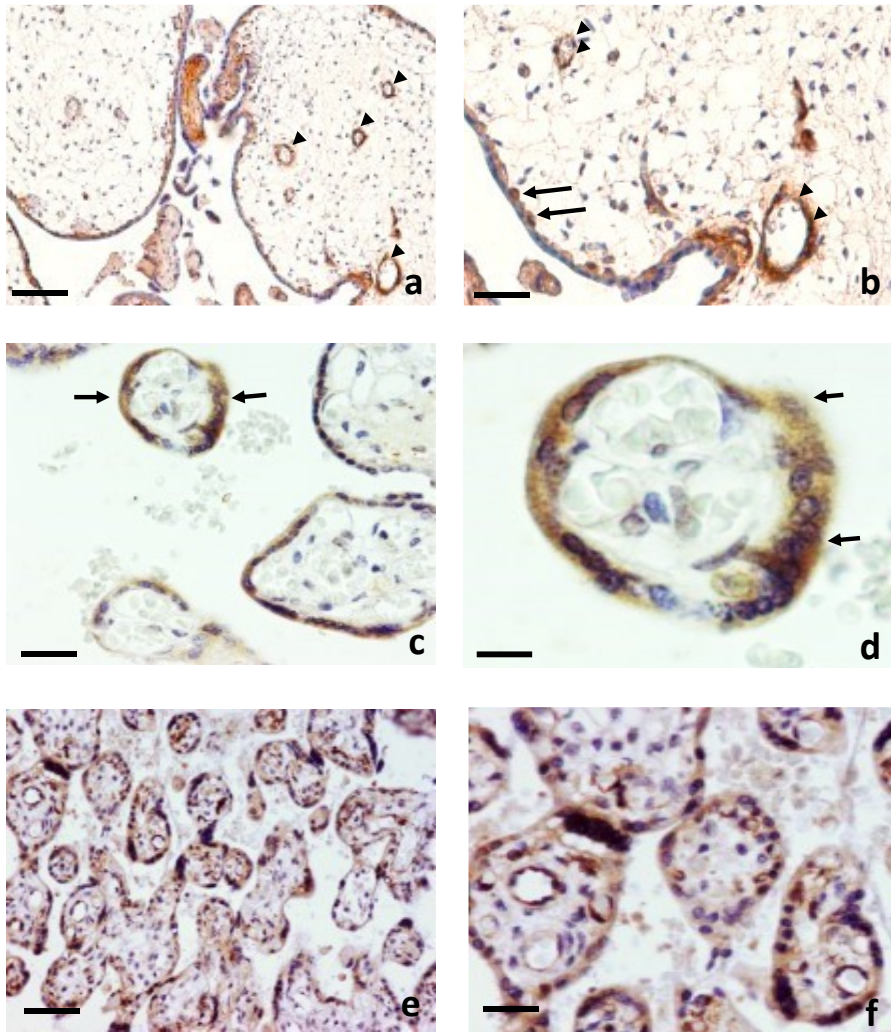
Figure 8: Immuno-localisation of HtrA1 in placentas of first and third trimester of normal pregnancy (a-d) compared to placentas from pregnancy complicated by Preeclampsia (e,f). Placental villi from first trimester show an evident staining in villous cytotrophoblast (b: arrows) as well as in the endothelium for HtrA1 (a,b: arrowheads). In third trimester placental villi Htra1 immunostaining was present in the syncytiotrophoblast (c,d: arrows) while fetal vessels are mainly negative for HtrA1. In placental villi of pregnancy complicated by PE, HtrA1 immunostaining was present in the syncytiotrophoblast and in fetal vessels (e,f). a, c, e: bar = 100 µm; b,d,f: 50 µm.

4.2 HtrA1 plasma levels

No difference in women's characteristics, between healthy pregnant and PE cases were found with regards to age at pregnancy, BMI variation after pregnancy, smoking, nutritional habits, physical activity and caesarian delivery. A significant elevation in BMI, before and after pregnancy, was found in PE cases compared to normal pregnancy. Moreover, in pregnancy affected by PE, it was found a shorter gestational age at delivery and higher log HtrA1 than healthy women (***Figure 9***). Newborns' birth weight and Apgar Score was the same in both cases. No immediate neonatal complications were observed in PE cases, except one newborn with jaundice. Instead, in healthy women, fourteen newborns were affected of immediate neonatal complications, i.e. 7 jaundices, 3 sepsis, 3 congenital organ defects (2 kidneys, 1 genital), 1 hyperglycemia.
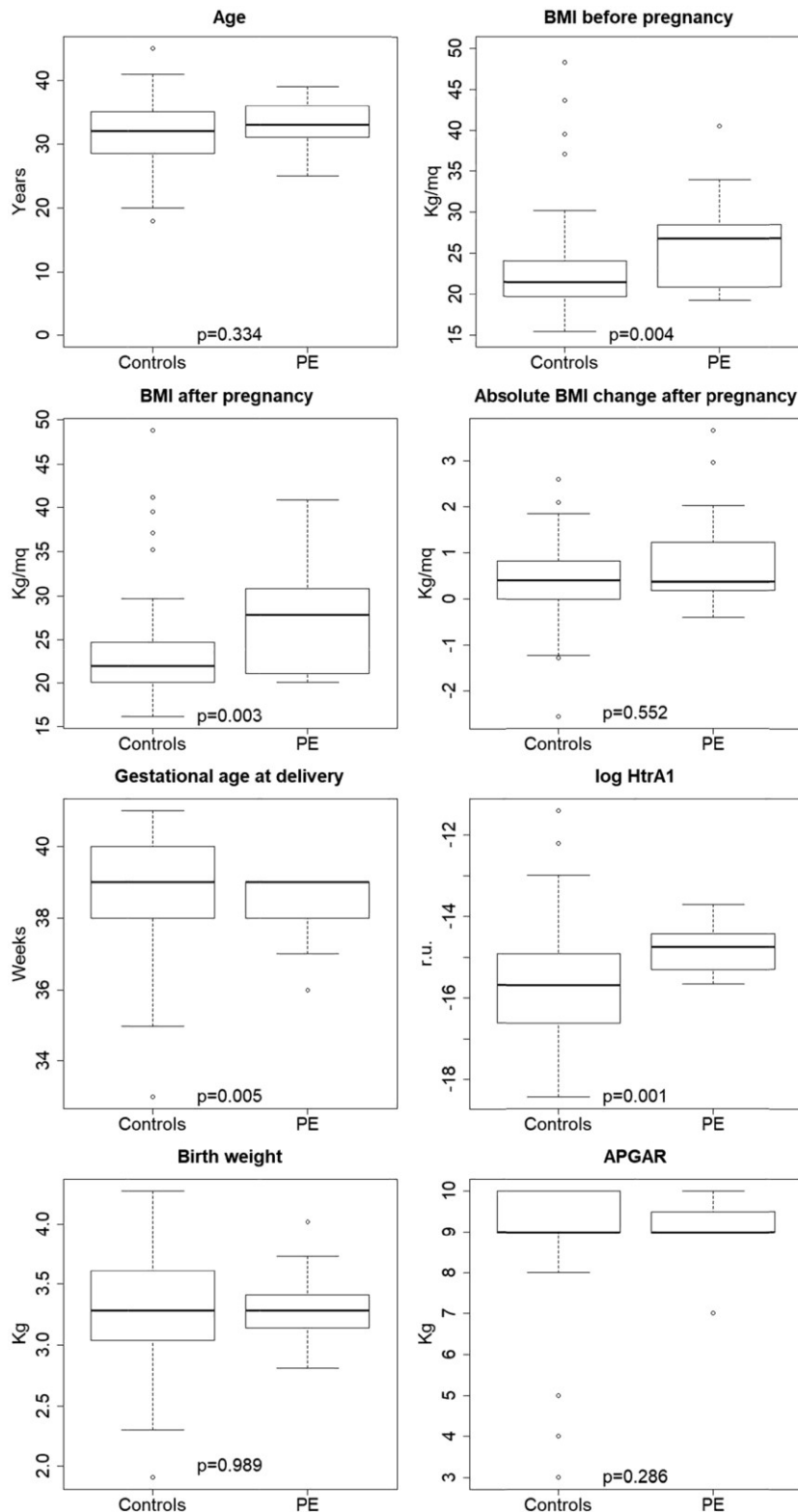
Figure 9: Demographic and clinical characteristics of the population considered in the study according to its health status. The box blots show the results concerning maternal age, BMI before and after pregnancy, absolute BMI change after pregnancy, maternal gestational age at delivery, logHtrA1, birth weight and APGAR index. PE cases showed significantly higher BMI, before and after pregnancy, shorter gestational age at delivery and higher log HtrA1 than healthy women. No significant difference was found in newborns' birth weight and Apgar Score. p-values refer to Wilcoxon rank sum test.

Table 1 shows the results of the multiple logistic regression analysis. PE was significantly associated with log HtrA1 values, BMI before pregnancy and gestational age at delivery. In particular, the probability of developing PE increased about 90% for every added unit of log HtrA1 and about 10% for every added unit of BMI, while decreased of about 37% for every week added of gestational age at delivery.

	OR	95% CI	P
log HtrA1	1.90	1.12; 3.40	0.021
BMI before pregnancy (kg/m2)	1.10	1.00; 1.20	0.044
Physical activity (yes vs no)	1.54	0.44; 6.10	0.514

Table 1: *Hosmer and Lemeshow goodness of fit test: Chi-square with 8 df, χ2=6.04, p=0.642. LR test: Chi-square with 6df, χ2=17.55, p=0.007.*

The ROC curve (***Figure 10***) showed that the model had a good accuracy in predicting preeclampsia, with an AUC equal to 0.83 (95%CI: 0.74–0.92).
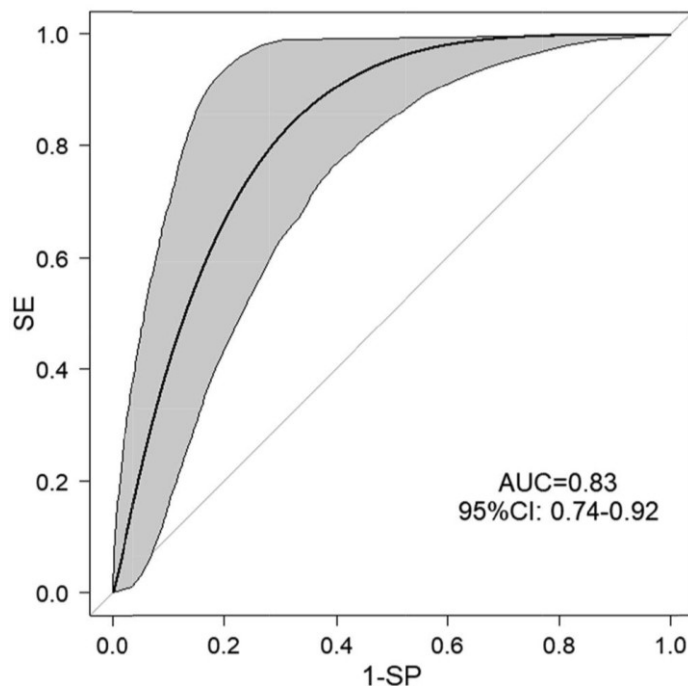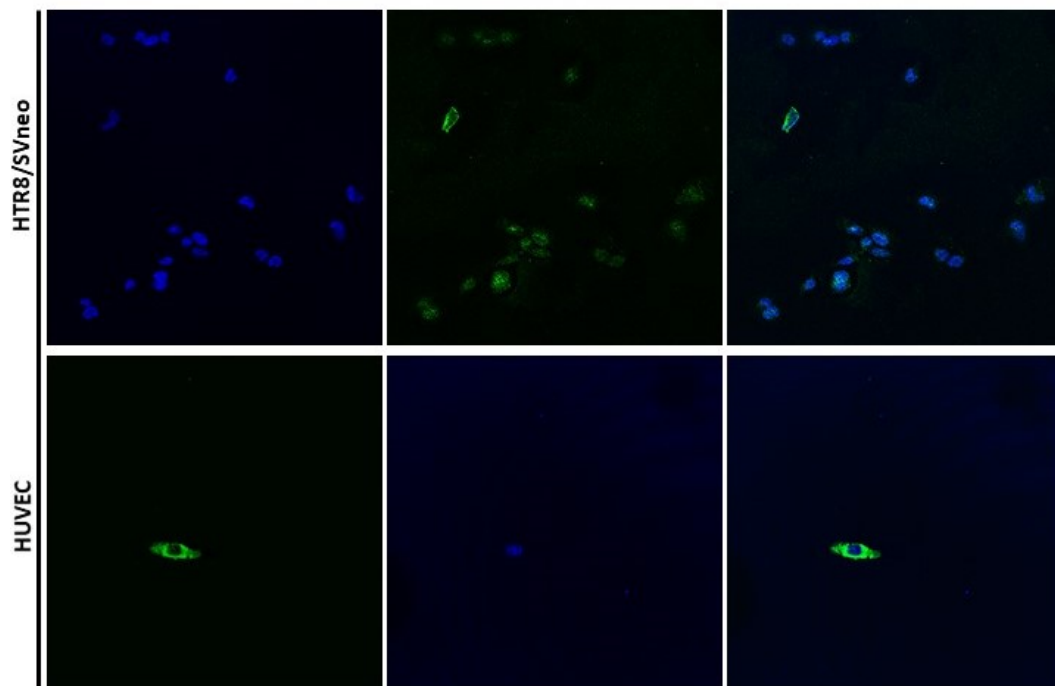

Figure 10: ROC curve for the predictiveness of preeclampsia. ROC curve was estimated including in the model logHtrA1, BMI before pregnancy, Gestation at delivery (variables with p < 0.10). SE: Sensitivity; SP: Specificity; Grey area identifies 95% confidence interval of ROC. AUC: Area Under Curve; 95%CI: 95% Confidence Interval.
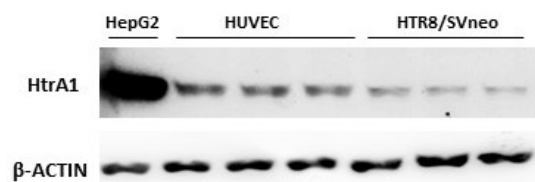
4.3 HtrA1 protein expression in HTR8/SVneo and HUVEC cell lines

Using immunofluorescence analysis, we assessed HtrA1 cellular localization in HTR8/SVneo and HUVEC cell lines, normally used as *in vitro* models of villous cytotrophoblast and endothelial fetal vessels respectively. Immunofluorescence staining localized HtrA1 expression in the cell cytoplasm in both cell lines (***Figure 11 a***). HtrA1 was expressed in HTR8/SVneo and HUVEC cell lines. HepG2 cell line was used as positive control (as showed in manufacture's instruction). Both cell lines showed a specific band at 55 kDa (***Figure 11 b***).

*Figure 11: HtrA1 protein expression in HUVEC and HTR8/SVneo placental cell lines. a) immunofluorescence analysis of HTRA1 showed a citoplasmatic localization in both cell lines. b) Western Blotting analysis confirmed HtrA1 protein expression in both cell lines showing a band at molecular weight of 55kDa confirmed by HepG2 cell line used as positive control as suggested by manufacture's instruction.*

4.4 Heparin increases HtrA1 expression in HUVEC and Htr8/SVneo cell lines

ASA and heparin were not cytotoxic in all concentrations tested, while curcumin significantly lowered cell viability at a concentration of 20 µM tested by MTT (**Figure 12**).
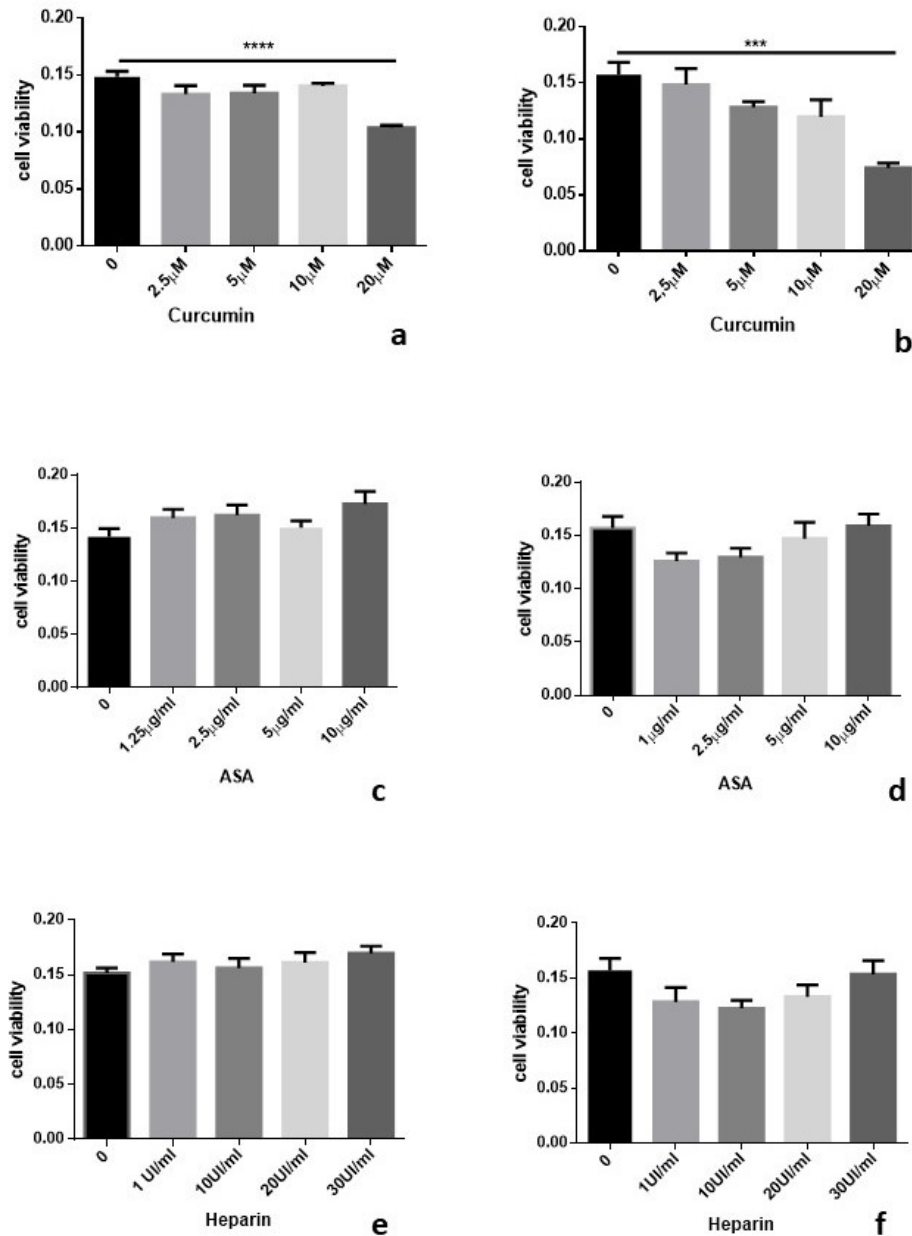
Figure 12: cytotoxic analysis of different concentrations of curcumin, ASA and heparin in HTR8/SVneo (a,c,e) and HUVEC (b,d,f) cell lines. No cytotoxic effects have been found for ASA and heparin in both cell lines while curcumin showed a significant cytotoxic effect at 20 µM in both cell lines.

Only heparin treatment at 48 hours showed a statistically significant increase in HtrA1 expression in both cell lines (***Figure 13***).   No significant differences were found after curcumin and ASA treatments.
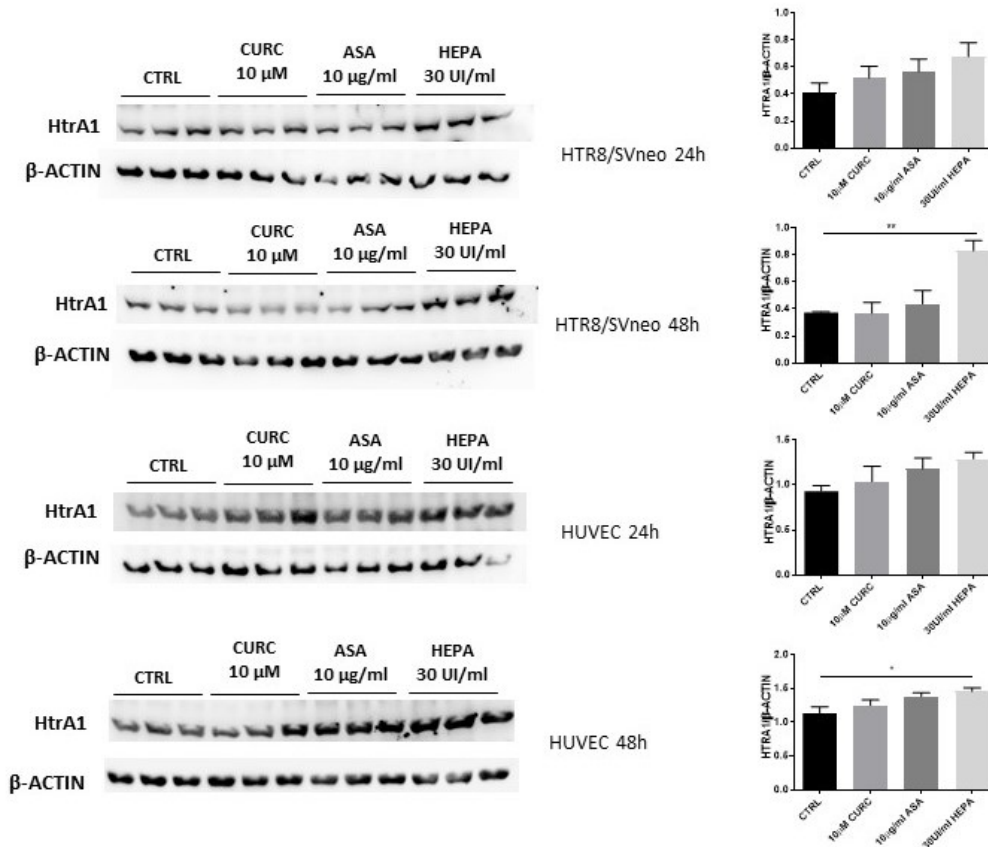
Figure 13: HtrA1 protein expression in HTR8/SVneo and HUVEC cell lines treated with curcumin (CURC), ASA and heparin (HEPA) for 24 and 48h. No statistically significant differences have been found in curcumin and ASA treatment in both cell lines for 24 and 48 h. A statistically significant increase of HtrA1 expression has been found in both cell lines treated with heparin for 48h while no differences have been highlighted at 24h.

Chapter 5: Discussion

Our data confirm previous findings concerning HtrA1 expression in normal and PE human placentas [37]. Interestingly, fetal vessels were positive for HtrA1 in PE placentas while they were negative in age matched controls. We hypothesized that the positiveness of the vessels in PE placentas of third trimester of gestation could be due to late development of the fetal vascular tree in this kind of placentas as previously described [44]. So, we can speculate that the fetal vessels in earliest phases of gestation are mainly negative for HtrA1. In addition, it is known that HtrA1 is a secreted protein and it was found an increase of HtrA1 levels in maternal plasma from first to third trimester of normal gestation when the placental fetal vessels became negative for HtrA1. These data suggest that HtrA1 detected in maternal plasma could be derived from endometrium due to the negative staining of the fetal vessels. Interestingly, Nie et al demonstrated that HtrA1 is up-regulated in both endometrial glands and decidual cells during endometrial preparation for embryo implantation suggesting a possible involvement of maternal HtrA1 in placental development [33]. At present, although the etiology of PE is still unknown, it has been proved that impairment of trophoblast invasion during early placentation process plays a key role in the onset of this pathology. In fact, PE is characterized by a shallow invasion of uterine spiral arteries of endometrium by extravillous trophoblast, which impairs arterial remodeling increasing maternal blood resistance causing systemic hypertension [45]. Recently it is more and more evident that, although PE symptoms appear after 20th weeks of gestation, the pathology begins to develop before this period [46]. Therefore, HtrA1 could be considered a useful candidate as early marker of PE. Knowing whether a pregnant woman will develop PE from first weeks of gestation (before 12 weeks) makes it possible to manage women at high risk of PE before the appearance of symptoms in order to prevent damage to the placenta and consequently to the fetus. In order to evaluate the possible role of HtrA1 as an early marker of PE, we investigated HtrA1 levels in healthy pregnant women at first trimester of gestation and followed until delivery. Interestingly, we found significant higher HtrA1 plasma levels in the first trimester maternal plasma of women who subsequently developed PE compared to control. Since it is impossible to recruit PE placentas before 20th of

gestation as symptoms appear after, we can only hypothesize that the PE placentas of the first trimester of gestation could not express or express low levels of HtrA1 in fetal vessels compared to normal one. This suggest that HtrA1 in maternal plasma could originate from the endometrium due to immature placental development. Our predictive model of PE showed a good accuracy having a ROC curve with an AUC equal to 0.83.

These results may have a strong clinical relevance, in terms of PE prevention and saving of economic resources, since HtrA1 could be tested by ELISA that is a noninvasive and low-cost assay, which could be easily included in clinical practice [47].

ASA and low molecular weight heparin are routinely used to treat pregnant women at high-risk of developing PE but the effects of these two molecules in placental tissues are unknown. In order to investigate the effect of these two compounds on HtrA1 modulation, we used HUVEC and Htr8 SV/neo cell lines as model of fetal vessel and villous cytotrophoblast respectively. Moreover, since PE is characterized by oxidative damage due to the production of reactive oxygen species (ROS), we tested the action of curcumin, a natural and well-known antioxidant compound, in modulating HtrA1 expression.

Interestingly, we detected a statistically significant increase of HtrA1 protein expression in both cell lines treated with low weight heparin for 48 hours while we did not find any statistically significant differences in HtrA1 protein expression with curcumin and ASA treatments. These data together suggest that heparin administration during first trimester of pregnancy could increase HtrA1 expression in fetal vessels and in villous cytotrophoblast ameliorating placental development.

In conclusion, we proposed a predictive model of PE, that include HtrA1 detection by ELISA, in order to ensure an early treatment to pregnant women. In addition, we demonstrated that the low weight heparin treatments routinely administrated to manage PE patients increase HtrA1 expression in *in vitro* models of human placental tissues suggesting that HtrA1 can be considered a key molecule in normal and pathological placental development.

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