Dissertation

THE ROLE OF PLACENTAL MATRIX METALLOPROTEINASES 14 AND 15 IN INFLAMMATION-ASSOCIATED PREGNANCY DISEASES

submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgment has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice and Ombuds Committee at the Medical University of Graz”.

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Table of contents

Declaration i

Acknowledgements ii

Abbreviations and Definitions 1

Abstract 5

Zusammenfassung 7

1. Introduction 9

1.1. Human placenta 9

1.1.1. First trimester placenta 9

1.2. Trophoblast invasion and pregnancy complications 10

1.2.1. Preeclampsia (PE) 11

1.2.2. Maternal obesity 12

1.3. Matrix metalloproteinases (MMPs) 13

1.3.1. MMP structure and activation 13

1.3.2. MMP regulation 15

1.3.3. MMPs in the first trimester of pregnancy 16

2. Hypothesis and objectives 17

3. Material and Methods 18

3.1. Ethics statement 18

3.2. Tissue collection 18

3.3. Human first trimester placental chorionic villi explants 18

3.4. Cell culture 19

3.4.1. Human first trimester trophoblast isolation and culture 19

3.4.2. Culture of ACH-3P cell line 20

3.5. Immunohistochemistry 21

3.5.1. Immunofluorescence double staining 21

3.6. RNA isolation and RT-qPCR 23

3.7. Protein isolation and Western blotting 24

3.8. Zymography 25

3.9. Enzyme-linked immunosorbent assay (ELISA) 25

3.10. Transwell invasion assays..26

3.11. Chick chorioallantoic membrane (CAM) assay 26

3.12. Statistical analysis..26

4. Results...28

4.1. MMP15 in human first trimester placenta 28

4.1.1. Localization 28

4.1.2. Function 34

4.2. ET-1, MMP regulation and trophoblast function...37

4.2.1. ET-1 and ACH-3P cell proliferation and viability 37

4.2.2. ET-1 regulation of MMP14 and MM15 in ACH-3P cells 38

4.2.3. ET-1 regulation of MMP14 and MMP15 in human first trimester trophoblasts 40

4.2.4. Identification of ETR subtypes involved in MMP regulation 45

4.2.5. Functional effects of ET-1 on first trimester placenta...48

4.2.6. Modulation of ET-1 mediated MMP14 and MMP15 downregulation by TNF-α ...51

4.2.7. Modulation of ET-1 mediated MMP14 and 15 downregulation by low oxygen tension 53

4.3. MMP14 and MMP15 regulation by obesity in human first trimester placental tissue55

4.3.1. MMP14 and MMP15 expression in lean and obese placental tissue 55

4.3.2. MMP14 and MMP15 protein levels in lean and obese placental tissue...............57

5. Discussion...62

5.1. MMP15 is involved in trophoblast invasion..62

5.2. The interplay between ET-1, inflammation and low oxygen tension in MMP14 and MMP15 regulation in human first trimester trophoblasts..63

5.2.1. ET-1 as a regulator of MMP14, MMP15 and TIMPs 63

5.2.2. MMP14/MMP15 downregulation is mediated via ETBR 65

5.2.3. ET-1 downregulates trophoblast outgrowth and invasion in early pregnancy......66

5.2.4. TNF-α enhances ET-1-mediated MMP15 downregulation.................................67

5.2.5. Low oxygen tension as a negative regulator of ET-1 67

5.3. Obesity alters placental MMP14 and MMP15 already during the first trimester of pregnancy 70

5.4. Summary, importance of the major findings and limitations 71

6. References 73

Abbreviations and Definitions

ACH-3P: Human first trimester trophoblast cell line

Act-MMP: Active-MMP

ADAMs: A disintegrin and metalloproteinases

ANOVA: Analysis of variance

AP-1: Activator protein-1

A.U.: Arbitrary units

BCA: Bicinchoninic acid

BMI: Body mass index

CAM: Chick chorioallantoic membrane

Cat: Catalytic domain

Cc: Cell columns

CD: Cluster of differentiation

cDNA: Complementary deoxyribonucleic acid

Cyt: Cytoplasmic domain

DAPI: 4',6-diamidino-2-phenylindole

DMEM: Dulbecco's Modified Eagle's medium

ECM: Extracellular matrix

EGF: Epidermal growth factor

ELISA: Enzyme-linked immunosorbent assay

ET-1: Endothelin-1

ETR: Endothelin receptor

EVT: Extravillous trophoblast

GA: Gestational age

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GDM: Gestational diabetes mellitus

GW: Gestational week

GPI: Glycophosphatidylinositol

HBSS: Hanks' Balanced Salt solution

hCG: Human chorionic gonadotropin

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Hin: Hinge region

HLA-G: Human leukocyte antigen-G

HxD: Hemopexin domain

iEVT: Interstitial extravillous trophoblast

IgG: Immunoglobulin G

IL: Interleukin

KCM: Keratinocyte medium

kDa: Kilodalton

K7: Cytokeratin 7

K18: Cytokeratin 18

M: Molar

MMPs:	Matrix metalloproteinases
MT-MMPs:	Membrane-type matrix metalloproteinases
µl:	Microliter
µm:	Micrometer
µM:	Micromolar
ng/ml:	Nanograms/mililiter
NK-cells:	Natural killer cells
nM:	Nanomolar
O_2:	Oxygen
PE:	Preeclampsia
PFA:	Paraformaldehyde
Pro:	Pro-domain
Pro-MMP:	Inactive matrix metalloproteinase
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
RPL30:	Ribosomal protein L30
RT-qPCR:	Reverse transcription-quantitative polymerase chain reaction
SD:	Standard deviation
sEng:	Soluble endoglin
Sflt-1:	Soluble fms-like tyrosine kinase-1
siRNA:	Small interfering RNA

TGF-β: Transforming growth factor-β

TIMPs: Tissue inhibitors of matrix metalloproteinases

TM: Transmembrane domain

T1D: Type-1 diabetes

TNF-α: Tumor necrosis factor-α

VEGF: Vascular endothelial growth factor

VT: Villous trophoblast

Abstract

During the first trimester of pregnancy trophoblast cells invade the uterine wall and remodel the spiral arteries, allowing an adequate blood supply to the fetus. Shallow trophoblast invasion is associated with pregnancy complications such as preeclampsia (PE). Other PE hallmarks are endothelin-1 (ET-1) upregulation, inflammation and hypoxia. Moreover, the risk for developing PE is increased in other pro-inflammatory conditions such as maternal obesity.

Trophoblast invasion is determined by matrix metalloproteinases (MMPs). Two members of this protease family, MMP14 and MMP15, have been identified in first trimester trophoblasts, but the actual role of MMP15 still remains unknown. Hence, we hypothesized that MMP15 is key player for first trimester placental function, and that MMP14 and MMP15 are regulated by the PE stimuli mentioned above as well as by maternal obesity.

The present study aimed to determine the role of MMP15 in first trimester trophoblast biology, and the effect of PE stimuli and obesity on MMP14 and MMP15 levels during the first trimester of pregnancy. For this purpose, MMP15 localization and function were investigated using immunofluorescence and human first trimester chorionic placental villi explants, respectively. Isolated first trimester trophoblasts were incubated with ET-1 (10nM or 100nM), tumor necrosis factor (TNF)-α (25ng/ml) or exposed to different oxygen tensions (1%, 2.5% and 20% O_2). Then, MMP14 and MMP15 expression and protein levels were measured by RT-qPCR and Western blotting, respectively. ET-1 functional consequences were determined using chorionic villi explants and transwell invasion assays. Finally, MMP14 and MMP15 expression and protein levels were also measured in placental tissue from lean and obese pregnant women.

The key findings of the study were: i) MMP15 is exclusively localized to invasive trophoblasts and is involved in trophoblast invasion; ii) ET-1 decreases MMP14 and MMP15 expression and protein levels in human first trimester trophoblasts and hinders trophoblast invasion; iii) TNF-α enhances ET-1-mediated MMP15 downregulation; iv) low oxygen tension (1% O_2) abolished the effect of ET-1 on MMP14 and MMP15 downregulation; v) maternal obesity correlated with an upregulation of MMP14 and a downregulation of MMP15 protein levels.

In conclusion, MMP15 is crucial for trophoblast invasion. Together with MMP14, MMP15 needs to be tightly regulated for an adequate placental function. Alterations in the first trimester placental milieu toward a pro-inflammatory environment lead to MMP14 and MMP15 dysregulation, resulting in impaired trophoblast invasion, and might, therefore, be one of the causes underlying pregnancy complications such as PE.

Zusammenfassung

Während des ersten Trimesters der Schwangerschaft invadieren spezielle Plazentazellen, die Trophoblasten, in die Uteruswand ein und modifizieren die Spiralarterien, wodurch eine ausreichende Blutversorgung des Fötus ermöglicht wird. Verminderte und oberflächliche Trophoblastinvasion ist mit Schwangerschaftskomplikationen wie Präeklampsie (PE) assoziiert. Andere PE Stimuli sind erhöhte Expression von Endothelin-1, Entzündung und Hypoxie. Zudem ist das Risiko für die Entwicklung von PE in anderen entzündlichen Erkrankungen wie mütterlicher Fettleibigkeit erhöht.

Die Trophoblastinvasion wird durch Matrixmetalloproteinasen (MMPs) reguliert. Zwei Mitglieder dieser Protease-Familie, MMP14 und MMP15, wurden in Trophoblasten während des ersten Trimesters identifiziert, aber die tatsächliche Rolle von MMP15 ist noch unbekannt. Wir erstellen die Hypothese, dass MMP15 ein wichtiger Akteur für die plazentale Funktion im ersten Trimester ist, und dass MMP14 und MMP15 durch die oben genannten PE Stimuli sowie durch mütterliche Fettleibigkeit reguliert werden.

Hierzu wurde die Lokalisation und Funktion von MMP15 mnittels Immunfluoreszenz an humanen Plazenta-Zotten des ersten Trimenons (Explants) untersucht. Trophoblasten, die aus Plazenten des ersten Trimesters isoliert wurden, wurden mit ET-1 (10nM oder 100nM), TNF-α (25ng/ml) oder unterschiedlichen Sauerstoffkonzentrationen (1%, 2,5% und 20% O_2) behandelt, und MMP14- und MMP15 mRNA und Protein wurden durch RT-qPCR bzw. Western-Blotting quantifiziert. Funktionelle Effekte von ET-1 Behandlung wurden mittels Zotten Explants und Transwell-Invasion Assays bestimmt. Schließlich wurde die Expression von MMP14 und MMP15 auch im Plazentagewebe von schlanken und fettleibigen schwangeren Frauen gemessen.

Die wichtigsten Ergebnisse der Studie waren: i) MMP15 ist ausschließlich auf invasive Trophoblasten lokalisiert, und ist an der TrophoblastiInvasion beteiligt; ii) ET-1 vermindert MMP14- und MMP15- mRNA und Protein Expression in Trophoblasten des ersten Trimenons, und verhindert auch die TrophoblastiInvasion; iii) TNF-α verstärkt die ET-1-vermittelte MMP15-Downregulation; iv) niedrige Sauerstoff-konzentration (1% O_2) beseitigtdie

Wirkung von ET-1 auf MMP14- und MMP15-Regulation; v) maternale Adipositas korreliert mit einer Erhöhung von MMP14 und einer Verminderung von MMP15.

Die Ergebnisse zeigen, dass MMP15 entscheidend für die TrophoblastiInvasion ist. Zusammen mit MMP14 muss MMP15 für eine adäquate Plazentafunktion genau reguliert werden. Ein entzündliches plazentares Milieu im ersten Trimester führt zu MMP14- und MMP15-Dysregulation, was eine beeinträchtigten Trophoblastinvasion bewirkt, und daher eine der Ursachen für Schwangerschaftskomplikationen wie PE sein könnte.

1. Introduction

1.1. Human placenta

The human placenta is a fast developing and highly specialized villous organ located at the feto-maternal interface. It fulfils a wide spectrum of functions that are essential for normal growth and development of the fetus (1). The placenta is involved in nutrient and oxygen transport. It also removes carbon dioxide and other metabolic waste products and acts as a barrier in the defense against pathogens and xenobiotics. Together with its metabolic and protective role, the placenta also synthesizes and secretes a number of hormones, cytokines and growth factors critical for a successful pregnancy (2). Although the placenta is a plastic organ which develops to accommodate the fetal demands along pregnancy, the first trimester is critical for placental and embryonic development (3).

1.1.1. First trimester placenta

During the first trimester of pregnancy, specialized placental cells, i.e. trophoblasts, differentiate following two pathways that are essential for placental function: i) villous trophoblasts (VT), and ii) extravillous trophoblasts (EVT). VT are proliferative cells which can also fuse to form the syncytiotrophoblast, a syncytium that represents the classical placental barrier and is involved in the transport of maternal nutrients to the fetal circulation and the secretion of pregnancy hormones (4) .

In those villi in contact with the uterine basement membrane, i.e. anchoring villi, VT proliferate forming cell columns. Differentiation into EVT occurs at the tip of the cell columns, where EVT acquire an invasive phenotype migrating and invading into the decidua. This results in the anchoring of the feto-placental unit to the uterus (5). EVT also reach the uterine spiral arteries and remodel them into wide, low resistance vessels. This is necessary for the establishment of an adequate blood supply to the fetus (6) (Fig. 1).

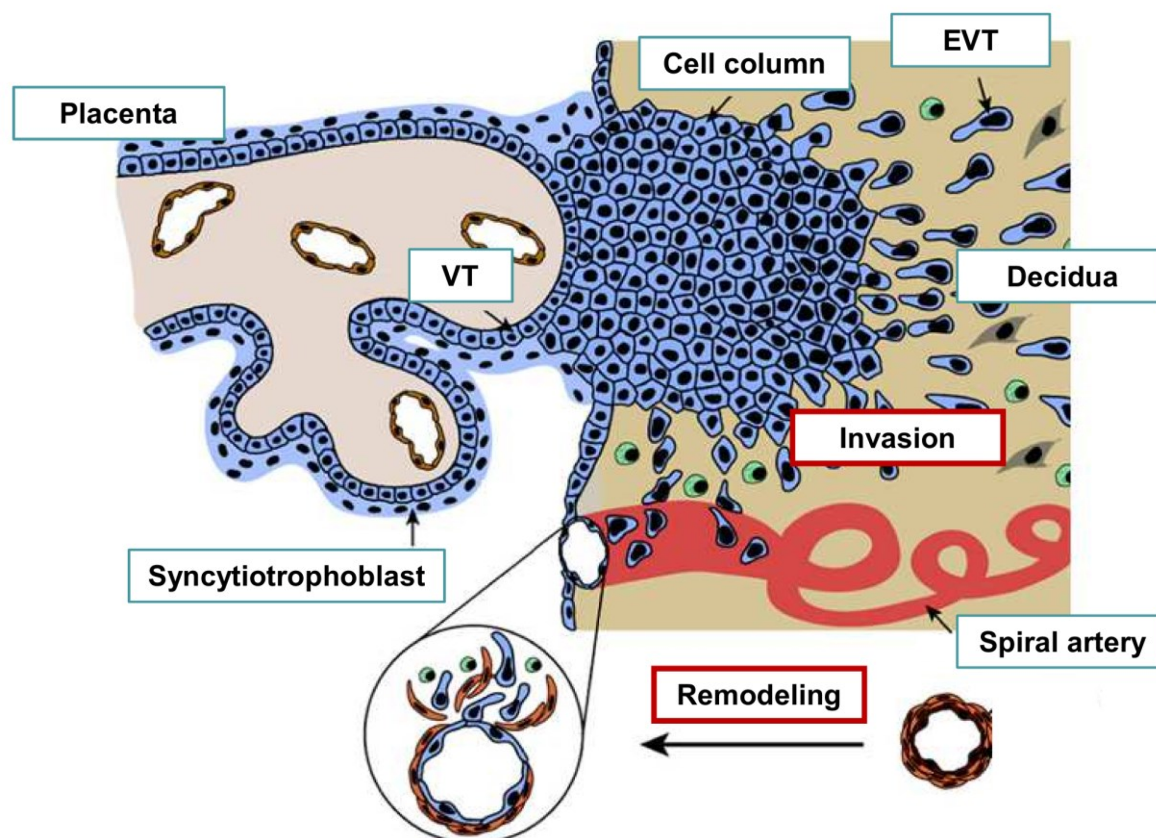

Figure. 1. Schematic representation of a human first trimester placental villous in contact with the decidua. The different trophoblast cell subpopulations are shown: villous trophoblasts (VT), syncytriotrophoblast and extravillous trophoblast (EVT). Trophoblast invasion and the remodeling of the spiral arteries are highlighted in red. Adapted from (5).

1.2. Trophoblast invasion and pregnancy complications

Trophoblast migration and invasion as well as the remodeling of the spiral arteries are required for a successful pregnancy. Therefore, trophoblast invasion is tightly regulated both temporally and spatially. Several cytokines and hormones including interleukin (IL)-6, IL-10, tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β influence the invasive potential of trophoblast cells (7). Moreover, inflammatory processes can alter the concentration of these molecules, thus conditioning the process of invasion (8).

1.2.1. Preeclampsia (PE)

PE is a pregnancy complication characterized by the onset of proteinuria and hypertension in normotensive pregnant women. It affects 2-8% of pregnancies worldwide and is considered one of the major causes of maternal morbidity and mortality (9). Furthermore, an effective treatment to tackle PE has still not been found (10).

According to its onset, PE can be subdivided into early- (before gestational week (GW) 34) or late-onset PE (appearing in GW 34 and onwards). Late-onset PE is considered a maternal disorder, whereas early onset PE has been associated with shallow trophoblast invasion and impaired spiral artery remodeling. Thus, placental malfunction is central to early-onset PE and it already begins during the first trimester of pregnancy (11-14). However, PE clinical features appear first during the second trimester of pregnancy (9), with the majority of the studies being performed in term placental tissue. Hence, more studies addressing PE during the first trimester, i.e. the time-window when PE originates, are necessary.

Inflammation is one of the hallmarks of PE, with several cytokines such as IL-6, TNF-α and IL-1β being dysregulated in this condition (15). As mentioned above, these cytokines also regulate trophoblast function. Therefore, understanding the impact of PE classical stimuli in first trimester trophoblast biology might help revealing the molecular mechanisms underlying this complication (16).

Insufficient spiral artery remodeling results in placental ischemia and compromises oxygen delivery to the fetus (17). In response to this hypoxic environment, placental mediators are released into the maternal circulation causing endothelial dysfunction. Because of this, endothelial dysfunction indicators such as soluble endoglin (sEng), soluble fms-like tyrosine kinase-1 (sflt-1) and endothelin-1 (ET-1) have gained relevance as biomarkers for PE (18-20). A summary of the crosstalk of PE stimuli is shown in Fig. 2.

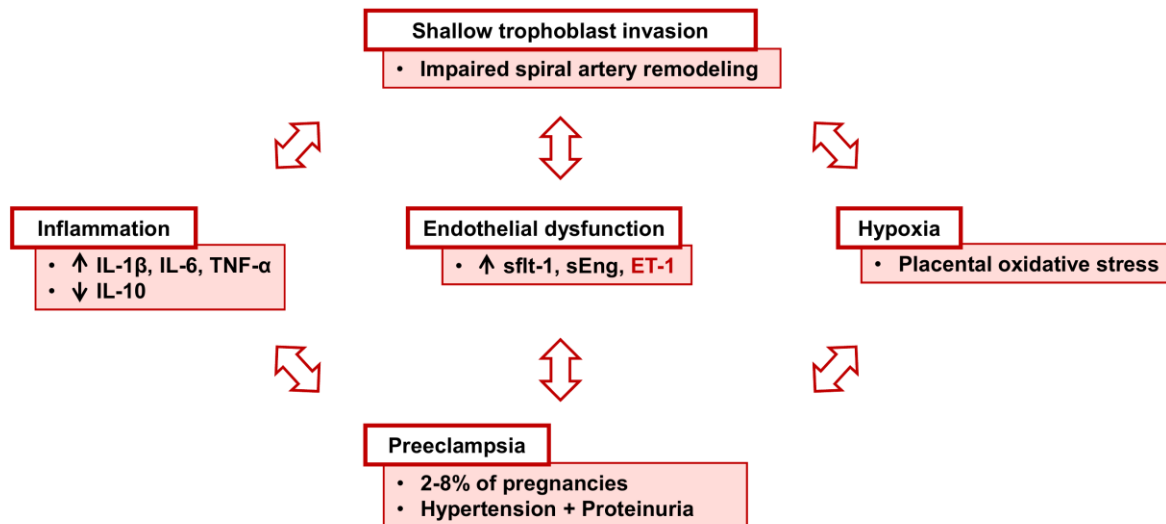

Figure. 2. Preeclampsia (PE) characteristic stimuli and their crosstalk. Shallow trophoblast invasion results in impaired spiral artery remodeling, which can promote inflammation, endothelial dysfunction and hypoxia. All these factors can also influence trophoblast invasion and are the major triggers of PE.

ET-1 is a 21-aminoacid peptide mainly known to act as a potent vasoconstrictor via its two G-protein coupled receptors (ETRA and ETRB) (21). Several studies have shown that ET-1 also plays an active role in the regulation of cell proliferation, migration and invasion (22). ET-1 can be found in maternal plasma, but it is also actively secreted by trophoblast cells, and ETRA and ETRB have been reported in placental tissue (23). Interestingly, PE has been associated with increased levels of ET-1 at term of gestation (24). ET-1 levels are also increased during the first trimester of pregnancies further complicated with PE (25). Therefore, ET-1 might be one of the molecules involved in the pathophysiology of PE already in the first trimester of pregnancy.

1.2.2. Maternal obesity

Maternal obesity is a condition known to affect pregnancy outcome. Compared with women with normal body mass index (BMI, 18.5-24.9), obese pregnant women (BMI≥30) have an increased risk of developing other pregnancy complications such gestational diabetes (GDM) and PE (26, 27). Despite the increase in the number of overweight (BMI=25-29.9) and obese women of reproductive age, and the raise in women with pregravid BMI within the overweight range (27), the effects of obesity in placental function remain poorly characterized.

Maternal obesity is characterized by low-grade inflammation (28, 29). Maternal TNF-α levels have been shown to correlate positively with BMI in pregnancy (30). Moreover, the number of CD14+ and CD68+ macrophages, which secrete pro-inflammatory cytokines such TNF-α, IL-1 and IL-6, is three times higher in placenta from obese women when compared to lean women (31). Since trophoblast invasion is affected by inflammation, studies addressing the effect of obesity in first trimester placenta are needed.

1.3. Matrix metalloproteinases (MMPs)

Trophoblast invasion is determined by the ability of EVT to secrete an extensive repertoire of proteases including cathepsins, serine-proteases, a disintegrin and metalloproteinases (ADAMs) and matrix metalloproteinases (MMPs) (32, 33). The latter have gained considerable attention in placental research and are the focus of this study.

MMPs are a family of 24 endopeptidases capable of virtually degrading all the constituents of the extracellular matrix (ECM). They are referred as zinc-dependent proteinases due to the presence of a zinc atom in their catalytic domain. Attending to their substrate specificity MMPs have been classified into different groups: collagenases, gelatinases, stromelysins and matrilysins (34, 35).

1.3.1. MMP structure and activation

Despite their substrate specificity, all MMP family members present a similar structure, and differences are only found at the level of their domain composition. The classical MMP domains consist of a pro-domain, a catalytic domain, a hinge region and a hemopexin domain (36). Although MMPs are secreted in their vast majority, some members of the MMP family can also be localized in the plasma-membrane. These Membrane-type (MT)-MMPs contain a transmembrane and a cytoplasmic domain (MMP14, MMP15, MMP16 and MMP24) or are anchored to the membrane via a glycosylphosphatidylinositol (GPI)-residue (MMP17 and MMP25) (37). MMP domain organization is shown in Fig. 3.

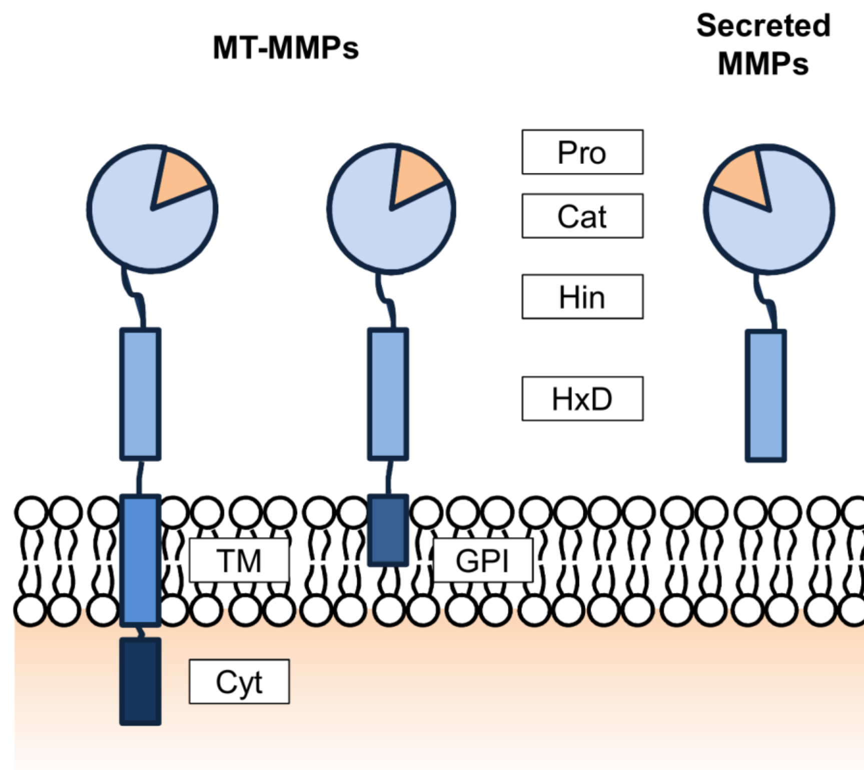

Figure 3. Secreted and MT-MMP domain assembly. Pro: pro-domain; Cat: catalytic domain; Hin: hinge region; HxD: hemopexin domain; TM: transmembrane domain; GPI: glycophosphatidylinositol; Cyt: cytoplasmic domain.

Both secreted and MT-MMPs are synthesized in their latent form, i.e. as zymogen, due to the inhibiting pro-domain at their C-terminus. Protease inhibition is achieved by interaction between a cysteine residue in the pro-domain and the zinc atom in the catalytic domain. Pro-domain removal by other proteases is required for MMP activation (38). Alternatively, a conformational change within the pro-domain or its interaction with reactive oxygen species (ROS) also result in MMP activation (39). MT-MMP pro-domain contains a recognition sequence for Golgi-associated pro-protein convertases. This allows their activation in the intracellular compartment (39-41) prior to their translocation to the membrane. Once in the membrane, MT-MMPs can undergo autocatalysis or are shed by other proteases. Autocataysis leads to their inactivation (42, 43), whereas MT-MMP shedding results in a soluble and active form of the enzyme secreted into the extracellular space (44).

Although MMP activity resides at the catalytic domain, the rest of the domains are also required for MMP functioning. The hemopexin domain has been

suggested to enable MMP oligomerization through protein-protein interactions (45), whereas the cytoplasmic domain and the GPI anchor in MT-MMPs are involved in cell signaling transduction (46) and might regulate MT-MMP trafficking to the membrane (47).

1.3.2. MMP regulation

MMPs orchestrate crucial processes for cell survival and function including proliferation, apoptosis, migration and invasion. Since imbalances in their expression and activity lead to pathological conditions, MMPs are tightly regulated (48). MMP regulation occurs at different levels: i) gene expression, ii) pro-MMP activation, and iii) MMP inhibition (49).

Attending to their basic promoter organization, i.e. the presence of a TATA box and a proximal activator protein 1 (AP-1) binding site, MMPs have been classified into three different categories: i) MMPs containing both a TATA box and an AP-1 element; ii) MMPs with a TATA box but lacking the AP-1 element; and iii) MMPs without TATA box and AP-1 element (50). The presence or absence of these cis elements on the promoter sequence modulates MMP response to certain stimuli. For instance, cytokines and growth factors such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), TGF-β or TNF-α only regulate MMPs within the first group (39). However, this classification is artificial and might not directly reflect MMP regulation. This is due to the presence of non-canonical elements within the promoter sequence of different MMPs, which contribute to their fine-tuned regulation (50, 51).

Finally, MMP inhibition can be considered as the last level on MMP regulation. Broad-range endopeptidase inhibitors such as α_2-macroglobulin are thought to be important for MMP inhibition in body fluids (52). However, MMP inhibition relies predominantly in MMP-specific inhibitors referred as tissue inhibitors of MMPs (TIMPs). TIMPs are a family of four proteins which specifically bind to the catalytic domain of MMPs, inhibiting their activity (53). Not all TIMP members are equally effective in MMP inhibition. For instance, TIMP1 is a weak inhibitor of MT-MMPs. Thus, TIMP regulation might also fine-tune MMP activity (54)

1.3.3. MMPs in the first trimester of pregnancy

Due to their importance in the degradation of the ECM, the role of MMPs in the first trimester of pregnancy has been mainly characterized in the context of trophoblast invasion (35). Among other substrates, MMPs degrade collagen IV, laminin, vitronectin and fibronectin, all of them ECM components found in the uterine wall and the spiral arteries (55). Moreover, several cytokines involved in trophoblast invasion such TNF-α and TGF-β can be activated by MMPs (56-58), which in turn regulates trophoblast function (59, 60).

Studies linking MMPs and trophoblast invasion have traditionally focused on MMP2 and MMP9 (61-63). In contrast to secreted MMPs, MT-MMPs can be located in the membrane in specific regions, allowing a very-directed pericellular degradation of the ECM. Moreover, MT-MMPs are central for tumor invasion and metastasis (47). Hence, the study of MT-MMPs and their regulation in the first trimester of pregnancy might be of help to understand the molecular mechanisms underlying trophoblast invasion.

From the MT-MMP sub-family, only MMP14 has been analyzed in depth in human first trimester placenta. Placental MMP14 is involved in different processes such as cell proliferation, migration and invasion as well as angiogenesis (64-67). MMP14 also regulates MMP2 activation. This occurs through a complex mechanism requiring TIMP2. At low levels, TIMP2 recruits pro-MMP2 to the membrane area where MMP14 dimers are localized. From this dimer, one MMP14 molecule interacts with the TIMP2-MMP2 complex, being inhibited, whereas the second MMP14 molecule will activate pro-MMP2 (42). Other classical substrates of MMP14 are collagen I, vitronectin and fibronectin, ECM components that need to be degraded to allow trophoblast invasion (35).

We have previously characterized MT-MMP expression in isolated human first trimester trophoblasts, observing that only MMP14 and MMP15 are expressed in these cells (68) (Fig. 4). Interestingly, MMP15 degrades collagen I and IV, laminin and fibronectin (68), and it also activates MMP2 (69) and its expression has been found in EVT (70). However, its role in placental function during the first trimester of pregnancy remains unknown.

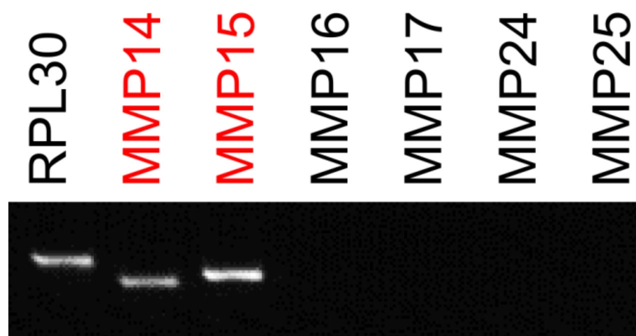

Figure 4. RT-PCR analysis of MT-MMP expression in primary human first trimester trophoblast. Ribosomal protein L30 (RPL30) was used as housekeeping gene. Adapted from (68).

2. Hypothesis and objectives

Considering the importance of ECM matrix degradation in the process of trophoblast invasion, and that a dysregulation of trophoblast invasion is associated with pregnancy complications such as PE, we hypothesized the following:

i) MMP15 is a novel protease crucial for placental function during the first trimester of pregnancy

ii) PE stimuli, including ET-1, inflammation and hypoxia, regulate MMP14 and MMP15 levels in human first trimester trophoblasts, entitling functional consequences for trophoblast invasion

iii) Conditions dealing with low-grade inflammation such as maternal obesity regulate placental MMP14 and MMP15 levels already in the first trimester of pregnancy.

This study aims to:

i) Determine MMP15 localization and function in first trimester human placenta

ii) Characterize the effect of ET-1 on MMP and TIMP levels in human isolated trophoblast, its role in trophoblast invasion and its modulation by TNF-α and low oxygen tension

iii) Determine MMP14 and MMP15 levels in first trimester human placental tissue from lean and obese pregnant women

3. Material and Methods

3.1. Ethics statement

This study was approved by the institutional review board and ethical committee of the Medical University of Graz (24-129 ex 11/12), the Medical University of Vienna (084/2009) and the University of British Columbia (H13-00640). Signed informed consent was obtained from the pregnant women.

3.2. Tissue collection

Human first trimester placental tissue (n=115) was collected after pregnancy termination for psychosocial reasons. For those studies involving whole placental tissue, samples were washed in Hanks' Balanced Salt solution (HBSS, Gibco, Invitrogen, Carlsbad, CA, USA), snap-frozen in liquid nitrogen and maintained at -80°C until further use, or fixed with 4% (w/v) paraformaldehyde (PFA). For trophoblast isolation, samples were maintained in Keratinocyte Medium (KCM, Gibco) supplemented with 1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Gibco) at 4°C until cell isolation was performed.

3.3. Human first trimester placental chorionic villi explants

First trimester placental chorionic villi explants were prepared as described elsewhere (71). Briefly, healthy looking undamaged villi from the periphery were dissected under the microscope and maintained in Dulbecco's Modified Eagle's (DMEM)/Ham's F12 medium (Gibco) without serum supplementation. Two different ECMs were used consisting of Matrigel (Corning, Bedford, MA, USA) or collagen I (Corning), the latter prepared by mixing collagen I with 10x DMEM (Gibco, 1:10, v/v) and 7.5% sodium bicarbonate (Sigma Aldrich, St. Louis, MO, USA, 1:5, v/v). ECM solution (20µl or 200µl for collagen I and Matrigel, respectively) was added to the center of 12mm transwells placed in 24-well plates, and incubated at 37°C for gel formation. 400µl DMEM/Ham's F12 medium were added to the bottom well and the dissected villi were then carefully placed on the top of the ECM and incubated overnight without medium to facilitate anchorage.

For MMP15 studies, experiments were performed under 3% O_2. DMEM/Ham's F12 medium containing non-targeting siRNA (control) or two different siRNAs targeting MMP15 (si5- and si6-siRNA, G24324, Qiagen, Hilden, GE) was added for 24h. Thereafter, fresh medium was added and outgrowth was monitored under the microscope for 72h. Trophoblast outgrowth was measured as the length between the margin of the villi and the front of the migrating sheet from 3-5 different points or as the outgrowth area. Image acquisition and analysis were performed using a microscope (Nikon SMZ 745, Vienna, AU) coupled to a camera (Moticam 580, Hong Kong, CH) and the ImageJ software, respectively.

For ET-1 studies, villi were incubated in DMEM/Ham's F12 medium without (control) or with 100nM ET-1 (Sigma Aldrich) for 24h. Trophoblast outgrowth was quantified as described above.

3.4. Cell culture

3.4.1. Human first trimester trophoblast isolation and culture

Primary human trophoblasts were isolated from first trimester placentas from GW 7 to 12. GW was determined based on ultrasound measurement of the crown-rump length. When required, only samples from similar GW (i.e. 7+8, 9+10 or 11+12) were pooled together prior isolation.

Trophoblast isolation was performed following established protocols (72). Briefly, tissue was washed with cold sterile saline and villi were isolated and digested with Dispase/Dnase (Gibco) and Trypsin (Gibco). Cells were centrifuged in a Percoll gradient (Gibco) and incubated with magnetic beads coupled with anti-CD-90 and anti-CD-45 (Dako, Glostrup, DK) antibodies to remove fibroblasts and common leukocyte-antigen expressing cells, respectively. The cells obtained constituted a mixture of VT and EVT. Purity and viability of isolations was assessed by immunostaining for cytokeratin 7 (K7, Dako, 1:750) and human leukocyte antigen (HLA)-G (BD-Biosciences, Bedford, MA, USA, 1:500), and human chorionic gonadotropin (hCG) secretion (Dade Behring, Deerfield, IL, USA), respectively. Only viable isolations with a purity ≥ 95% were used.

Isolated trophoblasts were seeded in 12 well-plates pre-coated with gelatin at a density of 1.5×10^6 cells/well, and cultured for 48h in a humidified incubator

(37°C, 5% CO_2) in KCM containing penicillin/streptomycin (Gibco), supplements (Gibco) and 10% (v/v) fetal calf serum (FCS, Thermo Scientific, Rockford, IL, USA). Prior to treatments, trophoblasts were washed with HBSS and maintained under low serum conditions (2%, v/v) for 24h.

Trophoblasts were incubated in the absence (control) or presence of 10nM or 100nM ET-1 for 24h. To characterize the ETR subtype involved in mediating ET-1 effects, trophoblasts were pre-incubated with two selective ETR antagonists for 2h: BQ-123 (Tocris, Bristol, UK, 1.4nM and 11.2nM) for ETRA, and BQ-788 (Tocris, 1.2nM and 9.6nM) for ETRB. These concentrations were chosen after establishing a standard curve with concentrations below and above the K_i of both antagonists (73, 74). After pre-incubation with the appropriate antagonist, cells were supplemented with 100nM ET-1 for 24h.

In another set of experiments, trophoblasts were treated with TNF-α (Sigma Aldrich, 25ng/ml) singly or in combination with 100nM ET-1. To assess the interplay between low oxygen tension and ET-1, trophoblasts were cultured under three different oxygen tensions (1%, 2.5% and 20% O2) in the absence or presence of 100nM ET-1 for 24h.

3.4.2. Culture of ACH-3P cell line

The human first trimester trophoblast cell line ACH-3P (59) was cultured in Ham's F12 medium (Gibco) with penicillin/streptomycin (Gibco) and 10% (v/v) FCS. Cells were seeded at a density of 5000 cells/well in 6-well plates (Thermo Scientific) pre-coated with gelatin (Sigma Aldrich) and maintained for 48h. Prior to treatments, cells were washed with HBSS and maintained under low serum conditions (2% v/v) for 24h. Thereafter, cells were incubated in the absence or the presence of 10nM or 100nM ET-1 (Sigma Aldrich) for 24h, 48h and 72h.

For viability and proliferation studies, cells were detached with accutase (Gibco) and the cell number was determined using the CASY cell counting system (Schärfe System GmbH, Reutlingen, GE). This technology allows to discriminate between live and dead cells due to their difference in electrical conductivity.

3.5. Immunohistochemistry

Immunohistochemical staining was performed on 4% PFA fixed, paraffin embedded human first trimester placental villi (GW 7-8) sectioned at 5µm. Slides were deparaffinized in xylene and rehydrated with decreasing concentrations of ethanol. For antigen retrieval, tissue sections were submerged in 10mM Sodium Citrate Buffer (0.05% (v/v) Tween 20, pH 6.0) and microwaved for 15min in a domestic microwave oven. Slides were allowed to cool for 45min at room temperature prior to immunohistochemistry.

Immunohistochemistry was performed using the Ultravision LP detection system (Thermo Scientific) according to manufacturer's guidelines. Sections were washed with 0.01M Phosphate Buffered Saline (PBS, pH 7.4) and blocked with hydrogen peroxide (12min) and Ultra-V (7min). Anti-MMP15 monoclonal antibody (Millipore, Billerica, MA, USA, 1:1250) was diluted with Dako Cytomation Antibody Diluent with Background Reducing Components (Dako), and sections were incubated with the primary antibody for 1h at room temperature. Primary Antibody Enhancer (30min) and HRP-Polymer (20min) were subsequently added. Thereafter, slides were incubated with 3-amino-9-ethylcarbazol (AEC, Thermo Scientific) for 10min. Sections were rinsed with PBS between each incubation step and counterstained with Mayer's Hematoxylin and mounted with Kaiser's glycerol gelatin (Merck Corp., Rahway, NJ, USA). A mouse IgG antibody (Millipore) was used as negative control.

Image acquisition and analysis was performed using a Leica DM4000 B microscope (Leica, Cambridge, UK) equipped with Leica DFC 320 video camera.

3.5.1. Immunofluorescence double staining

Immunofluorescence staining was performed on 4% PFA fixed, paraffin embedded human first trimester placental villi (GW 7-11) and decidua samples (GW 7-10) sectioned at 5µm. Deparaffinization and antigen retrieval was performed as described in section 3.5. Primary antibodies used for immunofluorescence staining are summarized in table 1.

Table 1. Primary antibodies used for immunofluorescence

Primary antibody	Host	Dilution
MMP15 (Millipore, MAB3320)	Mouse	1:1250
HLA-G (Exbio, 11449-C100)	Mouse	1:100
Cytokeratin 7 (Ventana, 790-4462)	Rabbit	1:75
Cytokeratin 7 (Santa Cruz Biotech, C2206)	Mouse	1:100
Ki67 (Thermo Scientific, RM-9106-S0)	Rabbit	1:100
Caspase-cleaved cytokeratin 18 (Roche, 14533800)	Rabbit	1:25

Sections were rinsed with PBS and blocked with 5% normal goat serum (Thermo Scientific) for 1h before the appropriate combination of primary antibodies was added. Slides were incubated overnight at 4°C in a dark moist chamber. After serial washing steps, slides were incubated with the appropriate secondary antibody (Table 2).

Table 2. Secondary antibodies used for immunofluorescence

Anti-mouse IgG Alexa Fluor (Thermo Scientific)
Fluor 488 (green, 11001)
Fluor 568 (red, 11004)
Anti-rabbit IgG Alexa Fluor (Thermo Scientific)
Fluor 488 (green, 11008)
Fluor 568 (red, 11011)

When both primary antibodies had a common host, slides were blocked first with normal horse serum (Thermo Scientific) for 30min and incubated with the first primary antibody for 1h at room temperature. After serial PBS washing, slides were incubated with the appropriate secondary antibody for 1h. Thereafter, sections were rinsed with PBS and blocked again for 30min using normal mouse serum (Thermo Scientific) before the second primary antibody and the subsequent appropriate secondary antibody were added. Negative controls were incubated with the appropriate IgG fractions (Millipore).

Slides were mounted with Prolong gold anti-fade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies-Invitrogen, Carlsbad, CA, USA) to counterstain the nuclei. Image acquisition and analysis was performed using a Zeiss Axio Z1 microscope equipped with an Axiocam (Zeiss, Jena, GE) and with the ZEN Software (Zeiss), respectively.

3.6. RNA isolation and RT-qPCR

Total RNA was isolated from human first trimester chorionic villi and primary human first trimester trophoblasts using the RNeasy mini kit (Qiagen) according to manufacturer's guidelines. RNA quality and integrity was determined using a QIAxpert device (Qiagen), and 250ng of RNA were reversely transcribed to cDNA using SuperScript II Reverse Transcriptase (Life Technologies) as per manufacturer's guidelines. cDNA was subjected to real-time quantitative PCR (RT-qPCR) using FAM-labelled TaqMan gene expression assays (Life Technologies, Table 3) and TaqMan universal PCR master mix (Life Technologies).

Table 3. Taqman gene expression assays used for RT-qPCR

Gene	Taqman gene expression assay
MMP14	Hs01037003_g1
MMP15	Hs00233997_m1
TIMP1	Hs00355335_g1
TIMP2	Hs00234278_m1
TIMP3	Hs00927214_m1
TIMP4	Hs00162784_m1
HLA-G	Hs00365950_g1
β-actin	Hs01060665_g1
XIST	Hs01079824_m1
DDX3Y	Hs00965254_gH

Components were mixed according to the manufacturer's instructions and amplified in a final volume of 20μL per well (96-well plates; Life Technologies) using the CFX96 real-time PCR detection system (BioRad Laboratories, Hercules, CA, USA). Ct values were automatically generated by the associated software and relative gene expression was calculated by the standard $2^{-\Delta\Delta Ct}$ method, with β-actin or HLA-G as the reference gene. When required, fetal sex was determined by RT-qPCR based on the expression profile of XIST (X-linked gene) and DDX3Y (Y-linked gene).

3.7. Protein isolation and Western blotting

Protein lysates from first trimester placental tissue, isolated primary trophoblast and ACH-3P cells were prepared in RIPA buffer (Sigma Aldrich) containing protease inhibitors (Roche, Mannheim, DE). Protein concentration was determined by bicinchoninic acid (BCA) assay (Thermo Scientific) according to manufacturer's guidelines. Protein lysates were mixed with Laemmli buffer (Sigma Aldrich) and denatured at 96°C for 5min. Equal amounts of total protein (3-10 μg/well depending on the starting material) were loaded onto 10% SDS-PAGE gels (BioRad Technologies) and resolved at 140V for 1h. Proteins were transferred to a nitrocellulose membrane (BioRad Technologies) and nonspecific binding sites were blocked for 1h with 5% non-fat dry milk (BioRad technologies) in tris-buffered saline (TBS) + 0.1% (v/v) Tween 20 (Sigma Aldrich). Thereafter, membranes were incubated with the appropriate primary antibody overnight at 4°C. The primary antibodies used are summarized in Table 4.

Table 4. Primary antibodies used for Western blotting

Primary antibody	Host	Dilution
MMP14 (Millipore, AB6004)	Rabit	1:1250
MMP15 (Millipore, MAB3320)	Mouse	1:500
HLA-G (BD Biosciences, 557577)	Mouse	1:1000
GAPDH (Novus Biologicals, NB300-221)	Mouse	1:20000
β-actin (Abcam, ab8227)	Mouse	1:25000

Blots were washed three times with TBS and incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (Biorad Technologies). After washing, immunolabeling was visualized using Supersignal West Pico/Femto Chemiluminescent Substrate (Thermo Scientific) and Chemidoc XRS software (BioRad Technologies). Band densitometry was performed using the Alpha Digidoc software (Alpha Innotech Corp., Innsbruck, AUT), with HLA-G, β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as loading control.

3.8. Zymography

Cell supernatants of human primary first trimester trophoblasts were collected and total protein concentration was determined by BCA assay. Zymography was performed according to manufacturer's guidelines (Life Technologies). Briefly, equal amounts of protein (6µg/well) were mixed with Tris-Glycine SDS sample buffer (Life Technologies), loaded onto 10% Tris-Glycine gels containing 0.1% gelatin (Life Technologies) and resolved at 125V for 2h. Gels were incubated with zymogram renaturing buffer (Life Technologies) for 30min followed by an equilibration step in developing buffer (Life Technologies) for 30min. Fresh developing buffer was added and gels were maintained overnight at 37°C. Gels were stained with Coomassie Brilliant Blue (Sigma Aldrich) for 30min and de-stained for 50min in methanol:acetic acid (2:1). Gelatinolytic activity was visualized as transparent bands (Chemidoc XRS Software) and band densitometry was determined using the Alpha Digidoc software (Alpha Innotech Corp.).

3.9. Enzyme-linked immunosorbent assay (ELISA)

Endogenous ET-1 levels were measured in cell supernatants of trophoblasts cultured under 1%, 2.5% and 20% O_2. After collection, supernatants were concentrated through column centrifugation (Sartorius Stedim, Goettingen, DE) to a final volume of 500µl. ET-1 ELISA (R&D, Abingdon, UK) was performed following manufacturer's guidelines. Total protein concentration in the supernatants was determined by the BCA method and used for normalization.

3.10. Transwell invasion assays

Trophoblast invasion was quantified using a classical transwell invasion assay. Transwell inserts (12mm, 12µm pore-size, Millipore) were placed into a 24-well plate and pre-coated with 1mg/ml fibronectin (Millipore). After 1h incubation at 37°C to allow fibronectin polymerization, 400µl DMEM/Ham's F-12 medium supplemented with 10% (v/v) FCS were added to the bottom well. Isolated trophoblasts were re-suspended in 300µl DMEM/Ham's F-12 medium without FCS in the absence (control) or presence of 100nM ET-1, and seeded in the upper chamber. After 48 hours, the inserts were washed twice with PBS and fixed with ice-cold methanol. Non-invading cells were removed with a cotton swab. The transwell membranes containing the invading cells were removed and mounted onto slides with Prolong gold anti-fade reagent with DAPI to stain the cell nuclei. Five different fields per membrane were counted under the microscope with a magnification of 100x.

3.11. Chick chorioallantoic membrane (CAM) assay

The *ex ovo* CAM assay was tested as a model to study trophoblast invasion. Fertilized white leghorn chicken (*Gallus domesticus*) eggs (Schropper GmbH, Gloggnitz, AUT) were incubated at 37.6°C and 75% humidity. After 3 days, the egg shell was cracked and the embryo was transferred to a sterile dish and incubated under the same conditions. On day 10, two silicones rings (on-plants) were placed on the CAM. Isolated trophoblasts ($1x10^6$ cells) were resuspended in 15µl KCM medium without or with 100nM ET-1. Cells were seeded on the on-plants alone or with 5µl Matrigel or gelatin. After 48h, the on-plants and the surrounding CAM were removed, fixed (4% PFA) and paraffin embedded. Immunohistochemical staining for K7 (Dako, M7018) was performed as described in section 3.5.

3.12. Statistical analysis

Statistical analyses were performed using Sigmaplot (version 12.5) and GraphPad (Version 5.01). Results are representative of at least three independent

experiments, i.e. isolations from different placentas. Data are expressed as mean ± SD or minimum and maximum. Due to the small sample size, the Shapiro-Wilk test was used to test for normality. When data were normally distributed, statistical significance was determined using t-test and one-way ANOVA analysis. When appropriate, i.e. control versus treatment, paired t-test and one-way ANOVA with repeated measures were used. Data not-normally distributed were analysed using non-parametric t-test. Correlations were determined by Pearson’s correlation coefficient. A probability of $p<0.05$ was considered significant.

4. Results

4.1. MMP15 in human first trimester placenta

4.1.1. Localization

MMP15 localization in human first trimester placental villi was firstly assessed by immunohistochemistry. MMP15 was exclusively found in the cell columns, preferentially located to the invasive front (Fig. 5). This suggests that EVT are the main trophoblast sub-population expressing MMP15. In order to confirm this, a double immunofluorescence staining with HLA-G, a classical EVT marker, and MMP15 was performed. Immunofluorescence revealed a co-localization between MMP15 and HLA-G (Fig. 6). This co-localization was found in human first trimester placenta tissue form different gestational weeks, showing that MMP15 is only found in EVT along the first trimester of pregnancy.

To determine whether MMP15 is found in EVT invading into the decidua, i.e. interstitial extravillous trophoblast (iEVT), a similar double immunofluorescence staining was performed in human first trimester decidua. MMP15 also co-localized with HLA-G in decidua samples (Fig. 7), revealing that invasive trophoblasts are the only cell type expressing MMP15 in both human first trimester placenta and decidua.

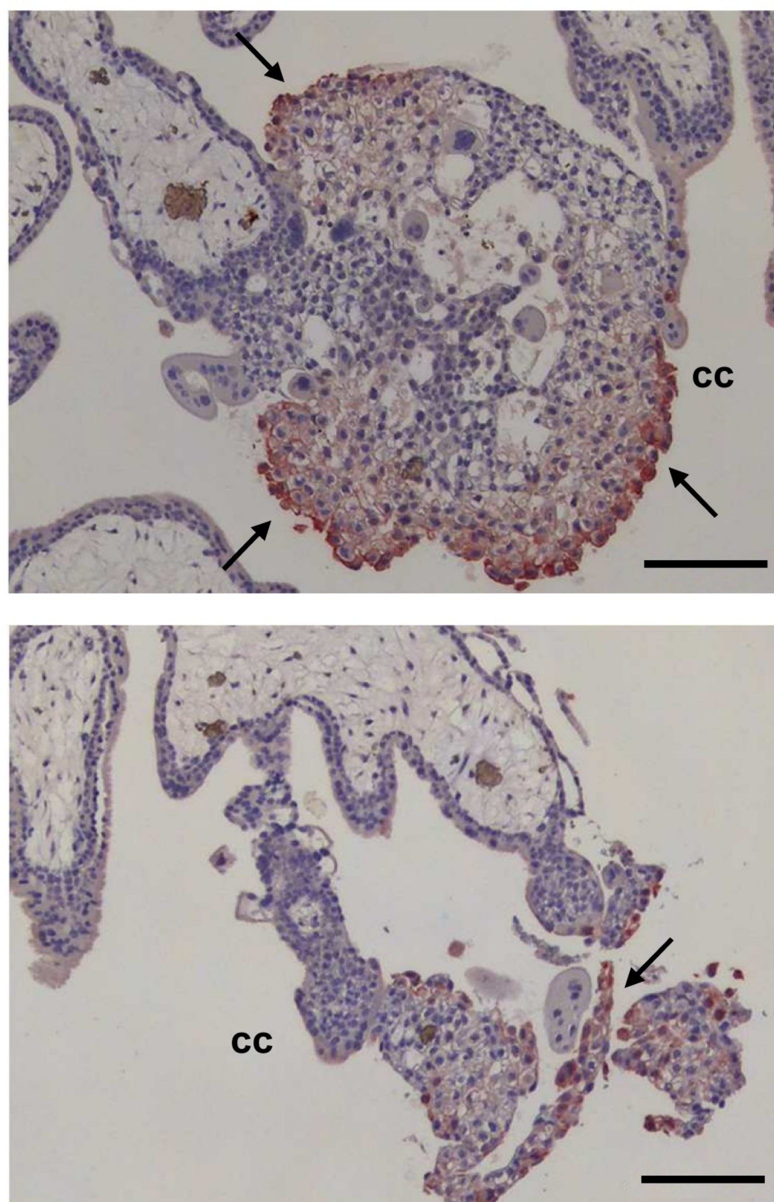

Figure 5. MMP15 immunohistochemical staining in human first trimester placental tissue. MMP15 immunostaining was performed in serial sections of human placental villi (GW 7-8, n=2). MMP15 was found in the cell columns (cc), preferentially localized to the invasive front (arrows). Scale bar: 200µm.

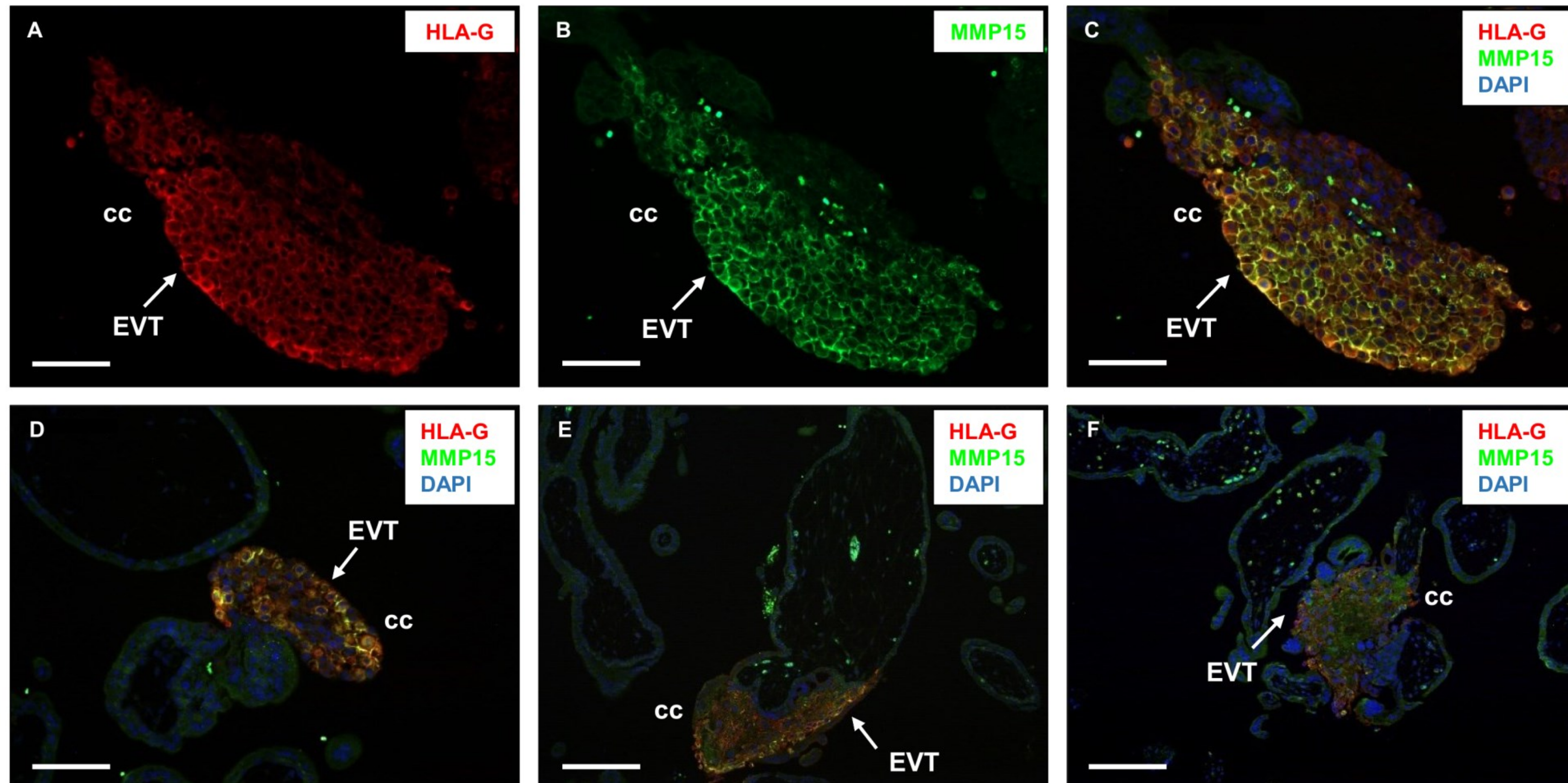

Figure 6. MMP15 immunofluorescence staining in human first trimester placental tissue. Immunofluorescence was performed in serial sections of human placental villi (GW 7-10, n=6). **(A)** Extravillous trophoblast (EVT) were identified by HLA-G immunofluorescence staining (red). **(B)** MMP15 localization (green) was found in the cell columns (cc). **(C-F)** Image overlay revealed a co-localization of MMP15 (green) and HLA-G (red) in human placental villi from different GW (6, 8, 9 and 10, respectively). Nuclei were counterstained with DAPI. Scale bar: 100µm (A-D) and 200µm (E and F).

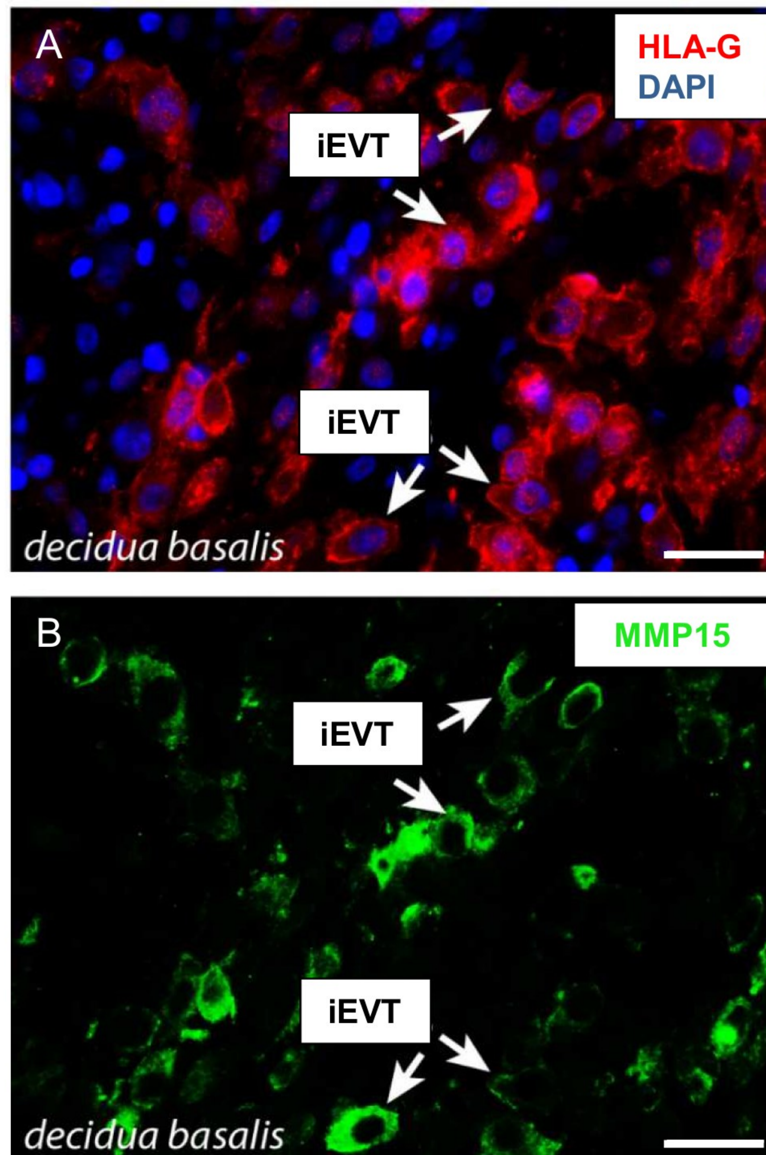

Figure 7. MMP15 immunofluorescence staining in human first trimester decidua. **(A)** Interstitial extravillous trophoblast (iEVT) were identified by HLA-G immunofluorescence staining (red) in serial sections of human decidua (GW 7-10, n=4). Nuclei were counterstained with DAPI. **(B)** iEVT were positive for MMP15 (green). Scale bar: 50µm.

To confirm whether HLA-G is a reliable marker for MMP15 expression and protein studies, MMP15 and HLA-G expression was assessed in human first trimester placental tissue. RT-qPCR analysis revealed a significant correlation between both genes (Fig. 8). Thus, HLA-G was subsequently used for normalization.

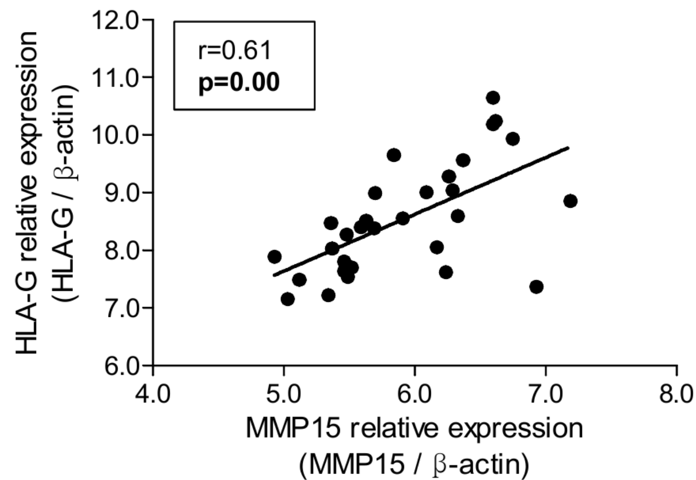

Figure 8. MMP15 and HLA-G correlation in human first trimester placental tissue. MMP15 and HLA-G expression was determined by RT-qPCR. Results were normalized to β-actin expression using the $2^{-\Delta\Delta Ct}$ method. n=30

At the cellular level, MMP15 mRNA and protein levels were determined in isolated human first trimester trophoblasts. RT-qPCR results showed that MMP15 expression is found in isolated trophoblasts and is not altered through the first trimester of pregnancy (Fig. 9). At the protein level, pro- and active (act)-MMP15 were detected by Western blotting. In parallel to the mRNA results, total, pro- and act-MMP15 were not modified along gestation (Fig. 10)

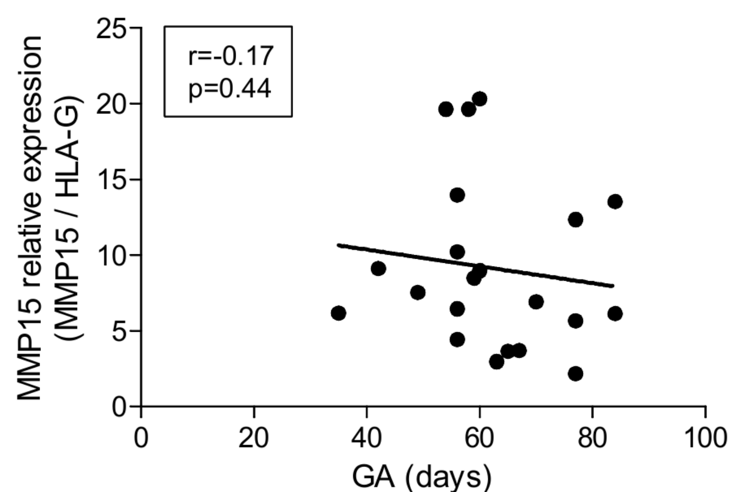

Figure 9. MMP15 expression in human first trimester trophoblasts from different gestational ages (GA). MMP15 expression was determined by RT-qPCR. Results were normalized to HLA-G using the $2^{-\Delta\Delta Ct}$ method. n=21

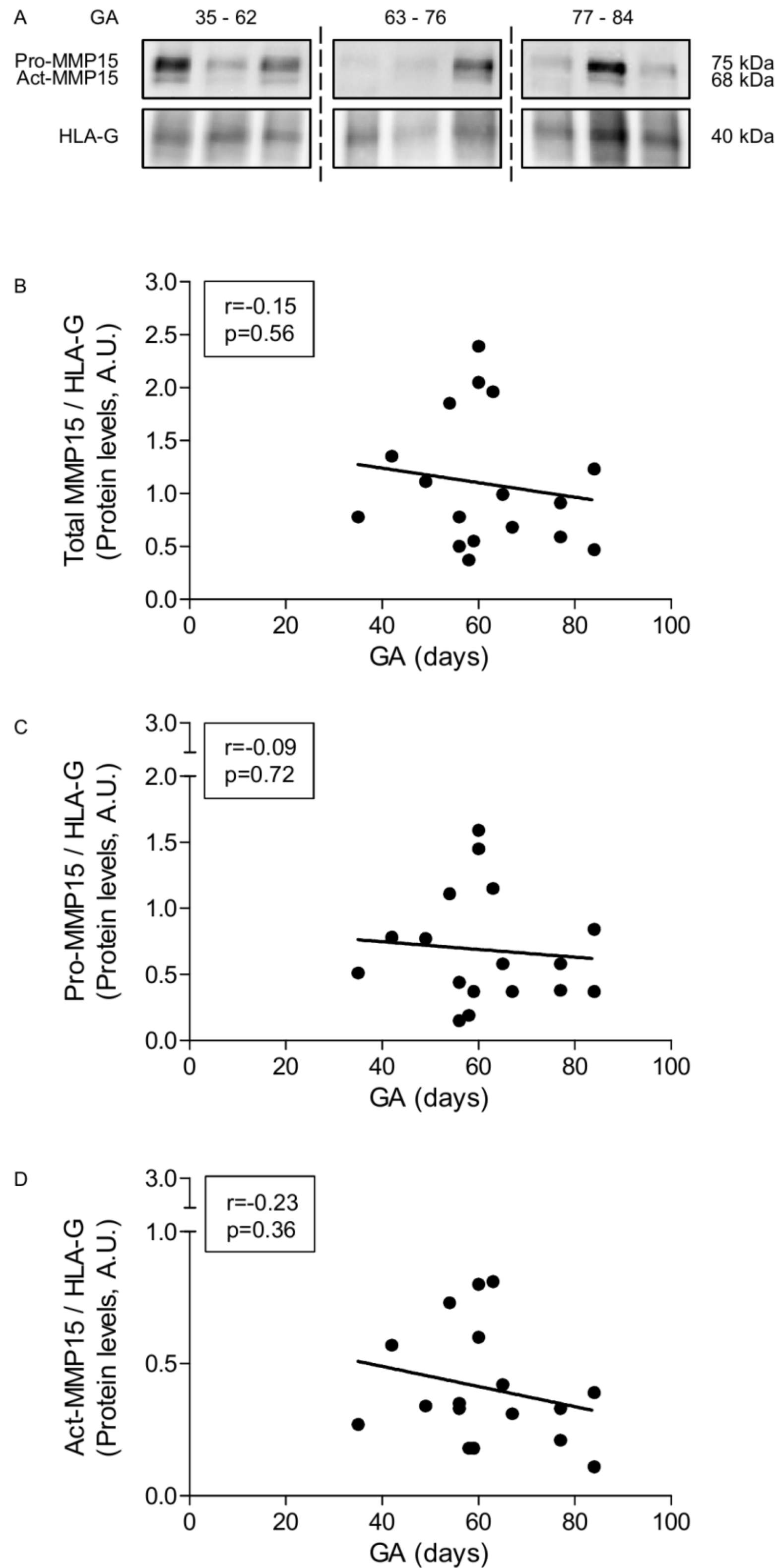

Figure 10. MMP15 protein levels in human first trimester trophoblasts from different gestational ages (GA). (A) Western blotting analysis revealed two bands corresponding to inactive (pro) and active (act) MMP15. Total MMP15 refers to both bands considered together. **(B-D)** Correlation between MMP15 protein levels and GA. Results were normalized to HLA-G protein levels. n=17

4.1.2. Function

To determine whether MMP15 plays a role in trophoblast function during the first trimester of pregnancy, human placental chorionic villi were placed on matrigel and transfected with two different siRNAs (si5-siRNA and si6-siRNA) to knockdown MMP15 (Fig. 11A – 11C). After 72h, trophoblast outgrowth, proliferation and apoptosis were determined.

Trophoblast outgrowth was measured as the distance from the villous margin to the outer edge of the migrating cell sheet. Trophoblast outgrowth area was also determined (Fig. 11D – 11F). Non-targeting (NT)-siRNA was used as control. In comparison with NT-siRNA, MMP15 knockdown significantly reduced trophoblast outgrowth length (-35%; $p \leq 0.001$ and -26%; $p<0.05$; si5-siRNA and si6-siRNA, respectively, Fig. 11G) and outgrowth area (-43%; $p \leq 0.001$ and -36%; $p \leq 0.01$; si5-siRNA and si6-siRNA, respectively, Fig. 11H). MMP15 knockdown efficiency was confirmed by RT-qPCR. Transfection with both siRNAs induced a downregulation of MMP15 expression (-60% and -54%; $p \leq 0.01$; si5-siRNA and si6-siRNA; respectively) when compared to NT-siRNA (Fig. 11I).

Trophoblast proliferation was determined by Ki67 immunofluorescence staining, a marker of cell proliferation (Fig. 12A – 12D). Proliferation was calculated as a percentage of Ki67 positive cells relative to cytokeratin 7 (K7) positive cells. MMP15 knockdown had no effect on trophoblast proliferation.

Similarly, trophoblast apoptosis was assessed by caspase-cleaved cytokeratin 18 (K18) immunofluorescence staining (Fig. 12E – 12H). Apoptosis was calculated as a percentage of caspase-cleaved K18 positive cells relative to K7 positive cells. MMP15 knockdown did not affect trophoblast apoptosis.

Altogether, these results suggest that MMP15 plays a critical role in trophoblast migration and invasion. However, other biological functions such as proliferation and apoptosis are not regulated by MMP15 in first trimester trophoblast.

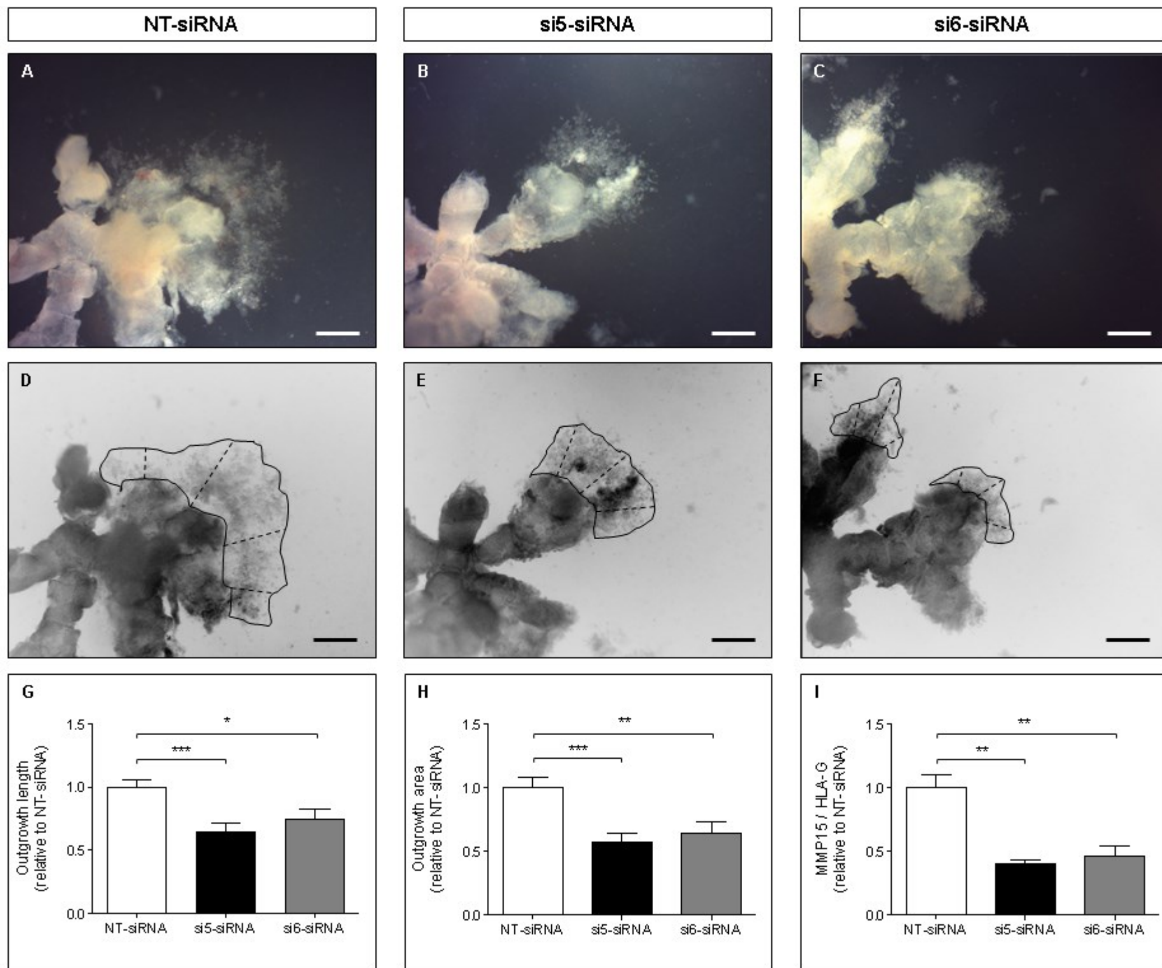

Figure 11. Effect of MMP15 knockdown in trophoblast outgrowth. (A-C) Human dissected chorionic villi from first trimester placentas (GW 8-10, n=4) were placed on matrigel and incubated for 24h with non-targeting (NT)-siRNA (control) or with two different siRNAs targeting MMP15 (si5-RNA and si6-RNA). **(D-F)** Trophoblast outgrowth was measured after 72h as the length from the villous tip to the distal edge of the outgrowth sheet (dotted line). The outgrowth area was also quantified (solid line). **(G and H)** Results were normalized to NT-siRNA, arbitrarily considered as 1. **(I)** MMP-15 knock down was confirmed by RT-qPCR. *$p<0.05$; **$p\leq0.01$; ***$p\leq0.001$

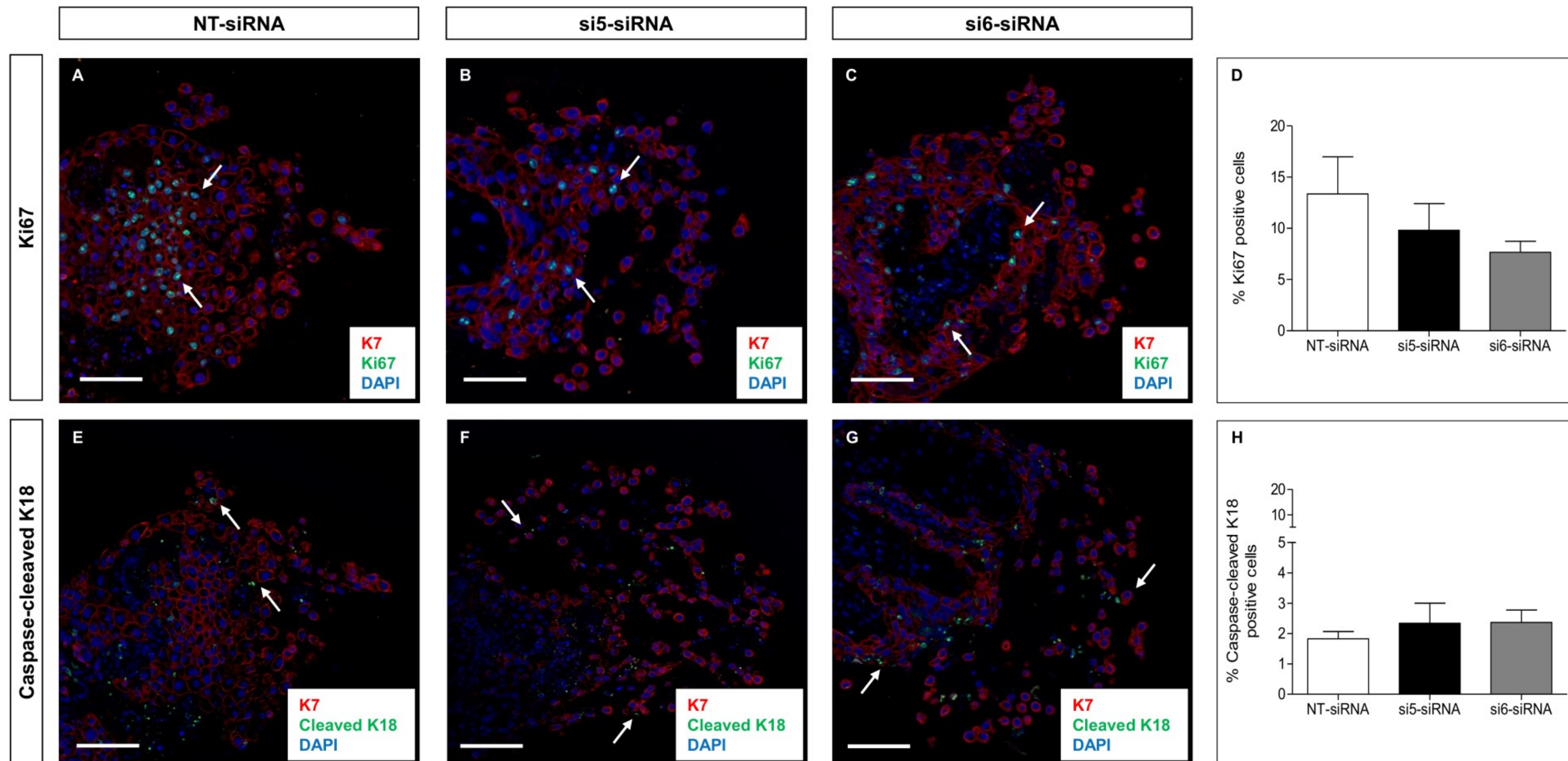

Figure 12. Effect of MMP15 knockdown in trophoblast proliferation and apoptosis. Human dissected chorionic villi from first trimester placentas (GW 8-10, n=4) were incubated for 24h with non-targeting (NT)-siRNA or with two different siRNAs targeting MMP15 (si5-RNA and si6-RNA). **(A-C)** Proliferative trophoblasts were identified by double immunofluorescence staining for cytokeratin 7 (K7, red) and Ki67 (green). **(F-G)** Apoptotic trophoblasts were identified by double immunofluorescence staining for K7 (red) and caspase-cleaved cytokeratin 18 (Cleaved K18, green). **(D)** Trophoblast proliferation and **(H)** trophoblast apoptosis were quantified as the percentage of Ki67 and cleaved K18 positive cells relative to K7 positive cells, respectively.

4.2. ET-1, MMP regulation and trophoblast function

4.2.1. ET-1 and ACH-3P cell proliferation and viability

ACH-3P cells were cultured in the absence (control) or presence of ET-1 (10nM and 100nM) for 24h, 48h and 72h. After each time-point, cell number was determined using the CASY cell counting technology. Cell proliferation was calculated as the number of viable ACH-3P cells along the different time-points (Fig. 13A). Cell viability was expressed as the percentage of viable cells relative to the total cell number (Fig. 13B). Both ET-1 concentrations had no effect on ACH-3P cell proliferation or viability.

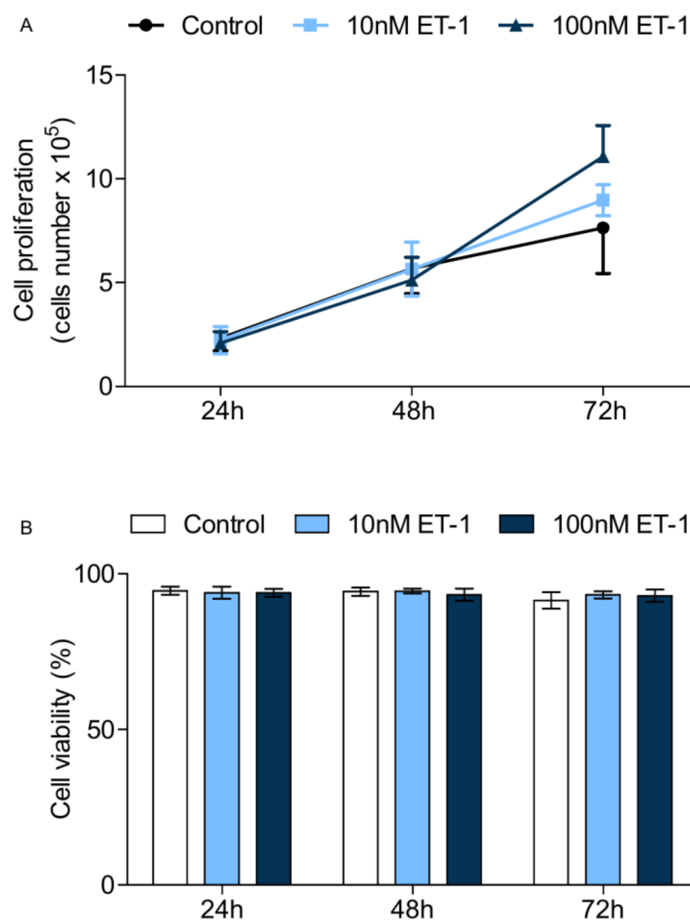

Figure 13. Effect of ET-1 on ACH-3P cell proliferation and viability. Cells were incubated in the absence (control) or the presence of different concentrations of ET-1 (10 and 100nM) for 24h, 48h or 72h. **(A)** Cell proliferation was expressed as the number of viable cells/ml. **(B)** Cell viability was measured as the percentage of viable cells relative to the total number of cells. Results are the mean of three independent experiments ± SD.

4.2.2. ET-1 regulation of MMP14 and MM15 in ACH-3P cells

ACH-3P cells were incubated in the absence (control) or the presence of ET-1 (10nM and 100nM) for 24h or 48h. MMP14 and MMP15 protein levels were determined by western blotting. A single band corresponding to active MMP14 (act-MMP14, 58 kDa) was detected (Fig. 14A and 14D). Two bands were detected for MMP-15 corresponding to the inactive (pro-MMP-15, 75 kDa) and the active (act-MMP15, 68kDa) form of the enzyme (Fig. 14B and 14E).

ET-1 induced a dose-dependent downregulation of MMP14, with 100nM decreasing MMP14 protein levels after 24h (-41%; $p<0.05$; Fig. 14C) and 48h (-40%; $p\leq0.001$; Fig. 14F). ET-1 had no effect of MMP15 protein levels after 24h (Fig. 14C). However, 100nM ET-1 downregulated total MMP15 protein levels after 48h (-22%; $p<0.05$). When analyzed separately, only act-MMP15 accounted for this downregulation (-41%; $p<0.05$), whereas pro-MMP15 levels were not affected by ET-1 (Fig. 14F).

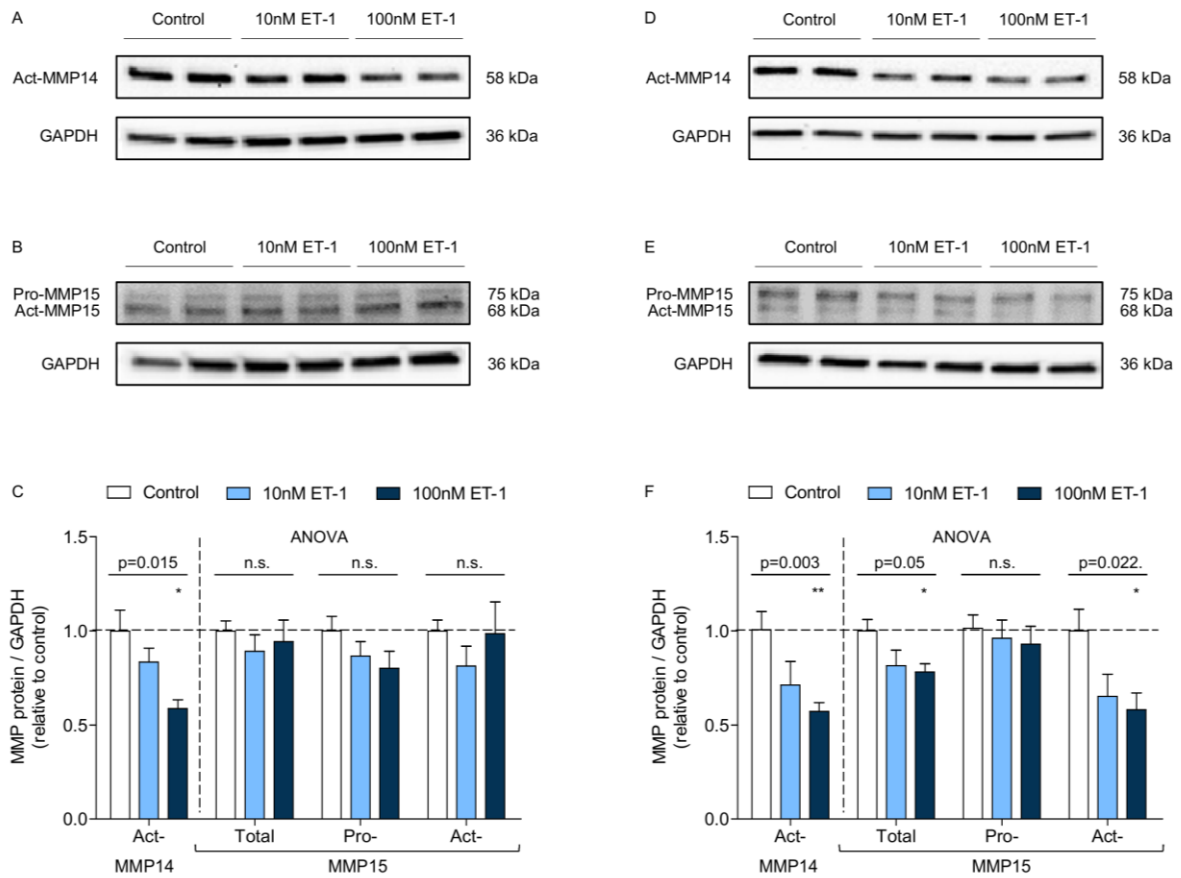

Figure 14. Effect of ET-1 on MMP14 and MMP15 protein levels in ACH-3P cells. Cells were incubated in the absence (control) or the presence of 10nM or 100nM ET-1 for 24h **(A-C)** or 48h **(D-F)**. MMP14 and MMP15 levels were determined by Western Blotting. A single band corresponding to active MMP14 was observed (act-MMP14, **A and D**). Both pro- and active (act)-MMP15 were detected **(B and E)**. Total MMP15 refers to both bands considered together. Results were normalized to GAPDH and calculated relative to the controls, arbitrarily considered as 1 **(C and F)**. Each condition was assayed in quadruplicates. Results are the mean of three independent experiments ± SD.*$p \leq 0.05$, **$p \leq 0.001$

4.2.3. ET-1 regulation of MMP14 and MMP15 in human first trimester trophoblasts

Isolated human first trimester trophoblasts were incubated for 24h in the absence (control) or the presence of 10nM and 100nM ET-1. MMP14 and MMP15 expression was determined by RT-qPCR in early first trimester trophoblasts (GW 7+8). ET-1 had a dose-dependent effect, with 100nM ET-1 decreasing MMP14 (-21%; p≤0.01) and MMP15 (-26%; p≤0.001) mRNA levels (Fig. 15A). The expression of the four members of the TIMP family (TIMP1 to 4) was also assessed in the same samples. ET-1 had a dose-dependent effect on TIMP regulation, with TIMP3 and TIMP4 mRNAs levels being upregulated by 100nM ET-1 (+47% and +39%, respectively; p<0.05; Fig.15B).

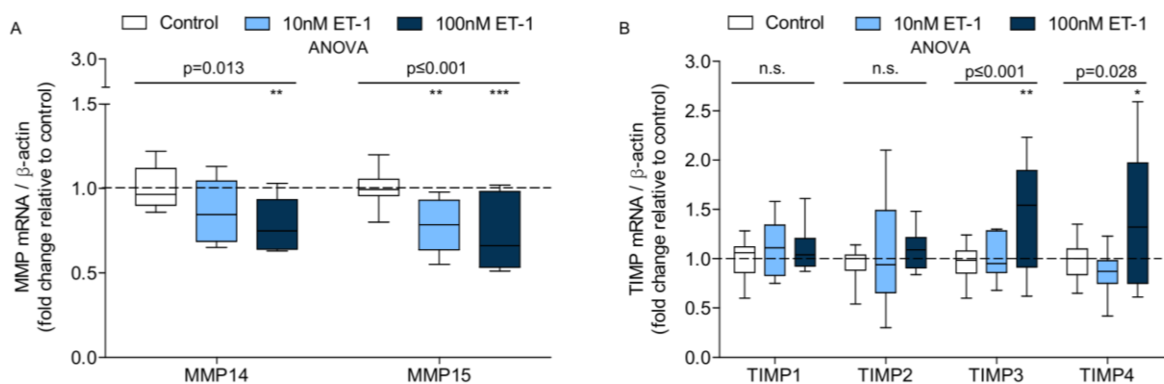

Figure 15. Effect of ET-1 on MMP14, MMP15 and TIMP expression in human first trimester trophoblasts. Trophoblasts (GW 7+8, n=5) were incubated in the absence (control) or presence of ET-1 (10 and 100nM) for 24h. MMP14, MMP15 **(A)** and TIMP **(B)** expression was determined by RT-qPCR. Results were normalized to β-actin using the $2^{-\Delta\Delta Ct}$ method and calculated relative to the controls arbitrarily considered as 1. Data are representative of four experiments and each condition was measured in triplicates. GW: gestational week, n.s.: non-significant. *p<0.05; **p≤0.01; ***p≤0.001 vs. control. Adapted from (75).

MMP14 and MMP15 protein levels were measured by Western blotting. For this set of experiments, trophoblasts were separated into early (GW 7+8) and mid (GW 9+10) first trimester trophoblasts. A single band corresponding to active MMP14 (act-MMP14, 58 kDa) was detected. Two bands were detected for MMP-15 corresponding to the inactive (pro-MMP-15, 75 kDa) and the active (act-MMP15, 68 kDa) form of the enzyme (Fig. 16A).

In concordance with the mRNA data, ET-1 also had a dose-dependent effect on MMP14 and MMP15 protein levels in early first trimester trophoblasts, with 100nM ET-1 reducing act-MMP14 (-18%; $p<0.05$) and total MMP15 (-22%; $p\leq0.01$). This downregulation was mainly accounted for pro-MMP15 (-27%; $p\leq0.001$), with act-MMP15 protein levels also being downregulated but not reaching significance (-17%; $p=0.07$; Fig. 16B).

In mid first trimester trophoblast ET-1 induced a similar decrease in act-MMP14 protein levels. However, this downregulation was not significant (-15%; $p=0.11$). The ET-1 effect on MMP15 downregulation was also maintained in mid first trimester trophoblast, with total (-24%; $p<0.05$), pro- (-25%; $p<0.05$) and act-MMP15 (-28%, $p\leq0.01$) being downregulated to a similar extent (Fig. 16C).

HLA-G protein levels were assessed by Western blotting to determine whether ET-1 affects trophoblast differentiation. HLA-G was not altered by ET-1 treatment, revealing that ET-1 only regulates MMP14 and MMP15 levels *per se* (Fig. 17).

Since first trimester placental tissue is a limited material, MMP14 and MMP15 regulation by ET-1 was only investigated after 24h. This time-point was established after measuring MMP14 and MMP15 protein half-life by blocking protein synthesis with cyclohexamide. These experiments showed that after 24h, MMP14 and MMP15 protein levels drastically dropped (Fig. 18). Thus, 24h is an adequate time point to study changes in MMP14 and MMP15 protein levels in trophoblasts.

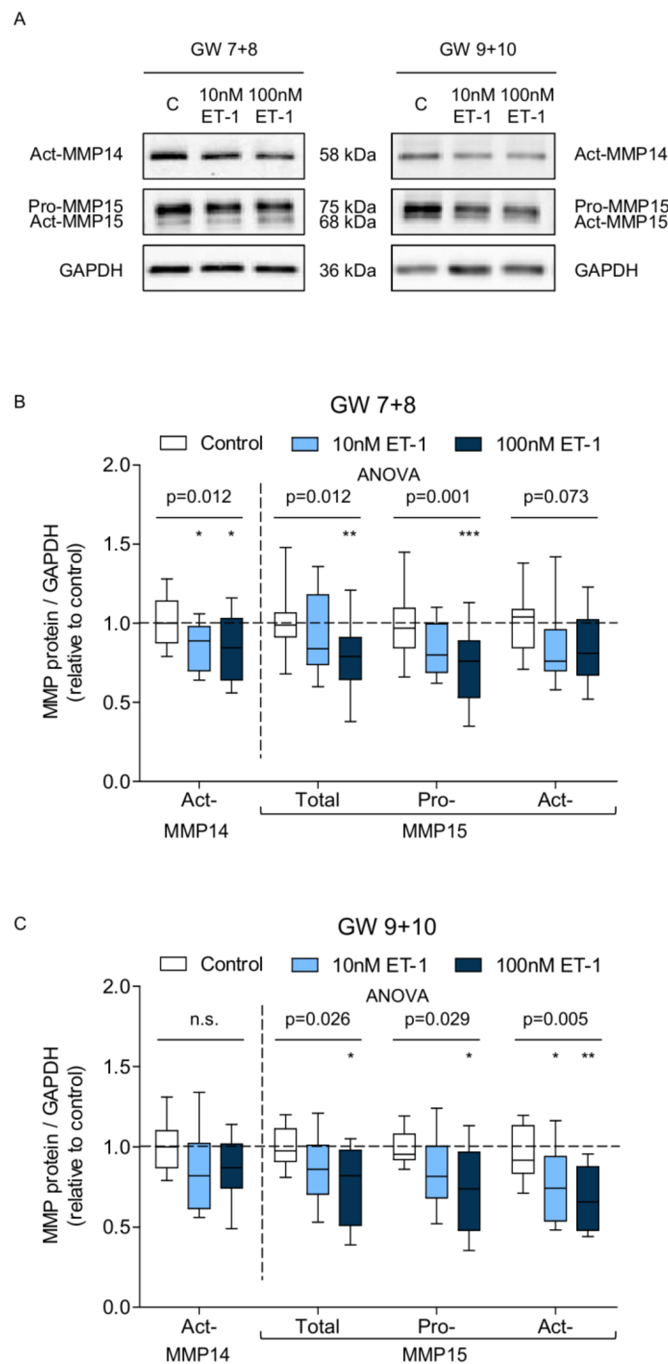

Figure 16. Effect of ET-1 on MMP14 and MMP15 protein levels in human first trimester trophoblasts. Trophoblasts were incubated in the absence (control) or presence of ET-1 (10nM and 100nM) for 24h. **(A)** MMP14 and MMP15 protein levels were determined by Western blotting. A single band corresponding to active MMP14 was observed (act-MMP14). Both pro- and active-MMP15 were detected. Total MMP15 refers to both bands considered together. For the analysis, trophoblast were separated according to gestational week (GW 7+8, n=9, **B**; and 9+10, n=4, **C**). Results were normalized to GAPDH and calculated relative to the controls, arbitrarily considered as 1. Data are representative of four experiments and each condition was assessed in triplicates. GW: gestational week, n.s.: non-significant. *p<0.05; **p≤0.01; ***p≤0.001 vs. control. Adapted from (75).

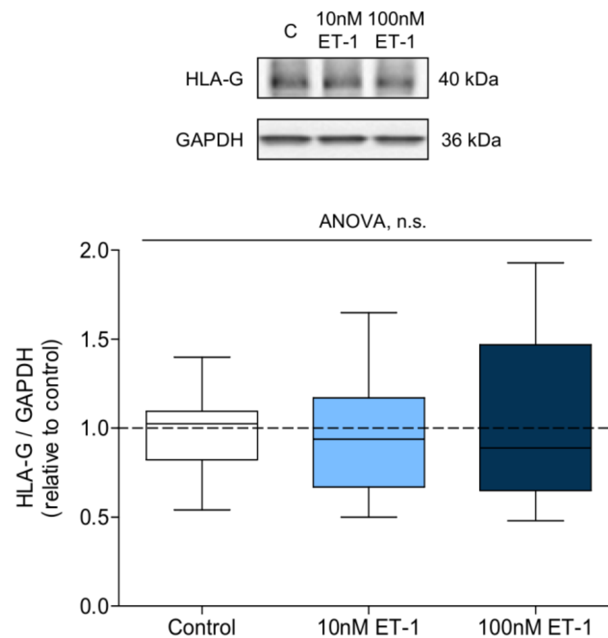

Figure 17. Effect of ET-1 on HLA-G protein levels in trophoblasts. Trophoblasts (GW 7-11, n=4) were incubated in the absence (control) or presence of ET-1 (10 and 100nM) for 24 hours. HLA-G protein levels were determined by Western blotting. Results were normalized to GAPDH and calculated relative to the controls, arbitrarily considered as 1. Data are representative of four experiments and each condition was measured in triplicates. n.s.: non-significant. Adapted from (75).

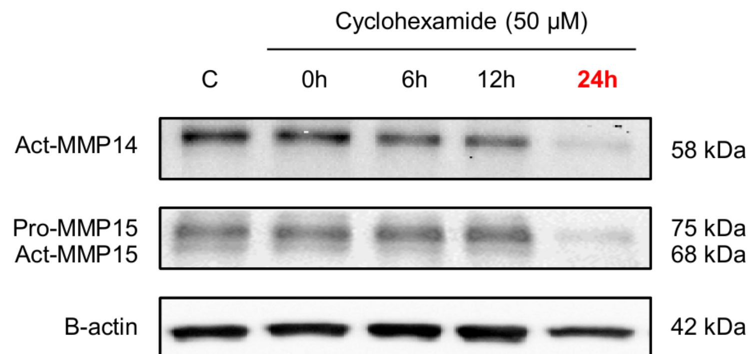

Figure 18. MMP14 and MMP15 half-life in human first trimester trophoblasts. Protein synthesis was blocked with 50µM cyclohexamide (CHX). Protein lysates were obtained at the time of (0h) and 6h, 12h and 24h after CHX addition. Trophoblasts maintained in culture for 24h in the absence of CHX were considered as control (C). MMP14 (active form: act-MMP14) and MMP15 (pro- and act-MMP15) protein levels were determined by Western blotting. β-actin was used as loading control.

To determine whether ET-1 also regulates MMP14 and MMP15 activity, zymography was performed. This technique allows the detection of MMP2, which is activated by MMP14 and MMP15. Pro-MMP2 (72 kDa) and act-MMP2 (66 kDa) were detected and the ratio between both was calculated as a measure of MMP14 and MMP15 activity. This ratio was increased in trophoblasts treated with both ET-1 concentrations (+26% vs. control; $p<0.05$) due to a decrease in act-MMP2, suggesting a downregulation of MMP14 and MMP15 activity (Fig. 19).

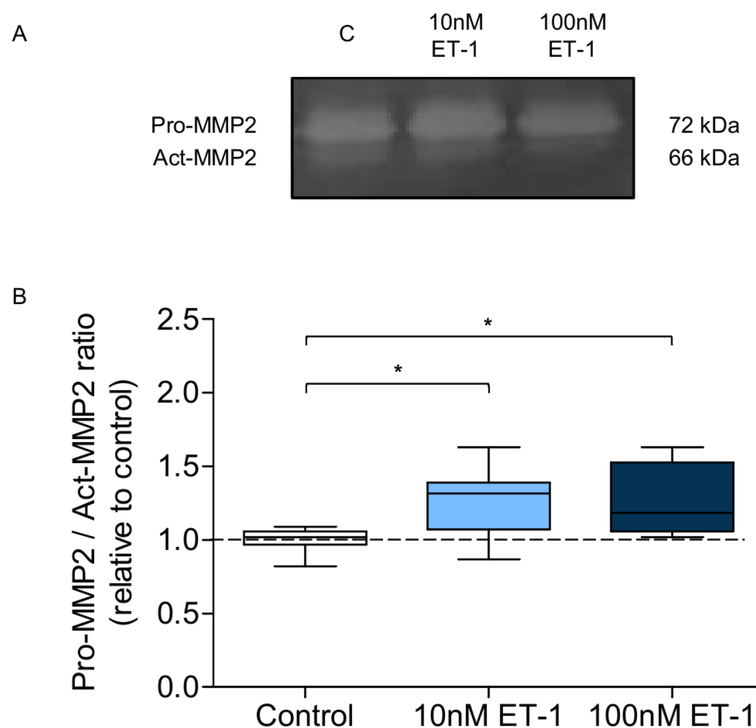

Figure 19. Effect of ET-1 on MMP2 activation in trophoblasts. Trophoblasts (GW 7-11, n=6). were incubated in the absence (control) or presence of ET-1 (10nM and 100nM) for 24h. **(A)** MMP2 protein levels were detected using zymography. **(B)** The ratio between pro- and active (act)-MMP2 was calculated as a measure of MMP2 activation. Results were calculated relative to the controls, arbitrarily considered as 1. Data are representative of four experiments and each condition was measured in triplicates. *$p<0.05$

4.2.4. Identification of ETR subtypes involved in MMP regulation

Two selective ETR antagonists (BQ-123 and BQ-788 for ETRA and ETRB, respectively) were used to determine ETR involvement in ET-1-mediated MMP regulation. Receptor-blocking studies have to consider endogenous production and release of ET-1, representing autocrine effects, as well as effects induced by exogenous ET-1, representing endocrine effects.

Incubation of human first trimester trophoblasts (GW 11-12) with BQ-123 or BQ-788 in the absence of ET-1, i.e. blocking the effect of endogenous ET-1, did not regulate act-MMP14 or total, pro- or act-MMP15 protein levels (Fig. 20 and Fig. 21, respectively).

ETRA inhibition with BQ-123 enhanced the effect of exogenous ET-1 on act-MMP14 (by 16%) and total MMP15 (by 15%) (Fig. 20; 100nM ET-1 vs. 11.2nM BQ-123 + 100nM ET-1; $p<0.05$). By contrast, the effect of exogenous ET-1 on act-MMP14 and total MMP15 downregulation was abolished by ETRB inhibition with both BQ-788 concentrations (Fig. 21). This was also observed for pro- and act-MMP15 when analyzed separately.

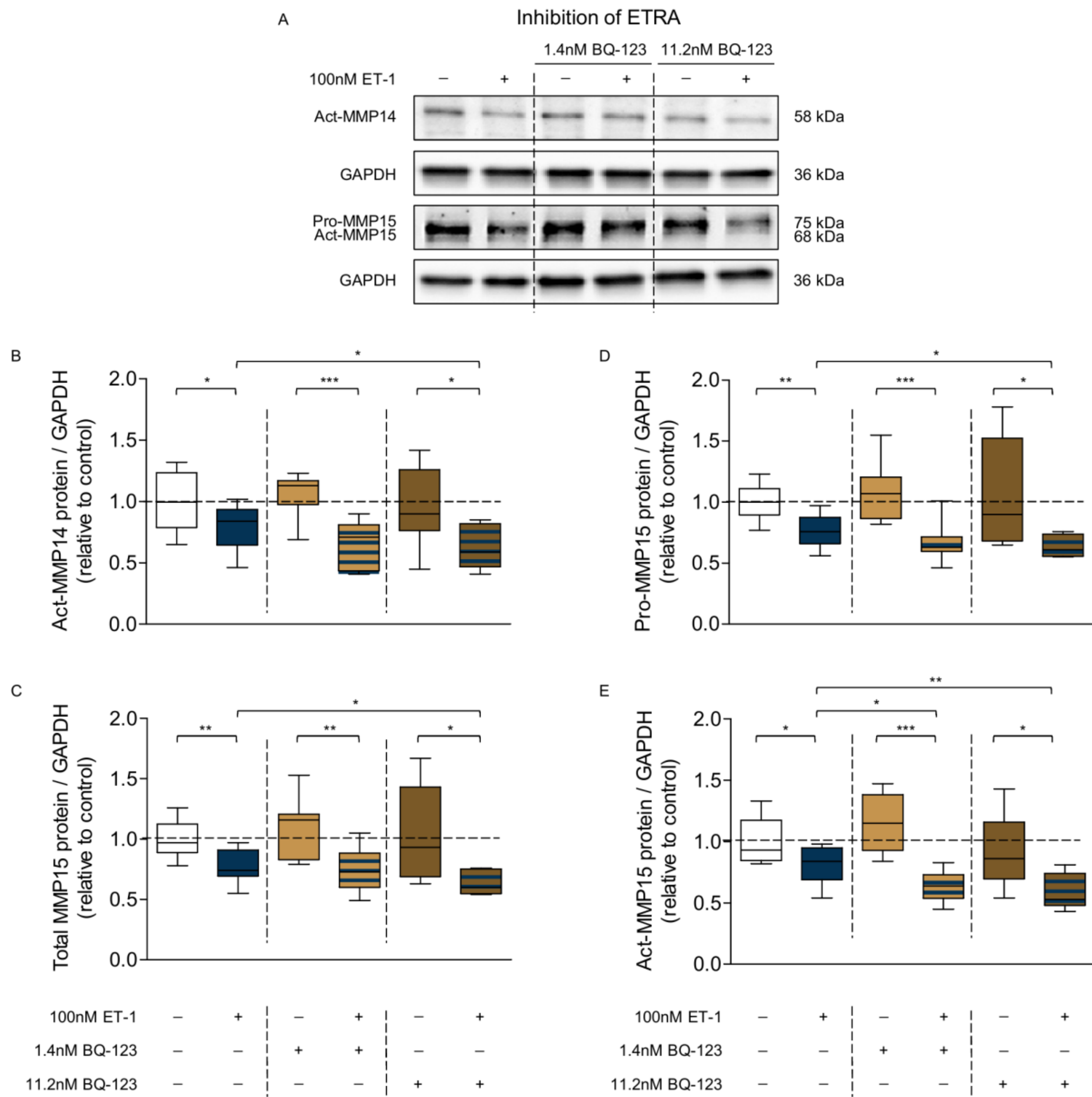

Figure 20. Effect of ET receptor (ETR) A blockade on MMP14 and MMP15 protein levels in trophoblasts. Trophoblasts (GW 11+12, n=7) were pre-incubated with BQ-123, a selective ETRA antagonist, for 2 h prior to the addition of 100nM ET-1. **(A)** MMP14 and MMP15 protein levels were determined by Western blotting. A single band corresponding to active MMP14 was observed (act-MMP14). Both pro- and active (act)-MMP15 were detected. Total MMP15 refers to both bands considered together. **(B-E)** Results were normalized to GAPDH and calculated relative to the controls, arbitrarily considered as 1. Data are representative of four experiments and each condition was measured in triplicates. *p<0.05; **p≤0.01; ***p≤0.001. Adapted from (75).

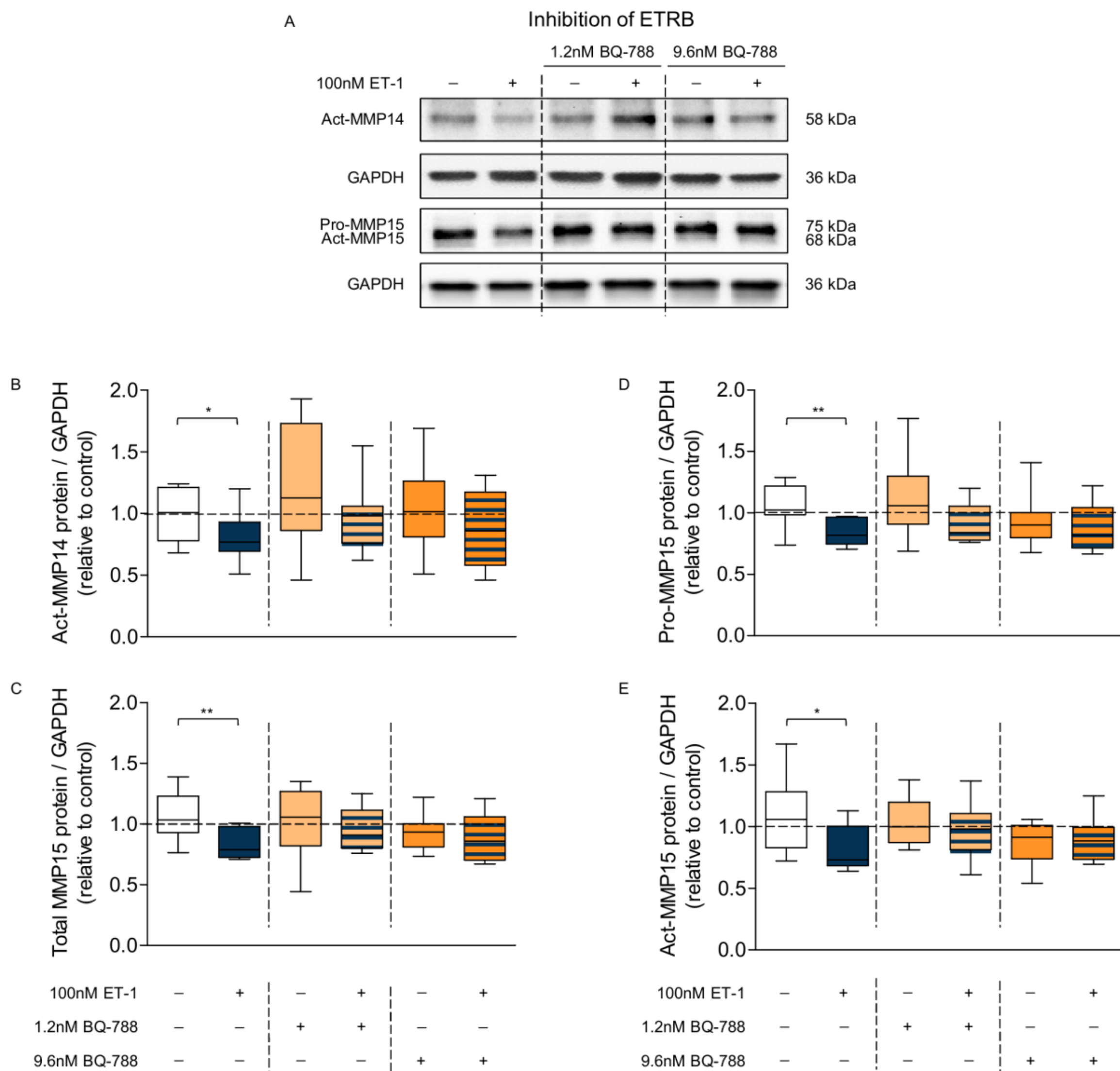

Figure 21. Effect of ET receptor (ETR) B blockade on MMP14 and MMP15 protein levels in trophoblasts. Trophoblasts (GW 11+12, n=7) were pre-incubated with BQ-788, a selective ETRB antagonist, for 2 h prior to the addition of 100nM ET-1. **(A)** MMP14 and MMP15 protein levels were determined by Western blotting. A single band corresponding to active MMP14 was observed (act-MMP14). Both pro- and active (act)-MMP15 were detected. Total MMP15 refers to both bands considered together. **(B-E)** Results were normalized to GAPDH and calculated relative to the controls, arbitrarily considered as 1. Data are representative of four experiments and each condition was measured in triplicates. n.s.: non-significant. *$p<0.05$; **$p\leq0.01$. Adapted from (75)

4.2.5. Functional effects of ET-1 on first trimester placenta

The effect of ET-1 on trophoblast migration and invasion was assessed following three different approaches. Firstly, human first trimester chorionic villi were incubated in the absence (control, Fig. 22A) or presence (Fig. 22B) of 100nM ET-1 for 24h. Trophoblast outgrowth was measured as the distance from the villous margin to the outer edge of the migrating cell sheet. ET-1 induced a decrease in trophoblast outgrowth (-24%; $p \leq 0.01$; Fig. 22C). However, trophoblast outgrowth is dependent on both, trophoblast proliferation and migration, and might not directly reflect trophoblast invasion. Thus, transwell invasion assays were additionally performed with isolated human first trimester trophoblast. Invasion assays revealed that ET-1 directly decreased trophoblast invasion to a similar extent (-26%; $p \leq 0.01$; Fig. 22D).

A third approach based on the CAM assay was intended (Fig. 23). Human isolated trophoblasts were seeded on the upper layer of the CAM (Fig. 23A). Despite attaching to the membrane (Fig. 23B and 23C, black arrows), the vast majority of trophoblasts failed to invade. Therefore, the low cell number of invasive trophoblasts (Fig. 23C, red arrows) did not allow an appropriate quantification of trophoblast invasion.

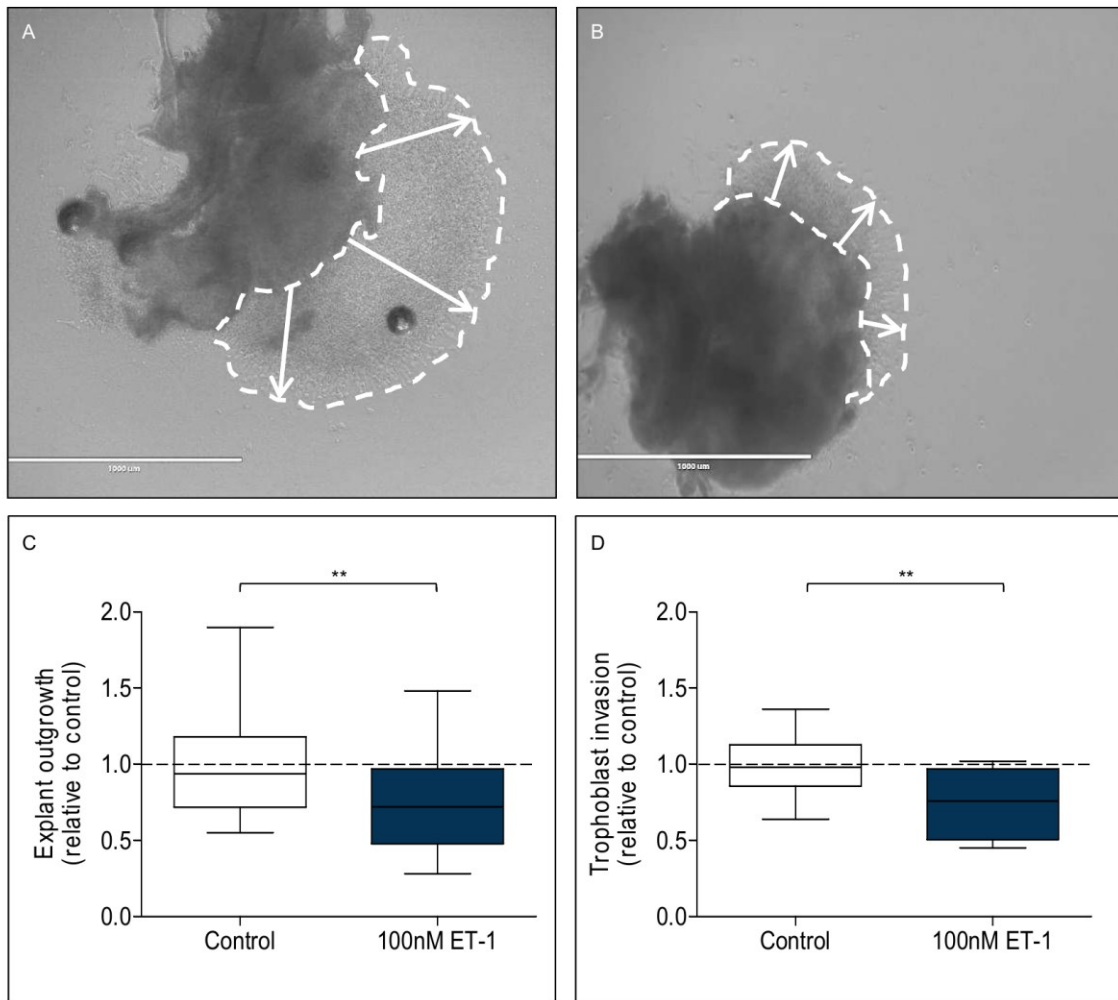

Figure 22. Effect of ET-1 on trophoblast outgrowth and invasion. Dissected chorionic villi from first trimester placentas (GW 7-11, n=4) were cultured on collagen I in the absence (control, **A**) or presence of 100nM ET-1 **(B)** for 24h. Explant outgrowth was measured as distance from the villous tip to the distal edge of the outgrowth sheet (arrows, A and B). Ten villi were used for each condition. For the invasion assay, isolated trophoblasts (GW 7-11, n=4) were seeded in transwell inserts pre-coated with fibronectin. Cells were incubated in the absence (control) or presence of 100nM ET-1 for 48h. Invading cells were stained with DAPI and quantified. Each condition was assayed in triplicates. **(C and D)** For both assays results were calculated relative to the controls, arbitrarily considered as 1. Data are representative of at least 4 experiments. $^{**}p \leq 0.01$. Adapted from (75).

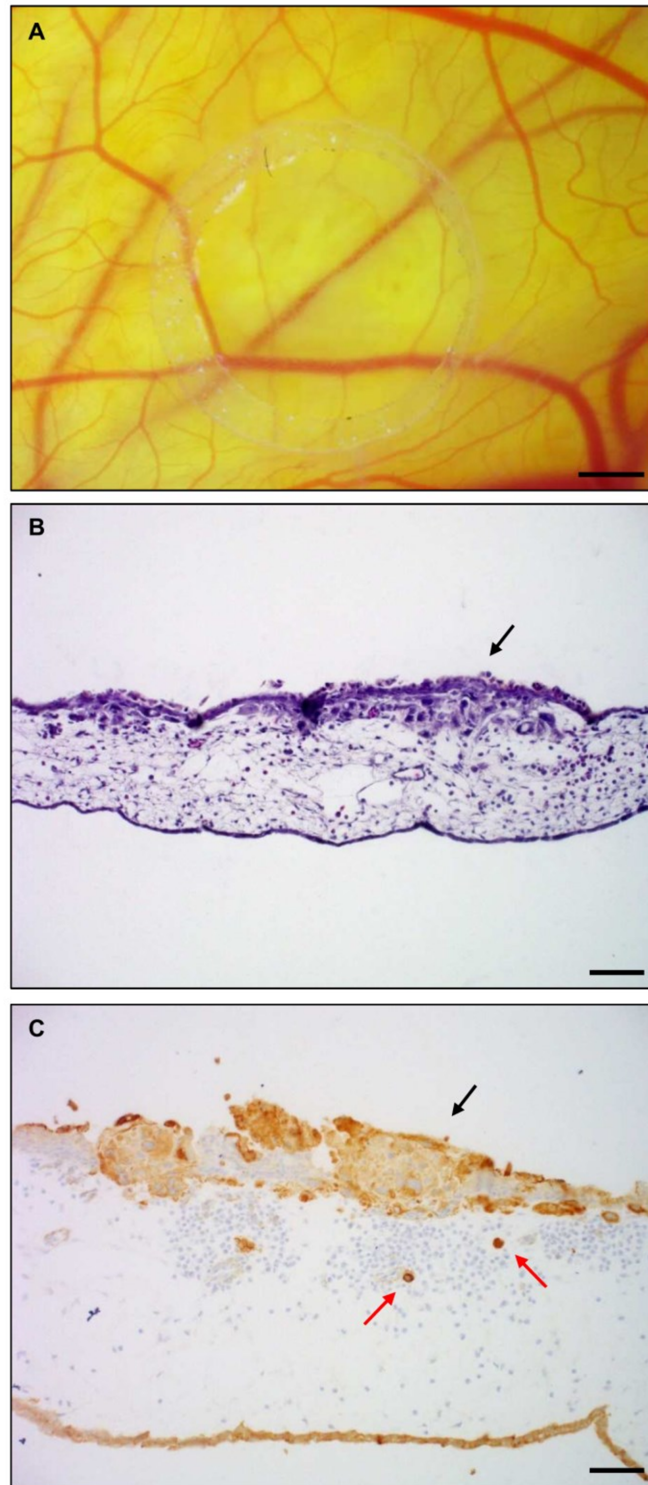

Figure 23. The chick embryo chorioallantoic membrane (CAM) assay as a model to study trophoblast invasion. (A) Trophoblasts (GW 7-10, n=3) were seeded on on-plants consisting of silicone rings placed on the CAM, and cells were allowed to invade for 48h. **(B)** Hematoxylin and eosin and **(C)** cytokeratin 7 staining were performed in CAM serial sections to identify trophoblasts. Black arrow: non-invasive trophoblasts. Red arrow: invasive trophoblasts. Scale bar: 1000μm (A) and 50μm (B and C).

4.2.6. Modulation of ET-1-mediated MMP14 and MMP15 downregulation by TNF-α

TNF-α was used to evaluate whether inflammation modulates the ET-1 effect on MMP14 and MMP15 regulation previously described (Fig. 24). When administered alone, TNF-α had no impact on act-MMP14 or total MMP15 protein levels. In combination with ET-1, TNF-α did not modulate the effect of ET-1 on MMP14 downregulation. By contrast, TNF-α enhanced ET-1-mediated total MMP15 downregulation by 10% (Fig. 24B; 100nM ET-1 vs. 100nM ET-1 + 25ng/ml TNF- α; $p<0.05$). This was also observed for pro- and act-MMP15 (Fig. 24C; +18% and +12%, $p<0.05$, respectively) when considered separately.

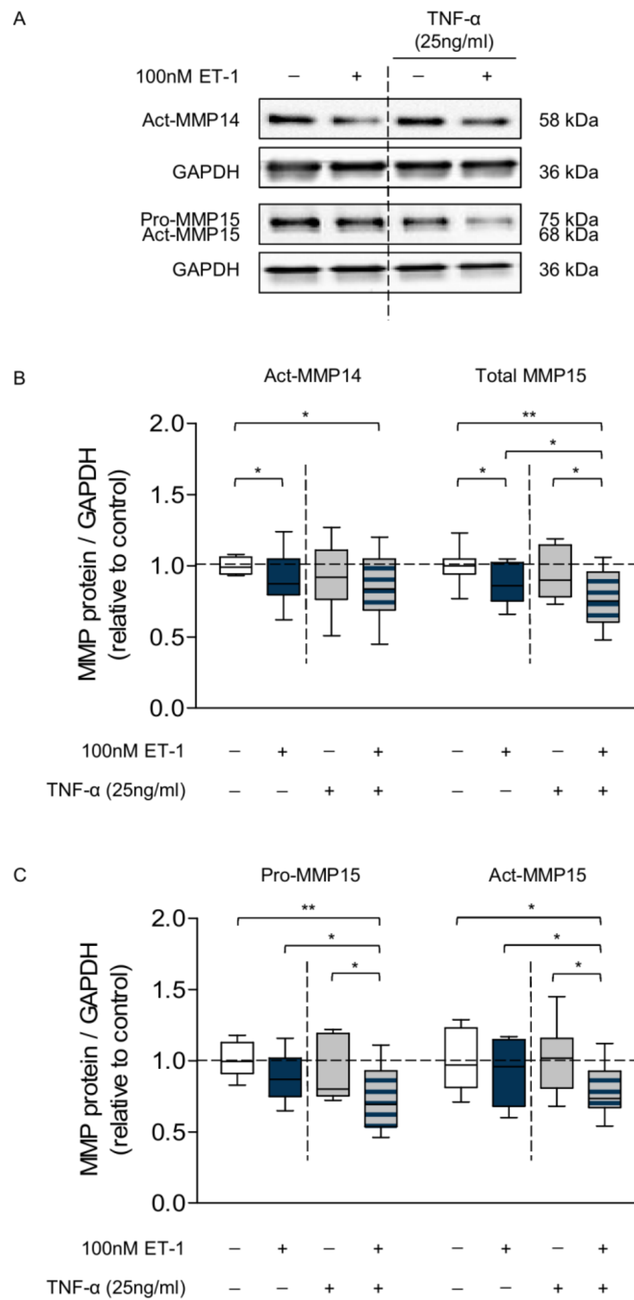

Figure 24. Effect of TNF-α on MMP14 and MMP15 regulation by ET-1 in human first trimester trophoblasts. Trophoblasts (GW 7+8, 9+10 and 11, n=5) were incubated in the absence (control) or presence of TNF-α (25ng/ml), 100nM ET-1 or the combination of both for 24 h. **(A)** MMP14 and MMP15 protein levels were determined by Western blotting. A single band corresponding to active MMP14 was observed (act-MMP14). Both pro- and active (act)-MMP15 were detected. Total MMP15 refers to both bands considered together. **(B and C)** Results were normalized to GAPDH and calculated relative to the controls, arbitrarily considered as 1. Data are representative of four experiments and each condition was measured in triplicates. *$p<0.05$; **$p\leq0.01$. Adapted from (75)

4.2.7. Modulation of ET-1 mediated MMP14 and 15 downregulation by low oxygen tension

In order to analyze whether oxygen modulates the effect of ET-1 on MMP14 and MMP15 protein levels, human first trimester trophoblasts were incubated in the absence (control, C) or the presence or 100nM ET-1 under three different oxygen tensions: i) 1% O_2, mimicking hypoxia, ii) 2.5% O_2, reflecting O_2 levels similar to those found in first trimester placental tissue *in vivo*, and iii) 20% O_2, i.e. the oxygen tension used for trophoblast isolation (Fig. 25).

In comparison to 20% O_2, trophoblasts cultured under both low oxygen tensions had lower act-MMP14 (-40%; $p<0.05$; and -16%; $p=0.22$; vs. 1% and 2.5% O_2, respectively) and pro-MMP15 (-45%; $p\leq0.001$; and -36%; $p\leq0.01$; vs. 1% and 2.5%, respectively) protein levels (Fig. 25A – 25C).

Both low oxygen tensions abolished the effect of ET-1 on act-MMP14 downregulation (Fig. 25A and 25B). Interestingly, 1% O_2 also abolished ET-1 mediated MMP15 downregulation, whereas 2.5% O_2 enhanced this downregulation by 20% (from -37% under 20% O_2 to -53% under 2.5% O_2; $p<0.05$) (Fig. 25A and 25C).

To determine whether low oxygen tension regulates endogenous ET-1 production, ET-1 levels were determined in cell supernatants of control trophoblasts, i.e. not supplemented with exogenous ET-1. An upregulation of ET-1 secretion was found under 1% O_2 (Fig. 25D, +50% and +55% vs. 1% and 2.5% O_2, respectively; $p<0.05$).

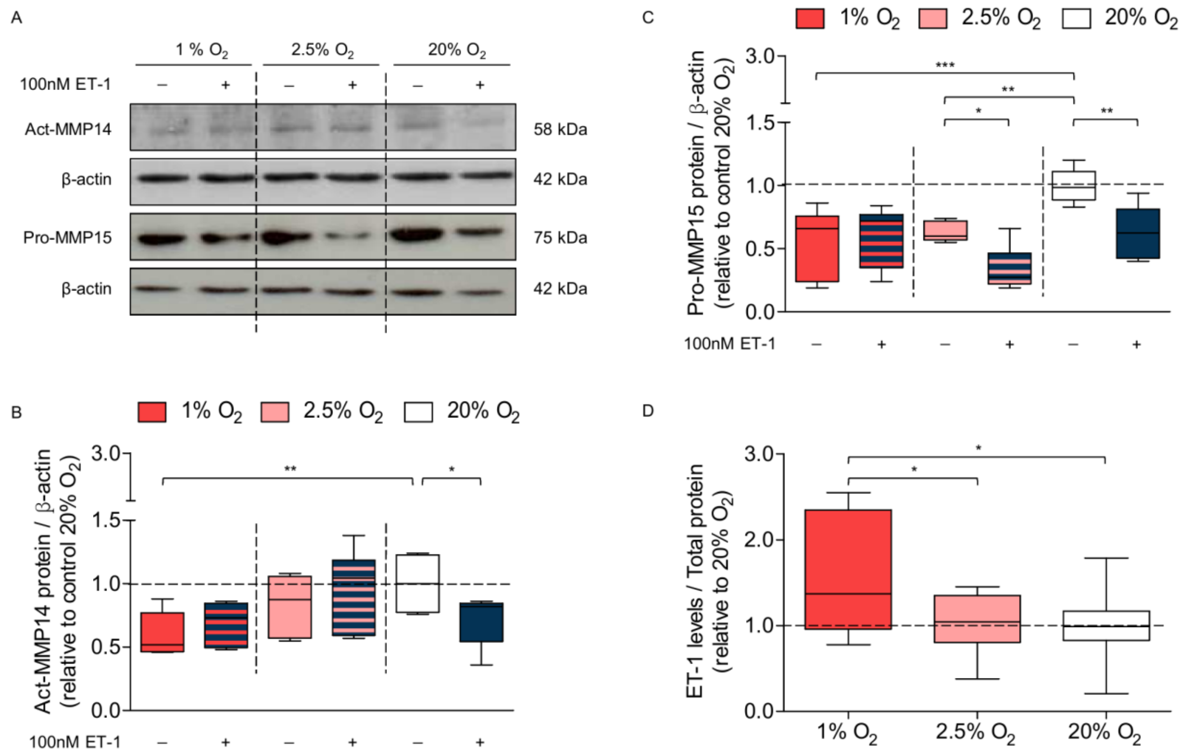

Figure 25. Effect of low oxygen tension on MMP14 and MMP15 regulation by ET-1 in human first trimester trophoblasts. After isolation, trophoblasts (GW 7+8, 9+10 and 11, n=12) were maintained under three different oxygen tensions: 1%, 2.5% and 20% O_2. Trophoblasts were incubated in the absence (control) or presence of 100nM ET-1 (blue) for 24h. **(A)** MMP14 and MMP15 protein levels were determined by Western blotting. A single band corresponding to active MMP14 was observed (act-MMP14). Both pro- and active (act)-MMP15 were detected. Total MMP15 refers to both bands considered together. **(B and C)** Results were normalized to β-actin and calculated relative to the controls, arbitrarily considered as 1. **(D)** ET-1 levels were determined by ELISA in the supernatants from cells not supplemented with exogenous ET-1. Results were normalized to total protein and calculated as fold change relative to the control (20% O_2). Data are representative of 4-6 experiments and each condition was measured in triplicates. *p≤0.05; **p≤0.01; ***p≤0.001. Adapted from (75).

4.3. MMP14 and MMP15 regulation by obesity in human first trimester placental tissue

4.3.1. MMP14 and MMP15 expression in lean and obese placental tissue

MMP14 and MMP15 mRNA levels were analyzed in human first trimester placental tissue from lean (BMI: 18.5 – 24.9) and obese (BMI≥30) women (Table 5).

Table 5. First trimester placental tissue parameters. (***p≤0.001)

	Lean (n=18)	**Obese (n=12)**
BMI		
Mean ± SD	**19.8 ± 1.5**	**34.4 ± 4.3*** **
Range	18.5 – 24.9	30.1 – 45
Gestational age (days)		
Mean ± SD	64.6 ± 12.8	58.1 ± 12.3
Range	49 - 82	36 - 74
Fetal sex		
Ratio (male:female)	4:5	7:5

Maternal obesity did not alter MMP14 or MMP15 expression (Fig. 26). To rule out a possible effect of GA or fetal sex, lean and obese samples were further stratified attending to these parameters. However, GA and sex did not affect MMP14 or MMP15 expression (Fig. 27).

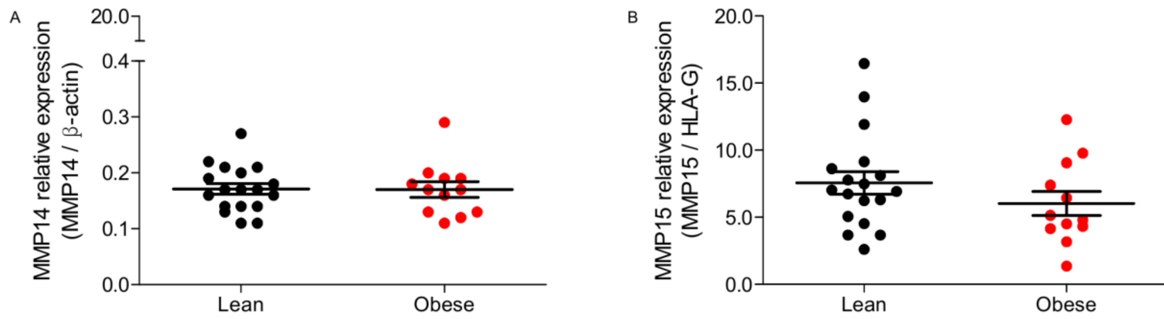

Figure 26. MMP14 and MMP15 expression in lean and obese first trimester placental tissue. MMP14 **(A)** and MMP15 **(B)** expression was determined by RT-qPCR. Results were normalized to β-actin or HLA-G using the $2^{-\Delta\Delta Ct}$ method. n_{lean}=18; n_{obese}=12

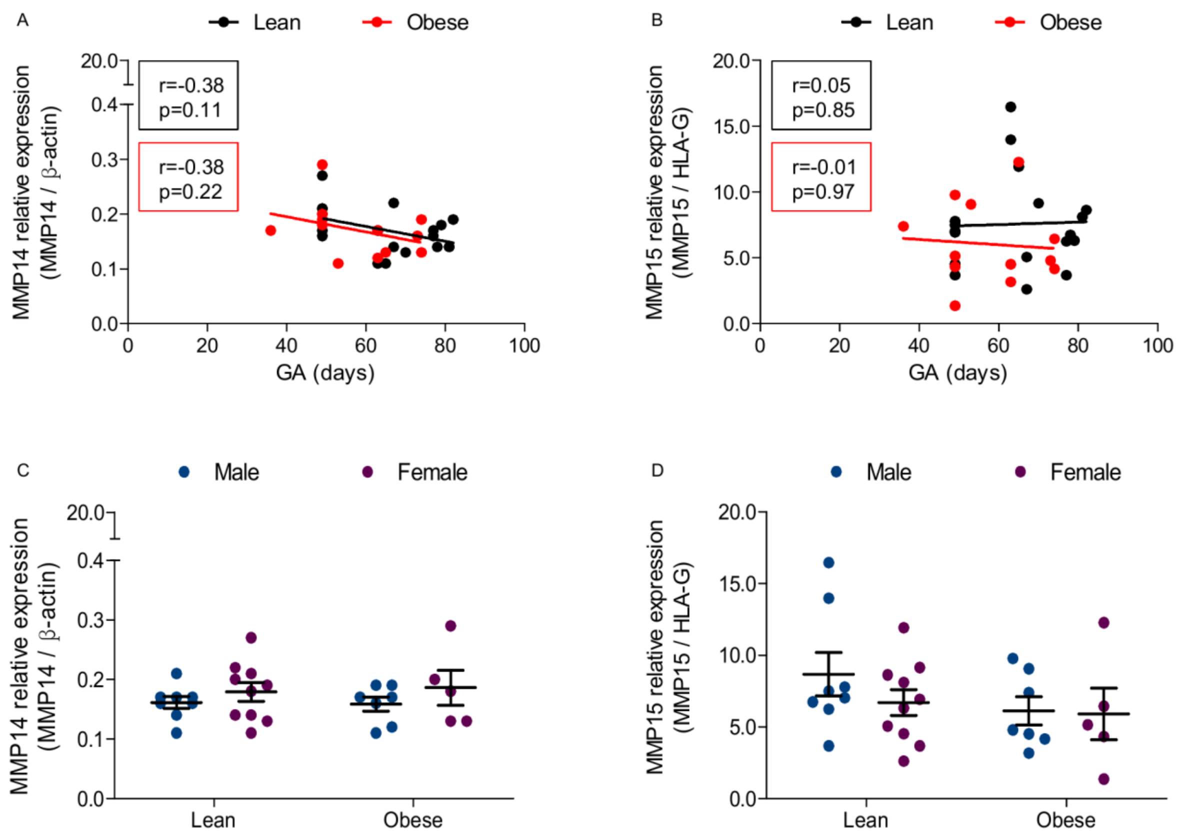

Figure 27. Correlation between gestational age (GA), fetal sex, obesity and MMP14 or MMP15 expression in first trimester placental tissue. Lean and obese samples were stratified according to GA **(A and B)** or fetal sex **(C and D)**. MMP14 (A and C) and MMP15 (B and D) expression was determined by RT-qPCR. Results were normalized to β-actin or HLA-G using the $2^{-\Delta\Delta Ct}$ method. $n_{lean+male}$=8; $n_{lean+female}$=10; $n_{obese+male}$=7; $n_{obese+female}$=5

4.3.2. MMP14 and MMP15 protein levels in lean and obese placental tissue

MMP14 and MMP15 protein levels were evaluated by Western blotting in the same lean and obese samples used for the expression studies. Two bands were detected for MMP14 and MMP15, corresponding to the inactive (pro-MMP14, 65kDa, and pro-MMP-15, 75 kDa) and the active (act-MMP14, 58 kDa, and act-MMP15, 68kDa) form of both enzymes (Fig. 28A and 30A). Total MMP14 levels were upregulated by maternal obesity (+41%; $p<0.05$). This upregulation was also observed when pro- (+35%; $p=0.07$) and act-MMP14 (+47%; $p<0.05$) were analyzed separately (Fig. 28B – 28D).

GA did not alter total, pro- or act-MMP14 protein levels in lean or obese placental samples (Fig. 29A – 29C). However, pro-MMP14 was significantly higher in female obese samples when compared to male obese (Fig. 29E), suggesting a plausible influence of fetal sex in pro-MMP14 protein levels within the obese cohort.

In the case of MMP15, obesity did not alter total, pro- or act-MMP15 protein levels (Fig. 30). Interestingly, when lean and obese samples were stratified according GA, a negative correlation between GA and total-MMP15 ($r=-0.59$; $p=0.06$) was found only within the obese cohort (Fig. 31A). Similar results were observed for pro- ($r=-0.55$; $p=0.08$) and act-MMP15 ($r=-0.65$; $p=0.03$) (Fig. 31B and 31C). These results suggest that obesity might trigger a MMP15 downregulation along the first trimester, otherwise not found in healthy pregnancy. Fetal sex had no impact on MMP15 protein levels (Fig. 31D – 31F).

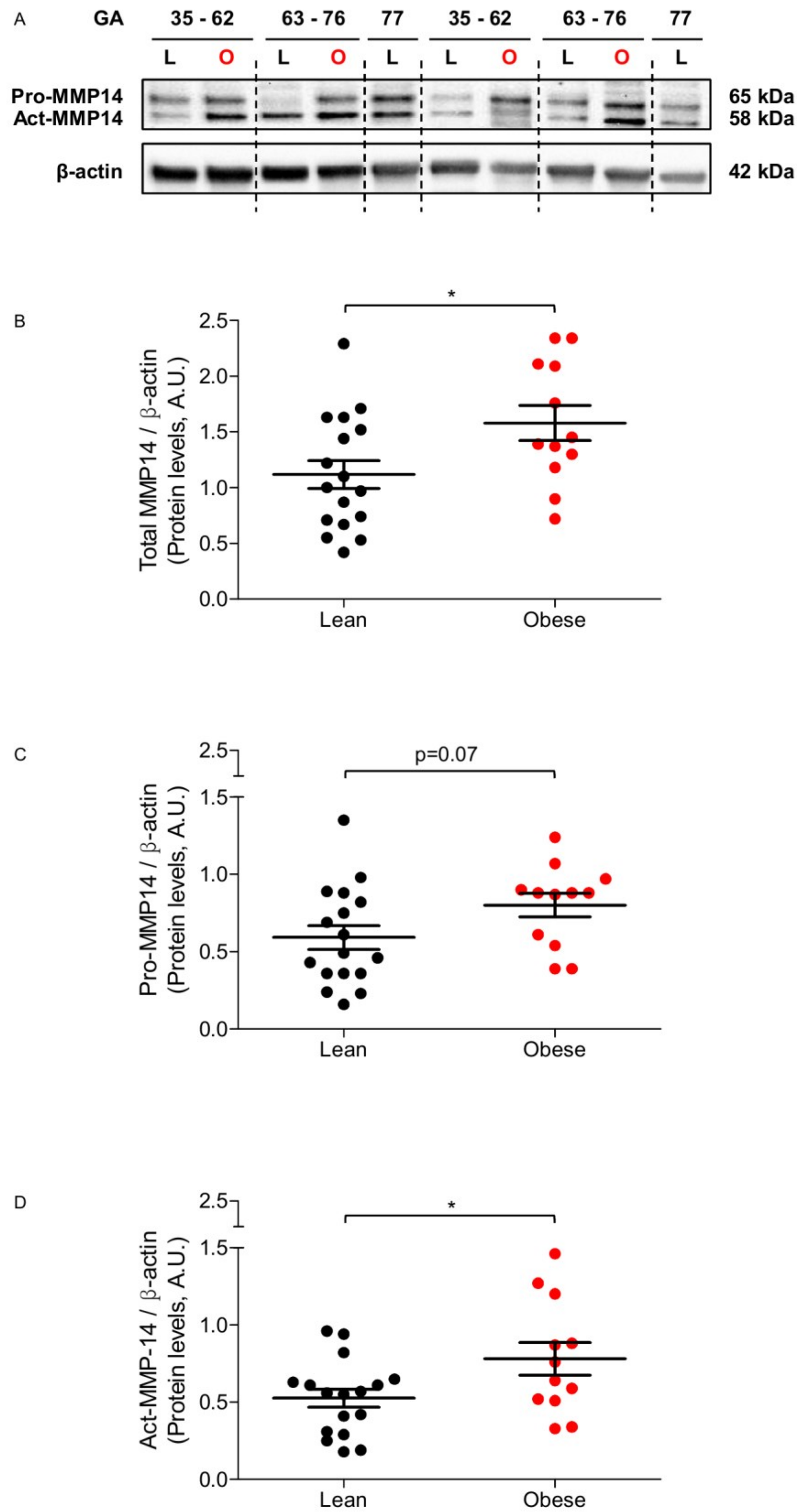

Figure 28. MMP14 protein levels in lean and obese first trimester placental tissue. (A) Western blotting analysis revealed two bands corresponding to inactive (pro) and active (act)-MMP14. Total MMP14 refers to both bands considered together. **(B-D)** Results were normalized to β-actin. n_{lean}=17; n_{obese}=12; *p<0.05

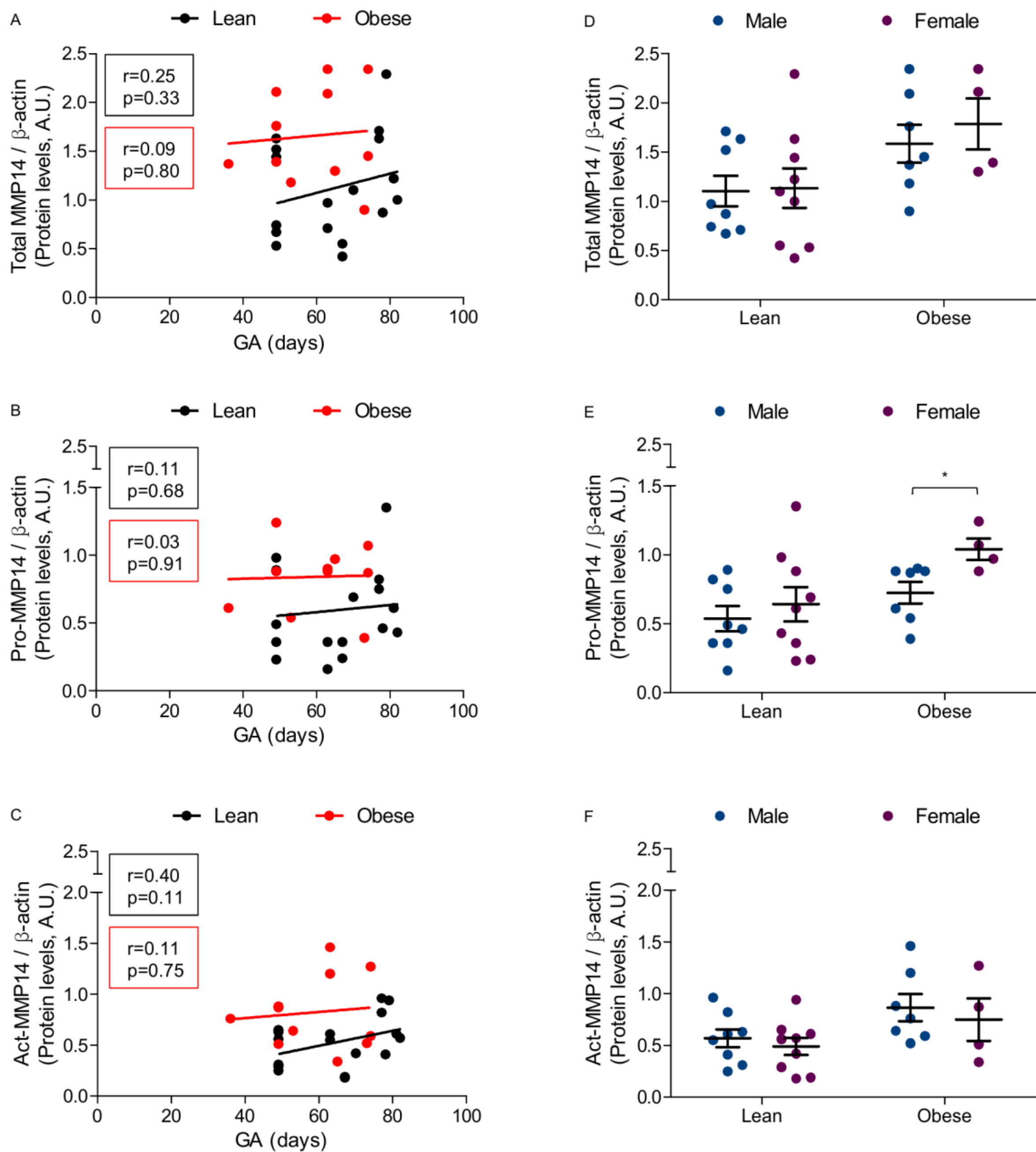

Figure 29. Correlation between (GA), fetal sex, obesity and MMP14 protein levels in first trimester placental tissue. Lean and obese samples were stratified according to GA (days) **(A-C)** or fetal sex **(D-F)**. Total, pro- and act-MMP14 levels were determined by Western blotting and normalized to β-actin. $n_{lean+male}$=8; $n_{lean+female}$=9; $n_{obese+male}$=7; $n_{obese+female}$=4

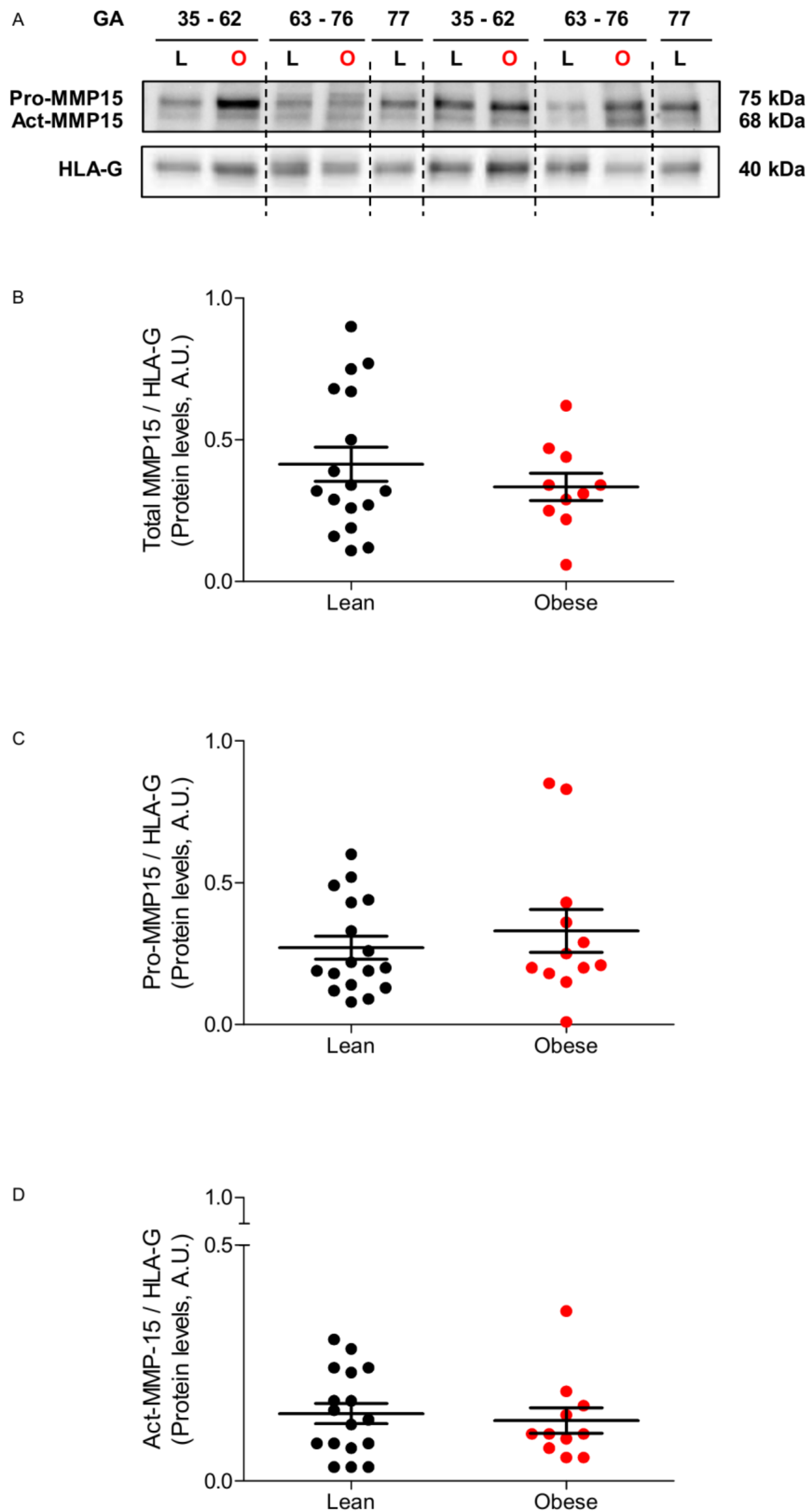

Figure 30. MMP15 protein levels in lean and obese first trimester placental tissue. (A) Western blotting analysis revealed two bands corresponding to inactive (pro) and active (act) MMP15. Total MMP15 refers to both bands considered together. **(B-D)** Results were normalized to HLA-G. n_{lean}=17; n_{obese}=12

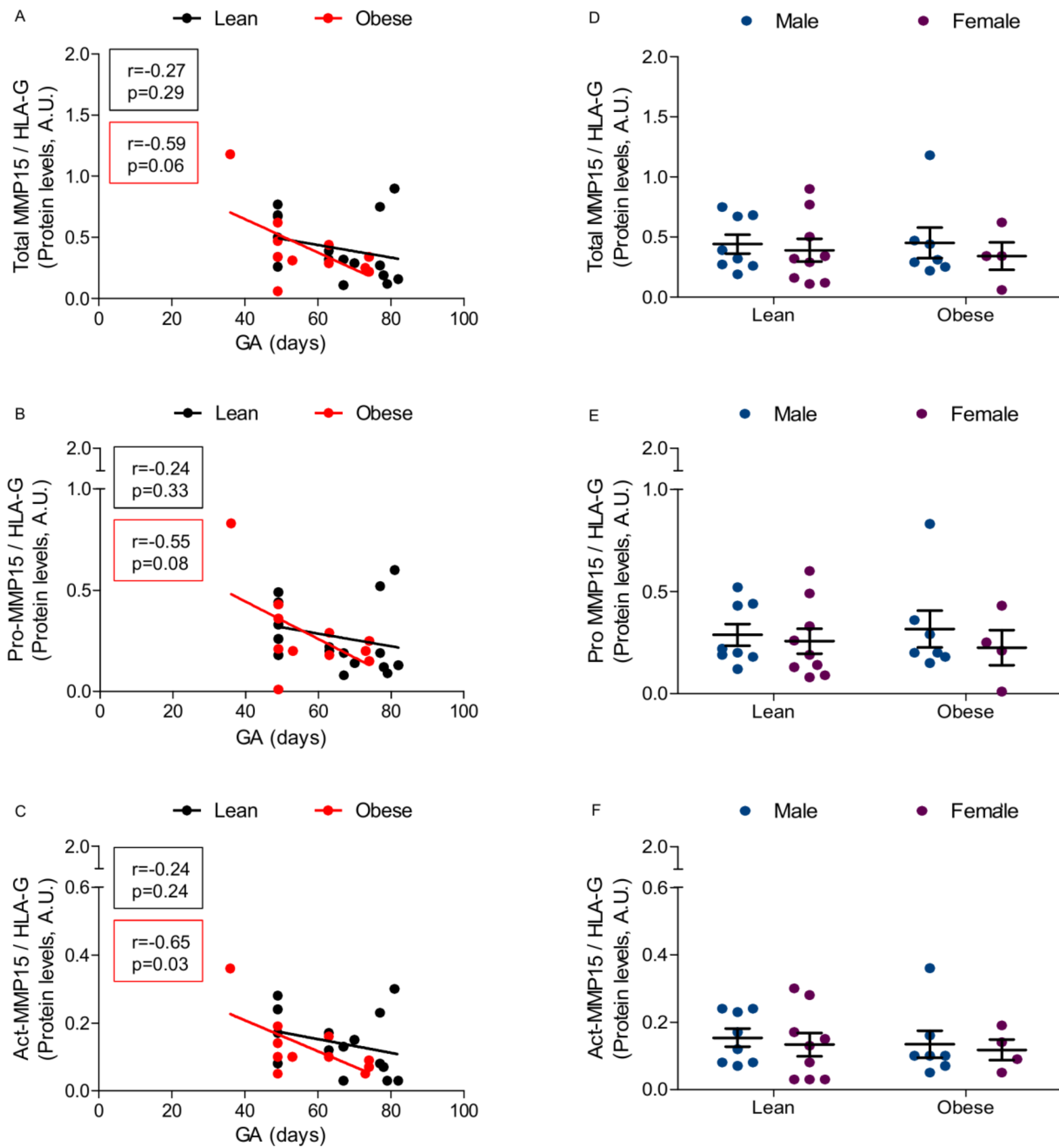

Figure 31. Correlation between gestational age (GA), sex and obesity on MMP15 protein levels in first trimester placental tissue. Lean and obese samples were stratified according to GA (days) **(A-C)** or sex **(D-F)**. Total, pro- and act-MMP15 levels were determined by Western blotting and normalized to HLA-G. $n_{lean+male}$=8; $n_{lean+female}$=9; $n_{obese+male}$=7; $n_{obese+female}$=4

5. Discussion

5.1. MMP15 is involved in trophoblast invasion

Despite an increase in the number of studies addressing placental function during the first trimester of pregnancy, the actual mechanisms governing trophoblast invasion are still not fully understood. Likewise, it is widely assumed that MMPs are central to the process of trophoblast invasion (33, 35). However, studies addressing placental MMPs have focused on the soluble members of this family, i.e. MMP2 and MMP9 (61, 76), and the importance of MT-MMPs in trophoblast biology has only recently emerged (68).

From the two members of the MT-MMP subfamily found in first trimester trophoblasts, i.e. MMP14 and MMP15 (68), only MMP14 has been characterized in depth (64, 66, 77). Although MMP15 was firstly described in human first trimester placenta in 1995 (78), and its expression has been found in EVT (70), its importance for placental function has not been previously studied.

We found that in human first trimester placental tissue MMP15 is only localized within the cell columns. More specifically, MMP15 co-localized with HLA-G, a classical marker of EVT (79), whereas VT or the syncytiotrophoblast were negative for MMP15. This contrasts with MMP14 localization in first trimester placenta, which is found not only in EVT but also in VT, the syncytiotrophoblast and in endothelial cells of the fetal capillaries (66). MMP15 was also found in trophoblasts which have already reached and invaded into the *decidua basalis* (iEVT), but not in any other decidual cell type. Moreover, MMP15 expression and protein levels remained constant in human isolated trophoblast along the first trimester of pregnancy. Therefore, its localization and expression pattern suggested a role of MMP15 in trophoblast invasion.

This was confirmed by knocking down MMP15 in human placental chorionic villi, which resulted in a downregulation of trophoblast outgrowth. Since outgrowth is also dependent on VT proliferation, we ruled out an effect of MMP15 knockdown on trophoblast proliferation. MMP15 has been shown to have an anti-apoptotic effect in other cell types (80). We analyzed whether this was also the case in

human first trimester trophoblasts and we found that MMP15 knockdown did not affect apoptosis in our study.

Altogether, our results suggest that MMP15 is restricted to the invasive compartment in human first trimester placenta. Indeed, and despite the repertoire of proteases found in trophoblast (including other members of MMP and the ADAM family among others (70, 81)) MMP15 appears to be crucial for trophoblast invasion.

5.2. The interplay between ET-1, inflammation and low oxygen tension in MMP14 and MMP15 regulation in human first trimester trophoblasts

Among other functions, ET-1 has been described as a regulator of cell proliferation, migration and invasion in several cell types (82-84). In pregnancy, ET-1 is known to be central in the pathology of PE (20) and its upregulation during the first trimester has been linked to an increased risk of subsequently developing PE later in pregnancy (25).

Here, we assessed whether ET-1 is able to regulate MMP14 and MMP15 in human first trimester trophoblast entitling functional consequences, and whether other PE stimuli such us inflammation and hypoxia also play a role in this regulation.

5.2.1. ET-1 as a regulator of MMP14, MMP15 and TIMPs

ET-1 has been shown to regulate MMPs in several cell types. However, these data refer mainly to MMP2 and MMP9 (85, 86) and a link between ET-1 and MT-MMPs was still missing. Thus, we assessed the role of ET-1 on MMP14 and MMP15 regulation.

We showed that in human first trimester trophoblasts ET-1 downregulates both MMP14 and MMP15 expression. This was paralleled by a decrease in MMP14 and MMP15 protein levels. Remarkably, only active MMP14 was detected, which has been previously described in isolated trophoblasts (64). Both,

pro-MMP15 and active MMP15 were observed. MT-MMPs are activated in a two-step process with Golgi-associated pro-protein convertases (PCs) generating an intermediate product, which is further activated by autocatalysis or other proteases (87). A family of seven PCs with different affinities in terms of MMP14 activation has been described (88), whereas the specific mechanism of MMP15 activation by PCs remains unknown. Our data suggest that different members of the PC family might be involved in MMP14 and MMP15 activation.

The effect of ET-1 on MMP downregulation was maintained along the first trimester of pregnancy. Moreover, HLA-G protein levels where not affected by ET-1, suggesting that ET-1-mediated MMP downregulation is not related to changes in trophoblast cell subpopulation, i.e. trophoblast differentiation.

We also characterized the effect of ET-1 on MMP14 and MMP15 protein levels in ACH-3P cells, a human first trimester trophoblast cell line (59). Although MMP14 protein levels were downregulated to a similar extent when compared to primary trophoblasts, ET-1-mediated MMP15 downregulation only appeared after 48h (vs. 24h in primary cells). This time shift might be due to differences in endogenous ET-1 production and ETR between primary trophoblast and trophoblast cell lines (89, 90), suggesting that results in these cell lines need to be carefully analyzed. Since ACH-3P cells remain proliferative in culture, we assessed whether a regulation of cell proliferation and/or viability might underlay MMP downregulation. However neither cell proliferation nor viability was affected by ET-1.

MMP activity does not only rely on MMP expression and protein levels. TIMPs, the natural inhibitors of MMPs, play a key role in the regulation of MMP activity (91). Thus, we also evaluated whether TIMP expression is modulated by ET-1 in human first trimester trophoblast. From the four members of the TIMP family only TIMP3 and TIMP4 mRNA levels were found to be upregulated by ET-1. Interestingly, an upregulation of TIMP3 and TIMP4 has been described in preeclamptic term placental villi (92, 93) . Thus, this downregulation might already begin during the first trimester of pregnancy due to ET-1 upregulation, which results in MMP inhibition and could hinder trophoblast invasion.

We observed that ET-1 might directly reduce MMP14 and MMP15 activity, since ET-1 downregulated MMP2 activation, a process mediated by MMP14 and MMP15. Interestingly, TIMP4 binding to MMP14 has been shown to be enough to abolish MMP2 activation (42, 94), pointing out that ET-1-mediated TIMP4 upregulation might also hinder MMP2 activation.

Our data suggest that in human first trimester trophoblasts ET-1 downregulates invasion-promoting MMPs and upregulates invasion inhibiting TIMPs, altering trophoblast functionality. Nevertheless, an in depth analysis of the downstream signaling pathways triggering MMP downregulation by ET-1 in trophoblast is still necessary.

5.2.2. MMP14/MMP15 downregulation is mediated via ETBR

The ET/ETR system plays a role in pregnancy under normal and pathological conditions. During the first trimester, ETRA and ETRB are expressed in VT, whereas ETRB is predominantly found in EVT, and has been related to trophoblast invasion (95). Changes in ETR levels through normal pregnancy have been described, with a preponderance of ETRB over ETRA as well as higher levels of both ETRs in first trimester vs. term trophoblasts (96).

ETR dysregulation is also involved in pregnancy complications. ETRA is upregulated in placental tissue from first trimester delayed miscarriages (97), whereas ETRB expression is upregulated in PE (98), where it has been shown to be involved in ET-1-mediated endoplasmic reticulum stress (99, 100).

Our data showed that the effect of ET-1 on MMP14 and MMP15 downregulation occurs through ETRB, since ETRB blocking abolished ET-1-mediated MMP downregulation. Moreover, ETRA blocking, i.e. allowing a higher ET-1 fraction to be bound to ETRB, enhanced MMP downregulation.

Our data support the involvement of ETRB in invasion and suggest that ETRB might already play a role in the pathogenesis of PE in the time-window when this complication originates. However, further studies analyzing ET-1 and ETR dynamics during the first trimester of pregnancy are still required to draw firm conclusions.

5.2.3. ET-1 downregulates trophoblast outgrowth and invasion in early pregnancy

To confirm whether ET-1 directly regulates trophoblast function in the first trimester of pregnancy, we used three different functional assays. Firstly, we confirmed that ET-1 downregulated trophoblast outgrowth from human placental chorionic villi. However, this method alone does not allow discrimination between trophoblast proliferation and migration/invasion. Therefore, a classical transwell invasion assay was performed, where ET-1 elicited a similar response, hindering trophoblast invasion.

The use of the CAM assay was intended to further confirm these results. The CAM assay is regularly used to determine the angiogenic potential of molecules on the CAM vasculature, and has recently emerged as a useful tool to study cell invasion (101). Nevertheless, trophoblast failed to invade through the CAM, pointing that this model might not be suitable to study trophoblast invasion.

Although the role of ET-1 in cell migration and invasion has been mainly studied in the context of tumor biology (22), few studies have addressed this question in first trimester trophoblast. Cervar (95) showed that a high dose of ET-1 (10µM) increased trophoblast invasion in primary isolated trophoblasts *in vitro*. The same authors have shown that ET-1 has a bimodal effect on trophoblast biology, with lower ET-1 concentrations inducing the opposite effect (102).

ET-1 regulates trophoblast functions in an autocrine (trophoblast-secreted ET-1) and in a paracrine (exogenous ET-1 supply from maternal plasma) manner. However, autocrine and paracrine ET-1 concentrations lie in the femtomolar (23) and the picomolar (25) range, respectively. Thus, the ET-1 concentrations used in the present study (100nM) are in line with other studies assessing ET-1 biological functions (103, 104) and might be mimicking the pathophysiological situation *in vivo* more accurately.

5.2.4. TNF-α enhances ET-1-mediated MMP15 downregulation

TNF-α levels in maternal plasma are upregulated in the third trimester of pregnancies complicated with PE (105). This dysregulation might already begin in the first trimester, since VT, EVT and uterine Natural killer (NK)-cells secrete TNF-α during this period, and TNFR is highly expressed in EVT (106). Indeed, TNF-α regulates aspects of trophoblast biology such as migration and invasion (76, 107). However, the role of TNF-α on MMP expression in first trimester trophoblast remains controversial. Several publications have addressed this question showing contradictory results for different MMPs (64, 108).

Our results revealed no effect of TNF-α on MMP14 or MMP15 protein levels. However, TNF-α enhanced the effect of ET-1 on MMP15 downregulation. Interestingly, TNF-α has been shown to increase ET-1 expression (109, 110), whereas ET-1 itself stimulates the secretion of TNF-α (111). This suggests a complex interplay between ET-1 and inflammation, which in turn might fine-tune the proteome expressed in trophoblast. Thus, a pro-inflammatory environment during the first trimester of pregnancy might further contribute to the impairment of trophoblast biology induced by ET-1.

5.2.5. Low oxygen tension as a negative regulator of ET-1

The placental environment during the first trimester of pregnancy is characterized by low oxygen tension. Oxygen concentrations rise from the placental villi (2-3% O_2) into the decidua (5% O_2) (8, 112). This is required for a successful pregnancy since trophoblast differentiation depends on this gradient. In fact, only those cells at the tip of the cell columns, i.e. in contact with the decidua, lose their proliferative capacities and become invasive (113). Oxygen has also been described as a regulator of MMP expression (114), and hypoxia is considered to contribute to the etiology of PE (17, 20).

We observed that MMP14 and MMP15 protein levels were reduced under low oxygen tension in human first trimester trophoblasts. This suggests that, as a result of trophoblast differentiation into invasive cells, increasing oxygen concentrations also triggered an upregulation of MMP14 and MMP15, pointing out again their importance for trophoblast invasion.

Low oxygen tensions differentially regulated the effect of ET-1 on MMP14 and MMP15 protein levels. Under 2.5% O_2, only MMP15 was downregulated, suggesting that MMP15 might be preferentially regulated by ET-1 *in vivo*. Contrarily, ET-1-mediated MMP14 and MMP15 downregulation was abolished under 1% O_2.

Hypoxia has been described to upregulate ET-1 secretion (115), and the ET-1 promoter is highly responsive to hypoxia (116). We confirmed that 1% O_2 increased ET-1 secretion in human first trimester trophoblast. Thus, low oxygen tension might increase ET-1 levels above its bimodal threshold, eliciting a negative feedback mechanism.

Fig. 32 summarizes the interplay between ET-1, TNF-α and hypoxia in MMP regulation and trophoblast function.

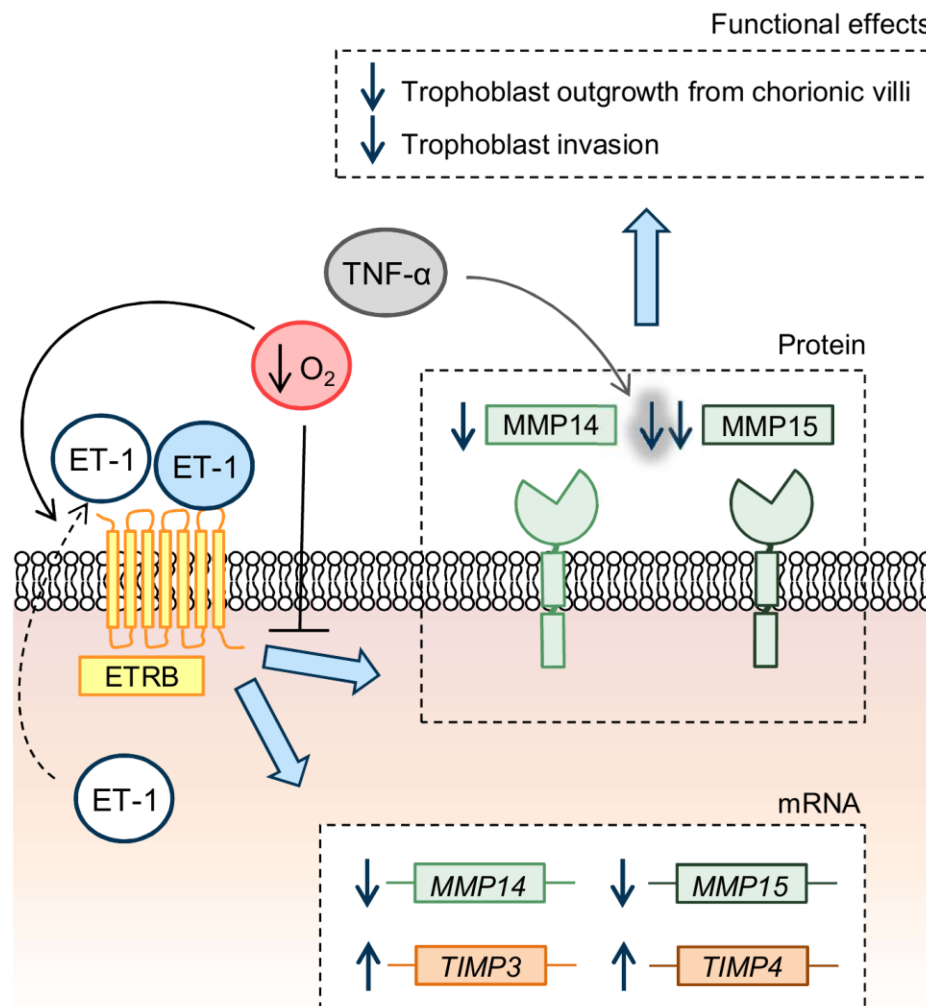

Figure 32. ET-1, TNF-α and O_2 interplay and its effect on MMP14 and MMP15 regulation in first trimester trophoblasts. Exogenous ET-1 (blue filling) upregulates TIMP3 and TIMP4 expression and downregulates MMP14 and MMP15 mRNA and protein levels via ETRB. ET-1-mediated MMP15 downregulation is enhanced by TNF-α. Low oxygen tension triggers a feedback mechanism upregulating endogenous ET-1 levels (white filling) above its physiological threshold, abolishing ET-1 mediated MMP downregulation. Adapted from (75).

5.3. Obesity alters placental MMP14 and MMP15 already during the first trimester of pregnancy

Obesity is considered a risk factor for the development of other pregnancy complications such as miscarriage and PE (117). However, the impact of obesity in human first trimester placental function remains poorly characterized. In this study, we analyzed the role of obesity in the regulation of MMP14 and MMP15 placental levels during the first trimester of pregnancy.

No differences were found in MMP14 expression, whereas MMP14 protein levels were upregulated in obese placental tissue. These results are in concordance with previous literature showing that leptin increases trophoblast invasion via upregulation of MMP14 in HTR-8/SVneo cells, i.e. a cell line that is used as first trimester trophoblast model (118). Moreover, an upregulation of MMP14 levels has been also described in first trimester placental tissue from type-1 diabetes (T1D) patients (64). T1D like obesity is characterized by a pro-inflammatory environment (119), which might underpin the observed changes in MMP14 levels. However, this downregulation cannot be solely attributed to trophoblasts, since MMP14 is also found in other placental cell types such as endothelial cells (66).

MMP15 expression was unaltered by obesity if analyzed independent of gestational age. Interestingly, MMP15 protein levels decreased throughout the first trimester of pregnancy only within the obese cohort, but not in placental tissue from lean women. This suggests that mid first trimester placenta might be more susceptible to the changes induced by obesity. Firmer conclusions about MMP15 and obesity in late first trimester placenta could not be drawn due to the lack of material. Nevertheless, our data shows the importance to consider gestational age as a continuous variable. Studying a single time point in early pregnancy might oversimplify the biological complexity of the first trimester of pregnancy.

Since our previous results showed MMP15 localization only in EVT, we can argue that obesity might entitle functional consequences for trophoblast invasion. However, further studies characterizing MMP15 and trophoblast invasion in isolated cells from lean and obese placentas are necessary.

Maternal obesity has been shown to differentially impact placental physiology according to fetal sex (120). Therefore, we also analyzed whether this is the case for MMP regulation in the first trimester of pregnancy. Only pro-MMP14 was differentially regulated by obesity in placental tissue from male and female fetuses, whereas no correlation between MMP15, obesity and fetal sex was found. However, firmer conclusions cannot be drawn due to the small sample size after stratifying the data.

Altogether, our data suggest that obesity plays a role in MMP regulation in the first trimester of pregnancy. Other factors such as gestational age and fetal sex might affect the impact of obesity in first trimester placental physiology and need to be accounted for. More studies addressing the functional consequences of obesity and its pro-inflammatory environment in the first trimester of pregnancy are guaranteed.

5.4. Summary, importance of the major findings and limitations

In the present study I have shown that MMP15 is exclusively localized to EVT and that it is involved in trophoblast invasion. Like MMP14, MMP15 is downregulated by ET-1 via ETRB, which in turn hinders trophoblast invasion. Other characteristic PE stimuli, such as low oxygen tension, abolished the ET-1 effect on both MMPs, whereas TNF-α only fine-tunes ET-1 mediated MMP15 downregulation. MMP14 and MMP15 are also differentially correlated with maternal obesity during the first trimester of pregnancy, with MMP14 protein levels being up-regulated and MMP15 protein levels decreasing toward the end of the first trimester only within the obese cohort.

My results expand the current knowledge of the protease repertoire involved in trophoblast invasion, and contribute to the understanding of the molecular mechanisms regulating trophoblast invasion in pro-inflammatory conditions, such as PE and maternal obesity. I showed that ET-1 mediated MMP14 and MMP15 downregulation occurs via ETRB. Thus, modulation of the ET/ETR system might open new therapeutic strategies to tackle PE. My finding that maternal obesity modulates MMP14 and MMP15 suggests that this condition

might dysregulate trophoblast invasion in the first trimester, prompting further complications later in pregnancy. This warrants further studies.

The main limitation of the present study is the lack of an in-depth analysis of the down-stream signaling pathways involved in MMP14 and MMP15 regulation. First trimester placental tissue is difficult to avail and the number of primary trophoblasts that can be isolated from this tissue is limited. Other pro-inflammatory factors altered in PE and maternal obesity that might regulate MMP levels and trophoblast invasion have not been analyzed here.

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