Thesis

PLACENTAL AUTOPHAGY AND INFLAMMATION IN HUMAN PREGNANCY

submitted by

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Graz, 24.07.2025

Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm, that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

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Graz, 24.07.2025 Tamara Bauer-Schnaitl m.p.

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Zusammenfassung in Deutsch

Die Plazenta ist ein lebenswichtiges Organ, das während der Schwangerschaft eine physiologische Verbindung zwischen Mutter und Fötus herstellt. Eine normale Funktion der Plazenta ist für die Entwicklung des Fötus, die Anpassung des Körpers der Mutter und den Erhalt einer gesunden Schwangerschaft unerlässlich. Autophagie und Entzündungen spielen eine Schlüsselrolle in den molekularen Mechanismen, die zur Gesundheit bzw. Erkrankung der Plazenta beitragen, und somit weitreichende Auswirkungen auf den Fortpflanzungserfolg haben. In dieser Arbeit werden der biologische Hintergrund, die Bedeutung und die Wechselwirkungen dieser beiden Prozesse untersucht.

Zunächst werden die Struktur und die Funktionen der menschlichen Plazenta erläutert, wie ihre Rolle beim Nährstoffaustausch, der Hormonproduktion, der Immunmodulation und der Ausscheidung von Abfallstoffen. Das Verständnis ihres Aufbaus ist für die Untersuchung der molekularen Mechanismen, die ihre Funktion beeinflussen, von entscheidender Bedeutung.

Die Autophagie ist ein Abbauprozess auf zellulärer Ebene. Die Autophagie im Plazentagewebe ist unter anderem für die zelluläre Homöostase, die Differenzierung der Trophoblasten und die Anpassung an hypoxischen und metabolischen Stress essenziell. Eine Dysregulation der Autophagie wurde auch mit Funktionsstörungen und Erkrankungen der Plazenta wie Präeklampsie und Intrauteriner Wachstumsrestriktion (IUGR) in Verbindung gebracht. In diesem Zusammenhang werden Entzündungsprozesse in der Plazenta beschrieben. Während lokale, kontrollierte Entzündungen ein normaler Teil der Plazentaentwicklung, vor allem während der Einnistung und der Wehen, sind, können unkontrollierte oder chronische Entzündungen schädlich sein. Die Plazenta muss die mütterliche Immuntoleranz mit schützenden Immunreaktionen ausgleichen. Dieser Prozess wird durch Zytokine, Immunzellen und Trophoblastzellen vermittelt. Ein Ungleichgewicht in diesem System kann die Plazentafunktion stören und zu unerwünschten Schwangerschaftskomplikationen führen.

In den Resultaten werden die zentralen Ergebnisse vorgestellt, in denen die Wechselwirkungen zwischen Autophagie und Entzündungsprozesse in der Plazenta sowie deren Auswirkungen auf Schwangerschaftskomplikationen hervorgehoben werden. Die Ergebnisse zeigen, dass Autophagie Entzündungsreaktionen beeinflussen kann. Umgekehrt kann eine übermäßige Entzündung Autophagieprozesse beeinflussen, was zu einer

Dysfunktion der Trophoblasten führt. Diese bidirektionale Beziehung ist im Zusammenhang mit Schwangerschaftskomplikationen von großer Bedeutung.

Zusammenfassend lässt sich sagen, dass eine koordinierte Autophagie und Immunantwort für eine gesunde Plazentafunktion unerlässlich sind. Störungen dieser Prozesse verursachen schwerwiegende geburtshilfliche Komplikationen. Zukünftige Forschungsergebnisse in diesen Bereichen können potenzielle Ansatzpunkte für zukünftige diagnostische und therapeutische Ansätze bieten.

Abstract in English

The placenta is a vital organ that establishes a physiological connection between the mother and fetus throughout gestation. Its proper function is essential for fetal development, maternal adaptation, and maintaining a healthy pregnancy. Autophagy and inflammation play key roles in the molecular mechanisms that contribute to placental health and disease, which has wide-reaching implications for reproductive success. This thesis explores the biological background, significance, and interaction of these two processes in human pregnancy. It highlights their roles in normal physiology and their contribution to pregnancy-related complications.

The text discusses the structure and function of the human placenta, emphasizing its roles in nutrient exchange, hormone production, immune modulation, and waste elimination. Understanding its architecture is essential for investigating how molecular mechanisms affect placental performance.

Autophagy is a cellular degradation process that is essential for adapting to stress and removing damaged organelles. Placental autophagy is shown to be essential for cellular homeostasis, trophoblast differentiation, and adaptation to hypoxic and metabolic stress. Defective autophagy has also been linked to placental dysfunction and disorders such as preeclampsia and intrauterine growth restriction (IUGR).

Along these lines, the inflammatory landscape of the placenta is discussed. While localized, controlled inflammation is a normal part of implantation and labor, uncontrolled or chronic inflammation can cause harm. The placenta must balance maternal immune tolerance with protective immune responses, a process mediated by cytokines, immune cells, and trophoblast signaling. An imbalance can disrupt placental function and contribute to adverse pregnancy outcomes.

The central findings are presented in the *Results* section, which highlights the interplay between autophagy and inflammation in the placenta and their impact on pregnancy-related complications. The results demonstrate that autophagy can modulate inflammatory responses. Conversely, excessive inflammation can impair autophagic flux, resulting in trophoblast dysfunction. This bidirectional relationship is particularly significant in the context of pregnancy complications.

In conclusion, coordinated autophagy and immune responses are essential for healthy placental function. Disturbances of these processes may underlie major obstetric disorders. Further research offers potential targets for future diagnostic and therapeutic approaches.

Details of previous publications

As a third author together with Prokesch et al.:

- Placental DAPK1 and autophagy marker LC3B-II are dysregulated by TNF-α in a gestational age dependent manner (1)

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Abbreviations

- *Atg1…Atg1 gene*
- Atg1…Atg1 protein
- *BECN1*…Beclin-1 gene
- BECN1…Beclin-1 protein
- CMA…chaperone-mediated autophagy
- CTB…cytotrophoblast
- DAPK1…Death-associated protein kinase 1
- DCs…dendritic cells
- DIC…disseminated intravascular coagulation
- DNA…desoxyribonuclein acid
- DRAM…DNA-damage regulated autophagy modulator
- enEVT…endovascular extravillous trophoblast
- ER…endoplasmatic reticulum
- EVT…extravillous trophoblast
- FIRS…fetal inflammatory response syndrom
- Gcn2… general control nonderepressible 2
- GDM…gestational diabetes mellitus
- GFP…green fluorescent protein
- GM-CSF…granulocyte-macrophage colony-stimulating factor
- GRO-α…growth regulated protein alpha
- hCG…human chorionic gonadotropin
- HDP…hypertensive disorders during pregnancy
- HIF1α…hypoxia-inducible factor-1α
- HLA…human leukocyte antigens
- HPL…human placental lactogen
- iEVT…intravascular (interstitial) extravillous trophoblast
- i.e. …id est
- IFN…Interferon
- IL…Interleukin
- ISSHP…International Society for the Study of Hypertension in Pregnancy
- IUGR…Intrauterine growth restriction

- KIRs…Killer-cell immunoglobulin-like receptors
- LC3B-II = MAP1LC3B…Microtubule-associated protein 1 light chain 3 beta
- MCP-1…monocyte chemotactic protein-1
- MHC…major histocompatibility complex
- monocyte-like THP-1 cells
- mRNA…mitochondrial ribonuclein acid
- mTOR…mechanistic target of rapamycin
- NF-κB…Nuclear factor kappa B
- NK..natural killer cells
- NLRs…NOD-like receptors
- OTR…oxytocin receptor
- p53…tumor protein p53
- PAS…pre-autophagosomal structure
- RB1CC1/FIP200…RB1-inducibel coiled-coil 1
- RNA…ribonuclein acid
- sFLT-1…soluble fms-like tyrosine kinase 1
- SNARE…soluble N-ethylmaleimide-sensitive-factor
- STB…syncytiotrophoblast
- t-SNARE…target synaptosome-associated protein receptor
- TEM…transmission electron microscopy
- Th cells…T helper cells
- TLRs…Toll-like receptors
- TNF …Tumor necrosis factor
- Tor…target of rapamycin
- Ubl…ubiquitin-like
- *ULK1*…ULK1 gene
- ULK1…ULK1 protein
- uNK…uterine natural killer cells
- v-SNARE…vacuole/vesicle synaptosome-associated protein receptor

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1. INTRODUCTION

1.1. Background and significance of placental autophagy and inflammation in reproduction

Autophagy is a fundamental cellular process, ensuring cellular homeostasis and survival during stressful conditions. In reproduction, autophagy plays a crucial role in maintaining gamete quality, supporting the development of the embryo, and regulating placental function.

Inflammation also plays an integral part in various reproductive processes, particularly during embryo implantation, placentation, and labor. The well-balanced interplay between autophagy and inflammatory processes ensures normal reproductive functions and successful pregnancy outcomes. A deeper understanding of these interactions could provide possible therapeutic strategies for reproductive health challenges.

1.2. Importance of placental function in human pregnancy

Although the placenta has a transient existence, it is one of the most important organs because without it the development of the fetus, and consequently the human being, would not be possible. It is a vital organ not only supporting the development of the fetus but also maintaining maternal well-being and health throughout pregnancy. Next to its principal function of supplying the fetus with oxygen and nutrients, its hormones also have profound effects on the mother's metabolism. Nonetheless, of all human organs, the placenta is the most poorly understood, which begs the question: why? There is no doubt that a human being cannot exist without a brain. If you type the keyword "brain" into PubMed, you get 2,478,839 results, whereas the keyword "placenta" yields 121,301 results. Even the keyword "spleen" has almost twice as many results, 222,257 to be exact. It is also common knowledge that you can live without a spleen, but without a placenta, human life is impossible. As is the case almost everywhere in today's society, the gender gap in medical research leads to real-life disadvantages for female patients in addition to a general misogyny (2). This indicates the significance of ongoing research on placental development and placentation, as it involves a series of highly coordinated processes that ensure the survival and growth of the fetus as well as the health and survival of the mother. Pathologies in placental function are

associated with severe pregnancy complications such as preeclampsia, intrauterine growth restriction (IUGR), or stillbirth, highlighting the importance of understanding these mechanisms for advancing perinatal medicine and emphasizing this significance to ensure optimal pregnancy outcomes.

1.3. Thesis aims

The main question I addressed is how inflammation and placental autophagy affect the development of the human placenta and the embryo, especially during early pregnancy. I also investigated their impact on pregnancy pathologies. Inflammation and autophagy are fundamental biological processes. They regulate cellular homeostasis, immune responses, and tissue integrity. These mechanisms play a critical role in the development of the fetus, maternal-fetal immune tolerance, and adaptation to environmental stressors in the human placenta.

Placental development and placentation involve a series of highly coordinated processes that ensure fetal survival and growth, such as trophoblast invasion, spiral artery remodeling or syncytiotrophoblast formation, as discussed in more detail in the third chapter of my thesis. The disruption of any one of these steps can lead to complications during pregnancy or miscarriage, highlighting the importance of understanding these mechanisms to advance perinatal medicine. The aim of my thesis was to explore and review the current scientific literature and data and its concepts related to placental autophagy and inflammation in human pregnancy. I also aimed to explore the interplay between inflammation and placental autophagy with a focus on their impact on placental function and pregnancy outcomes and to summarize the current understanding.

2. MATERIAL AND METHODOLOGY

Material

The core of this thesis focuses on the systematic research, analysis and comparison of the available primary scientific literature regarding the question of how inflammation and autophagy processes influence the human placental tissue and the course of pregnancy. By way of introduction, I summarized the fundamentals of placental function and important cellular processes of the placenta, including basic molecular mechanisms of autophagy and inflammation from medical textbooks, journals and e-books, which inform the first few

chapters. In total eight books were selected and included for the basic knowledge in this thesis. An overview of the selected literature is provided in Table 1.

What follows is a review of the state of the art of current research on placental morphology and development. Several articles served as sources, the majority of which I have collected from the meta-database PubMed because of its extensive coverage of biomedical literature and clinical research.

Table 1 shows the bibliographic details of the eight selected books forming the basis of the literature review

Authors	**Title**	**Journal**	**Year**	**Volume (Issue)**	**Pages**
Benirschke K, Burton GJ, Baergen RN	Pathology of the Human Placenta	Springer	2012	6th ed.	941
Moore K, Persaud TVN, Torchia MG, Viebahn C	Embryologie: Entwicklungsstadien - Frühentwicklung - Organogenese - Klinik	Urban & Fischer Verlag/ Elsevier GmbH	2013	6th ed.	601
Ovalle WK, Nahirney PC	Netter's Essential Histology	Elsevier Ltd	2013	2nd ed.	517
Hartmann M, Pabst MA, Dohr G	Zytologie, Histologie und Mikroskopische Anatomie: Licht- und elektronenmikroskopischer Bildatlas	Facultas	2010	5th ed.	144
Junqueira LCU, Carneiro J, Kelley RO, Junqueira LCU	Histologie: mit 14 Tabellen	Springer	2002	5th ed.	482
Boyd JD, Hamilton WJ	The Human Placenta	Heffer	1970		404
Sadler TW	Taschenlehrbuch Embryologie	Thieme	2014	12th ed.	536
Qin ZH	Autophagy: Biology and Diseases: Basic Science	Springer	2020	1st ed.	742

Methodology:

A systematic search strategy was used to ensure the comprehensive review of the existing literature. My focus was to identify relevant peer-reviewed articles, clinical trials and reviews that shed light on the molecular mechanisms, possible clinical implications and

pathological aspects of this interaction between placental autophagy and inflammation in human pregnancy. Special emphasis was put on recent publications until April 30th, 2025. They were retrieved from the electronic database PubMed, primarily by using following keywords:

- Autophagy (80,830 results) → 16,537 reviews
- Autophagy marker (8,674 results) → 408 reviews
- Placental autophagy (455 results) → 76 reviews
- Autophagy in pregnancy (914 results) → 123 reviews
- Autophagy and inflammation (9,865 results) → 3,086 reviews
- Autophagy and inflammation and placenta (55 results) → 15 reviews
- Autophagy and inflammation and immunity (2,657 results) → 1,126 reviews
- Autophagy and cytokines (6,921 results) → 1,161 reviews
- Placental inflammation (6,118 results) → 1,170 reviews
- Placental immune tolerance (1,646 results) → 614 reviews
- Autophagy and pregnancy complications (463 results) → 73 reviews
- Autophagy and placenta (423 results) → 66 reviews
- Autophagy and placentation (455 results) → 76 reviews
- Autophagy and trophoblast function (240 results) → 37 reviews
- Autophagy and preeclampsia (153 results) → 42 reviews
- Inflammation and preeclampsia (2,647 results) → 624 reviews
- Autophagy and IUGR (64 results) → 10 reviews
- Inflammation and IUGR (603 results) → 121 reviews

In total, using the defined keywords, 25,375 reviews were found. As shown in figure 1, this resulted in 55 selected articles and 8 selected books that were included in this thesis.

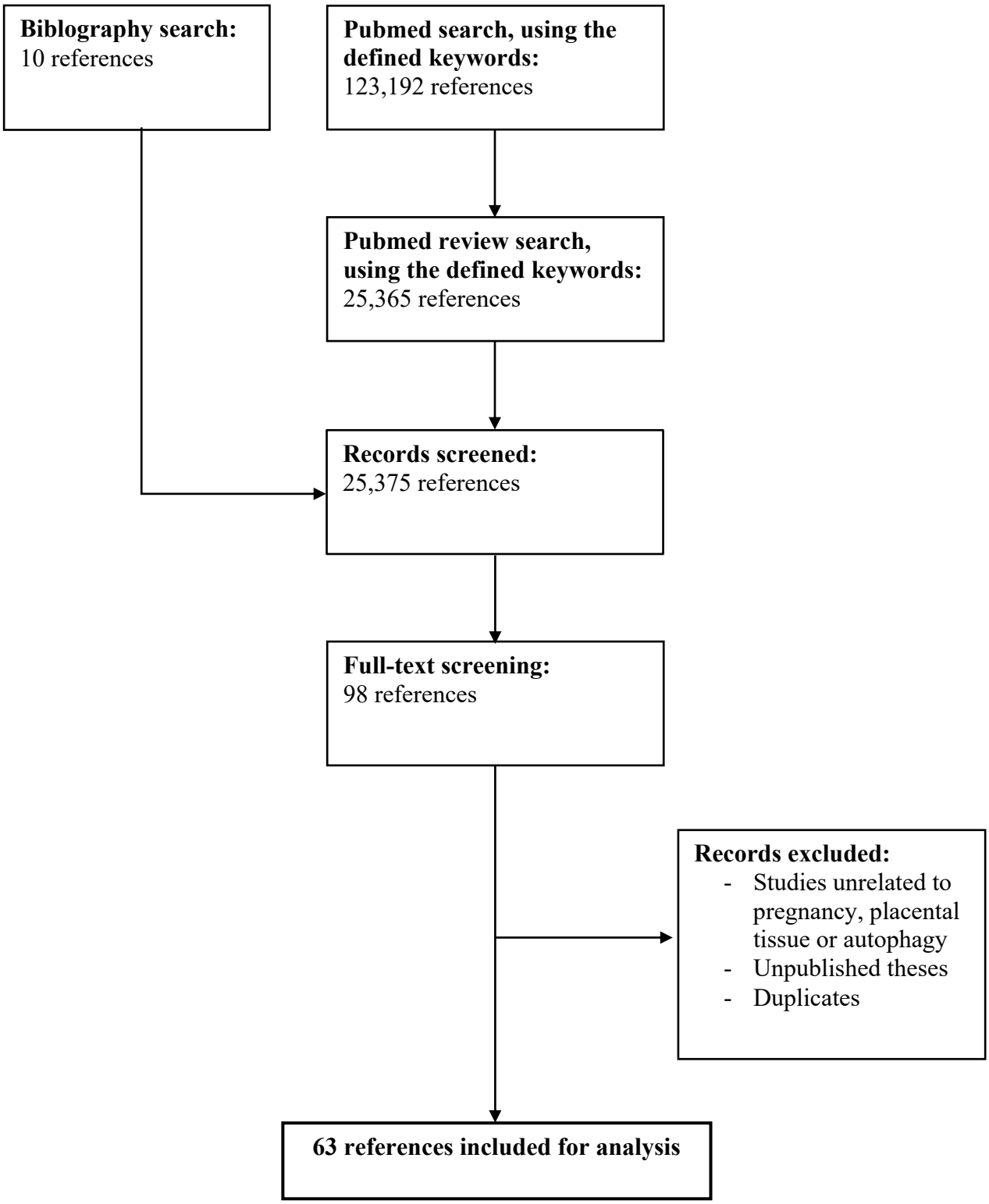

Figure 1 Flow-chart of the literature review

Inclusion and exclusion criteria

Articles were included if they met the following criteria:

- Focus on the role of autophagy in the placenta during human pregnancy
- Investigation of the relationship between autophagy and inflammation
- Research conducted on yeast, on mice or relevant in-vitro models of human pregnancy
- Research conducted with human subjects
- Peer-reviewed original articles from journals indexed in PubMed

Articles were excluded if they met the following criteria:

- Studies unrelated to pregnancy, placental tissue or autophagy
- Unpublished theses
- Duplicates
- Not English

Screening and selection process

The search results were imported into the Zotero reference management tool. Duplicate uploads were removed, and titles and abstracts were checked for relevance. Full-text articles were then assessed using the inclusion and exclusion criteria. Additional sources from various articles were used as necessary to describe the current state of the art. However, only sources mentioned or cited in previously selected articles matching the inclusion and exclusion criteria were followed up on. This structured, systematic approach ensured the inclusion of relevant studies. A PRISMA flowchart (Fig. 1) was generated to illustrate the selection process.

Finally, I included findings from a study that I published as a co-author, entitled "Placental DAPK1 and autophagy marker LC3B-II are dysregulated by TNF-α in a gestational age-dependent manner (1)." In this article, Prokesch et al. (2017) analyzed the potentially cytotoxic influence of the pro-inflammatory cytokines TNF- α and IL-6 on the gene expression profile of 84 key genes involved in the regulation and execution of autophagy processes in the early human placenta (1). These data and findings were compared

with the currently available data in a separate subsection, and the previously reviewed and analyzed literature was placed in the context of the published paper.

Notes on the writing process

Writing my thesis involved several instructive stages of research, critical analysis and the synthesis of complex biomedical literature to better understand the individual concepts of autophagy and inflammation in the placental context. Throughout this process, emphasis was placed on maintaining scientific accuracy while ensuring readability and logical flow. Key challenges included managing a large amount of literature, assessing the methodological quality of the studies, and presenting molecular mechanisms in a clear and concise manner. I would like to mention the following points to ensure good readability:

- Symbols for genes are in italics (*Atg1*), symbols for proteins are not (Atg1). Human genes are capitalized (*ULK1*), as are their proteins (ULK1). For example, Atg1 is not the same as ULK1; they are related proteins that both play a role in autophagy. Atg1 is a protein kinase found in yeast; the human orthologs are ULK1 and ULK2.
- A combination of landmark studies and newer findings has been included in this thesis to provide both historical context and current relevance.
- Specific biomedical terms, such as *maternal-fetal, placenta* and *autophagy,* are consistently defined and used throughout the text to avoid ambiguity.

3. PLACENTAL HOMEOSTASIS, AUTOPHAGY, AND INFLAMMATION

3.1. The human placenta

Definition and function

The human placenta is a complex organ distinct from other organs, in that it takes on almost all functions of most fetal organs during pregnancy (3). As a temporary, maternal-fetal or fetal-maternal organ that grows throughout pregnancy, it is always associated with the uterine endometrium joining the mother with the developing embryo or fetus. From the beginning, it is essential to all physiological interactions between mother and embryo, as it represents the location of the maternal-fetal contact zone (4). It is involved in many important tasks, such as nutrition, metabolism, thermo-regulation, waste elimination, immunity, and certain endocrine functions as well (5).

To approach a definition of this unique organ, it is important to delineate the correct term. Accurate terminology is crucial when defining the placenta. Referring to either the "fetal placenta" or the "maternal placenta" to describe different parts of the placenta may cause confusion and lead to misinterpretation, as it is ultimately impossible to separate the maternal from the fetal components of the human placenta. For accuracy, I advocate using the term "maternal-fetal," as it reflects the conventional order found in scientific literature and emphasizes the maternal environment as the primary physiological and immunological context of fetal development. This terminology is widely accepted in the field of obstetrics, immunology, and developmental biology, ensuring clarity and consistency. (3)
To ensure brevity, however, the term "placenta" will be used from this point forward.

Structure

A full-term placenta is a disc-shaped organ that averages 15 to 25 cm in diameter and 2 to 3 cm in thickness. It weighs between 400 and 600 grams (5). There are two main components that make up the placenta: the fetal component and the maternal one, both enclosing the intervillous space. The chorionic plate with its branching chorionic villi and its two chorioamniotic membranes (chorion and amnion) represents the fetal section of the placenta. The decidua basalis represents the maternal, uterine surface of this organ (6). These two components cannot be separated, neither in structure nor in function. For example, the basal

plate (maternal section) consists not only of maternal cells but a mixture of endometrium-derived (maternal) cells and trophoblastic (fetal) cells (3).

Important steps in placental development and placentation

Trophoblastic differentiation begins after the successful implantation of the embryo in the endometrium of the uterus. This marks the beginning of the placental development and the formation and remodeling of the villi. (7)

The process of decidualization, or the transformation and remodeling of the endometrium, happens in response to the implantation of the blastocyst. The endometrium of the uterus contains spindle-shaped endometrial stromal fibroblasts. During decidualization these cells transform into secretory decidual cells, and the endometrium is then referred to as the decidua. The decidua can be divided into three parts: the decidua basalis, the decidua capsularis and the decidua parietalis. The decidua basalis is located beneath the implantation site and is the most important region for placentation. In this thesis, the name “decidua” principally refers to this region. The decidua capsularis grows over the superficial pole of the implanted blastocyst. The remaining part of the uterine wall is made by the decidua parietalis. At around 12 to 14 weeks of pregnancy, the decidua capsularis and parietalis fuse, obliterating the uterine lumen. Decidualization is influenced by maternal hormones, such as progesterone and estrogen. It occurs to a limited extent during the secretory phase of the menstrual cycle in anticipation of implantation. The transformation of the endometrial fibroblasts into epitheloid decidual cells is irreversible. Therefore, if there is no pregnancy, it must be shed during menstruation. However, if pregnancy occurs, the decidua provides an immunologically tolerant environment that allows the trophoblast to invade in a regulated fashion. (3)

During the implantation of the blastocyst in the decidua basalis of the endometrium, the embryo consists of three main components: the embryoblast (the inner cell mass), the blastocoel or blastocyst cavity, and the trophoblast. The embryoblast and the blastocoel are both surrounded by the trophoblast, a specialized cell line which develops into a large part of the placenta (8). The trophoblast proliferates and differentiates itself into two layers: the cytotrophoblast (CTB), or Langhans cells, which consists of an irregular layer of mononuclear cells forming the inner layer of the trophoblast and the syncytiotrophoblast (STB), which assembles the outer layer by syncytial fusion of mononucleated cells. The CTB provides the stem cells that undergo proliferation and differentiation before finally

fusing with the STB. This process is necessary for the growth and regeneration of the STB (3). In the fusion of CTB, a real syncytium is formed. A syncytium is a multinucleated barrier or layer without intercellular boundaries. The CTB undergoes rapid division to permit the expansion of the STB (8). The STB itself is in a terminally differentiated post-mitotic state. The absence of mitotic figures within the STB may be an adaptation that reduces the risk of malignant change at the maternal-fetal interface. Due to its position, the STB is involved in many essential placental functions, such as active transport, the synthesis of peptide and steroid hormones, metabolic regulation, protection against xenobiotics, and immunological defense (3,9). To carry out its functions, the STB has well advanced and well-equipped organelles, such as endoplasmatic reticuli, Golgi complexes, a great deal of mitochondria, and fat droplets containing cholesterol. Therefore, it secretes hormones essential for pregnancy, such as progesterone and estrogen, as well as human chorionic gonadotropin (hCG) and human placental lactogen (HPL). It also possesses many irregularly formed microvilli and pinocytic vesicles that intensify its ability to exchange substances. After the implantation of the blastocyst in the decidua basalis of the endometrium, the STB actively invades the wall of the uterus (8). This early STB appears to be the only tissue capable of penetrating the uterine epithelium (10). Huppertz et al. have shown through *in vitro* studies using placental cells and tissues that, contrary to STB, mononucleated cytotrophoblasts are unable to invade an intact monolayered epithelium. They used villous explants from the first trimester to confront them with either term amnion or decidua, also from the first trimester. Despite their structural similarity to a simple monolayered epithelium like the uterine epithelium, the outgrowing mononucleated extravillous trophoblasts failed to invade the epithelial layer (11,12). The STB can penetrate the decidua, forming an interface with the maternal blood stream and establishing initial maternal-fetal interactions. This gives the developing embryo access to nutrition. Irregular extra-cytoplasmatic spaces appear in the STB due to proteolytic activity. These spaces get bigger and come to form lacunae (8). These lacunae or intervillous space between the decidua basalis and the chorionic plate is filled with secretions of the endometrial gland and tissue fluid during the first trimester of human pregnancy (3). The STB grows bigger, so its destructive activity reaches the capillaries of the endometrium. The walls of the capillaries and venules rupture, resulting in the formation of a hemochorial placenta, as maternal blood flows into the newly formed lacunae and comes into direct contact with the fetal chorion, though no fluid is exchanged (8). Now, the basis of the uteroplacental circulation is formed and the maternal blood circulates through the

intervillous space surrounding the vascularized placental villi during the second and third trimester of pregnancy (3).

Following implantation, the formation of chorionic villi represents a critical step in the development of the placenta. Primary villi, consisting of cytotrophoblasts and surrounding STB, develop into secondary villi, which also contain mesoderm. Cells of the extraembryonic mesoderm differentiate into endothelial cells and form blood vessels, which are the basis for the capillary networks of the villi. After the emergence of fetal capillaries in the mesenchymal the tertiary or placental villi are formed on the 17^{th} to 22^{nd} day of embryo development. This creates the basis of the maternal-fetal exchange of substances by increasing the surface area and vascularization (7). The fetal villous capillaries soon connect to the vessels that develop in the chorionic plate and in the body stalk, which contains the umbilical cord vessels. These vessels then join the intraembryonic circulatory system, which subsequently establishes a connection between the embryo and the placenta (13).

Basic structure of the villous tree

The different types of villi have various structural and functional specializations. While they differ from one another, all villi share a fundamental structure: they are covered by the STB, which has a layer of CTB underneath it and a stromal core. In early gestation, the CTB forms a complete layer that disperses as gestation advances. However, the cells stay connected by elongated cell processes. The separation between the STB and the CTB from the stromal core of the villi is formed by the trophoblastic basement membrane. The stromal core of the villi consists of connective tissue cells, villous macrophages, connective tissue fibers, and ground substance. (3)

As previously stated, the STB can only expand through the fusion-based recruitment of underlying progenitor CTB cells. This process occurs throughout gestation; therefore, the nuclei will be of various ages. This produces a spectrum of contrasting appearances, ranging from euchromatin to dense heterochromatin condensations. The nuclei within the STB have an euchromatic appearance in early pregnancy when they are randomly dispersed and contain a prominent nucleolus. As gestation progresses, many nuclei of the STB show heavily condensed heterochromatin with an involuted nucleolus. These nuclei often cluster together to form syncytial knots, which may appear as gentle elevations on the villous surface. These knots are known as a sign of epithelial turnover and therefore trophoblast maturation and aging. The heterochromatic appearance of the nuclei within syncytial knots,

along with the absence of transcriptional activity, has led to the hypothesis that syncytial knots undergo apoptotic changes and are shed from the surface of the villi as a part of a regular 3-to-4-week cycle of the STB. An estimated 3 g per day is shed into the maternal circulation at term but also clumps of STB nuclei are transported into the maternal circulation in the early stages of pregnancy. There have been the suggestions that it may serve to induce immune tolerance, although there is the absence of expression of MHC class I or class II antigens. The activation of an apoptotic cascade may be necessary for CTB fusion. However, the completion of apoptosis is temporarily delayed by 3 to 4 weeks due to high levels of the anti-apoptotic Bcl-2 protein transferred from the CTB cytoplasm. Some of the morphological observations do not align with the apoptosis hypothesis, so it remains to be seen whether these nuclei are truly apoptotic. While apoptosis may not play a central role in STB biology under normal conditions, apoptotic processes are activated during pathological pregnancies. An increased number of apoptotic nuclei has been observed in the placentas of cases with IUGR, and an even greater increase has been observed in preeclampsia. Additionally, morphological evidence of autophagic vacuoles near syncytial knots suggests that autophagy may occur and that the constituents of the nuclei may be recycled within the STB. Syncytial knots are uncommon before 32 weeks of gestation and are most prevalent in post-mature placentas, with an increase in their frequency at 42 weeks. In term of their correlation with other placental pathologies, a minimal increase is seen in preeclampsia. Syncytial knots also seem to be more common in areas of reduced fetal perfusion. (3,9)

Placental barrier

Until the 25th week of gestation, the placental barrier consists of five layers derived from fetal tissue and separating the maternal from the fetal blood:

- Syncytiotrophoblast (STB)
- Cytotrophoblasts (CTB)
- Basal membrane
- Connective tissue in the villi
- Fetal capillary endothelium

Following the 25th week of pregnancy, histological modifications lead to the partial disappearance of CTB, resulting in an irregular, thinned layer of the STB. Thus, the placental barrier is thinned down to three layers: the STB, fetal connective tissue (also called the

interstitial space), and the fetal capillary endothelium. In certain regions, the STB is in direct contact with the fetal capillary endothelium, separated only by a shared basal membrane. Looking at the STB through an electron microscope reveals that its apical surface is densely covered by microvilli. This structural feature enhances the surface between the fetal and maternal circulatory system. Towards the end of the third trimester, fibrinous material forms on the surface of the villi as an expression of placental aging. This fibrinous material can be intensely stained with eosin and is likely to reduce transplacental transfer. (4)

Placental circulation

Establishing maternal circulation presents a major hemodynamic challenge to a hemochorial placenta. There is the maternal-fetal interface, with maternal blood directly surrounding the trophoblast surface. To facilitate effective transplacental exchange, the maternal and fetal placental circulations must be synchronized (7). An important step in establishing the maternal blood flow into and through the intervillous space of the placenta is the dissolution of the endovascular trophoblast plugs resulting in the onset of maternal blood flow including blood cells (12). More detail on the endovascular trophoblast cells can be found below. Once the maternal blood enters the intervillous space, it is temporarily outside the maternal circulation. The maternal blood enters the intervillous space in about 80 to 100 endometrial spiral arteries through the openings in the CTB (4). Before the maternal spiral artery remodeling takes place, the trophoblast taps into branches of the maternal uterine arteries, where the bloodstream has a higher pressure than in the fetal vascular system. Because of the higher pressure in the maternal bloodstream there is a risk of compressing fetal capillaries within the terminal villi, which could disrupt umbilical circulation and the formation of vasculosyncytial membranes. To prevent this from happening, the uterine arteries undergo dilatation during pregnancy to meet the metabolic demands of the fetal-placental unit. This process is facilitated by endocrine and local flow-dependent mechanisms. It is described as maternal spiral artery remodeling (7). So, this invasion of CTB leads to dilatation of the maternal spiral arteries. Consequently, the arteries with an initially small diameter and a high vascular resistance become vessels with a larger diameter and a reduced vascular resistance. This increases blood flow into the intervillous space, thereby enhancing the nutrient and oxygen content in the placenta. As a direct consequence, the supply of nutrients and oxygen to the embryo or fetus is adequate and optimized (13). The increase in placental oxygen pressure from 20 to 60 mmHg is an important stimulus for the differentiation of both villous

and extravillous trophoblasts (EVT). Furthermore, maternal blood flow into the intervillous space at the end of the first trimester establishes direct physical contact between the maternal immune cells and the placental STB (12).

The remodeling of the spiral arteries involves the loss of smooth muscle cells from its walls and their replacement by a fibrinoid material, conducted either through dedifferentiation or apoptosis. Although the exact molecular mechanisms remain unclear, what it is known is that there are two phases to this process. The initial stage is endocrinally mediated, which is followed by a second phase driven by EVT. Deficient spiral artery remodeling has been associated with obstetric disorders, for example preeclampsia. Malperfusion of the placenta caused by deficient spiral artery remodeling induces oxidative stress. This, in turn, triggers the release of pro-inflammatory cytokines and angiogenic regulators from the STB, leading to maternal endothelial activation and potential dysfunction and thus the preeclamptic syndrome. (7)

Trophoblast development and invasion

Following the attachment of the blastocyst to the uterine epithelium and thus, the initiation of the implantation, trophoblastic differentiation takes place. This involves the development of the multinucleated STB, which acts as an epithelial covering for the placental villi, as well as the development of an extravillous subset of trophoblast cells outside the placental villous tissues. These two types of trophoblast cells differ from each other in morphology, function, expression and profile. (12)

EVT are common during the first trimester of pregnancy but can also be found later on. They originate from the tips of the anchoring villi that attach the villous trees to the endometrium. After proliferating, some of the CTB penetrate through the STB around day 14 after conception (pc) and come into direct contact with the maternal uterine tissue. These cells migrate away from the placenta, either through the lumens of the spiral arteries or through the endometrial stroma. Consequently, they are no longer found within the placental villous tissues. That is why they are then referred to as “extravillous trophoblasts” or EVT. As soon as they enter the maternal uterine tissues, they encounter and interact with maternal immune cells, particularly uterine natural killer cells (uNK), which are part of the innate immune system. (7,12)

In section 3.3. more detailed description of the interactions between these cells can be found.

All EVT originate from the trophoblast cell columns located between the tips of the anchoring villi and the decidual tissues. Trophoblast proliferation in these column-shaped structures can only be found in the proximal part of the anchoring villi, which is close to the basement membrane. Following proliferation, the daughter cells lose contact with the basement membrane and exit the cell cycle. They are pushed forward in the cell column due to proliferation pressure from above. Once they reach the distal end of the column, they come into contact with maternal tissues, thus beginning to invade the uterus. The height of the cell columns changes during pregnancy. At the start, the cell columns are tall due to high proliferation pressure. However, by the end of the first trimester, this proliferative pressure appears to decrease. One possible explanation for this is the increased inflow of maternal blood into the placenta at this time, which is accompanied by an increase in placental oxygen pressure. By around 20 weeks after conception, the columns have shortened, and most of them have disappeared by the end of the pregnancy. An important characteristic of the EVT is that they possess either proliferative activity or invasive behavior. They do not show both characteristics simultaneously. This is a key difference between trophoblast invasion and malignant tumor cells. As they travel through the cell column, the EVT adopt a new phenotype by undergoing the first step of differentiation, which involves leaving the cell cycle and losing their proliferative activity. This occurs as soon as the cell loses contact with the basement membrane of the anchoring villus. Upon reaching the distal end of the cell columns, they undergo further differentiation to form various subtypes of extravillous trophoblasts and initiate active invasion of the uterine tissues. (3,12)

Huppertz et al. described three different morphological phenotypes of EVT: large EVT with a polygonal shape, small EVT with an elongated spindle shape and giant EVT with multiple nuclei (12). All these EVT subtypes have different functions, as morphology is always linked to function. The large EVT are the most frequently determined EVT, which used to be referred to as X-cells. The large polygonal EVT is the predominant subtype of the extravillous trophoblast at the time of delivery. Their relative number increases from 45% at the end of the first trimester to 69% at the mid-gestation stage to 89% at term. They can typically be found in the basal plate of a term placenta, as well as along the route of invasion into the inner third of the myometrium. Their main function appears to act as a glue fixing the placenta to the uterine wall. The small EVT are common in early pregnancy. Their relative number decreases from 55% at the end of the first trimester to 31% at the mid-gestation stage to 11% at term. They appear to be highly invasive, hence their main

occurrence in the first trimester. Conversely, large EVT invade neither as rapidly nor as deeply as small cells. Once the small EVT have reached their target location, they may undergo further differentiation into the larger subtype upon invading the myometrium. The third subtype are the giant EVT containing two or more irregularly shaped nuclei of varying sizes and they may contain more than ten nuclei. These giant non-invasive EVT stay at their sites until delivery. As they are mainly located at the border between the decidua and the myometrium, they are rarely found at the basal plate of a delivered placenta. It appears that this subtype originates from the fusion of mononucleated EVT throughout pregnancy. Interestingly there is no fusion between two mononucleated villous trophoblasts. As previously mentioned, small EVT can invade very deeply and rapidly. However, the potential depth of invasion that a single small EVT can achieve is limited once it fuses with another mononucleated EVT or with the large subtype. This development of the giant cells may serve to control trophoblast invasion. (3,12)

EVT can be further subclassified into interstitial trophoblast cells (iEVT) and endovascular trophoblast cells (enEVT). In early pregnancy, enEVT can invade maternal blood vessels and accumulate in their lumens, which creates a kind of "trophoblastic plug" structure that maintains a low-oxygen environment during embryonic and placental growth and development (14). This plugging of spiral arteries by enEVT, which keeps the oxygen partial pressure below 20mmHg prior to 10 weeks of gestation, may support the following key developmental processes, among others: reduction of oxidative stress; enhanced embryonic growth; sufficient nutrient supply via histiotrophic nutrition; and immune protection. Reducing oxidative stress with low oxygen levels protects the developing embryo from oxidative damage and teratogenic effects. Under hypoxic conditions, embryonic cells proliferate faster and more efficiently. Despite the low oxygen levels, the embryo is nourished by maternal plasma ultrafiltrate and uterine gland secretions. Immune protection is a direct consequence of the absence of maternal blood cells in the intervillous space, preventing exposure of the developing placenta and embryo to circulating maternal immune cells (12). Additionally, enEVT can invade and remodel the spiral arteries by replacing the arterial vascular endothelial cells to enhance placental blood flow and thereby promote fetal development, while iEVT can invade the myometrium of the uterus, anchoring the placenta to the uterine wall (14).

Huppertz et al. described three invasive pathways through which the trophoblast can invade uterine tissue, in addition to remaining in the decidual stroma (12). First, it can invade

deeply to reach the inner third of the myometrium; this was previously referred to as "intravascular EVT" or iEVT (for instance in the book *Autophagy: biology and diseases*, edited by Zheng-Hong Qin), although it is now called "interstitial trophoblast cells." This term better describes this subset of EVT and will be used from this point on. The second route is through the uterine spiral arteries. In this case, the term "endovascular trophoblast" or enEVT is only used for EVT that are inside the lumen of spiral arteries, as well as trophoblasts which are in the walls of these arteries, as described in the previous paragraph. There is another subset of EVT called "endoglandular trophoblast," which, as a third invasive pathway, reaches uterine glands through the interstitium of the decidua to be also found in the lumen of these glands replacing uterine epithelial cells. It remains unclear whether these two populations of endovascular and endoglandular trophoblasts differ from one another from the outset, or whether some of trophoblasts just change direction during their invasion of the uterine stroma. (12,14)

Further details on EVT are provided in chapters 3.2.2. and 3.3. for a comprehensive overview.

3.2. Autophagy in the placenta

3.2.1. Basics of autophagy

Definition

Autophagy is an important intracellular recycling process that maintains cellular homeostasis by removing damaged organelles, misfolded proteins, and pathogens. The word "autophagy" comes from the Attic Greek and means "eating of self" or "self-devouring"; "auto" means "self" and "phagy" means "eating." The name highlights the process's role in cellular self-digestion. This "self-eating" process provides the elimination of dispensable or dysfunctional cellular components or structures through a lysosome-dependent regulated mechanism (15). Eukaryotic cells use two main systems for intracellular protein degradation: the autophagy-lysosomal system and the ubiquitin-proteasome system. Proteasomal degradation recognizes ubiquinated, short-lived proteins, while the lysosomal-mediated pathway targets long-lived proteins through a complex process. During this process, cytosolic components, including damaged organelles, are delivered to lysosomes via autophagosomes, and extracellular materials are delivered via endocytosis (16).

Autophagy is an evolutionarily conserved, essential cellular pathway present in all eukaryotic cells and in multiple other cell types, from yeast to mammals (17,18). In mammals, this degradation of certain cellular components takes place in lysosomes. Conversely, in yeast and plants it happens in vacuoles (19).

It is induced in response to various stimuli, including extracellular stress such as nutrient limitation or amino acid starvation, hypoxia, growth factor withdrawal, overcrowding and high temperature or intracellular stress conditions such as accumulation of damaged and/or superfluous organelles and cytoplasmic components, endoplasmatic reticulum stress, mitochondrial damage and as a response to various immune stimuli. This is one of its primary roles in unicellular organisms like yeast. Next to its participation in the degradation of long-lived proteins and cytoplasmic organelles, autophagy also contributes to cellular homeostasis and biosynthesis, serving as a catabolic pathway in cells and cell death. Autophagy plays a crucial role in cellular survival by maintaining the cellular homeostasis, quality control, cell growth control, antiaging mechanisms, innate immunity, and cellular damage protection, especially in mammalian systems. It therefore plays a potential role not only in physiological but also in pathological processes resulting from the deregulation of autophagy. This includes certain diseases such as cancer, cardiomyopathy,

muscular diseases, and neurodegenerative disorders as well as developmental disorders and immunity malfunctions. (17,20,21)

Historical aspects and landmarks

The 2016 Nobel Prize in Physiology or Medicine was awarded to Yoshinori Ohsumi *"for his discoveries of mechanisms for autophagy"* (22). Since then, the medical community has seen an increased interest in autophagy. It has become the subject of extensive research in numerous scientific fields. Our understanding of the molecular process of autophagy is very young; it was discovered some sixty years ago. The Belgian cytologist and biochemist Christian René de Duve, who among other things, discovered lysosomes, which are organelles with a lytic function (hence the name), was awarded the Nobel Prize for Physiology or Medicine in 1974. He coined the terms "autophagy" and "autophagic vacuoles" and described his concept of autophagy as the degradation of cytoplasm and organelles by single or double-layered membrane vesicles known as "autophagosomes." All his discoveries led to autophagy becoming part of the lysosome-related nomenclature (15). Later on, it came to be referred to as a type-II-programmed cell death (17). However, since autophagy is known to promote cell survival and rarely causes cell death, this nomenclature is no longer accurate (23).

In the 1990s, Ohsumi et al. identified multiple autophagy-related genes in a yeast model, for example the first yeast autophagy gene, *Apg1*, now called *Atg1*. This gene encodes the Atg1 protein with its kinase activity playing a key role in nutrient-starvation-induced autophagy. To date, 42 *Atg* genes have been identified. The abbreviation *Atg* (AuTophaGy) was first used by Klionsky et al. in the early 2000s, who not only edited a new journal called *Autophagy* but also did a lot of research on the interactions between the proteins encoded by these genes, their functions in autophagy and the molecular mechanisms of autophagy. (18)

Types of autophagy

There are at least three known types of autophagy: macro-, micro-, and chaperone-mediated autophagy (CMA) which share the common fate of lysosomal degradation and its possible recycling of the degraded cargo. (18–20)

Macroautophagy involves the formation of a double-membrane vesicle that contains sequestered parts of the cytoplasm. It is subdivided into a selective and a non-selective type.

Having completed the fusion of the double-membrane vesicle with the lysosome, which is now called autophagosome, the inner vesicle or autophagic body will be delivered into the lumen of the degradative compartment. In selective macroautophagy various autophagic adaptor proteins or receptors identify specifically recognized cargo proteins, whereas in the non-selective type randomly chosen parts of the cytoplasm are sequestered into autophagosomes and transported for further lysosomal degradation (19,20). Macroautophagy can therefore function in a non-selective way or target specific organelles, ribosomes, and protein aggregates for degradation. For example, xenophagy describes the selective autophagy of intracellular pathogens. Its research suggests that autophagy may act as a defense mechanism against bacteria and viruses (14). However, some of the different specific mechanisms which regulate selective autophagy are not fully understood. As macroautophagy can use selective and non-selective mechanisms to degrade larger structures, so does microautophagy (24).

Microautophagy is characterized by an inward membrane deformation of the lysosomal limiting membranes. This invagination, protrusion, and/or septation of the lysosomal membrane generates intraluminal vesicles containing cytosolic cargo, which are eventually degraded and broken down in the lysosomes. (19,20)

Chaperone-mediated autophagy (CMA) works without membrane deformation. Instead, it involves the transportation of the cytosolic cargo proteins directly into lysosomes with the help of a special cytosolic molecular chaperone and a lysosomal membrane protein (19). Nevertheless, macroautophagy is the most studied type of autophagy and is usually referred to simply as autophagy, I will therefore use the term “autophagy” for this type hereafter.

During autophagy, which is acutely induced by starvation or stress, intact organelles like mitochondria and parts of the cytosol are enclosed in a double-membrane vesicle called autophagosome. The autolysosome is then formed from the completed autophagosome formation. This autophagosome formation fuses with an endosome or lysosome to degrade its contents using the cargo of lysosomal hydrolases to enable their degradation. The resulting amino acids and other by-products are transported back into the cytosol via membrane permeases and transporters for reuse to build macromolecules, for metabolism and for cellular recycling. This is the main mechanism for eukaryotic cells to degrade, remove and recycle larger harmful or unwanted substances, for example damaged mitochondria, excess peroxisomes, excess ribosomes, endoplasmatic reticulum, endosomes,

lipid droplets, and intracellular pathogens (14). Because of this basic mechanism, autophagy plays an important role in cellular homeostasis. It has been shown that there is a connection between the uncontrolled accumulation of such substances and either insufficient or excessive autophagy, which can compromise cell physiology as well as normal cellular function and therefore needs to be properly regulated. A dysregulation of autophagy is associated with physiological and various pathophysiological states, such as neurodegeneration, cancer, aging, infection and immune response, development, cardiovascular diseases and other diseases, especially when it is insufficient or excessive (25).

Molecular mechanisms and physiology

As genetic analysis of yeast is a comparably simple procedure, the molecular components of autophagy were initially mainly studied and recorded in yeast. This provided the basis for identifying the morphology, molecular machinery and biological functions of autophagy in higher eukaryotes, such as mammalian cells. Nowadays the mechanisms of autophagy are studied in a broad spectrum of organisms (26). Numerous autophagy genes identified in yeast have potential orthologs in higher eukaryotes. Several of these orthologs have already been demonstrated to be important for autophagy in organisms such as plants, flies, mammals, and others. There is also strong evidence that the inactivation of these orthologs in higher eukaryotes results not only in the conservation of autophagy function but also in potentially important roles for the autophagy machinery in many aspects of development, including normal reproductive growth, stress-induced adaptations, ageing, cell death and cell growth control (20).

To keep the scope of this work within reasonable limits, the following summary of the molecular processes of autophagy is mainly based on work on yeast autophagy proteins. With this background knowledge, a more detailed discussion of autophagy, particularly during differentiation and development in higher eukaryotes, is possible. This may lead to a better understanding of one of the core subjects of my work, placental autophagy.

Autophagy and its related processes can be broken down into multiple steps. The framework of breaking down the process of autophagy into different steps resulted from the analysis of the yeast *Atg* mutants, as shown in fig. 2 (17,27).

These include (17):

I. Induction
II. Cargo selection and packaging
III. Vesicle nucleation
IV. Vesicle expansion and completion
V. Retrieval
VI. Vesicle targeting, docking, and fusion
VII. Breakdown of the intralumenal vesicle and its cargo and its recycling

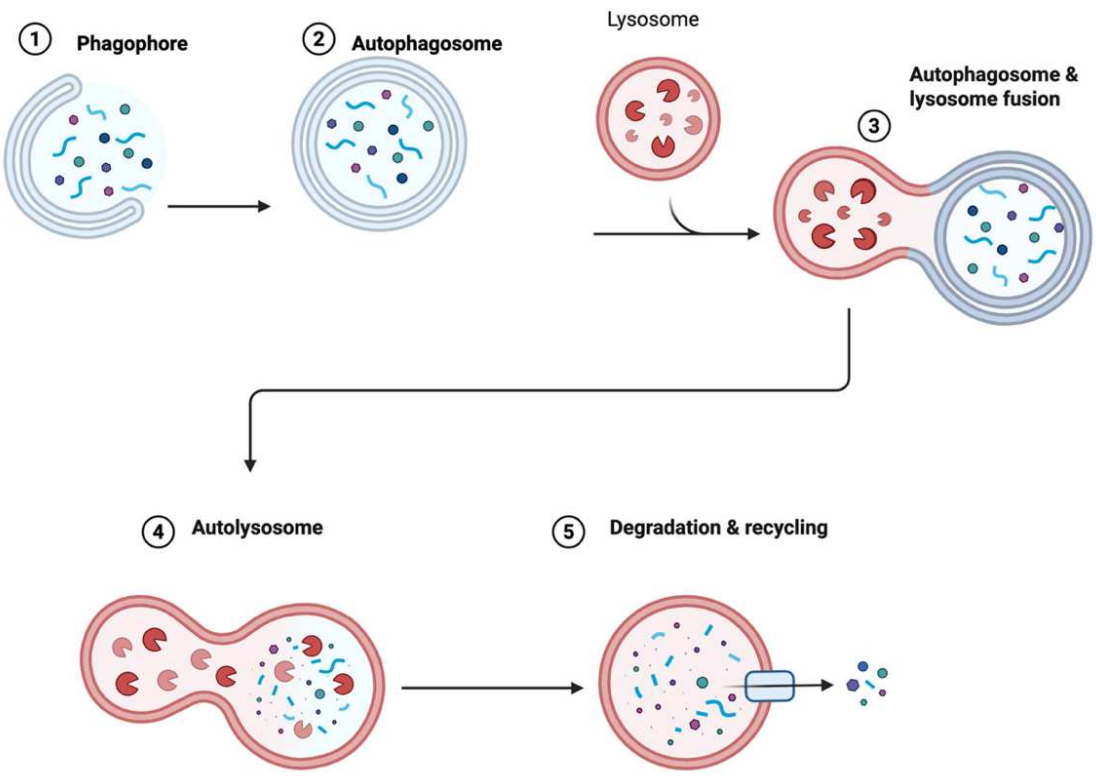

Figure 2: Main steps of the autophagic process; created in BioRender. Bauer-Schnaitl, Tamara (2025) https://BioRender.com/rmgo9a4 (27)

Induction

This Atg1 complex binds at the pre-autophagosomal structure (PAS) before the initiation of autophagy, thus forming the phagophore, i.e., the isolation membrane. Proteins of the Atg1 complex in yeast include Atg1-Atg13-Atg17-Atg29-Atg31. In yeast, the PAS is probably assembled *de novo*. In mammalian cells, this Atg1 complex consists of Atg1 homologs ULK1 or ULK2, the mammalian homolog of Atg13 ATG13, and RB1-inducible coiled-coil

1 (RB1CC1/FIP200), which in combination with the protein ATG101 trigger the formation of the autophagosome. However, the origin of the autophagosomal membrane in mammals is still controversial. (28)

The protein kinase Tor (for "target of rapamycin") is one of the key components for the initiation of autophagy. As a negative regulator it normally inhibits autophagy under nutrient-rich or basal conditions, by acting in two ways. First, the hyperphosphorylation of the autophagy protein Atg13 reduces the affinity of Atg13 for the Atg1 kinase, with which it interacts. Atg1 is a serine-threonine kinase. Their reduced interaction could inhibit autophagy (29). It is also unclear whether Tor is directly involved in the phosphorylation of Atg13 or needs other Atg proteins to act on. Furthermore, when Tor is inhibited by starvation or treatment with rapamycin, it can induce partial dephosphorylation of Atg13 allowing autophagic induction. The main type of starvation which has the most significant impact on Tor is nitrogen starvation. Another starvation-induced kinase is Gcn2, which also plays a role in autophagy induction. However, other stress factors, such as carbon starvation, hypoxia, and oxidative stress can also trigger autophagic induction. The regulatory elements responsible for controlling the induction of autophagy under these various stress conditions remain unidentified. Second, Tor controls the phosphorylation of several effectors by acting in a signal transduction cascade, which is responsible for the regulation of both the transcription and the translation of certain proteins. Some of these proteins are necessary for the autophagy process (17,20).

Cargo selection and packaging

Although autophagy has been viewed as a non-selective process involving randomly chosen cytoplasmic material to be indiscriminately sequestered into the cytosolic autophagosome, certain forms of autophagy are very specific in selecting its special cargo material. In pexophagy, organelles such as peroxisomes, but not other organelles, are targeted (17). In this selective form of autophagy the growing phagophore membrane can specifically target and interact with chosen organelles and protein structures (24). Not only peroxisomes, but several other organelles have been identified to represent this special cargo material, such as mitochondria (mitophagy), lysosomes (lysophagy), the endoplasmatic reticulum (ER-phagy or reticulophagy), and the nucleus (nucleophagy). By having these selective autophagy pathways, it is required for some to have specific cargo receptors and/or adaptors (28).

Vesicle nucleation

Nucleation, with its specific components as the starting point of vesicle formation is the least understood step in this process. The origin of the autophagosome membrane is also unclear, although the ER is thought to play an important role in supplying the membrane for the vesicle formation process (17). However, there are other potential sources involved in the formation of autophagosomes, for example ER-Golgi intermediate compartments, ER-mitochondria junctions, mitochondria, Golgi-endosomal membranes or the plasma membrane (28). It is also quite possible that vesicle formation happens de novo in autophagy-related processes. On the one hand, the vesicle does not appear to be formed by extrusion from the surface of an organelle. On the other, it is not attached to an organelle. The result is an autophagosome with a double membrane. The contents of the autophagosome lumen would become equivalent to the lumen of an organelle or the extracellular space (17,20). There is another structure that is not yet well understood. The pre-autosomal structure (PAS) is thought to play an important role as a site for vesicle formation during autophagy (20).

Vesicle expansion and completion

Vesicle formation in autophagy pathways involves expansion and sealing, supported by ubiquitin-like protein systems. While the exact mechanism remains unclear, vesicles may form either through a pre-existing membrane deformation with possibly no expansion step or by sequential membrane addition, which then allows the membrane to grow, constituting the expansion phase. Expansion refers to membrane growth, and completion marks the sealing of the vesicle, separating its cargo from the cytosol. Key proteins in these steps include two ubiquitin-like (Ubl) protein systems involved in novel conjugation reactions (17,30). These two Ubl proteins are Atg8 and Atg12. Both participate in novel conjugation reactions. Atg8 first undergoes proteolytic processing and then modifies the lipid phosphatidylethanolamine. Atg12 is covalently connected to Atg5. Although the exact purpose of the conjugation reactions is not known, the proteins Atg12-Atg5 are seen as potential candidates for a transient coat complex involved in autophagy. This transient coat is formed by such protein components playing a central role in most vesicle budding processes (20).

Retrieval

In autophagy, specific components are recycled after vesicle formation utilizing a retrograde trafficking process to retrieve components. This reutilization is essential to maintaining efficiency and function. For example, many receptors often detach from their cargo and return to their original compartment to be reused for a new round of loading. Although some autophagy proteins are involved in vesicle formation, they do not appear to be associated with the completed autophagosome, suggesting that they may be retrieved at some point before or after vesicle completion. Similarly, proteins which are important for the identity or function of a compartment, such as v-SNAREs, must be sent back to their donor membrane to maintain compartmental integrity. Another instance is the transmembrane protein Atg9, which is involved in vesicle formation and is recycled with the help of Atg2 and Atg18. It has been suggested that this type of retrieval pathway is evolutionarily conserved because the autophagy proteins Atg18 and Atg9, for example, have orthologs in higher eukaryotes. One exception is Atg8 protein, as it is associated with the completed autophagosome, making it a useful autophagy marker in yeast and in higher eukaryotes. (17,20)

Vesicle targeting, docking, and fusion

The timing of the vesicle fusion with the vacuole or lysosome must be regulated. There is a risk of cargo being left in the cytosol, if this process begins before the double-membrane vesicle is complete. The result of the autophagosome fusing with a lysosome is a degrading structure called “autolysosome” or “autophagolysosome.” The fusion process of autophagosomes with vacuoles may also include a set of SNARE proteins, as some molecular genetic studies have indicated. (17,20)

Breakdown of the vesicle and its cargo

Once the fusion of the double-membrane autophagosome with the vacuole is complete, the single-membrane autophagic body, and thus the autophagolysosomal contents can be degraded. This breakdown of the vesicle is one of the main purposes of autophagy, at least in yeast, to degrade cytoplasm and therefore successfully recycle macromolecules for reuse in the production of essential components during nutrient stress. The vesicle lysis step is dependent on an acidic pH of the vacuole lumen and proteinase B, which activates vacuolar

zymogens playing a direct role in the degradation process. Also Atg15 is homologous to a family of lipases and may function in this manner. (17,20)

Assessment of autophagy in mammalian cells

There are different methods to assess autophagy in mammalian cells. The most standard and reliable method is the conventional transmission electron microscopy (TEM) despite the limitations of being time-consuming and requiring highly skilled experts. Also, the differentiation between autophagosomes, which are single-membrane structures and autophagolysosomes, which are double-membrane structures is difficult by TEM. This is why the term autophagic vacuole was coined to describe these structures together. Another reason why TEM cannot be objectively quantitative is that it is possible to mistake other organelles such as the ER for autophagic vacuoles. (31,32)

LC3 is a specific marker of autophagy for its role during autophagosome genesis, and it can be localized in any types of autophagic membrane during the autophagic process, for example the phagophore, autophagosome, and autophagolysosome, as it intervenes in the late stages of autophagosome formation. LC3 is a mammalian ortholog/homolog of the yeast autophagy-related gene 8 or Atg8. It is the most used marker to assess autophagy in cells. First, there is Pro-LC3. It is converted to LC3-I by an Atg4 family protease after synthesis. When autophagy takes place, LC3 is activated and LC3-I is converted to LC3-II with conjugation to phosphatidylethanolamine by the activity of Atg3 and the Atg12-Atg5-Atg16L1 complex. This allows LC3-II to be localized in the membranes of the autophagosome. At the end of the autophagic process LC3-II is also degraded in autophagolysosomes. The conversion of LC3-I to LC3-II or the amount of LC3-II can be used to monitor autophagy. It indicates the formation of autophagosomes. It is important to consider that elevated levels of LC3-II may reflect either an upregulation of autophagosome formation or reduced turnover. As autophagy is a highly dynamic process, a so-called autophagic flux, regulated by both the on and off rate, the amount of LC3-II does not always correlate with overall autophagic activity or flux at a given time point. LC3 can be observed by immunofluorescence using anti-LC3 antibodies or green fluorescent protein (GFP), which is commonly used for *in vitro* assessment of autophagy and real-time observation of GFP-LC3 localization. Another example of the use of LC3 are GFP-LC3 transgenic mice for *in vivo* analysis of autophagy. There is a novel tandem fluorescent tagged LC3 method, where red fluorescent protein is also fused with GFP-LC3. One advantage of this red

fluorescent protein is that it is resistant to lysosomal degradation. Depending on the stage of autophagy, autophagosomes appear as yellow dots, while mature autophagolysosomes show up red due to the quenching of GFP in the acidic milieu of the lysosome. (31,32)

Another marker of autophagy is p62, or sequestosome 1. It is an adaptor molecule that is involved in targeting cargo for autophagosomes by interacting with polyubiquinated protein aggregates and facilitating their degradation at the lysosome through direct binding to LC3 via a short LC3 interaction region. This autophagy marker is itself degraded by autophagy. Thus, p62 accumulation may represent defects in the selective autophagy of ubiquinated aggregates. However, an additional method is needed to measure autophagy, because not only is there a possible increase in p62 transcription by oxidative stress, but p62 itself is degraded by the ubiquitin-proteasome system as well as autophagy, and therefore its level may be increased when the proteasome is inhibited. (31,32)

3.2.2. The role of placental autophagy in human pregnancy

There is growing evidence that autophagy has many important functions during human reproduction, especially in embryogenesis, implantation, placentation, and in the maintenance of pregnancy (16). Some autophagy-related proteins present in the human placenta are BECN1, LC3B and DRAM. BECN1 plays a role in promoting the synthesis and growth of pre-autophagosomal membranes, LC3B was discussed in the previous chapter and DRAM is a lysosomal protein regulating autophagy in a p53-dependent manner. These three Atg-related proteins were the focus of the study by Hung et al. who demonstrated autophagy by transmission electron microscopy and immunofluorescence in the placentas at early and late gestation (33). BECN1, LC3B and DRAM were consistently expressed in the placentas examined, but no significant differences were found in the protein levels of these molecules in placentas at early, mid and late gestation with uneventful vaginal deliveries, suggesting that autophagy plays a role in the development of the placenta. Autophagy markers were found in CTB, STB, EVT, and decidual stromal cells (32,33).

One function of autophagy in placental development may be to support EVT that invade the maternal decidua basalis into the first third of the uterine myometrium at the implantation site, as well as migrate along the lumina of the spiral arterioles under nutrient-poor and hypoxic conditions in early pregnancy (16). The observation of autophagy in EVT could already be made from the seventh week of gestation and even under physiological hypoxia

in these cells. Nakashima et al. were able to show for the first time the important role of autophagy in trophoblast functions under physiologically hypoxic conditions and described it as "*an indispensable mechanism to promote EVT-invasion as well as crosstalk between EVT and the endothelium at the fetal-maternal interface under physiological hypoxia during early pregnancy,*" which could indicate a physiological required role of autophagy during normal placentation *in vivo*, especially in trophoblast functions, including invasion and vascular remodeling in EVT (34). Further evidence for this involvement of autophagy was provided by Aoki et al. using a mouse model with *Atg7* knockout placentas, meaning the *Atg7* gene was deleted in trophoblast cells but not fetuses, which enabled them to demonstrate that these *Atg7* knockout placentas lead to a restricted placental growth suggesting that the deficiency of autophagy in placenta is a risk factor for poor placentation (35). Tsukamoto et al. found that autophagy plays an important role during the post-fertilization stage of the oocyte (36). They generated autophagy-deficient mice by using oocyte-specific Atg5 knockout mice. The oocytes lacking Atg5, and therefore autophagy, were fertilized normally but failed to develop beyond the four- and eight-cell stages when fertilized by Atg5-null sperm and resulted in embryonic lethality. However, they could develop further in these stages if they were fertilized by wild-type sperm (36). These two examples were chosen to highlight the importance of autophagy during some of the earliest stages of human pregnancy, showing the dynamic changes of autophagy along the development of the embryo from activation immediately after fertilization to downregulation later.

As early as the one- to four-cell stage, about 4 hours after sperm entry, autophagy is triggered by inhibition of the mTOR pathway. One possible role for autophagy at this stage is to degrade superfluous maternal material and organelles from the fertilizing sperm. After this oocyte to zygote transition stage, where autophagic protein turnover may be a critical determinant of normal embryonic development, involving many changes, including protein synthesis, protein and RNA degradation, and organelle remodeling, there is a gradual decline in autophagy-related gene transcription through the morula and blastocyst stages. The cause of the downregulation during this period remains to be determined (31). One explanation is that downregulation prevents the destruction of critical factors of further embryonic development (37). Oh et al. suggest that this suppression of autophagy may be associated with the preparation for implantation and invasion into the maternal uterus (31).

Autophagy also takes place in the trophoblast cells of term-placental villi (23). As described in more detail in chapter 3.1, trophoblasts are the major cell types in the placenta. As previously mentioned, they can be further differentiated into cytotrophoblasts (CTB), the syncytiotrophoblast (STB) and extravillous trophoblasts (EVT) (14). Earlier in this chapter the importance of autophagy for EVT invasion and interaction with the endothelium under physiological hypoxia in early pregnancy was outlined, highlighting its critical role in normal placentation (34). Autophagy is enhanced in cells undergoing differentiation into EVT during the initial stages of pregnancy. Conversely, reduced autophagic activity in EVT, as well as reduced invasion and vascular remodeling activity was being observed in ATG4B-mutant EVT cells. This process appeared to be independent of hypoxia-inducible factor-1α (HIF1α) signaling, indicating that autophagy may regulate the activity of enEVT invasion independently of HIF1α. Enhanced autophagic activity appears to protect trophoblasts against external pathogens during STB formation. In addition, autophagy activity is induced in the placenta when the mother is malnourished, to maintain the energy supply of the fetus and especially its brain, by degrading intracellular substances of placental cells (14).

In their study, Avagliano et al. attempted to show the *in vivo* assessment of autophagy in humans at the maternal-fetal interface after implantation during the first trimester of pregnancy to verify its involvement in this early phase of pregnancy, and its different expression in normal pregnancy and spontaneous miscarriage (32). They performed immunohistochemical and immunofluorescence studies on all samples. Due to limited tissue availability, a subset of the population, was used for transmission electron microscopy evaluation, and some villous and decidual samples were prepared for Western blotting analysis. Avagliano et al. were able to detect signs of autophagy in villous and decidual samples from first trimester pregnancies by LC3 staining in villous STB and CTB by immunohistochemical method and by LC3 immunostaining in trophoblast anchoring columns and EVT. They found positive immunoreactivity in both basalis and in parietalis decidua and observed autophagosomes by electron microscopy in CTB, STB, and decidua to confirm the localization of autophagy. In addition, comparable LC3-II protein expression was detected in villous and decidual samples by Western blotting analysis. The results of the study showed that autophagy is constitutively expressed at the maternal-fetal interface, with higher expression in the CTB than in the STB under normal conditions. In their evaluation of autophagy in spontaneous miscarriage Avagliano et al. detected increased autophagy expression in villous samples due to an increase in autophagic vacuole concentration in STB.

They suggest that this increase reflects a prosurvival tendency of the cells in response to environmental changes, as autophagy is known to play a cytoprotective role in placental trophoblasts during stressful conditions. The authors detected autophagy markers in decidua samples, trophoblast anchoring columns, and EVT. Although autophagy expression appeared comparable in the decidual tissues from normal pregnancy and spontaneous miscarriage groups, there was a simultaneous increase in apoptosis and hypoxia in spontaneous miscarriage. They also observed elevated autophagy levels in villous tissue alongside reduced activity, but the decidual tissue showed the opposite pattern with low levels of autophagy and increased apoptosis, which may indicate a potential protective role of autophagy against hypoxia-induced apoptosis especially during spontaneous miscarriage, although further research is needed to confirm this hypothesis. Nevertheless, they were the first to demonstrate that spontaneous miscarriage samples show higher autophagy expression in villi. (32)

Later, autophagy is also involved in the regulation of labor. Autophagy was more pronounced in placentas from women who have given birth by caesarean section than in women who have given birth naturally (14). Although it is well known that blood flow to the placenta during the mechanically very stressful vaginal delivery is rather irregular due to muscle contractions, autophagy activity was higher in placentas from caesarean section than from vaginal delivery. One cause may be the use of anesthetics during caesarean section. Another cause of increased autophagy activity is thought to be the lower glucose and amino-acid levels detected in the cord blood of children delivered by elective caesarean section, which may be due to the mother's mandated fasting before surgery. This in turn is consistent with the activation of autophagy as a survival mechanism in the event of nutrient deficiency (14,38).

3.3. Inflammation in the placenta

3.3.1. The immune system in pregnancy

Pregnancy is a unique immunological state that protects both the mother and the fetus. The immunology of a normal pregnancy is highly complex, involving several different factors and processes. Rather than being suppressed, the maternal immune system undergoes dynamic modulation forming a responsive network or system capable of recognizing and adapting to environmental changes. This modulation of the immune system results in different responses, based on the stage of pregnancy and on the type of microorganism. The system must protect the mother against environmental threats while preventing damage to the fetus. This adaptive network of recognition, communication, trafficking and repair processes can also sound the alarm to ensure the well-being of both mother and fetus if necessary. In light of the increased knowledge gained in recent decades, the earlier description of the fetus as a semi-allograft and the assumption that the placenta is an allograft expressing paternal proteins which, according to various beliefs, assumptions and observations should be rejected by the maternal organism under normal immunological conditions has been revised. It is therefore important to understand the placenta as being more than just a transplanted organ. (39,40)

Mor et al. suggest that, while there may be active mechanisms preventing a maternal immune response against paternal antigens, the trophoblast and the maternal immune system have evolved to interact cooperatively to support each other and to ensure a successful pregnancy. A new paradigm is proposed: this is where the maternal immune response to microorganisms is influenced by signals from the fetal-placental unit. According to this view, the immunology of pregnancy results from the interaction, signals and responses between the maternal immune system and the fetal-placental immune system. Placental signals actively modulate how the maternal immune system responds to potential threats. They also suggest that the microenvironment created by the placenta affects the differentiation and function of immune cells that enter the implantation site. Their findings support the hypothesis that trophoblasts can transform immune cells into a trophoblast-supporting phenotype. Furthermore, Mor et al. demonstrate that conditioned media from trophoblasts stimulates the secretion of cytokines such as IL-6, IL-8, MCP-1 and GRO-α by monocyte-like THP-1 cells. The secretion of these cytokines promotes trophoblast

development and function. This interaction between trophoblast and immune cells during pregnancy can be divided into three distinct stages (39,40):

1. Attraction: initial recruitment of immune cells to the implantation site by chemokines secreted by the trophoblast
2. Education: differentiation of these recruited immune cells by regulatory cytokines produced by trophoblast cells
3. Response: the trophoblast-conditioned immune cells respond to signals of the local microenvironment and produce factors that support placental formation and function

Aldo et al. describe the characteristics of each of these stages using *in vitro* models of trophoblast-immune interactions, observing significant changes in endothelial cell phenotype upon differentiation in Matrigel. These changes may be necessary for the endothelium to direct trophoblast migration and transformation. For example, villi-like structures attract monocytes/macrophages, which then localize around trophoblast-derived tubes. These macrophages support the culture environment and promote trophoblast survival. Conversely, the absence of trophoblast-derived signals causes macrophages to remain on the surface without migrating through the Matrigel (41).

In general, the immune system protects the host against pathogens. Detection of and response to microbial invasion and the coordination of cell migration for surveillance depend on the innate immune system. Important adaptations of the immune system occur during pregnancy. During a normal pregnancy, there are numerous immune cells present in the human decidua. Most of these decidual leukocytes, roughly 70 percent, are NK cells and about 20 percent are macrophages (40). Lower levels of dendritic cells, mast cells, granulocytes and B cells can also be found in the placental bed (42). All these immune cells infiltrate the decidua during the first trimester. They also begin to accumulate especially around the invading trophoblast cells. Removal of immune cells does not support the pregnancy; it terminates it and leads to miscarriage. Removing macrophages, NK cells, or dendritic cells (DC) can negatively affect placental development, implantation, or decidua formation. Trophoblasts, for instance, are unable to reach the endometrial vascularity in the absence of NK cells, which can also lead to miscarriage, suggesting that these NK cells are important for the invasion of trophoblasts in the uterus (40). NK cells are classified as lymphocytes, which produce cytotoxic and cytokine functions. Their cell marker is CD56. They also lack CD3 expression, which is a T cell marker. There are two types of NK cells in humans: the cytotoxic NK cells, with around 90% of all NK cells, and the regulatory NK

cells, which produce cytokines. Uterine NK in the endometrium as well as in the placental bed are characterized as regulatory NK cells. However, they also express different markers in comparison to blood NK cells. For example, they express higher levels of Killer-cell immunoglobulin-like receptors (KIRs), which are important for uNK cell function, including the production of certain growth factors, trophoblast migration, and spiral artery remodeling (42).

Another factor that negatively affects placental development, implantation, or decidua formation is the absence of DCs. This absence prevents the formation of the decidua and thus the implantation of the blastocyst. That there are more immune cells at the implantation site is not due to a reaction to the fetus as a "foreign body." They are probably attracted to protect and facilitate the pregnancy. Therefore, the immune system at the implantation site is not suppressed but active and functional, if needed under cautious control, playing a central role in protecting the mother against environmental stressors while simultaneously protecting the fetus from potential harm. Hence, it is appropriate to speak of pregnancy as a unique state of the immune system that is modulated but not suppressed (40). It is important to note that during the first trimester no maternal blood including blood cells enters the intervillous space of the placenta, only maternal plasma is present until the end of the first trimester, which means that only a clear solution circulates through this space. This solution consists of blood plasma, filtered through the plugs formed by endovascular trophoblast and secreted from uterine glands, supplying a mixture of nutrients, growth factors, and cytokines to the developing villi of the first-trimester placenta (12).

Burton et al. have demonstrated the visualization of such secretion products in the intervillous space of first-trimester placental tissues. These products, secreted by the uterine glands, contribute to embryonic development after implantation. This leads to the concept of histiotrophic nutrition of the embryo during the first trimester transitioning into hemotrophic nutrition once maternal blood enters the intervillous space. Thus, in addition to endovascular trophoblasts opening spiral arteries, endoglandular trophoblasts appear to open uterine glands towards the intervillous space of the first-trimester placenta. Evidence of this is also provided by their ability to invade the decidual interstitium and uterine glands, to replace uterine epithelial cells and to localize within the lumen of the uterine glands. (43,44)

The absence of maternal blood cells also keeps any circulating maternal immune cells away from the developing villi. Maternal blood flows into and through the intervillous space of the placenta after the first trimester, allowing direct contact between circulating maternal

immune cells and the placental STB (12). The apical surface of the STB does not express major histocompatibility complexes (MHC) I or II. This is crucially important for immunological protection. Therefore, the villous surface is immunologically inert to maternal immune cells that circulate through the intervillous space (3).

Huppertz et al. describe seven different levels of immunologically significant aspects of interactions between the mother and the developing fetus (12,45):

1. Attachment and adhesion of the blastocyst to the uterine epithelium on day 6 to 7 pc
2. First erosion of maternal or uterine tissues by an invasive STB on day 7 to 9 pc
3. Invasion of EVT into the uterine decidua to reach the myometrium on gestational day 14 until delivery
4. Invasion of EVT into uterine spiral arteries as “endovascular trophoblast” or into uterine glands as “endoglandular trophoblasts” in gestational weeks 3 until delivery
5. Flow of only maternal plasma into the intervillous space of the placenta in gestational weeks 4 to 10 in the first trimester
6. Flow of maternal blood into the intervillous space of the placenta after the first trimester in gestational week 11
7. Deportation of trophoblast material into the maternal circulation from around gestational week 4 until delivery

Immunological interactions may occur at each of these stages. The interactions are being tightly regulated by control mechanisms to help preventing fetal rejection by the mother. Especially EVT encounter a variety of maternal immune cells along their path through maternal tissues coming into very close contact with them and interacting with them. However, detailed insights and the precise mechanisms underlying the interactions between fetal cells and maternal immune cells remain largely unknown. Nevertheless, when discussing the immunological interactions between trophoblasts and maternal cells, it is important to highlight more of the unique features of EVT. They are normal healthy cells that have differentiated from the trophectoderm. EVT express specific proteins, including oncofetal proteins like the oncofetal fibronectin and the blood group antigen precursor ‘i’ as well as unusual MHC molecules, notably the human leukocyte antigens (HLA) HLA-C, HLA-E and HLA-G. (12)

3.3.2. The role of inflammation in normal pregnancy

Inflammation is a physiological component of pregnancy. It plays an important role in implantation, placental development, and the birthing process. The inflammatory response is tightly regulated and phase-dependent. During the early stages of pregnancy, pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) facilitate trophoblast invasion and spiral artery remodeling.

The long-held view of pregnancy as a „Th-2“or anti-inflammatory state has been both supported and challenged. One reason for such contradictory findings may be that pregnancy has been oversimplified as a single immunological event in an anti-inflammatory condition, where changes in the type of cytokines produced can lead to miscarriage or complications during pregnancy, rather than recognizing it as a dynamic process involving at least three distinct immune phases (39,40,46):

1. “The open wound”: The first trimester of pregnancy until the very early second trimester and especially early implantation is characterized by a Th1 proinflammatory environment, dominated by Th1 cells and cytokines such as IL-6, IL-8 and TNF-α. During this time a strong inflammatory response is required. This strong response is triggered by the blastocyst invading the endometrial epithelium, resulting in tissue damage and disruption to the epithelium. This leads to the initiation of trophoblast invasion, replacement of the endothelium with trophoblast, and vascular remodeling. All these processes require an inflammatory environment to support tissue repair and immune regulation. As a result, many pregnant individuals experience systemic symptoms such as fatigue, fever and especially nausea, which is commonly referred to as “morning sickness.” These symptoms are a direct consequence of the inflammatory response as well as hormonal and metabolic changes among other factors. The whole body may feel unwell as it struggles to adapt to the presence of the fetus. Therefore, due to the prevalence of proinflammatory mediators, the first trimester is characterized as a proinflammatory phase.
2. “The good time”: The second immunological phase of pregnancy is often considered the most stable and favorable period for the pregnant woman. Maternal symptoms such as nausea and fever usually improve, partly because the immune response is no longer the main endocrine feature. It is marked by rapid fetal growth and development, as well as a symbiotic relationship between the mother, the placenta,

and the fetus. Immunologically, this phase is characterized by a shift towards an Th2 anti-inflammatory environment.

3. "Preparation for birth": During this final immunological phase of pregnancy, fetal development is complete, and all organ systems are prepared for life outside the womb. To prepare and initiate parturition, the maternal immune system reactivates a proinflammatory state. This is marked by an influx of immune cells into the myometrium, creating a proinflammatory environment that promotes and triggers uterine contractions, as well as the expulsion of the baby and the rejection of the placenta.

Therefore, pregnancy involves both proinflammatory and anti-inflammatory conditions that are regulated throughout gestation and depending on the stage of gestation (39,40,46). The numbers of uNK cells also varies depending on the stage of pregnancy. They increase in the placental bed of early human pregnancy in the first trimester, after which their number decreases, although their presence continues until the final stages of pregnancy. Macrophages are present in the placental bed throughout pregnancy. While the number of macrophages is high in early pregnancy, it declines as gestation progresses (42).

As our understanding of pregnancy immunology evolves beyond the classical Th1/Th2 paradigm and embraces new concepts, the importance of proinflammatory Th1 cytokines, such as TNF-α and IFN-γ, in supporting a successful pregnancy is gaining recognition. Therefore, at every stage of pregnancy, the maternal immune system and the placental trophoblast dynamically adjust a combination of modulating signals and responses to ensure an overall cooperative status. TNF-α levels rise progressively throughout pregnancy and further increase postpartum. Maternal body mass index (BMI) and age may influence maternal TNF-α levels during pregnancy. Elevated levels of TNF-α have been associated with several adverse pregnancy conditions, including gestational hypertension and gestational diabetes mellitus (GDM). They have also been shown to affect the biology of trophoblasts, including their migratory activity, syncytialisation, and endocrine function, during distressed pregnancies. Furthermore, elevated TNF-α may influence maternal-fetal communication by provoking a shift in the secretory profile of immune-modulating factors derived from the placenta, thereby affecting the activity of maternal immune cells. For example, the differentiation of peripheral blood monocytes into macrophages can be induced by trophoblast-derived factors, which also enhance the recruitment and differentiation of inducible regulatory T cells. It is now widely accepted that the placenta acts as an immune-

modulating organ regulating both the local immune response of cells present at the implantation site as well as the systemic immune response. (46)

As gestation progresses towards term, inflammatory activity increases, with an enhanced inflammatory response and proinflammatory cytokines actively remodeling and ripening the cervix, weakening, and ultimately rupturing the fetal membranes and activating myometrial contractility. Parturition, especially at term, is associated with a significant increase in the number of macrophages and neutrophils in both upper and lower segments of the myometrium. This influx of inflammatory cells is linked to an increase in the production of cytokine mRNA. A key feature of immune adaptation during pregnancy is the change in cytokine production. Cytokines can be structurally classified into four major groups: the 4α-helix family members (including IL-2, IFN-γ, and IL-10), the IL-1 family, the IL-17 family, and chemokines. However, from a functional perspective, it is more helpful to distinguish between just two groups: those associated with Th1 reactions, and those associated with Th2 reactions. Th1 reactions are associated with cell-mediated immunity, while Th2 reactions are associated with humoral immunity. Th1 cells and Th2 cells produce different types of cytokines and factors. Th1 cells produce cytokines such as IL-1, IL-2, IL-6, IL-12, IL-15, IL-18, IFN-γ, and TNF-α, which promote strong cell-mediated immune responses. In contrast, Th2 cells produce IL-4, IL-5, IL-10, IL-13, and GM-CSF, and play a key role in regulating humoral response. While local T cells contribute to cytokine production, the primary sources of Th2 cytokines are nonlymphoid tissues, particularly placental and decidual tissues. Trophoblast play a central role in this process. During a normal pregnancy, the so-called Th2 phenomenon may occur, shifting the balance of Th1/Th2 activity towards Th2 activity, which has the potential to protect the maternal-fetal relationship. However, infections or inflammatory stimuli can disrupt this immunological balance, resulting in a shift towards Th1 dominance. This Th1-driven response promotes the production of proinflammatory cytokines, which are associated with the development of pregnancy complications such as spontaneous miscarriage, preterm labor and/or pre-eclampsia. However, as mentioned above, it is misleading to simplify pregnancy as an isolated, single immunological event in a predominantly anti-inflammatory or Th2 state. The three aforementioned distinct immunological phases that occur during pregnancy must also be considered. (47)

A brief description of some important maternal and placental hormones, such as progesterone, estrogen and oxytocin, with their possible effects on inflammatory pathways

may also be instructive. Progesterone is essential throughout pregnancy, ensuring uterine quiescence for most of the gestation period. As a potent immunomodulator, progesterone inhibits mitogen-stimulated lymphocyte proliferation and reduces the production of proinflammatory cytokines by macrophages in response to bacterial products. It also promotes IL-10 production. However, progesterone function can be reduced by intrauterine infection or inflammation which increases the levels of proinflammatory cytokines, IL-1 and TNF-α, in gestational tissues such as fetal membranes, decidua, amniotic fluid, and myometrium. Estrogens play a role in preparing the body for labor by increasing contractility and excitability of the myometrium. The dynamic interplay between estrogen and progesterone, whereby estrogen stimulates, and progesterone relaxes uterine activity, regulates the timing and process of parturition. Estrogen also plays a key role in certain immune responses by inhibiting Th1 proinflammatory cytokines, such as IL-12, TNF-α, and IFN-γ, while stimulating Th2 anti-inflammatory cytokines, such as IL-10 and IL-4. Another important hormone is oxytocin, which is involved in the initiation of labor by stimulating uterine contractions. Its receptor, the oxytocin receptor (OTR), is upregulated in response to proinflammatory mediators associated with labor. The OTR promoter contains putative binding sites for transcription factors such as NF-κB, which are activated by proinflammatory cytokines, including IL-6 and IL-1β, that increase during labor. IL-1β, in particular, enhances OTR mRNA expression and promotes the nuclear translocation and DNA-binding activity of NF-κB, thereby amplifying uterine responsiveness to oxytocin Table 2 provides an overview of these hormones mentioned and their main functions. (47,48)

Table 2 Effects of maternal/placental hormones in inflammatory pathways – adapted and modified from Kalagiri et al. (48)

HORMONES	**EFFECTS**
Progesterone	• Blocks mitogen-stimulated lymphocyte proliferation • Prolongs survival of the allograft • Modulates production of antibodies • Decreases oxidative bursts of monocytes • Reduces proinflammatory cytokine production by macrophages in response to bacterial products • Favors IL-10 production • Reduced function in the face of intrauterine inflammation

	• Maintains uterine quiescence throughout most of gestation
Estrogen	• Critical role in some immune response and immune-related diseases • Inhibits Th-1 proinflammatory cytokines • Stimulates Th-2 anti-inflammatory cytokines
Oxytocin	• Oxytocin receptor positively regulated by inflammatory mediators of parturition • OTR promoter has transcription factor binding sites activated by IL-1β and IL-6

3.3.3. Key inflammatory pathways in the placenta

Several signaling pathways orchestrate inflammation in the placenta. As described before, early implantation is characterized by a proinflammatory state dominated by Th1 cells and cytokines such as IL-6, IL-8 and TNF-α, which are secreted both by endometrial cells and by immune cells recruited to the implantation site. The population of immune cells in the decidua consists predominantly of uNK cells, macrophages and dendritic cells (DCs) (39). uNK cells accumulate in the endometrium during the late secretory phase of the non-pregnant cycle, as well as at the implantation site in early pregnancy, where they are particularly numerous. They play a central role in regulating trophoblast migration and arterial remodeling by releasing cytokines and proteases in response to local signals. Contrary to their name, uNK cells do not kill invading trophoblasts. Instead, they support their migration and mediate the remodeling of the uterine arteries. There is a finely tuned interaction between the polymorphic HLA-C ligands expressed on trophoblasts and the killer-cell immunoglobulin-like receptors (KIRs) found on uNK cells. The nature of this HLA-C–KIR interaction significantly influences pregnancy outcomes; certain combinations are associated with an elevated risk of disorders such as miscarriage, pe-eclampsia, and fetal growth restriction. This highlights the importance of maternal-fetal immune compatibility in early gestation (7). uNK cells produce IL-8 and interferon-inducible protein-10 chemokines, which promote trophoblast migration and invasion. They also produce angiogenic factors that are essential for vascular growth and decidual development. DCs, a heterogenous group initiating and coordinating the innate adaptive immune response, accumulate in the uterus before implantation and stay in the decidua throughout gestation. Macrophages and DCs

may both play a key role in modulating the cytokine profile at the maternal-fetal interface. This contributes to the regulation of implantation, immune balance, and tissue adaptation during early pregnancy (39).

A regulated balance between pro- and anti-inflammatory mediators is essential for a healthy pregnancy. Kaislasuo et al. found that women who experienced early pregnancy loss between weeks 6 and 8 had significantly lower levels of the anti-inflammatory cytokine IL-10 than those with ongoing pregnancies. Conversely, elevated levels of the proinflammatory cytokine TNF-α were observed in individuals experiencing pregnancy loss between weeks 4 and 9. These results emphasize the importance of early immune regulation and imply that an imbalance in favor of proinflammatory substances transcribed by NF-κB result in early pregnancy failure. (49)

One of the key inflammatory pathways in the placenta is the nuclear factor-kappa B (NF-κB) pathway. It regulates the expression of pro-inflammatory cytokines as well as adhesion molecules, and it may play a protective role by stimulating anti-apoptotic cascades in response to embryonic stress. Production of NF-κB is elevated in the decidua prior to conception, and activation of NF-κB has been observed in trophoblast and decidual immune cells, particularly during the early and late stages of pregnancy. NF-κB regulates over 400 genes involved in inflammation, apoptosis, angiogenesis, and cellular responses to hypoxia and oxidative stress. Although levels of NF-κB must decrease to support maternal immunosuppression and maintain gestation during the final stages of physiological pregnancy, increased NF-κB activation during early pregnancy, causing hypoxia and inflammatory processes, is advantageous for implantation. For example, the stimulation of NF-κB is caused by the binding of ligands to NF-κB, such as cytokine receptors, Toll-like receptors (TLRs), NOD-like receptors (NLRs) and TNF receptors. (50)

4. RESULTS

4.1. Interplay between placental autophagy and inflammation

Protective versus pathological roles

Autophagy is essential to both innate and adaptive immunity. For instance, it contributes to cellular defense mechanisms that benefit the host against invasion of microorganisms by removing these invading pathogens (31). One example of this protective role of autophagy is the xenophagic capture and degradation of intracellular pathogens including bacterial and viral pathogens, called xenophagy. However, this process is not fully understood. The cellular mechanisms targeting intracellular bacteria and probably viruses to autophagosomal compartments resemble those involved in selective autophagy of endogenous cargo (51).

Human placental trophoblasts contribute actively to antiviral defense of the placenta against various viral infections. They can induce autophagy in nearby cells, providing a broad antiviral resistance and protecting the fetus from pathogens circulating in the maternal bloodstream (52). As suggested by Delorme-Axford et al., trophoblasts are resistant to viral infection and use a pathway to suppress viral infections systemically by transmitting this viral resistance to nonplacental cells (53). This may be owed to a unique mechanism that shields placental and maternal cells against viral infections during pregnancy, thereby enhancing the protection of the developing fetus. The transfer of the viral resistance happens via trophoblastic conditioned medium and trophoblastic exosomes among others. The position of trophoblasts at the maternal-fetal interface enables them to orchestrate key immunological and physiological interactions that support fetal development and maintain pregnancy. There they serve as both mechanical and immunological defensive barriers that prevent viral transmission to the fetus. Antiviral innate immune signaling is essential at the maternal-fetal interface, because transplacental viral infections from the maternal blood to the fetus can lead to severe pathological outcomes such as neurodevelopmental dysfunction, birth defects, and fetal death. Furthermore, maternal viral infections can negatively affect pregnancy even if they do not directly affect the fetus. Therefore, it is crucial to understand the antiviral defense mechanisms of trophoblast, as well as the strategies viruses use to evade them in order to develop possible therapeutic strategies that can prevent fetal disease (53).

Autophagy and cytokines

Both autophagy and cytokines influence each other, and both play important roles in maintaining a pregnancy. In his review article, Harris describes the regulation of autophagy by cytokines, as well as the regulation of cytokines by autophagy (21). As described, autophagy contributes to both innate immunity and adaptive immunity. In innate immunity, it eliminates intracellular bacteria directly, but many microorganisms target or modulate autophagy to survive. In adaptive immunity, it supports the processing and presentation of antigens expressed via MHC class I and II molecules. These molecules are also known as the human leukocyte antigen (HLA) complex in humans. Many cytokines, toll like receptors (TLR) and NOD-like receptor (NLR) ligands, are potent inducers of autophagy. TNF-α, for example, is a Th1 cytokine that stimulates autophagy in cells such macrophages, T lymphoblastic leukemic cells, vascular smooth muscle cells, and skeletal muscle cells, among others. However, the exact mechanism by which TNF-α stimulates autophagy remains unclear and may vary among different cell types. IFN-γ is another Th1 cytokine that induces autophagy, while IL-4 and IL-13, which are classical Th2 cytokines, inhibit it. Many other cytokines contribute to the process of autophagy. However, discussing all of them would go beyond the scope of this thesis. Autophagy itself not only responds to cytokine signaling but also directly influences cytokine transcription, processing and secretion, so it may have a specific role to play in the regulation of inflammatory cytokines. Impaired autophagy function has been associated with an increased secretion of proinflammatory cytokines such as IL-1α, IL-1β and IL-18. Autophagy may be involved in the regulation of TNF-α secretion by macrophages and dendritic cells. Many of these interactions between cytokines and autophagy are bidirectional, which highlights the important role of autophagy in immune defense, particularly against bacterial and viral infections, and in regulating inflammatory responses. For instance, autophagy may aid in clearing an infection caused by Mycobacterium tuberculosis by affecting Th1/Th2 polarization. However, autophagy may also play a key role in controlling inflammation and modulating the immune response through negative regulation of IL-1 and type 1 interferons. (21)

The role of the cytokines TNF-α and IL-6 during placentation

As previously discussed, TNF-α and IL-6 both play a role in modulating placental autophagy and inflammation. In this context, the findings of a study that I co-authored entitled

"Placental DAPK1 and autophagy marker LC3B-II are dysregulated by TNF-α in a gestational age-dependent manner" provide further insight (1).

In this study, Prokesch et al. investigated the potentially cytotoxic effects of the pro-inflammatory cytokines TNF- α and IL-6 on the gene expression profile of 84 key genes involved in the regulation and execution of autophagy in the early human placenta. They conducted the first study to examine the impact of IL-6 and TNF-α treatment on DAPK1, a regulator of apoptosis, autophagy and programmed necrosis and the autophagy marker LC3B in first trimester and term placental tissue. The researchers studied whether exogenously applied TNF-α and IL-6, which mirror increased maternal pro-inflammatory cytokines, affect the autophagy program at the transcriptional level in the first trimester human villous placenta. They discovered that elevated maternal TNF-α levels mediate the downregulation of DAPK1 in the placenta of the first trimester. To investigate this, human placental explants were incubated in the presence or absence of the proinflammatory cytokines TNF-α and IL-6. After 48 hours of culturing, analyzing the expression profile of 84 autophagy-related genes revealed a similar shift in the presence of TNF-α and IL-6 with more genes being downregulated than upregulated amongst the array of analyzed genes. The results of the autophagy PCR array showed significant downregulation of DAPK1 by TNF-α and IL-6. Staining in DAPK1 was detected in placental macrophages and the villous trophoblast layer using immunohistochemistry on first-trimester placental explants. The villous trophoblast layer exhibited more intense staining for CTB compared to STB. Western blot analysis of first trimester placental explants for LC3B revealed a reduction in LC3B-II levels following TNF-α treatment. This decline in autophagic activity aligns with the overall downregulation of autophagy-related genes, including *DAPK1*, in response to TNF-α. Prokesch et al. further evaluated the effects of TNF-α on term placental tissue. Although they observed a trend towards reduced DAPK1 protein levels, this decrease did not reach statistical significance. Interestingly, unlike in the first trimester, TNF-α exposure increased both LC3B-I and LC3B-II levels in term placental explants. They analyzed DAPK1 expression and the autophagy marker LC3B in placental tissue and primary trophoblasts isolated from placentas with preeclampsia. Immunohistochemical analysis revealed reduced DAPK1 staining in the STB of preeclamptic placentas compared to controls. Consistent with these findings, primary trophoblasts isolated from preeclamptic placentas exhibited decreased *DAPK1* mRNA expression compared to trophoblasts from healthy pregnancies. At the protein level, DAPK1 expression was also decreased, while both LC3B-I and LC3B-

II levels increased in preeclamptic placentas. Prokesch et al. concluded that elevated maternal TNF-α induces alterations in the expression profile of autophagy-related genes, particularly during the first trimester, where there is a higher proportion of downregulated than upregulated placental genes. Downregulation of DAPK1 expression may disrupt the balance between autophagy, apoptosis and programmed necrosis pathways, potentially resulting in different regulatory mechanisms of placental autophagy throughout gestation. (1)

4.2. The impact of autophagy and inflammation in pregnancy-related complications

Autophagy and inflammation have been implicated in regulating some pregnancy-related disorders. Notably, both of them play important roles in the pathophysiology of preeclampsia, a serious condition associated with preterm birth, intrauterine growth restriction (IUGR), and an increased risk of neonatal mortality (34). Compared to placentas from healthy pregnancies, preeclamptic placentas show reduced expression of autophagy markers such as LC3-II and Beclin-1. This disruption of autophagy may compromise the ability to defend the trophoblast against oxidative and inflammatory stress, which could contribute to the development of PE (54,55).

Preeclampsia, a common pregnancy-related complication and/or multifactorial disease, falls in the category of hypertensive disorders during pregnancy (HDP). It is a leading cause of increased maternal mortality during pregnancy. The International Society for the Study of Hypertension in Pregnancy (ISSHP) has updated the HDP definition to include chronic hypertension, white-coat hypertension, masked hypertension, gestational hypertension, and preeclampsia (56). Clinically, HDP presents with hypertension, edema, and proteinuria. The condition resolves once the pregnancy is terminated or completed, and the uterus is emptied of the fetus and placenta. Preeclampsia is also characterized by these components: elevated blood pressure, edema, and proteinuria. Though they may be variably expressed, all three components must be present. Clinical manifestations may develop slowly or very fast. If left untreated, preeclampsia can result in eclampsia and other serious complications, such as placental infarction, abruptio placentae, and fetal death. It may also lead to maternal death due to cerebral hemorrhage or disseminated intravascular coagulation (DIC) (3). Although delivery of the baby and placenta is the primary treatment for

preeclampsia, there is no medical treatment for women with severe preeclampsia. However, a prophylactic practice of aspirin administration has been introduced to prevent early-onset preeclampsia. Nakashima et al. describe two theories of the disorder: a two-stage disorder theory at the early stage as well as the late stage of pregnancy, which can be combined with two additional stages before conception and after implantation to form a four-stage disorder theory (57). At the early stage of pregnancy there is a reduction in placental perfusion. At the late stage, maternal systemic pathophysiological changes occur due to the release of soluble factors from stage one. The two additional stages can occur before conception, involving poor maternal tolerance to paternal antigens in sperm and after implantation, involving poor maternal immunoregulation of maternal-fetal antigens on trophoblast. While the role of inflammation in preeclampsia is well-established, clinical focus often centers on the causes of systemic inflammation rather than angiogenic imbalance. One key antiangiogenic factor, involved in disrupting placental angiogenesis, is soluble fms-like tyrosine kinase 1 (FLT-1), which is primarily secreted by STB. FLT-1 induction increases in response to STB stress, which can cause oxidative stress, autophagy, endoplasmatic reticulum stress, syncytial knots, apoptosis, necrosis and pyro-ptosis. Although placental nonsterile inflammation is caused by STB stress, the diffusivity of the placenta to systemic inflammation can be determined, because of the immune tolerance to paternal or fetal antigens during the early pregnancy period. (58). Maynard et al. used gene expression profiling to identify candidate factors produced by the placenta in preeclampsia (59). They found that mRNA for FLT-1 is upregulated, resulting in elevated FLT-1 production in the placentas of women suffering with preeclampsia. To test their hypothesis that these elevated circulating FLT-1 levels contribute to endothelial dysfunction, leading to an antiangiogenic state, endothelial tube formation was measured, which is known as an established *in vitro* model of angiogenesis. The results showed that serum from normotensive women promoted the formation of regular, tube-like structures by endothelial cells, whereas serum from women with preeclampsia significantly inhibited tube formation (59). Nakashima et al. also describe the importance of regulatory T cells (Treg cells) and maternal-fetal immune tolerance mediated by these cells as well as autophagy-mediated homeostasis in trophoblasts. They propose a possible interaction between autophagy and immune tolerance during pregnancy. Treg cells can induce for maternal-fetal immune tolerance, because they suppress the activity of CD8+ and CD4+ T cells (57). Aluvihare et al. demonstrate that maternal Treg cells are necessary for maintaining an allogeneic pregnancy by suppressing

autoimmune and aggressive allogeneic responses in mice pregnancies where the fetus and mother have different MHC. The absence of these maternal Treg cells leads to gestational failure due to immunological rejection of the fetus (60). Treg cells increase in the peripheral blood and decidua during human pregnancy (57). At the maternal-fetal interface, Treg cells in the decidua expand clonally in response to specific antigens. These clonally increased effector Treg cells are more prevalent in late pregnancy than in early pregnancy. In cases of miscarriage involving normal fetal chromosomes, the number of Treg cells decreases, while the proportion of clonal effector Treg cells remains unchanged. However, the number of clonal effector Treg cells is insufficient in the decidua of pregnancies presenting with preeclampsia, compared to those in normal pregnancies. These findings suggest that an adequate expansion of antigen-specific Treg cells is essential for maintaining a healthy pregnancy. Its deficiency may contribute to the sterile systemic inflammation observed in preeclampsia (61).

Decidualization is the process by which the endometrium becomes receptive to embryo implantation, which involves specialized endometrial stroma cells called decidual stroma cells (DSCs). These cells play a role in recruiting leukocytes and in inducing morphological vascular changes. Autophagy activation can be observed in DSCs. Treatment with low doses of rapamycin, an autophagy activator, appears to reduce fetal loss. This treatment increases adhesion molecules in uterine stromal cells and increases the number of resident NK cells. It also influences autophagy in these cells, although the exact process is not known. However, high doses of rapamycin impair fetal survival by inhibiting mTOR, a central regulator of cellular growth, proliferation, and protein synthesis (57).

The activation of autophagy in trophoblasts indirectly influences the function of NK cells at the local level. In women with unexplained spontaneous abortions, DSCs exhibit increased p62 expression, a protein degraded by the autophagy pathway, and decreased ATG5 expression, a crucial protein in autophagosome formation. This indicates inhibition of autophagy in DSCs in these cases. *In vitro* studies further demonstrate that decidual NK cells, which normally support early placentation through angiogenesis, fail to attach to DSCs from women with unexplained spontaneous abortions. Together, these findings suggest that proper regulation of autophagy at the maternal-fetal interface is critical for maintaining the balance between trophoblast and immune cell function necessary for a successful pregnancy (57). Increased expression of the p62 protein in the placenta of women with preeclampsia, along with decreased p62 mRNA levels compared to normal pregnancies, leads to the

inhibition of autophagy in preeclampsia (58). The autophagy substrate p62 is highly expressed in EVT cells derived from biopsies of preeclamptic placentas. This suggests that autophagy is inhibited in EVT in the presence of preeclampsia. (16). Additionally, serum from preeclamptic mice induces hypertension and proteinuria in interleukin-10 (IL-10) knockout mice, indicating that circulating factors, such as sFLT1 and soluble endoglin (sENG), may trigger preeclampsia-like symptoms *in vivo* (62). Nakashima et al. described an accumulation of protein aggregates in the placenta of pregnant and preeclamptic women because of dysregulated lysosomal biogenesis and trophoblast stress. Consequently, this process could be a contributor to the onset of PE (16).

5. DISCUSSION

This thesis investigates the increasingly recognized roles of autophagy and inflammation in placental biology and their influence on pregnancy outcomes. Despite remarkable advances in understanding the regulation of autophagy in non-placental tissues in recent decades, the specific mechanisms in the human placenta remain poorly defined. The mechanistic target of rapamycin (mTOR) is a central regulator of nutrient sensing in the placenta (57). However, its precise contribution to the balance of trophoblast function and, consequently, to fetal survival or demise, requires further clarification.

Autophagy has only recently been identified in human placental tissues, and its exact role in trophoblast homeostasis is unclear. Whether autophagy primarily acts as a protective mechanism to promote cell survival and adaptation to stress or contributes to placental dysfunction in pregnancy-related disorders remains an open question. Key issues that need to be addressed include the extent to which autophagy modulates apoptosis in the trophoblast layer, particularly in complicated pregnancies such as preeclampsia and IUGR, and whether autophagy helps to maintain placental function by recycling nutrients and regulating cellular stress responses. Nakashima et al. described the involvement of autophagy in the pathophysiology of preeclampsia; however, its effect on preeclamptic placentas remains unclear (16). In another study their findings indicate that inhibition of autophagy impairs EVT invasion and vascular remodeling. The authors propose that impaired autophagy may contribute to inadequate placentation (34). Additionally, Hung et al. claim that, compared to normal pregnancies, autophagy is more active in villous trophoblasts of placentas from pregnancies with preeclampsia and IUGR or IUGR alone (33). They detect a possible interplay between autophagy and apoptosis in IUGR, with p53 playing an important and complex role in regulating trophoblast cell turnover under hypoxic stress (63).

Current evidence suggests that autophagy may play a dual role in pregnancy. On the one hand, autophagy may support placental adaptations to various stressors encountered during gestation by inhibiting apoptosis and promoting homeostasis. On the other hand, dysregulation of autophagy could contribute to the pathogenesis of placental disorders. For example, some propose that self-destruction by autophagy has a unique role in protecting placental function during stressful periods, thereby optimizing fetal development. This protective mechanism is particularly critical in pregnancies complicated by preeclampsia or IUGR, where excessive inflammation and oxidative stress are common. In 2018, Aoki et al. presented the first report of autophagy deficiency leading to impaired placentation, which is

complicated by maternal hypertensive disorders due to trophoblast dysfunction. The authors suggested that placental autophagy is necessary for normal placentation. (35)

The dynamic interplay between autophagy and apoptosis in the trophoblast layer also requires further investigation. Disruptions in this balance could have significant implications for placental integrity and, consequently, fetal health. For instance, autophagy induction might counteract apoptosis in response to exogenous stressors, thereby sustaining trophoblast viability and placental function.

Furthermore, inflammation in the placenta is a finely tuned process that is essential for normal pregnancy progression. Aberrations in key inflammatory pathways are increasingly recognized as contributing factors to adverse pregnancy outcomes, including preeclampsia, IUGR, and preterm birth. Therefore, understanding how autophagy interacts with inflammatory pathways is a key element. Identifying these connections could reveal new therapeutic targets aimed at modulating inflammation and enhancing placental resilience under stressful conditions.

While much progress has been made in understanding the molecular basis of autophagy in non-placental tissues, its regulation and functional significance in the human placenta remain unclear. Future studies that systematically address these questions, particularly in the context of pregnancy complications, are essential for advancing research and developing clinically meaningful interventions.

5.1. Critical reflection / limitation in content and methodology

Although this thesis attempts to illuminate the relationship between autophagy and inflammation in the human placenta, several limitations in content and methodology must be acknowledged. First, the inherent complexity of placental biology makes it difficult to isolate specific pathways and interactions. Autophagy and inflammation are not isolated processes but rather integrated with multiple signaling pathways, cellular stress responses, and immune regulatory mechanisms. Consequently, the content of the different chapters may oversimplify the broader biological context. Additionally, the literature reviewed in this thesis largely consists of studies with *in vitro* models, animal experiments, or *ex vivo* placental tissue samples. While these models are indispensable for understanding molecular mechanisms, they may not fully capture the dynamic *in vivo* environment of the human placenta. An additional limitation relates to the heterogeneity within human pregnancies themselves. Factors such as maternal age, prior pregnancies, body mass index, pre-existing

medical conditions, and lifestyle habits can profoundly influence both autophagy and inflammatory responses. However, some of the included studies do not stratify their cohorts according to these variables, which may introduce confounding effects. These limitations underscore the need for future studies with more rigorous participant selection and stratification strategies to improve the reliability of findings.

Another methodological challenge is quantifying autophagic flux in human placental tissue. Mizushima et al. reported that that they were unable to measure autophagic flux in fixed tissues and that common autophagy markers do not work in tissue sections. Therefore, placenta-specific autophagy-deficient mice are necessary to evaluate the role of autophagy in placentation (35). Although markers such as LC3 accumulation and p62 degradation are often used to estimate autophagic activity, they provide only static snapshots that may not accurately reflect the dynamic nature of autophagic flux. Additionally, variability in tissue sampling, preservation methods, and assay standardization can complicate data interpretation further.

A major challenge in understanding the relationship between autophagy and inflammation is its bidirectional nature. Autophagy can modulate inflammatory responses, and inflammation can induce or inhibit autophagic pathways. Depending on the experimental context, current literature often presents conflicting results. For instance, some studies examining preeclampsia-related placental inflammation describe an increased autophagy activation as a protective response in the pathophysiology of preeclampsia, while others suggest the opposite. Such discrepancies may arise from differences in experimental design, sample processing, or severity of the disease. Therefore, caution is warranted when interpreting and generalizing such findings.

Finally, by its nature, this thesis is a literature review rather than a systematic review. Although efforts were made to incorporate a wide range of studies, potential biases in literature selection and interpretation cannot be fully excluded. Future systematic reviews and meta-analyses are needed to provide a more quantitatively robust assessment of the current state of knowledge.

This thesis highlights the roles of autophagy and inflammation in placental function and perinatal health; however, its content and methodology are limited by the complexity of biological processes, model-dependent findings, participant heterogeneity, and methodological constraints. Addressing these limitations through more integrative and

context-specific studies is crucial for improving our understanding of placental biology and its implications for maternal-fetal health.

6. CONCLUSION

The aim of this thesis was to highlight the roles of placental autophagy and inflammation, emphasizing their potential influence in normal pregnancies and pregnancy complications. A central challenge in this field is achieving a deeper understanding of the maternal-fetal immune interaction. This knowledge is essential for improving pregnancy outcomes as well as both maternal and fetal health. A more nuanced understanding of this interaction is necessary for developing targeted therapeutic interventions for pregnancy complications, and for informing public health policies, especially during pandemics, when pregnant women are particularly vulnerable.

Despite its importance in maintaining a successful pregnancy, the current understanding of autophagy in the placenta is still in its early stages, with many aspects yet to be explained. Further research into placental autophagy could offer valuable insights into the mechanisms underlying pregnancy complications associated with abnormal placental development. Advancing this knowledge could also pave the way for the development of innovative diagnostic and therapeutic strategies to improve both maternal and fetal health outcomes.

Accumulating evidence suggests that autophagy plays a crucial role in normal placentation and in the pathophysiology of obstetric disorders involving excessive inflammation. This dual role highlights the complex interplay between autophagy and inflammation in the placenta that may significantly influence the course of pregnancy and the development of complications, such as preeclampsia and IUGR. A better understanding of how autophagy modulates inflammatory pathways in the placenta could lead to new opportunities for intervention, especially in high-risk pregnancies.

Reproduction is a unique biological process involving complex events, such as gametogenesis, fertilization, and embryonic and placental development. Autophagy plays a fundamental role in these processes by maintaining cellular homeostasis, promoting cell survival, and mediating cellular interactions through the degradation of excess or damaged organelles or cellular components. This recycling and remodeling function is essential for normal placental development and for the overall success of reproduction.

Despite these insights, significant gaps remain in our understanding of the molecular mechanisms and functional significance of autophagy in reproductive biology. For example, the relationship between autophagy and the dynamic microenvironmental changes during gametogenesis is largely unknown.

Looking ahead, progress in understanding the multifaceted role of autophagy in placental and reproductive biology is expected to promote the development of innovative therapeutic strategies. Advances in this area could transform the management of pregnancy complications and reproductive disorders, ultimately improving maternal and fetal outcomes worldwide.

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