The role of endogenous pro-resolving lipid mediators in decidualization and pregnancy

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Resumo

A implantação embrionária é precedida por processos celulares vitais que promovem a reconfiguração do útero e são, largamente, influenciados pelo ciclo hormonal. Nos seres humanos, um dos eventos mais importantes é a diferenciação das células do estroma do endométrio em células deciduais. Este processo, conhecido como decidualização, inicia-se com o aumento dos níveis de progesterona, e envolve uma extensa reprogramação bioquímica conferindo às células deciduais um papel importante na recetividade uterina. É um processo suscetível ao ambiente inflamatório, que condiciona a proliferação e diferenciação das células do estroma do endométrio. Alterações na decidualização estão associados a uma diminuição da fertilidade. Assim, compreender o ambiente inflamatório associado à decidualização permitirá um maior conhecimento da saúde reprodutiva feminina, e ao mesmo tempo aprimorar o sucesso de intervenções clínicas personalizadas. Neste sentido, estudamos a expressão das enzimas 5-, 12 e 15-lipoxigenases, envolvidas no metabolismo de mediadores anti-inflamatórios, em tecidos maternos provenientes de interrupção voluntária da gravidez (controlo) e aborto espontâneo. Além disso, exploramos o envolvimento de quatro mediadores lipídicos com reconhecidas propriedades anti-inflamatórias, as lipoxinas A4 e B4 e resolvinas D1 e E2, na decidualização usando culturas primárias de células do estroma do endométrio, bem como, uma linha celular imortalizada de células do estroma, as St-T1b. Observamos um aumento significativo das enzimas lipoxigenases nas amostras de aborto espontâneo, em comparação com os eletivos, refletindo um desequilíbrio inflamatório profundo. Além disso, verificamos que as células natural killer deciduais têm um papel no controlo do ambiente inflamatório. Finalmente, observamos que os mediadores lipídicos estudados interferem com a decidualização em ambos os modelos celulares, além de induzirem alterações morfológicas profundas.

Abstract

Successful embryo implantation is preceded by a series of uterine remodelling processes and physiological changes driven by the hormonal cycle. One of the most crucial events is the transformation of endometrial stromal cells into decidual cells, known as decidualization, which is triggered by the increased levels of progesterone. Decidualization encompasses extensive genetic reprogramming, affecting hundreds of proteins and confers decidual cells with critical functions necessary for uterine receptivity. Aberrant decidualization is increasingly being recognized as an infertility factor, especially in cases of repeated pregnancy loss. As a multifaceted process, it can be impacted in several ways, one of which is the inflammatory environment in the uterus that directly affects the development of decidual cells. Performing thorough investigations into critical inflammatory mediators involved in decidualization is essential for advancing our knowledge of women's reproductive health. This thesis contributes to the advancement of knowledge in this field by quantifying the levels of lipoxygenase enzymes in samples from elective and spontaneous miscarriages. Additionally, it investigates the impact of four lipid mediators – lipoxins A4 and B4, and resolvins D1 and E2 – on the process of decidualization, utilizing primary endometrial stromal cells and an immortalized endometrial cell line, known as St-T1b cells. The research highlights a notable elevation in LOX enzymes in cases of spontaneous abortions compared to elective ones, underscoring a profound inflammatory imbalance. Furthermore, this thesis suggests a potential role for decidual natural killer cells in regulating the inflammatory milieu and, consequently, the extent of decidual cell differentiation. Lastly, incubation with these compounds results in a significant reduction in decidualization biomarkers in both St-T1b cells and primary cells, accompanied by marked morphological alterations.

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AA	Arachidonic acid
ALX	Lipoxin receptor
cAMP	Cyclic adenosine monophosphate
DHA	Docosahexaenoic acid
dNK	Decidual Natural killer
E2	Estradiol
EPA	Eicosapentaenoic acid
ESC	Endometrial stromal cell
FOXO1	Fork-head box protein 1
IFN- γ	Interferon-gamma
IGFBP-1	Insulin-like growth factor-binding protein 1
IL	Interleukin
LOX	Lipoxygenase
LX	Lipoxin
MMP	Metalloproteinase
MPA	Medroxyprogesterone acetate
NF-κB	Nuclear factor-kappa B
P4	Progesterone
PR	Progesterone receptor
PRL	Prolactin
ROS	Reactive oxygen species
RPL	Recurrent pregnancy loss
Rv	Resolvin

1. Introduction and objectives

1.1. Uterine structure and hormonal cycle

The uterus consists of two different regions: the uterine cavity and the cervix. Three layers comprise the uterine cavity. The outermost layer, perimetrium is made up of mesothelium and connective tissue. It is followed by the myometrium, which occupies a significant area of the uterus and plays a role in birthing contractions and menstruation. During pregnancy, it suffers hypertrophy and hyperplasia to accommodate fetal growth. The innermost layer, endometrium, consists of a luminal epithelial layer and a large stroma, presenting an abundant vasculature and glands, which suffer alterations in concordance with the uterine cycle. The endometrium can be further discriminated in two sections, the basal and functional regions; the latter is shed through menstruation while the former plays a vital role in regenerating the functional layer, on account of a relatively recently discovered stem cell population (1).

On average, a woman's cycle lasts 28 days (Figure 1) and can be divided into the ovarian and uterine cycle, which are interconnected. Estradiol (E_2) and progesterone (P_4) produced in the ovarian follicle trigger most of the events of the three uterine stages – menstruation, proliferative and secretory – through their receptors, estrogen receptor (ER-α, ER-β) and progesterone receptor (PR-A, PR-B) (2). As steroid hormones, E2 and P4 diffuse easily through the cell membrane, bind to nuclear receptors, and induce transcriptional changes. ER is expressed in the stroma and epithelium in the proliferative phase, inducing cell proliferation (3). The myometrium also expresses ER receptors and their effects peak in ovulation with strong uterine contractions, helping sperm cell movement through the female reproductive system (4). Cervical secretions decrease significantly in viscosity, an extremely important process to facilitate sperm entry (5). Estrogen mediates expression of PR in the endometrium and is indispensable in priming the uterus for pregnancy. A post-ovulatory rise in progesterone initiates the secretory phase and decidualization, described further below (6). There is a slight growth in uterine thickness due to estradiol, but this phase is best described by the increase in glandular growth and secretions, releasing important factors and nutrients for the implanting conceptus stimulated by progesterone (7). Uterine contractions decrease significantly, and cervical secretions increase in viscosity.

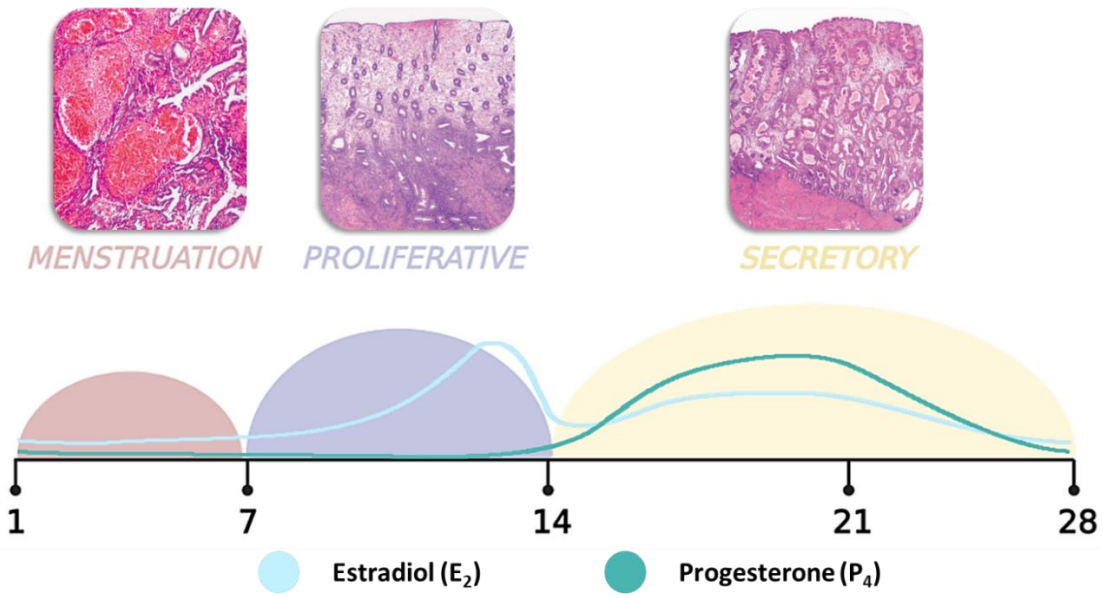

Figure 1. **The uterine hormonal cycle is comprised of approximately 28 days**. Fluctuations of estradiol (E2) and progesterone (P4) induce specific effects in all uterine layers, giving rise to three phases – menstruation, proliferatory and secretory. Representative images adapted from Histology at Yale, with permission.

1.2. Endometrial decidualization

Uterine remodeling and physiological changes that are determined by the hormonal cycle preface embryo implantation in humans. Endometrial stromal cells (ESCs) must proliferate and differentiate into decidual cells, a process termed decidualization (Figure 2). Through this transformation, decidual ESCs (dESCs) undergo genetic activation changes and acquire functions that are imperative to implantation and gestation. As such, it is comprehensible that abnormal decidualization may lead to implantation failures and infertility.

Decidualization occurs in species where there is invasion of the maternal layer by trophoblasts. Maternal blood comes in direct contact with the fetal chorion, known as hemochorial placenta. In this case, the maternal section of the placenta is equally shed at birth (8). Contrary to most species, human decidualization occurs spontaneously, independent of signaling by the conceptus. This means decidualization is not triggered with pregnancy, rather occurring in every hormonal cycle. Decidualized ESCs perform distinctive functions that contribute to uterine receptivity. These functions include facilitating and regulating trophoblast invasion and immunomodulation, which are crucial processes in establishing the immunological relationship between the mother and the conceptus (9). In addition, decidual cells display high oxidative resistance stress, in part, due to the higher expression of enzymes such as superoxide dismutase (10). Another

potential function attributed to decidual cells, as indicated by recent research, is their involvement in embryo biosensing, a topic that will be further investigated below.

Approximately six days post-ovulation, endometrial fibroblasts differentiate into large, rounded cells with a bigger cytoplasm and higher number of nucleoli, first appearing around the spiral arteries and later expanding to the entire functional endometrium (11). Endometrial stromal cells can be identified through their vimentin^{+}/desmin^{+}/cytokeratin^{-} expression as the trophoblast and epithelial cells express the opposite phenotype (12). The decidualized endometrium is transient and lasts around 4 days. This timeframe provides strong evidence for the concept of window of implantation, denoting a limited time where a blastocyst is able to implant, a theory that relies on the concept of a curated uterine environment. Decidualization occurs in response to the rise of progesterone, which increases local intracellular cyclic adenosine monophosphate (cAMP) levels. Deciduogenic signals, whether autocrine or paracrine, involve crucial contributions from epithelial cells (17). Additionally, there are other factors known to elevate cAMP levels, including relaxin, corticotropin-releasing hormone, and prostaglandin E2. In turn, inhibitory signals such as interferon-γ (IFN- γ) likely play a role in controlling decidualization (13).

Fork-head box protein-1 (FOXO1) is one of the earliest increasing factors in decidualization, responsible for the cell-cycle exit of stromal cells and commitment for differentiation and, thus, reconfiguration of genetic expression. Studies estimating dozens of altered genes with decidual stimulus (10,14,15). Protein synthesis and chromatin architecture remodeling are the main changes that occur during the process of decidualization (16). Additionally, decidualization is dynamic, with gene regulation fluctuating at different timepoints. Principal gene markers for decidualization commonly used in studies include FOXO1, prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP1), as some of the highest upregulated genes (13). Furthermore, dESCs development involves a mesenchymal-epithelial transition (18) with upregulation of conventionally epithelial genes, such as integrins, playing an extremely important role as adhesion factors, favoring recognition of the blastocyst.

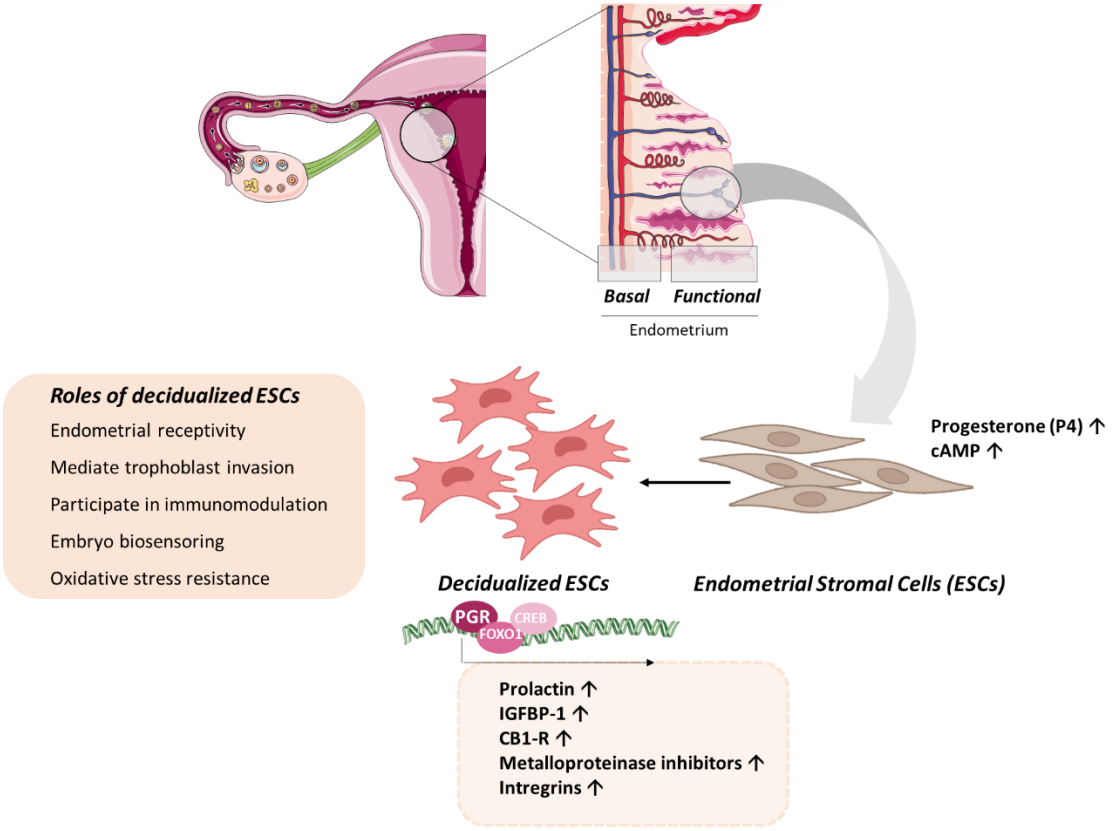

Figure 2. Endometrial decidualization. During early secretory phase, stromal cells respond to the rise of P4 and cAMP by undergoing genetic reconfiguration and differentiating into decidual cells, with a unique transcriptional network and critical roles in pregnancy.

Recent research has revealed that epigenetic mechanisms play a significant role in governing the expression of decidualization-related genes, showing that histone modifications occur throughout differentiation. Heightened levels of H3K27ac and H3K4me3 are primary facilitators of transcription (19), demonstrating a strong connection between gene regulation and global alterations in histone modifications in the decidualization process. However, despite this, the exact implications of epigenetic dysregulation leading to decidual insufficiency and implantation failure remain unclarified.

The first trimester maternal tissues have a large number of natural killer (NK) cells, termed decidual NK (dNK) cells, which make up 70% of the immune cell population in this tissue, mainly in the peri-vascular regions (20). Through the action of progesterone, proinflammatory cytokines and chemokines, especially CXCL10 and CXCL11 are believed to help recruit these cells (21). While most peripheral blood NK cells belong to the $CD56^{dim}/CD16^{+}$ subtype, dNK cells are $CD56^{bright}/CD16^{dim/-}$, a subtype characterized by higher cytokine production, but lower cytotoxicity (22). Research suggests this

subtype represents an earlier stage of NK cell maturation and the brightness of CD56 correlates with their activation potential (23). Activated $CD56^{bright}$ cells can function either as pro-inflammatory anti-tumoral effectors (24) or in a regulatory, immunotolerant context (25).

The importance of dNKs in implantation is uncontested, although complex and multifaceted. One of the main functions of dNKs is immune regulation and maintenance of maternal-fetal tolerance (26). By interacting with other immune cells, such as T-regulatory cells, they participate in the production of several cytokines and chemokines, such as IFN- γ, interleukin (IL)-10 and transforming growth factor-beta, which can modulate the immune population of the placenta. Further, dNKs secrete vascular endothelial growth factor, placental growth factor and angiopoietin-1/2, among other angiogenic factors, promoting the growth of blood vessels in the decidua and facilitating blood supply to the placenta. The production of matrix metalloproteases (MMPs) also underpins their possible role in extracellular matrix remodeling (27).

1.2.1. Clinical perspectives

Cyclic decidualization underlies menstruation. In the absence of pregnancy, the ovarian corpus luteum regresses and progesterone levels decline. In fact, the decidual phenotype is transient, and relies on maintenance by progesterone (28). Studies show the low levels of this hormone trigger rapid nuclear localization of FOXO1, increasing transcription levels of the inflammatory nuclear factor κB (NF-κB) pathway and apoptosis-associated factors, such as Bcl-2-like protein-11. Thus, uterine FOXO1 is the link between spontaneous decidualization and menstruation (28,29). The following reactions, including necrosis, immune cell influx, tissue breakdown and bleeding allow the disposal of the decidual layer, in the absence of pregnancy.

Menstruation could be colloquially thought of as “evolutionary regression”, but the advantage has been explained through several theories. Currently, there is very strong evidence for a role in embryo biosensoring for decidualized cells. As opposed to mice where an implanting blastocyst triggers the endometrial reaction and promotes adhesion independently of the viability or quality of the embryo (30), decidual cells have been shown to actively recognize their metabolic quality through crosstalk and modulate the uterine environment to promote or reject implantation. Brosens *et al*. elegantly showed that, when decidualized cells were exposed to conditioned medium from pre-implantantion *in vitro* embryos, a significant differential transcript regulation response was observed. Namely, healthy embryos triggered 15 transcriptionally altered genes,

whereas in the case of impaired embryos, as many as 449 genes exhibited alterations. Upon analyzing the regulated transcripts through gene ontology, it was demonstrated that healthy blastocysts elicited a discrete but implantation-positive response, whereas lower quality embryos triggered stress (30,31). Another study suggested decidual cells actively migrate towards and accept high-quality embryos (32). It is therefore suggested specialized dESCs select and commit to healthy pregnancies, conferring an evolutionary advantage. Embryos that do not successfully implant are shed during menstruation, before any detectable signs of pregnancy (33).

Another benefit of cyclic decidualization and menstruation is higher fecundity through preconditioning, which means menstruation prepares the uterus for a possible pregnancy involving similar processes such as production of reactive oxygen species (ROS), vascular changes and inflammation (34). Repeated shedding followed by reconstitution of the endometrial layer is accompanied by recruitment and activation of progenitor cells, improving the resilience and overall health of the uterus over time by expanding the residing population of mesenchymal and epithelial stem cells that are responsible for regenerating the endometrium (35). Such a complicated physiological orchestration provides even more reasons why studying maternal-factor infertility requires more studies, to enable personalized clinical care.

Disordered uterine differentiation may underlie a higher percentage of miscarriages than previously thought. While most miscarriages are caused by genetic embryo abnormalities (36), conditions such as recurrent pregnancy loss (RPL) suggest there are instances where the uterus fails to recognize the presence of an impaired embryo. In fact, RPL patients have been shown to express lower levels of decidualization markers PRL and IGFBP-1. However, the endometrium of these patients expressed higher levels of prokinetin-1, a key implantation molecule. Additionally, prokinetin-1 declined significantly later, compared to women who did not experience RPL (37). Further, the endometrium seems to be comparatively more receptive, as women with RPL tend to have shorter intervals between pregnancies preceding miscarriage. This paradoxical situation demonstrates how impaired decidualization and alterations in the implantation window can disable biological functions such as embryo quality selectivity.

Further, some pregnancy complications may have much earlier origins. Preeclampsia, or maternal hypertension, reflects poor spiral artery remodelling and less pronounced trophoblast invasion and implantation (38). Both symptoms can arise from defective programming of the uterus. A fascinating study by Garrido-Gomez *et al*. evaluated the decidual response from control women as well as women previously affected by

preeclampsia in pregnancy. In women with a history of preeclampsia, it was observed that ESC primary cultures exhibit a biochemical and morphological inability to decidualize, while also displaying significant alterations in gene expression. Moreover, conditioned medium from these cells did not promote trophoblast invasion in culture. These observations suggest the existence of a maternal factor for these conditions, that persists even after the occurrence of pregnancy complications (39).

1.3. Inflammation: a two-phase process

Inflammation is a reaction to tissue damage or foreign substances/organisms. However, its focus is the organism's return to physiological homeostasis. We now know that inflammation is tightly controlled and must be immediately followed by a resolution phase for this reason. Failure to dissipate the inflammatory environment is the basis of many chronic diseases, such as autoimmune conditions and fibrosis. Resolution was thought to be passive, which occurred upon depletion of all inflammatory factors in the tissue. The discovery of lipid mediators, as well as proteins such as annexin and neuromodulators, introduced the notion that resolution is an active process (Figure 3).

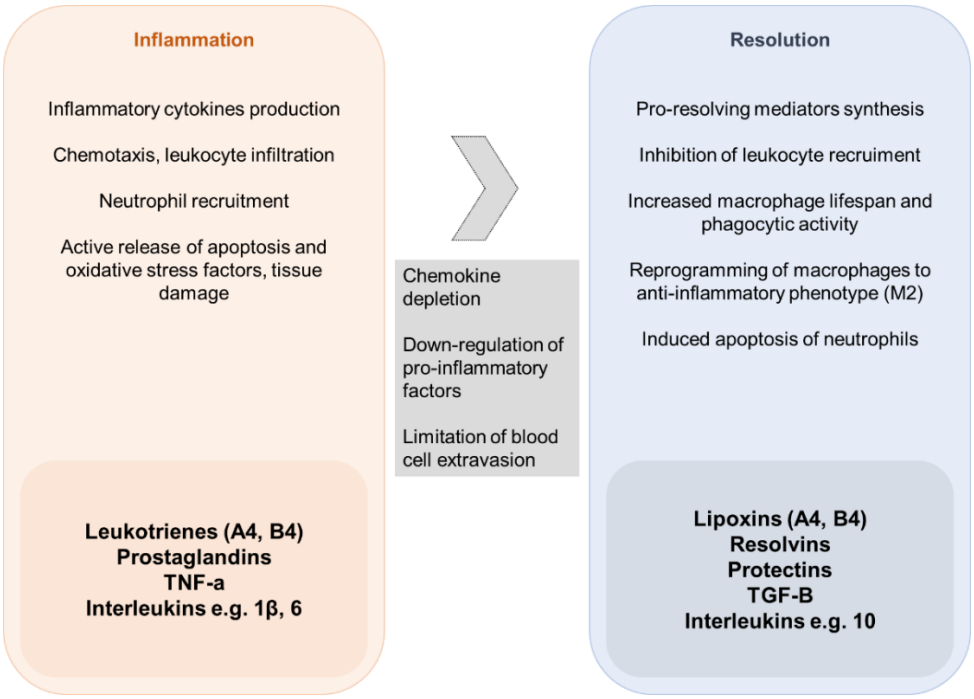

Figure 3. **Review of the inflammatory and resolution hallmark processes**. Local mediators effect a switch between immune cell activation profiles and enable a return to tissue homeostasis.

During inflammation, leukocytes are recruited through chemotaxis and activated to exert their functions, leading to tissue damage, oxidative stress, and apoptosis. Thereafter, anti-inflammation and pro-resolving factor levels increase, leading to leukocyte clearance, increased rate of M2 macrophage polarization – which is regarded as an anti-inflammatory macrophage – phagocytic activity and apoptosis of neutrophils (40). Lipid mediators play a crucial role due to their lipophilic properties. Their ability to diffuse easily across cell membranes allows rapid signaling and the propagation of effects within the tissue.

The biosynthesis of lipid mediators involves three fatty acids, namely arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are obtained through diet and metabolized into pro-resolving mediators (Figure 4). Different lipoxygenase (LOX) enzymes catalyze the conversion of fatty acids into various lipid mediators, leading to the production of lipoxins, resolvins and other related compounds. While AA metabolites are lipoxins (LX) A4 and B4, EPA produces E-series resolvins and DHA generates D-series resolvins (41). Lipid mediators are generally produced in stages by multiple cells, a process known as transcellular biosynthesis (42). As AA is released to the extracellular space, it is immediately taken up and used by cells, in two major routes. The first pathway involves 5-LOX that culminates in the production and release of the pro-inflammatory mediator LTA4, which can be converted to anti-inflammatory LXA4 and LXB4 by 12-LOX or 15-LOX. The second pathway involves the action of 15-LOX on AA, forming an intermediate non-bioactive molecule, 15S-H(p)ETE which can be converted by 5-LOX into LXA4 and LXB4. Additionally, mediators can arise from an alternative, aspirin-mediated process, involving the cyclooxygenase-2 (COX-2) enzyme (43).

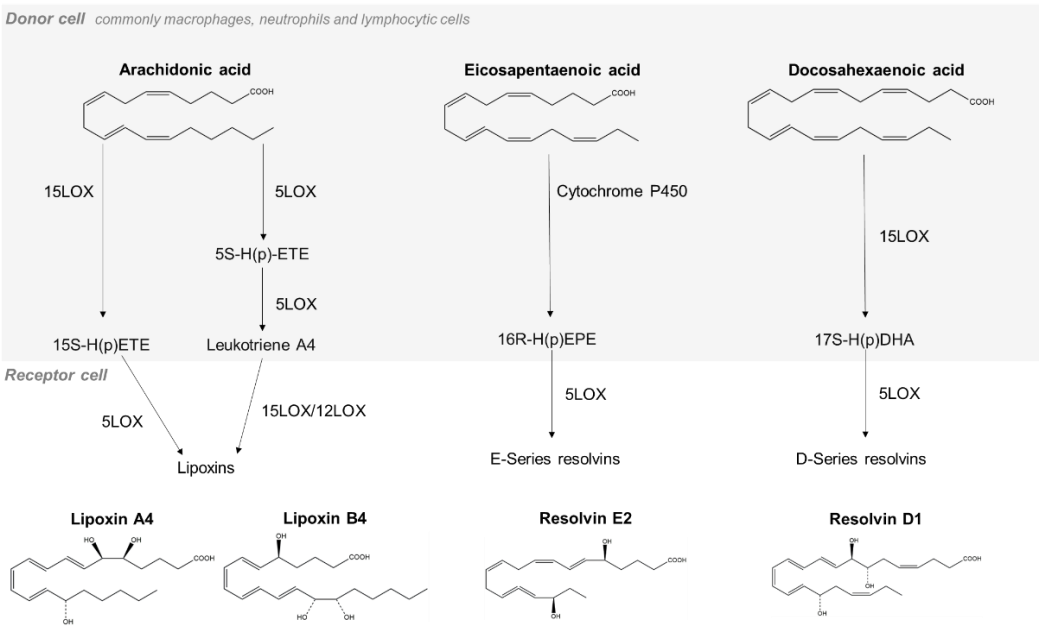

Figure 4. Biosynthesis pathway of lipid mediators. Lipid mediators, such as lipoxins (LX) A4 and B4 from arachidonic acid, E-series resolvins from eicosapentaenoic acid, and D-series resolvins from docosahexaenoic acid, are generated in a multi-stage process known as transcellular biosynthesis.

Lipoxins and resolvins are considered as specialized pro-resolving mediators because they inhibit inflammation signals, but also promote and activate resolution pathways. As such, they are one of the only mediators known to exhibit both properties, alongside protectins and maresins. Their effects are mainly propagated by binding to a G-coupled lipoxin receptor (ALX) (44), but they have been shown to interact with other receptors such as estrogen receptor, which is especially interesting in a pregnancy environment (45). Upon interaction with ALX, several cell reactions are triggered, such as the internalization of the receptor towards the perinuclear region. The impact of this interaction on the proliferative PI3K-AKT pathway varies depending on the cell type. Generally, in immune cells such as neutrophils, it exerts an inhibitory effect. However, in macrophages, it has a positive influence as ALX activation leads to an increased lifespan and M2 polarization, enhancing phagocytic activity (46,47). Finally, lipoxins control synthesis and release of pro-inflammatory cytokines by increasing the transcription level of suppressors of cytokine signaling and preventing transcription of inflammatory cytokines, namely IL-8 by inhibiting nuclear accumulation of NF-κB (48,49).

1.3.1. Inflammatory effects in decidualization and pregnancy

Decidualization is recognized to have two phases, with distinct transcriptional programs, as a result of the inflammation mediators produced locally. In early decidualization, the differentiation process triggers a pro-inflammatory response, dominated by STAT signaling. Studies supporting this have reported a beneficial role of inflammation, including higher decidual cell growth and increased success of *in vitro* fertilization following uterine scratch or biopsy (50). Further, uterine scar tissues from previous surgeries have been identified as a preferred implantation site (51). Salker *et al.* conducted a PCR array analysis and identified 70 upregulated inflammatory cytokines and receptors following 2 days of decidualization, compared to the control group. In the late phase, a state regulated by the NF-κB pathway emerges, characterized by increased NF-kB signaling, which has been previously linked to menstruation. After 8 days of differentiation, only 12 transcripts remained elevated while many were expressed below control levels, underpinning an anti-inflammatory effect (52).

Early decidualizing cells express IL-11 and IL-1β, both cytokines with pro-inflammatory activity (53,54), in a manner that coincides with IGFBP-1 and PRL rise (55). In fact, inhibition of IL-11 diminishes the expression of these decidualization markers, suggesting a role for inflammation in their differentiation (56). Decidualized ESCs also release IL-33, a cytokine belonging to the IL-1 family which triggers helper T cells to produce type 2 inflammation (52) – protective inflammation – through the induction of cytokines IL-4, IL-5, IL-9, and IL-13. It has also been described that the release of IL-33 is disordered in RPL, where prolonged expression extends the implantation window and renders the uterine environment less protective, increasing the probability of miscarriage (52). Interestingly, this also suggests that inflammation is necessary for the expression of receptivity factors.

The source for inflammatory factors promoting decidualization is complex and involves several cell types. Recently, an interesting theory emerged proposing the existence of a subpopulation of ESCs that do not differentiate, but through FOXO1 signalling, undergo acute senescence and become an important source for pro-inflammatory factors. These factors promote decidualization of neighbouring cells (57). Subsequently, elimination of senescent cells by dNK cell recruitment and activation culminates in endometrial remodelling, suggested by co-culture studies, where dNK cells cleared senescent cells and formed cavities in the decidual layer, which is beneficial for trophoblast invasion (58). In a pathological situation, inflammation persists beyond the timeframe, transmitting the senescence-associated phenotype to healthy cells (59).

Emerging research focusing on anti-inflammatory lipid mediators shed light on their potential role in implantation. Based on current understanding, processes such as ovulation, menstruation, embryo implantation and childbirth are all short-term yet essential inflammatory events. Among the various lipid mediators, LXA4 has garnered significant attention (Figure 5). LXA4 levels are dynamic and reflect the different phases of pregnancy. Low levels are observed in the follicular and early luteal phase, increasing significantly towards the end of the monthly cycle. Lower levels during early luteal phase could be reflective of its undesirable action at this time. Following implantation, it seems to be crucial for LXA4 levels to rise, as this promotes proper placental development and fosters a favourable environment (60). Additionally, Xu *et al.* showed that LXA4 interferes with the synthesis of many implantation factors, including matrix MMP-9, β-catenin and vimentin (61). Altered levels of LXA4 have been associated with spontaneous miscarriage (62,63).

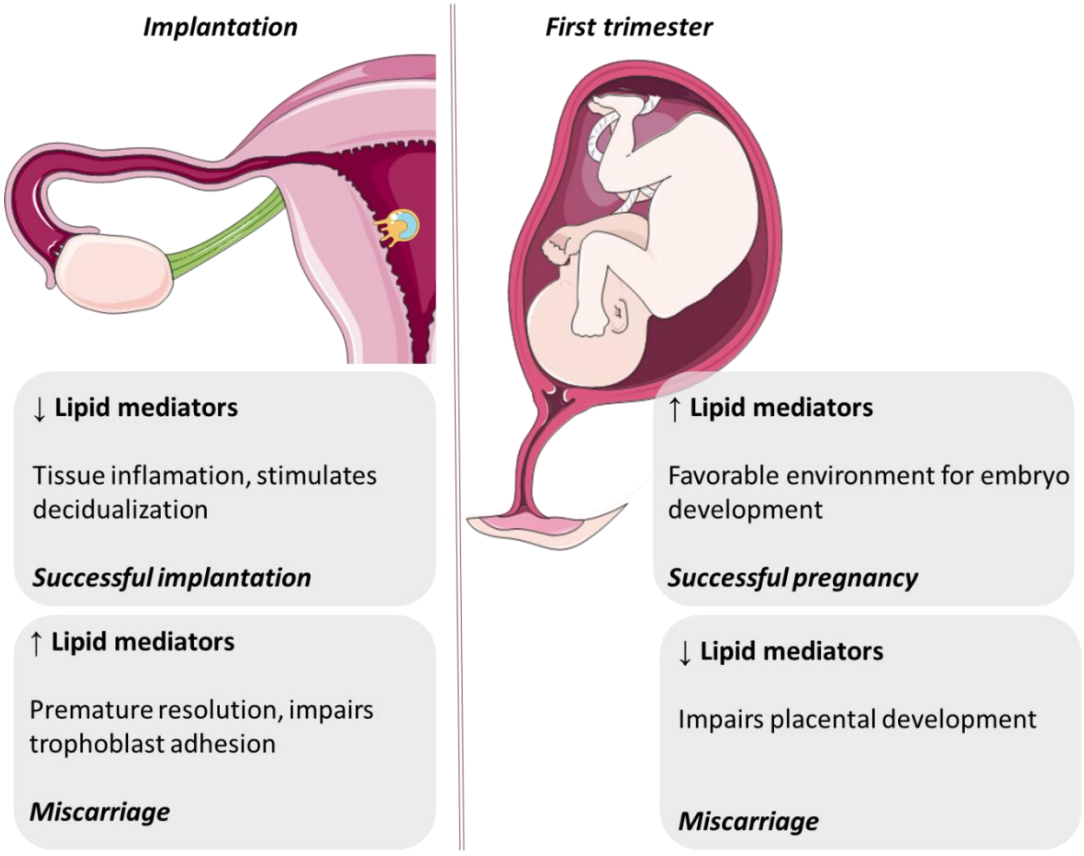

Figure 5. Current correlations between lipid mediator (LXA4) levels in pregnancy stages and their outcome.

2. Aims

Spontaneous miscarriages are often attributed to genetic defects in embryos that inhibit their progression to a full-term pregnancy. However, a significant percentage of abortions have no discernible cause, termed idiopathic abortions. There is a growing body of evidence pointing to maternal factors, especially uterine receptivity, as potential causes. Uterine receptivity is tightly linked to decidualization, a process regulated by inflammatory signals. Although the importance of inflammation in pregnancy is uncontested, it remains an underexplored area. This thesis seeks to advance our understanding of female reproductive research by investigating the inflammatory environment present in first-trimester maternal tissues.

To achieve such objectives, it is proposed to:

1) Study 12-, 15- and 5-LOX metabolic enzymes in first trimester human decidual tissues of gestational age obtained from pregnancies which were voluntarily terminated or from women who suffered a miscarriage.
2) Examine how LXA4, LXB4, RvD1, and RvE2 impact the differentiation of endometrial stromal cells *in vitro*, employing both primary endometrial cells and an immortalized endometrial cell line.

2. Methods

2.1. Tissue collection and processing

This research received approval from the ethics committee at Centro Materno Infantil - Centro Hospitalar do Porto. First-trimester human decidual tissues were collected with informed consent. The tissues were collected at the gestational age from 5 to 10 weeks in the group of pregnancies which were voluntarily terminated, for non-clinical reasons, or 5 to 12 weeks from women who suffered a miscarriage. Sample characterization was performed through statistical testing regarding age, gestational age, obstetrical history, and fertility history of both groups. These samples were processed for RNA and protein extraction.

2.2. RNA extraction and real-time qPCR analysis

TripleXtractor (GRISP, GB23.0100) was added to the tissues to lyse cells and release cellular contents. Following homogenization, chloroform was added to separate the samples in three phases, a translucent upper phase where RNA is present, a white interphase and a lower red phenol-chloroform phase, containing DNA and proteins. Following a 2-minute incubation with chloroform, the tubes were centrifuged at 12000 G for 15 minutes. Then, the upper phase was collected and transferred to a new tube, using a pipette. Isopropanol was added to precipitate RNA with a 10-minute incubation, followed by centrifugation at 12000 G for 10 minutes. The RNA pellet that formed was washed two times with 75% ethanol, each with a centrifugation step of 12000 G for 5 minutes. After the final washing step, tubes were carefully inverted and air-dried for 10 minutes, to remove ethanol residue. Finally, the washed RNA pellets were resuspended in DEPC-treated deionized water, and incubated at 55 ºC for 10 minutes, to promote dissolution. Concentration and purity were determined by measuring absorbance at 260 and 280 nm and calculating the A260/A280 ratio, using a NanoDrop ND-1000 spectrophotometer. This method relies on nucleic acids exhibiting maximum absorbance at 260 nm, whereas proteins are generally measured at 280 nm. Extractions performed along this thesis were routinely above 1.6 in the purity ratio. Due to RNA's instability, it must be converted into cDNA prior to any PCR analysis. This was performed using Xpert cDNA synthesis kit (GRISP, GK81.0100). The expression of LOX enzymes was evaluated in real-time qPCR reactions, using designed specific primers and our determined optimal annealing conditions, in Bio-Rad MiniOpticon Real-Time PCR Detection System (Table I). Two housekeeping genes were tested, β-actin and GAPDH,

which both produced similar results. Finally, levels of expression were determined relatively to the housekeeping β-actin, following the $2^{-\Delta\Delta ct}$ method.

Table I. Primers used for qPCR and amplification protocols.

Target	Sequence (Forward 5')	Sequence (Reverse 5')	Cycle conditions
IGFBP-1	GAGATAACTGAGGAGGAG	CCAAAGGATGGAATGATC	Initial step 95 ºC 3 min 40 cycles 95ºC 3s, 60 ºC 30s, 72 ºC 30s
PRL	GAGACACCAAGAAGAATCGGAA CATACAGG	TCGGGGGTGGCAAGGGAAGAA	Initial step 95 ºC 3 min 40 cycles 95ºC 3s, 59 ºC 30s, 72 ºC 30s
Actin	TGCCATCCTAAAAGCCACCC	AGACCAAAAGCCTTCATACATCTC	Initial step 95 ºC 3 min 40 cycles 95ºC 3s, 55 ºC 30s
12-LOX	GCCCAAAGCTGTGCTAAACC	TGGGGGAGGAAATAGAGCCT	Initial step 95 ºC 3 min 40 cycles 95ºC 3s, 60 ºC 30s
15-LOX	GCACCCAAGAGTACCAGTCC	GCTGTGAAAGACGACCCAGA	Initial step 95 ºC 3 min 40 cycles 95ºC 3s, 58 ºC 30s
5-LOX	AGGGGTCTGTTTTGTTGGCA	TGGCGCGGTGGATTCATAC	Initial step 95 ºC 3 min 40 cycles 95ºC 3s, 58 ºC 30s

2.3. Protein extraction and Western-blot analysis

For protein extraction, tissues were maintained in ice and lysed in Lysis buffer (1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, and 18 mM KH_2PO_4, pH=7.4, 1% Triton X-100) supplemented with protease inhibitors (Sigma Aldrich, P8340) and centrifuged at 12000 G for 10 minutes at 4 ºC. The supernatant containing the protein lysates was transferred to a new tube and stored at -80 ºC. Protein was quantified using the Bradford standard method, with known bovine serum albumin concentrations. Western blotting was performed by separating 25 µg of protein from each sample in a 10% polyacrylamide gel and transferring in a Bio-Rad semi-dry Trans-Blot Turbo system. The membranes were blocked for unspecific binding with 5% non-fat milk in TBS-Tween and primary antibodies targeting the LOX enzymes and the loading-control actin were incubated, overnight at 4 ºC. In the following day, the membranes were washed and incubated with the secondary antibody conjugated with horseradish peroxidase enzyme (Santa-Cruz, 516102), for 1 hour at room temperature. Chemiluminescent signal was visualized in Bio-Rad

ChemiDoc Touch Imaging System after incubation with substrate and signal enhancer in 1:1 ratio (Advansta, K-12045-D50).

2.4. Immunohistochemistry detection of LOX enzymes

Microscopic slides of first trimester placental tissues were prepared using a Leica RM2145 microtome. Paraffin-embedded tissues were sectioned to a thickness of 4 μM and placed in water heated at 40 ºC for approximately one minute, to minimize tissue folding which would impact staining procedures. Sectioned tissues were collected onto 3-aminopropyltriethoxysilane (Sigma A3648) coated microscopic slides and placed in an incubator at 42 ºC overnight to optimize adherence of the tissues.

Immunohistochemistry was performed using Vectastain® ABC-AP Kit (AK-5002-NB), following their guidelines. In short, tissues were dewaxed and hydrated in xilol and decreasing ethanol concentrations, with a final step in tap water, for approximately 20 minutes. Then, tissues were blocked in normal horse serum for 30 minutes at room temperature and incubated with primary antibody overnight at 4 ºC (Table II). The next day, tissues were washed in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and incubated with biotinylated secondary antibody for 30 minutes. After incubating with ABC-AP reagent containing avidin and biotinylated alkaline phosphatase, AP substrate solution (Sigma Aldrich, F4648) was added, and tissues were incubated for an appropriate amount of time for optimal signal to develop. Finally, a counterstain with hematoxylin was performed to enhance tissue visualization, and the slides were mounted in aqueous medium (Sigma Aldrich, C9368). This technique was additionally performed on our primary cell cultures to confirm expression of LOX enzymes directly in ESCs. In this case, cell-containing slides were fixed in methanol and blocked in 2% bovine serum albumin in PBS-Tween, following the same protocol thereafter.

Further, immunofluorescence was performed, with the aim of pinpointing cell-types expressing our target enzymes. In this case, tissues were dewaxed, hydrated, and blocked as described, and incubated with primary antibody for one hour at room temperature. Following, they were washed in PBS 1X and incubated with fluorescent secondary antibody for one hour. Tissues were once again washed, two times in PBS to prevent background staining, and finally incubated with a direct fluorochrome-marked antibody for an hour. Slides were mounted in DAPI-containing medium to stain nuclei (Sigma Aldrich, F6057). All slides were observed using a Nikon Eclipse CI fluorescent

microscope fitted with an excitation/emission filter array from Semrock and analyzed with Nikon NIS Elements Image Software. Further image editing was performed with ImageJ.

Table II. References for antibodies used for first trimester tissue immunohistochemistry techniques and their respective dilutions.

Primary Antibody	Dilution	Secondary Antibody	Dilution
12-LOX (Thermofisher, MA5-26911)	1:250	Goat anti-mouse IgG-FITC (Santa-Cruz, 2010) (IF)	1:250
15-LOX (Santa-Cruz, 133085)	1:250 (IF) 1:500 (IHC)	Horse anti-Mouse IgG-Texas Red (Vector Labs, TI-2000-1.5) (IF)	
5-LOX (Thermofisher, MA5-26829)	1:250	Vectastain ABC-AP Kit Biotinylated Antibody (AK-5002-NB) (IHC)	
CD56-PE (Biosciences, 345812)	1:200	-	-
CD68-FITC (Invitrogen, 11-0689-42)	1:200		

2.5. Cell culturing and isolation of primary cells

We established primary cultures of endometrial stromal cells by utilizing decidual tissue obtained from the term placenta of five healthy women at full-term pregnancy. Collected tissues were placed in normal saline and promptly transported to the laboratory within one hour. They were then thoroughly washed with PBS to remove any blood clots and kept on ice until further processing. In short, after extensive washing, the decidua was separated from the chorionic membrane and dissected. Tissues were further enzymatically dissociated by incubation with collagenase I at 1 mg/ml for an hour at 37 ºC with shaking. Following dissociation, blood cells were lysed using an ammonium chloride solution (0.8 g tissue culture grade NH_4Cl, 0.084 g $NaHCO_3$, 0.037 g EDTA in 100 ml water) for 5 minutes. This resulted in a loose cell suspension, which was filtered through a 40 µm nylon mesh (BD Biosciences, 352340). The filtrate was centrifuged at 280 G for 10 minutes to obtain a cell pellet. Finally, the isolated cells were plated. In the day after, medium was exchanged to remove non-adherent cells, which are mostly bone-marrow derived cells that do not adhere to plastic flasks.

The St-T1b cell line, as well as endometrial stromal cell primary cultures were used for experimentation. The telomerase-immortalized human endometrial stromal cell line was kindly supplied by Doctor Birgit Gellersen from Endokrinologikum Hamburg, Germany. Both cell groups were maintained in Dulbecco′s Modified Eagle′s Medium/Nutrient Mixture F-12 (DMEM/F12, Sigma Aldrich D2906), supplemented with sodium

bicarbonate at 1.2 g/l, fetal bovine serum 10% (PAN Biotech, P30-3031), antibiotic/antimycotic 1% (PAN Biotech, P06-07100), insulin 1 µg/ml (Sigma Aldrich, I0516) and estradiol 1 nM (Sigma Aldrich, E2758). According to cell exponential growth phases, experiments were initiated from the third passage of cells. The ST-T1b cell line present a particular limitation as their response to differentiation stimuli decreases along the passages, related to their expression of progesterone receptors. To counter this, cells were routinely frozen and maintained for a short number of passages. Conversely, primary cells do not face this problem. They are responsive and reliable, having received hormonal conditioning from the woman's physiological system and environment before harvesting. Following the mentioned isolation protocol, the primary cells were cultured until they reached exponential growth, around passage 3, and used for experiments.

2.6. Morphology and viability studies

To determine a concentration of the compounds that would be used for decidualization experiments but not impair the viability of cells, the MTT assay was performed. Cells were trypsinized at around 80-90% confluency and resuspended in medium. Using a Neubauer counting chamber and following the trypan blue method, cells were counted. When incubating with trypan blue, viable and healthy cells do not uptake the dye and show indirectly, as unstained cells. Cells that have lost membrane integrity and selective permeability are stained in blue. Using this method, the estimated number of cells were determined, and an equal number of cells were plated into each well. MTT assays were performed in 96-well plates with a density of 4000 cells/well. Each compound (LXA_4, LXB_4, RvD1, RvE2, Cayman Chemicals) was incubated in triplicate for three test concentrations (0.001 µM, 0.01 µM, 0.1 µM), for 24, 48 and 72 hours. Following incubation, 20 µl of MTT was pipetted into each well (final concentration of 0.5 mg/ml). The MTT method relies on the mitochondrial conversion of MTT into purple formazan crystals that are detectable spectrophotometrically. The intensity of the purple signal will directly correlate to cell density, and viability. Thus, we can distinguish, in relation to the control, wells that have lost cell density and integrity. MTT was incubated for two hours, then the crystals were suspended in a DMSO/isopropanol 3:1 solution, and the plate was read at 540 nm. Results were expressed as percentage of control and statistically analysed.

The morphology of the cells was evaluated by Giemsa staining. Cells were cultured in 24-well plates for Giemsa staining, to qualitatively assess their response to the compounds. Following a wash in PBS, the cells were fixed in methanol for 30 minutes at

4 ºC, and the plates were stained with Giemsa for one hour at room temperature. Following the staining, plates were washed in PBS 1X several times and mounted in DPX medium (Merck Chemicals, 10197905000). Representative images of each well were captured.

2.7. Decidualization assays

To evaluate the influence of lipid mediators in decidualization, the St-T1b cell line and primary cells were used for experimentation. Following trypsinization, as described, cells were plated at a density of 25.000 cells/well in 24-well plates. In this case, the medium used was not supplemented with estradiol and insulin, as estradiol stimulation would interfere with the validity of our results. The cells were decidualized by stimulation with 0.5 mM 8-Br-cAMP and 1 µM medroxyprogesterone acetate (MPA) for five days. This synthetic progesterone analogue is not degraded in culture; thus, it is able to maintain the cells for the entire experiment. Experiment design included non-differentiated and differentiated controls, followed by differentiation stimulus in the presence of each lipid mediator. RNA was extracted following the described protocol and converted into cDNA. Through real-time qPCR, using primers for the established decidualization markers (IGFBP-1 and PRL, Table I) we can evaluate and determine the expression levels relatively to the control, using actin expression as an endogenous housekeeping gene. Giemsa stainings were performed in parallel, exactly as described above, to evaluate the morphology of decidualizing cells.

2.8. Statistical analysis

The data was graphed and analyzed in GraphPad Prism (GraphPad PRISM v. 9.1, San Diego, CA, USA). The provided results were expressed as the mean±SEM (standard error of the mean) of at least three replicates for each experiment. $P<0.05$ values were considered statistically significant. The results from qPCR and Western-blot experiments were analyzed using the non-parametric Mann-Whitney test. Decidualization assays were first approached using normality tests. When normality was observed, data was analyzed through ANOVA and Dunnett's multiple comparisons test. Data that did not follow normal distribution was analyzed through Kruskal-Wallis test.

3. Results

3.1. Differential expression patterns for LOX enzymes in placenta

First trimester samples were obtained from two distinct groups, women who experienced miscarriages (sporadic), and a control group of women who voluntarily terminated their pregnancy (elective). The descriptive statistics for age and reproductive history of the women are shown in Table III, as well as the P-value pertaining to a t-Student test for each category. Miscarriage samples originate from significantly older women, which is consistent as the abortion probability increases with age. This is a limiting factor in this work, which has to be taken into consideration, as we cannot exclude genetic causes. Interestingly, miscarriage samples originate from women who exhibit higher rates of parity (number of deliveries at 24 weeks or more, irrespective of the viability of the fetus) and gravidity (number of times the woman has become pregnant). Once again, these differences are limiting, but are not entirely unexpected. As mentioned, studies suggest women who experience recurrent miscarriages actually have a less selective uterus, which favours pregnancy, disregarding potential physiological imbalances that could later have an impact on gestation (37).

Table III. Age and fertility history of women with elective or voluntary miscarriage, represented as mean ± standard deviation, or median (interquartile region). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Parameters	Elective (n=9)	Sporadic (n=18)	P value
Age (years)	27.0±8.7	35.7±3.5	0.0187*
Gravidity	1.0 (1.0-2.0)	3.0 (1.7-3.3)	0.0346*
Parity	0.0 (0.0-0.5)	1.0 (0.75-2.0)	0.0066**
Miscarriage	0.0 (0.0-0.0)	1.0 (1.0-2.0)	<0,0001***
Gestational age (weeks)	8.0 (6.0-10.0)	7.0 (6.0-8.0)	0.4429

LOX enzymes are typically associated with immune cells. However, studies have demonstrated their presence in epithelial cells and even in stromal cells of diverse tissues, such as the lung, brain, and intestine. In order to evaluate LOX enzymes expression profile in first trimester maternal tissue, immunohistochemistry was performed. The expression patterns of the enzymes differed. The immunohistochemical staining pointed to a higher expression in sporadic miscarriage samples (Figure 6).

12LOX was detectable throughout the tissue, seemingly expressed by various cell types, including endometrial stromal cells (ESCs) and trophoblastic cells in chorionic villi. A similar pattern was observed for 5LOX, though the levels of expression in stromal cells appear lower. In contrast, 15LOX displayed a notable and distinct pattern of expression, with ESCs showing markedly lower levels of expression. Moreover, 15LOX expression was observed in endothelial cells. Interestingly though, sparse cell clusters or solitary cells along the stroma exhibiting positive staining for 15-LOX were found, often adjacent to the vessels. Additional cells inside the vessel were positive for 15-LOX.

To confirm the staining of ESCs, primary cell cultures were concurrently subjected to staining using primary antibodies targeting the enzymes at both non-differentiated and differentiated stages. All enzymes were found to be expressed in ESCs. No discernible differences were apparent between the groups (Figure 7). Curiously, 12-LOX exhibited nuclear localization in all replicas of the assay, while 5 and 15-LOX appear distributed throughout the cytoplasm.

These results prompted further research to confirm the other cell type present. Immunofluorescence co-staining techniques were employed targeting the immune cells that could be expressing these enzymes. To achieve this, the tissues were co-stained for LOX enzymes as well as immune cell markers for macrophages (CD68) and dNKs (CD56). We confirmed that the isolated cells expressing 15LOX were $CD56^{+}$ (Figure 8) and no co-expression was observed in macrophages. The $CD56^{+}$ cells were found neighbouring the spiral arteries, which is consistent with previous research on the localization of dNKs (64). Consistent with our immunohistochemistry findings, there is an apparent increase in expression levels observed in sporadic miscarriage cases. Additionally, both 12LOX (Figure 9) and 5LOX (Figure 10) were detected throughout the tissue stroma and were also found to be expressed by both macrophages and dNKs, as indicated by their co-expression.

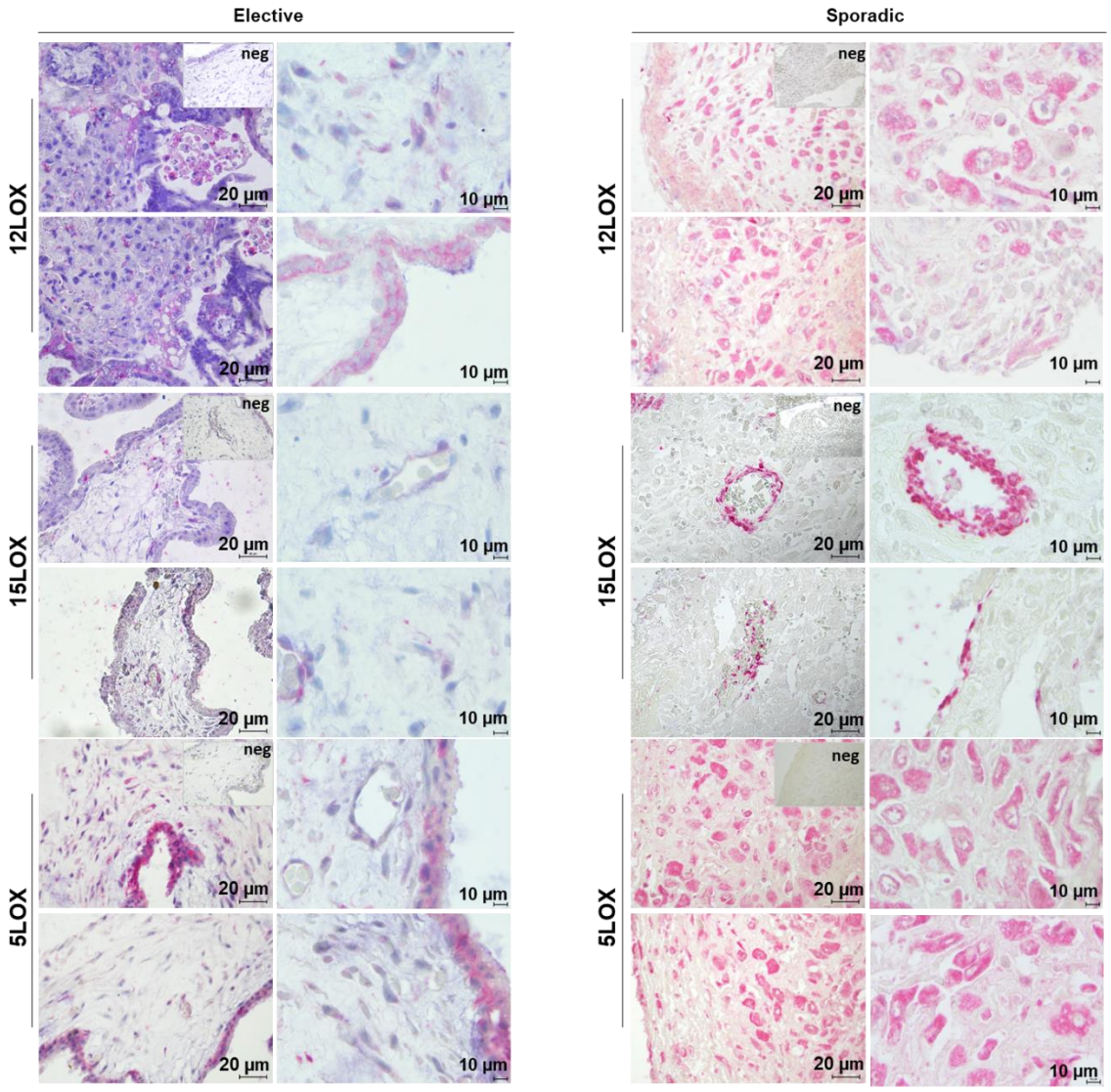

Figure 6. Immunohistochemistry staining of 12, 15 and 5-LOX in first trimester placenta of elective and sporadic miscarriages. Neg: negative control. Scale bars: 20 µm and 10 µm.

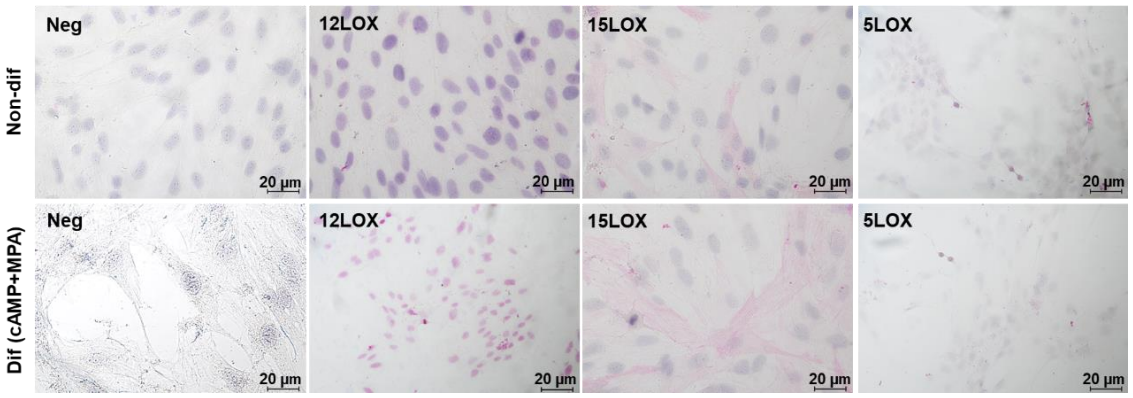

Figure 7. Immunocytochemistry staining of 12, 15 and 5-LOX in primary endometrial stromal cells. Scale bar: 20 µm. Neg: negative control; Non-dif: non-differentiated; Dif: differentiated. Scale bars: 20 µm.

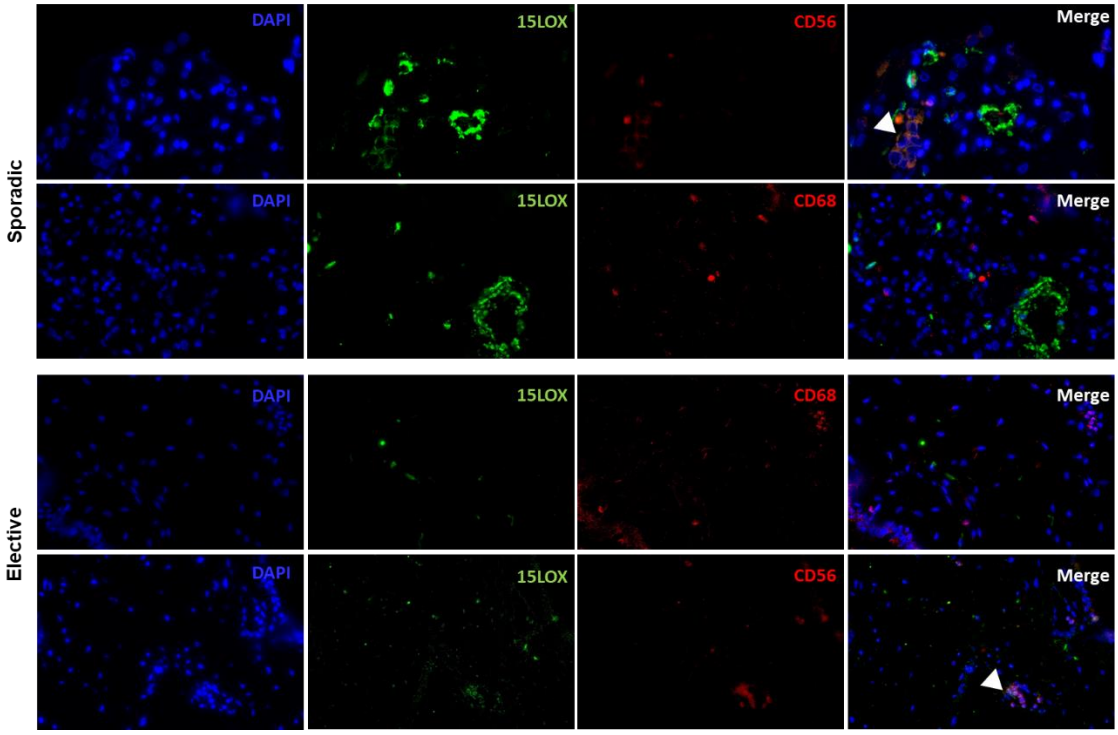

Figure 8. Imunofluorescence co-localization assay of 15-LOX enzyme with NK and macrophage-cell markers, CD56 and CD68, respectively. Staining was performed with anti-15LOX antibody (green), 40,6-diamidino-2-phenylindole (DAPI) (blue) and antibodies against CD56 or CD68 (red). Co-localization occurred in CD56+ cells (arrowheads), but not CD68+ cells.

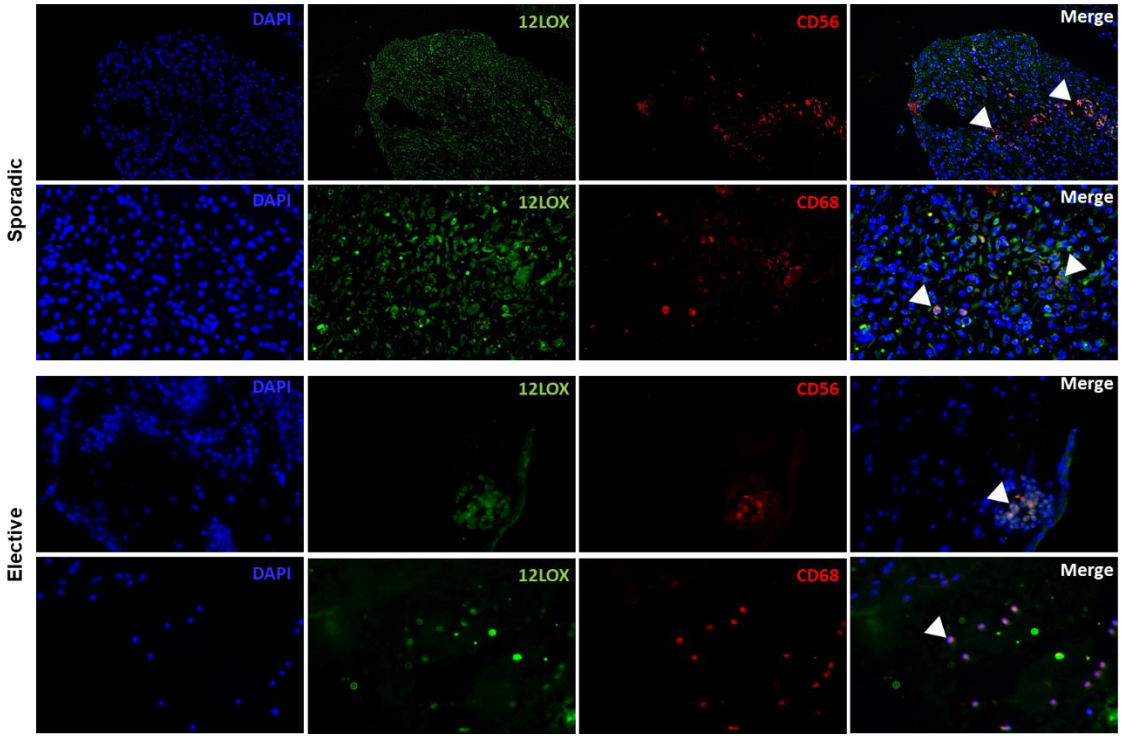

Figure 9. Imunofluorescence co-localization assay of 12-LOX enzyme with NK and macrophage-cell markers, CD56 and CD68, respectively. Staining was performed with anti-12LOX antibody (green), 40,6-diamidino-2-phenylindole (DAPI) (blue) and antibodies against CD56 or CD68 (red). Co-localization was observed for both CD56+ and CD68+ cells (arrowheads).

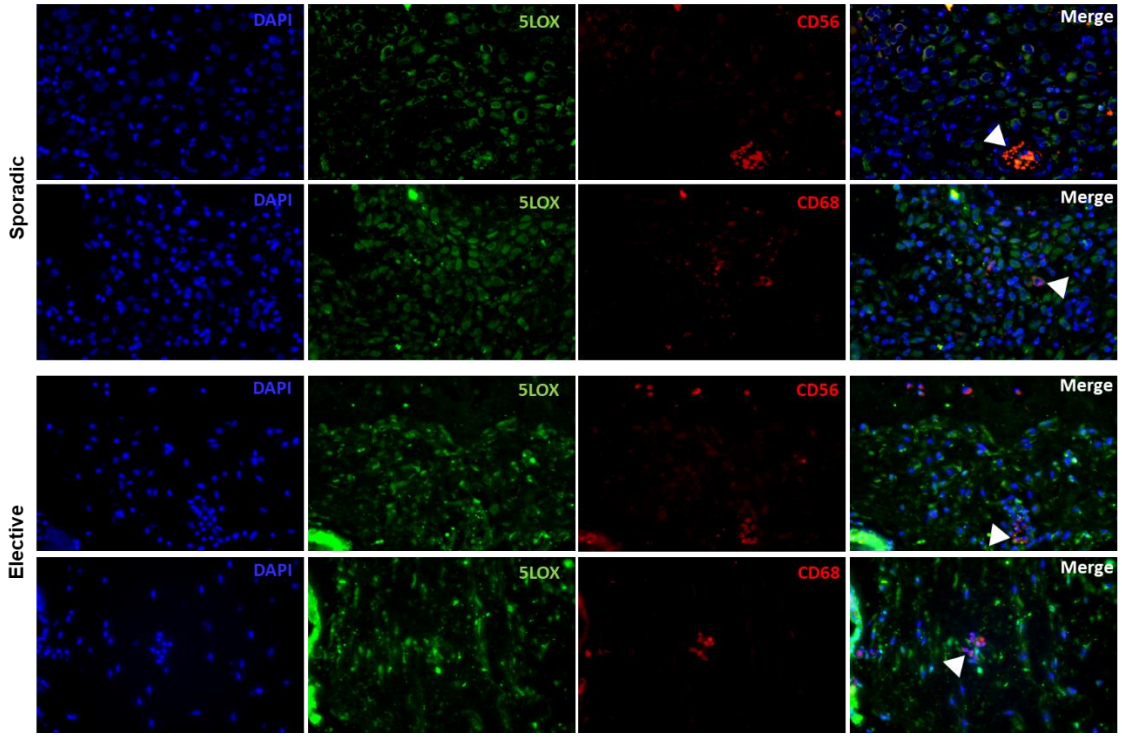

Figure 10. Imunofluorescence co-localization assay of 5-LOX enzyme with NK and macrophage-cell markers, CD56 and CD68, respectively. Staining was performed with anti-5LOX antibody (green), 40,6-diamidino-2-phenylindole (DAPI) (blue) and antibodies against CD56 or CD68 (red). Co-localization was observed for both CD56+ and CD68+ cells (arrowheads).

3.2. Higher levels of LOX enzymes in sporadic miscarriage

As mentioned previously, higher blood levels of lipoxins have been associated with miscarriage. To evaluate the local production of lipoxins and resolvins in first trimester maternal tissue, qPCR and Western Blot techniques were employed to detect the LOX enzymes responsible for their biosynthesis, at both the RNA and protein level. In terms of mRNA level, the expression was not significantly different between the groups (Figure 11C). Similarly, COX2, which participates in alternative pathways of synthesis, exhibited comparable expression. However, at the protein level, tissue samples from cases of sporadic miscarriage exhibited notably higher levels of 12LOX ($P = 0.0197$) and 15LOX ($P = 0.0026$), 8-fold and 17-fold higher, respectively. There were no discernible differences in the protein levels of 5LOX (Figure 11A, B). These enzymes play a role in the synthesis of D-series resolvins and lipoxins. These findings are consistent with the qualitative results obtained through immunohistochemistry.

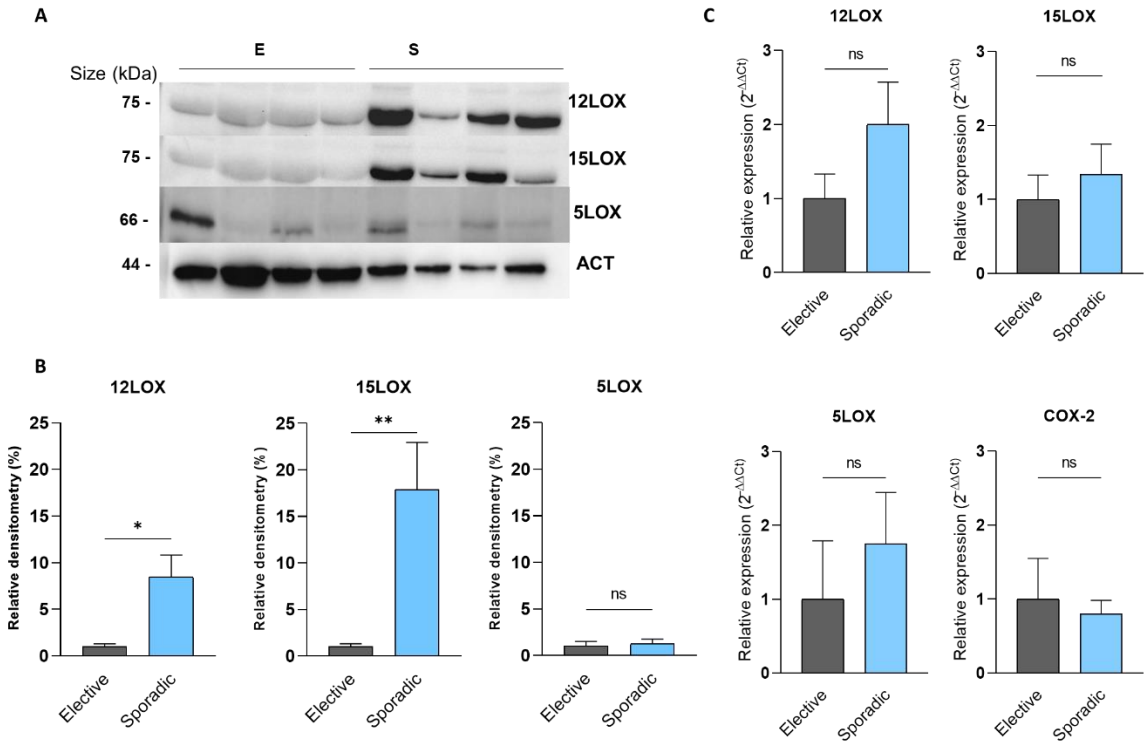

Figure 11. **LOX enzymes production in placental tissue of elective and sporadic miscarriage**. **A**. Representative Western-Blot images of protein bands following antibody detection. E: elective; S: sporadic. **B**. Corresponding densitometry graphs comparing protein levels relative to actin between the groups, obtained through ImageLab. *P<0.05; **P<0.01; ns: not significant. **C**. Plotted relative cDNA expression using the $2^{-\Delta\Delta ct}$ method.

3.3. Endogenous lipid mediators affect decidualization

To evaluate the potential impact of these compounds on the differentiation of ESCs, the St-T1b cell line as well as fresh primary cell cultures were maintained in culture until they reached exponential growth. Subsequently, they were routinely subjected to differentiation stimuli, with or without the presence of LXA4, LXB4, RvD1 and RvE2. On a first approach, we demonstrated that the presence of these compounds generally did not affect the viability non-differentiated cells (Figure 12A, B). However, RvE2 significantly affected the viability of non-differentiated St-T1b cells at a concentration of 0.1 μM, after 72 hours of incubation (P = 0.0076). Viability was not compromised in any tested scenario for primary ESCs (Figure 12B). Thus, for the decidualization assays, an intermediate concentration of 0.01 μM for the four compounds was used, ensuring that viability was not compromised.

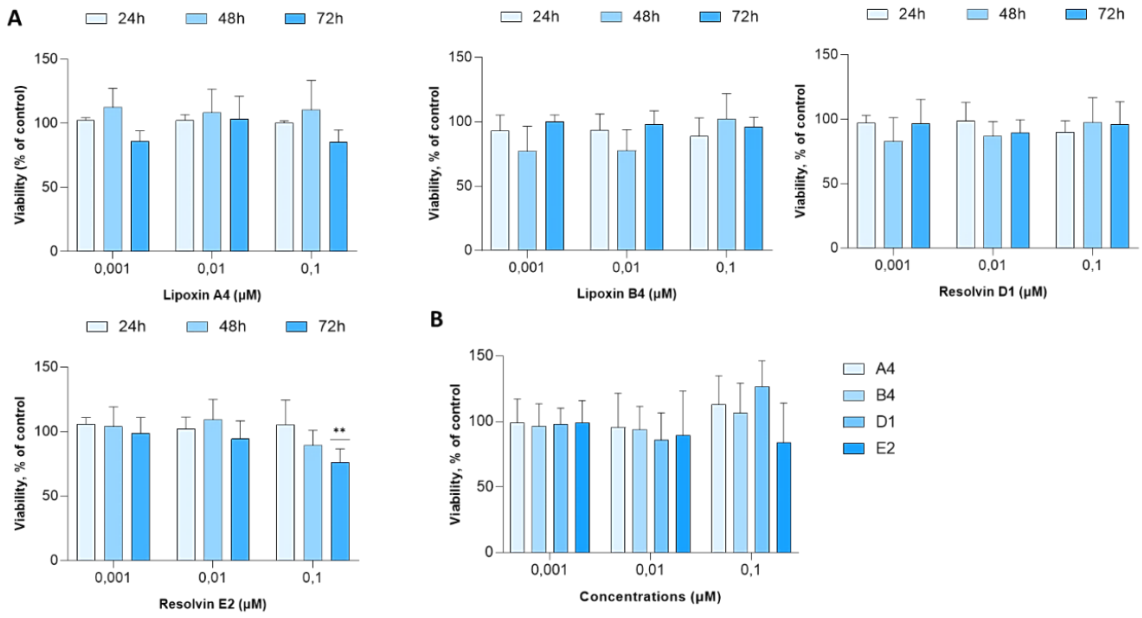

Figure 12. **MTT viability assays for both St-T1b and primary endometrial stromal cells**. **A.** Cells from St-T1b line were incubated with lipid mediators for 24, 48 and 72 hours in three concentrations (0.001, 0.01, 0.1 μM). **B.** Primary cells were incubated in a five-day period. Viability is expressed as a percentage of control. **P<0.01.

As previously outlined, cells were exposed to a decidualizing medium for a duration of five days, with the presence or absence of endogenous lipid mediators (Figure 13). Initial experiments were carried out for the Giemsa staining, aimed at a qualitative evaluation of decidualization. Generally, decidual cells are recognized by their expanded cytoplasm, heightened nucleolar density and extensions which resemble pseudopods. Specifically, cells undergoing decidualization in the presence of LXA4, RvD1 and RvE2 demonstrated a closer resemblance to the non-differentiated control group, featuring the prototypical fibroblastic shape of ESCs, a smaller cytoplasm and elongated nucleus (Figure 13A).

For quantitative experiments, cells were decidualized and lysed, and their RNA was extracted for the detection of two decidualization biomarkers, IGFBP-1 and PRL. Decidualization experiments (n = 7) were verified, having reached significance for both markers (IGFBP1, P = 0.0002 and PRL, P = 0.0019) when compared to non-differentiated cells. Differentiation was markedly reduced by the presence of LXA4 (IGFBP1, P = 0.02 and PRL, P = 0.008), RvD1 (IGFBP1, P = 0.002) and RvE2 (IGFBP1, P = 0.009 and PRL, P = 0.03) when compared to decidualizing cells (Figure 13B). The effect of RvD1 on PRL expression did not reach statistical significance (P = 0.07). Interestingly, only LXB4 did not affect decidualization at any point (Figure 13B).

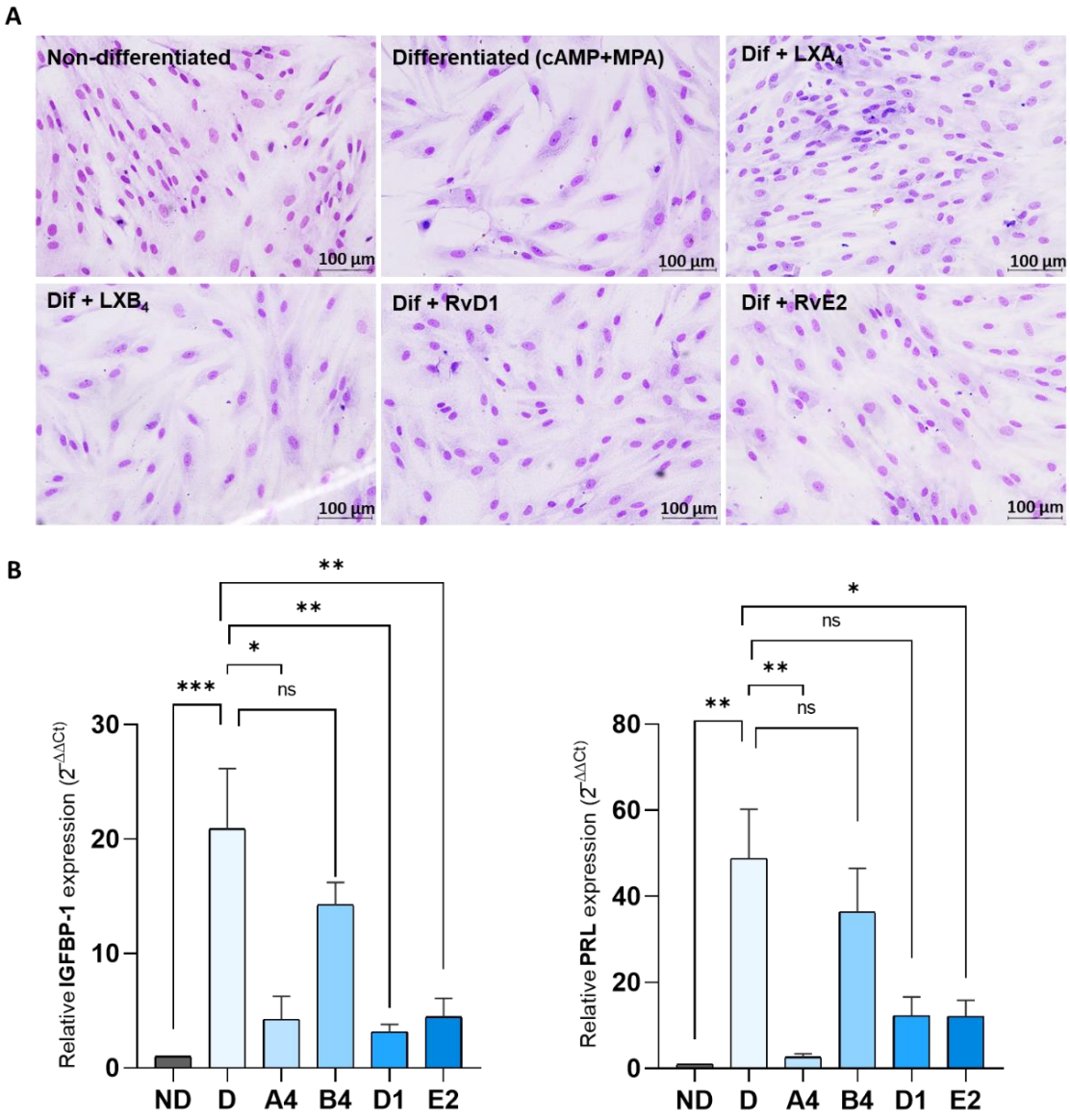

Figure 13. St-T1b decidualization assays. **A.** Representative images following decidual stimulus for each group, obtained with Giemsa staining. ND: Non-differentiated. D, Dif: Differentiated. ns: Not significant. Scale bar: 100 µm. **B.** Relative IGFBP-1 and PRL expression in each group relative to β-actin, using the $2^{-\Delta\Delta ct}$ method.

Decidualization experiments were repeated in the same fashion in the case of cultured primary cells. Giemsa staining revealed a similar pattern in disruption of typical decidual cell morphology (Figure 14A). Cells differentiated in the presence of LXA4, RvD1 and RvE2 were comparable to non-differentiated cells. The results obtained for relative PRL expression were consistent with these results, as well as the St-T1b cells. Decidualization was affected by the presence of LXA4 (P = 0.03), RvD1 (P = 0.008) and RvE2 (P = 0.006) and was not affected by LXB4 (Figure 14B). Conversely, IGFBP-1

expression did not differ from the positive control (D) in any case, which is unlike the results obtained with the St-T1b cell line.

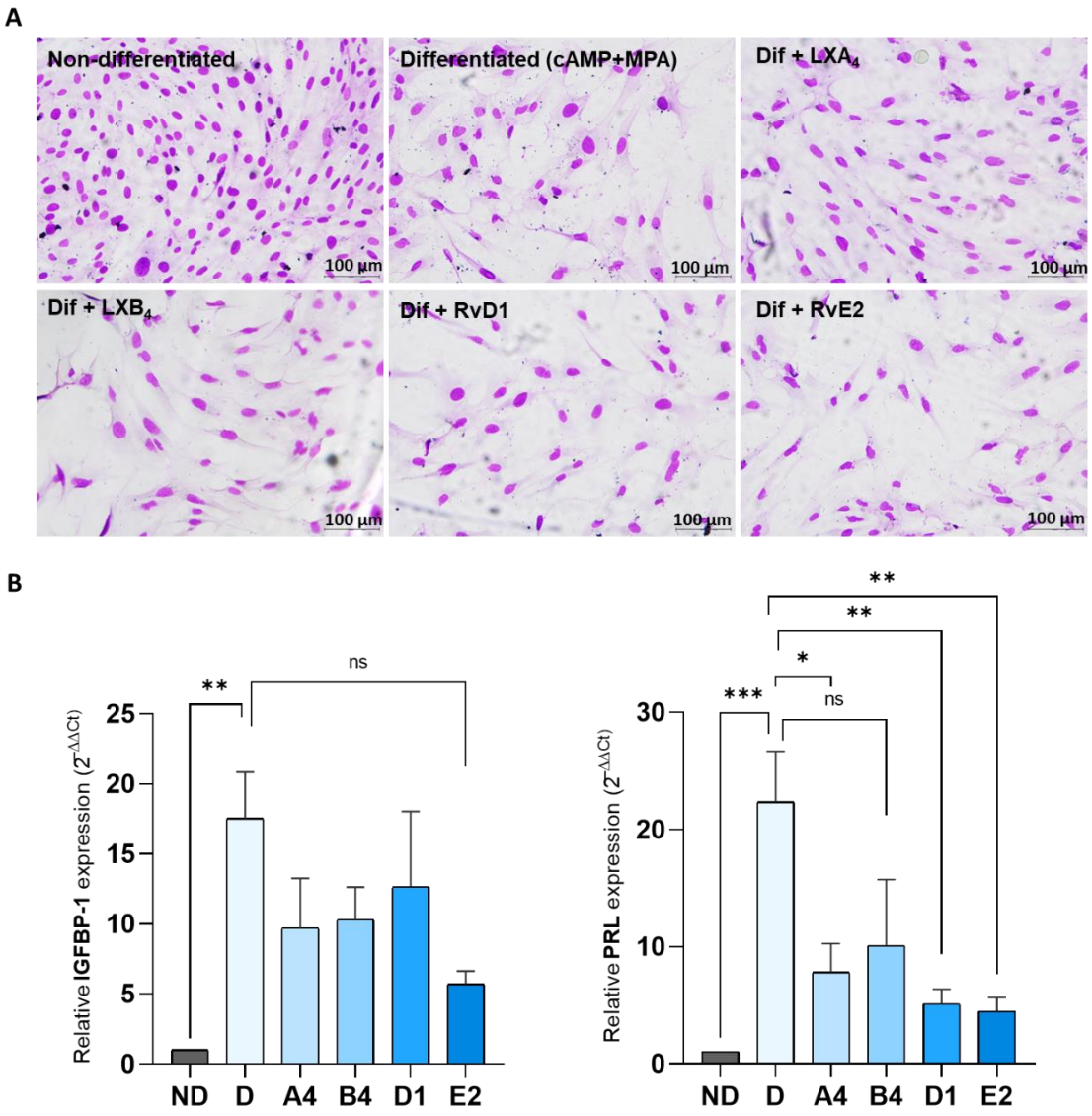

Figure 14. Primary ESC decidualization assays. **A.** Representative images following decidual stimulus for each group, obtained with Giemsa staining. ND: Non-differentiated. D, Dif: differentiated. ns: Not significant. Scale bar: 100 µm. **B.** Relative IGFBP-1 and PRL expression in each group relative to β-actin, using the $2^{-\Delta\Delta ct}$ method.

4. Discussion

Human reproduction is markedly inefficient. The likelihood of conceiving and reaching a diagnosed gestation is around 30-40% for each menstrual cycle (33). Early pregnancy loss is usually recognized within the first 12 weeks of gestation, which corresponds to the first trimester of pregnancy. During this particular timeframe, research indicates that approximately 40% of conceptions do not lead to a successful pregnancy, with a notable portion of these cases resulting in pre-clinical losses (65). The primary culprits behind first trimester loss are abnormal genetic makeup of the conceptus accounting for the majority of cases (50 to 80%). The most prevalent genetic anomaly is autosomal trisomy involving single chromosomes. These pregnancies typically miscarry early, often failing to implant (66). Conversely, cases where women undergo miscarriage, whether sporadic or recurrent, despite presenting a euploid pregnancy raise secondary causes, leading to the suspicion of uterine factors and endocrine dysregulation (67). Generally, these miscarriages occur at 5 to 10 weeks (68). These phenomena motivate research into the female physiology linked with uterine receptivity.

Defective decidualization has been consistently linked with recurrent miscarriage (37,69,70). One of the striking hallmarks of this relationship is the propensity for the uterine microenvironment to exhibit dysregulated inflammation. The delicate balance between pro-inflammatory and anti-inflammatory factors, if perturbed, can create a state of chronic inflammation in the tissue, potentially impacting implantation, placentation, and overall pregnancy viability. The specific mechanisms responsible for the disrupted uterine inflammation remain largely elusive. Further research is imperative to unveil these mechanisms, with the goal of developing targeted interventions aimed at enhancing pregnancy outcomes for individuals experiencing recurrent miscarriage.

Both 12-LOX and 15-LOX are responsible for metabolizing essential fatty acids into potent lipid mediators known as lipoxins and resolvins. These molecules exert anti-inflammatory effects, dampening the excessive immune response and activating the resolution of inflammation. Interestingly, though no transcriptional alterations were observed, sporadic abortion samples have significantly increased levels of 12LOX and 15LOX enzymes. This suggests that the control of 12LOX and 15LOX takes place primarily at the protein level, underlining the presence of an imbalanced inflammatory environment within uterine tissues during spontaneous abortion when contrasted with the conditions observed in normal first-trimester maternal tissues. Furthermore, as observed in other tissues, it is likely that LOX transcripts at the uterine level are subject to tight regulation, with their transcripts undergoing degradation until the enzyme is

needed. This was demonstrated to happen through nonsense-mediated decay of alternative transcripts (71), or through inhibition of translation by heterogeneous ribonuclear protein K (72). While we haven't fully understood these mechanisms for all lipoxygenases, nor in the case of ESCs, it does seem to indicate that when there are more of these enzymes at the protein level, it could be because the endometrium has a need for their byproducts, possibly as a response to excessive inflammation. In the case of sporadic abortion, upregulation of 12LOX may increase the conversion rate of leukotriene A4 to lipoxins (A4 and B4), counteracting the pro-inflammatory role of leukotriene. A higher level of 15LOX may increase the production of 15S-H(p)ETE and 17S-H(p)DHA, important precursors to lipoxins and D-series resolvins, respectively.

Subsequently, we explored the cellular localization of the LOX family in first-trimester maternal tissues by immunohistochemistry. We observed the presence of 12-LOX in various cell types, such as ESCs and trophoblast cells within chorionic villi. A similar expression pattern was noted for 5LOX. However, 15LOX exhibited distinct expression, lower in ESCs and noticeably present in endothelial cells and around spiral arteries. Our observations also unveiled the capacity for dNKs to express LOX. This suggests a new role for these cells, where they might have a regulatory function in inflammation processes within endometrium, which, in turn, could also impact ESC differentiation. On the other hand, the expression of 15LOX by $CD68^+$ macrophages were not found. It is important to distinguish between the two 15LOX genes, *15LOX-1* and *15LOX-2/LOX15B*. The antibody used targets 15LOX-1, which is more comprehensibly described. Accordingly, a 2019 study claimed 15LOX-2/LOX15B is constitutively expressed in macrophages, while 15LOX-1 is not (73). Incubation with IL-4 and IL-13, which promotes M2-macrophage polarization, leads to the expression of 15LOX-1 (74). This could explain the results obtained in immunofluorescence, as $CD68^+$ is a general monocyte and macrophage marker. In turn, 12LOX and 5LOX were expressed by both dNKs and macrophages.

Despite the lack of clarity on the uterine pathways they regulate, the presence of LOX enzymes seems to be crucial for implantation. In a research study involving ovariectomized mice with a knockout of *12LOX* and *15LOX* genes, a notable decrease of their metabolites occurred, followed by a reduction of 80% in implantation sites, compared with control mice (78). The expression of 12 and 15LOX enzymes in the uterus might be mediated by progesterone, further implicating them in ESC differentiation and preparation for pregnancy (78).

Given the observed disparity in the inflammatory environment within first-trimester maternal tissues between spontaneous abortion and elective pregnancy termination, this inflammatory imbalance could potentially impede stromal cell differentiation through the generation of lipoxins and resolvins during spontaneous abortion. Thus, to evaluate the potential impact of pro-resolving action on decidualization, ESCs were stimulated to differentiate *in vitro* in the presence of these compounds. Apart from LXB4, decidualization was significantly affected, albeit to a lesser degree in primary ESCs. Moreover, as the process of decidualization is known to create spaces between cells through selective apoptosis of stromal cells (75), facilitating implantation in an autocrine manner (76,77). The structural organization of the decidual endometrium, while often overlooked, plays a crucial role in promoting trophoblast invasion through the stroma. In light of these findings, both of these aspects may be adversely affected. It is intriguing to contemplate the potential role of immune cells in the imbalance of lipoxin and resolvin levels and their impact on uterine receptivity.

While this study is preliminary, it provides evidence that these lipid mediators play a role in early pregnancy and highlights the repercussions of their dysregulation. Future research aimed at elucidating the precise pathways influenced by lipoxins and resolvins in the development of dESCs would not only bolster their significance, but also strengthen the case for their involvement in miscarriage. Such studies may raise a potential for their use as biomarkers in clinical settings to evaluate the inflammatory state of the pregnant woman. A similar application has already been suggested for LXA4 as a biomarker in preeclampsia, a condition hallmarked by excessive inflammation (79).

5. Conclusion

Abnormalities in decidualization are an understudied factor of infertility. In a world where couples are increasingly struggling to conceive, it is imperative to increase our knowledge in the reproductive field and improve personalized care practices. This thesis puts forward pro-resolving lipid mediators as players capable of affecting decidualization. We also detected an unbalanced inflammatory state in sporadic abortion samples, characterized by in significantly higher levels of 12- and 15-lipoxygenase enzymes. Finally, we uncovered the expression of 15-LOX by dNK cells, revealing a possible new role for these cells in the context of uterine receptivity. Alterations in these lipid mediators raise the possibility of an imbalance in their regulatory mechanisms, which could potentially compromise the inflammatory response. This disruption likely encompasses a multifaceted array of mechanisms, implicating various cell types, including dNKs and macrophages. Consequently, such an imbalance may exert a ripple effect on decidualization, ultimately influencing the entirety of the reproductive process, culminating in pregnancy loss. It is the hope of this thesis to point towards relevant applications that could arise for these compounds, whether as biomarkers or therapeutics.

6. References

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