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INNATE LYMPHOID CELLS AT THE MATERNAL-FETAL INTERFACE. THE ROLE OF PROGESTERONE IN PRETERM BIRTH

VOLUME 1

Tese no âmbito do Programa Doutoral em Ciências da Saúde ramo Ciências Biomédicas orientada pela Professora Doutora Ana Luísa Fialho Amaral de Areia e pela Professora Doutora Anabela Mota Pinto, apresentada à Faculdade de Medicina da Universidade de Coimbra.

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Preamble

The purpose of the work presented in this thesis was to establish the role of innate lymphoid cells in preterm birth, through progesterone actions. There is growing amount of evidence in the literature highlighting the relevance of innate lymphoid cells amongst the diverse actions of the innate immune system. These range from their role in disease, namely in the response against intracellular pathogens and allergy; mediating inflammatory responses; in tissue remodeling and repair, amid others.

The nomenclature and characterization of these cells has been evolving in the last decade. Despite the fact that this group of cells now includes natural killer cells and lymphoid tissue inducer cells, this work focuses on the most recently discovered groups one, two and three innate lymphoid cells. It is in these latter groups that there is scarce data regarding their role in pregnancy.

Of all the different actions of innate lymphoid cells, their ability to mediate inflammation raised our interest. In fact, in pregnancy there are two major inflammatory events: implantation and labor itself. It was in this context that we sought out to study preterm birth as an inadequate or premature inflammatory event. The decision to include progesterone as key mediator in this process derived from the knowledge that progesterone is a key anti-inflammatory hormone throughout pregnancy, and because progesterone is currently administered to pregnant women who suffer from preterm labor, with good clinical outcomes, based on international guidelines.

The relevance of this work resides in the fact that the incidence of preterm birth has increased in developed countries regardless of multiple strategies carried out to avoid it. Moreover, this work deliberately raises several scientific questions important in the development of the immunology field.

The thesis presented herein, aims to contribute to a more complete understanding of the mechanism underlying preterm birth. Moreover, this work aims to expand our previous knowledge in the immunology of pregnancy, giving rise to the development of new clinical protocols by identifying new therapeutic targets. In doing so, the author aims to contribute significantly to promote the reduction in children morbidity and mortality and hospital costs.

Publications

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Acronyms

A

ACOG - American College of Obstetricians and Gynecologists

AHR - Aryl hydrocarbon receptor

B

BPgr – Before administration of progesterone

C

CD - Cluster of differentiation

CRTH2 - Chemoattractant receptor-homologous molecule expressed on T helper 2 cells

COX-2 – Cyclooxygenase 2

CLP- Common lymphoid progenitor

CHUC - Centro Hospitalar e Universitário de Coimbra

D

dNK - Decidual NK cells

DAMP - Damage-associated molecular patterns

E

ELISA - Enzyme-linked immunosorbent assay

E2 - Estradiol

Eomes - Eomesodermin

E46 - Estradiol receptors (E46)

EVT - Extravillous trophoblast

F

FTB – Full term birth

FMO - Full minus one

G

GATA3 - GATA binding protein 3

GvHD - Graft-*versus*-host disease

H

HLA - Human leukocyte antigen

hCG - Human chorionic gonadotropin

I

ILC - Innate Lymphoid cells

ILCPs - ILC precursors

IL – Interleukin

IFN-γ - Interferon gamma

IP-10 - Interferon gamma-induced protein 10

ILT–- Immunoglobulin (Ig)-like transcripts

IVF - *In vitro* fertilization

K

KIR - killer cell Immunoglobulin (Ig) -like receptors

L

Lin$^-$ - Lineage negative

LILR - Leukocyte immunoglobulin-like receptors

LTi - Lymphoid tissue inducer

M

MHC - Major Histocompatibility Complex

mPR - Membrane progesterone receptors

N

NK - Natural Killer cells

NCR - Natural cytotoxic receptor

NF-kB - Nuclear factor kappa B

NKp30 - Natural cytotoxicity triggering receptor 3

NKG2D - Killer cell lectin like receptor K1

P

PBS - Phosphate buffered saline

PTB - Preterm birth

PTL - Preterm labor

pPROM - Preterm premature rupture of the membranes

PGE2 - Prostaglandin E2

P4 - Progesterone

24h PGr -Twenty-four hours after the administration of progesterone

PREs - Progesterone response elements

PG - Prostaglandins

PRs - Progesterone receptors

PAQR - Progestin and adipoQ

PIBF - P4-Induced Blocking Factor

PGF2a - Prostaglandin F2a

PRRs - Pattern recognition receptors

R

RAG - Recombination activating gene

ROR - Retinoic acid–related orphan receptor

RORα - Retinoic-related orphan receptor alpha

RORC - RAR related orphan receptor C

S

SDF-1 - Stromal cell-derived factor 1

SLO - Secondary lymphoid organs

SPTB - Spontaneous preterm birth

T

T-bet: T-box transcription factor

Th - T helper cell

Tregs - T regulatory cells

U

uNK - Uterine Natural Killer

V

VEGF - Vascular endothelial growth factor

Summary

One of the most complex areas in perinatal-neonatal medicine remains the care of women anticipating a preterm delivery.

Preterm Birth (PTB), defined as delivery occurring before 37 completed weeks of gestation, globally represents 15 million babies born prematurely[1].

PTB can result from a range of causes such as exposure to environmental triggers, maternal stress, fetal or maternal genetic abnormalities, and hormonal imbalance, amongst others. Progesterone acts as an immunosteroid by contributing to the establishment of a pregnancy protective milieu. Previous results from our group have demonstrated the benefits of progesterone therapy in PTB, through the actions of membrane progesterone receptors (PRs) in regulatory T cells (Tregs) [2,3].

The act of giving birth is widely regarded as an inflammatory event[4]. Very likely, it is the immune response of the host that presumably leads to the inflammatory response and preterm labor[4].

Randomized studies and individual patient data meta-analysis have shown that in women with a short cervix, progesterone reduces PTB and adverse neonatal outcomes[5]. However, there is no evidence that women with PTB have lower progesterone levels, or that administration of progesterone vaginally increases its concentration in peripheral blood. Therefore, the mechanism by which a modest additional amount of progesterone could achieve its therapeutic effect is unclear, suggesting that it may be exerted locally[6].

Recently, a new cell type belonging to the innate immune system, termed innate lymphoid cell (ILC), was characterized. Functionally, ILC resemble T helper 1 (Th1), T helper 2 (Th2) and T helper 17 (Th17) cells and have revealed an essential role in the initiation, regulation and resolution of inflammation[7]. Dysregulation or expansion of pro-

inflammatory ILC populations may directly interfere with pregnancy, ultimately resulting in pregnancy loss or adverse outcomes.

In this work, we investigated the role of progesterone in spontaneous preterm labor (PTL), through the actions of ILC. Moreover, we analyzed the relative frequencies of ILC subsets in pregnancy and the levels of Interleukin (IL)-4, IL-17, IL-22, and interferon (IFN)-γ as inflammatory mediators. Besides, this work aims to expand our previous knowledge in the immunology of pregnancy, giving rise to the development of new clinical protocols by identifying new therapeutic targets. In doing so, the author aims to contribute significantly to promote the reduction in children morbidity and mortality and hospital costs. For this purpose, we included a study group composed of women with spontaneous PTL attending the Obstetric Department of Coimbra Hospital and University Centre (CHUC); and a control group comprising healthy pregnant women attending prenatal consultation in the same institution. Administration of natural progesterone was done, once daily, in a 200mg vaginal dosage in the study group. ILC were isolated and characterized from maternal peripheral blood, maternal-fetal interface, and cord blood samples, using flow cytometry. Plasmatic cytokines were determined in peripheral blood and cord blood samples by enzyme-linked immunosorbent assay (ELISA), at the time of labor.

Resumo

Uma das áreas mais complexas da medicina perinatal e neonatal continua a ser o cuidado de mulheres grávidas, antecipando o parto de uma criança prematura.

O parto pré-termo, definido como o parto que ocorre antes das 37 semanas completas de gestação, representa globalmente o nascimento de 15 milhões de crianças prematuras.

O parto pré-termo pode resultar de uma variedade de causas, como a exposição a fatores ambientais, *stress* materno, variações genéticas fetais ou maternas, desequilíbrio hormonal, entre outras. Neste contexto, a progesterona atua como um imuno-esteróide, contribuindo para um ambiente de proteção da gravidez. Dados publicados recentemente pelo nosso grupo demonstram os benefícios da terapia com progesterona no parto pré-termo, mediados pelos recetores da progesterona nas células T reguladoras.

O parto é amplamente considerado como um processo inflamatório. Muito provavelmente esta resposta inflamatória tem origem numa resposta imunitária por parte do hospedeiro.

Estudos randomizados e meta-análises individuais demonstraram que, em mulheres com colo do útero curto, a progesterona reduz o parto pré-termo e um resultado neonatal adverso. No entanto, não há evidências que mulheres com trabalho de parto pré-termo tenham níveis mais baixos de progesterona ou que a administração de progesterona por via vaginal aumente sua concentração no sangue periférico. Portanto, o mecanismo pelo qual uma quantidade adicional modesta de progesterona poderia alcançar um efeito terapêutico não é claro, sugerindo que este possa ser exercido localmente.

Recentemente, identificou-se um grupo de células pertencente ao sistema imunológico inato, denominadas células linfoides inatas, que funcionalmente se assemelham às células T auxiliar 1 (Th1), T auxiliar 2 (Th2) e T auxiliar 17 (Th17) e

revelaram um papel essencial na iniciação, regulação e resolução da inflamação. A desregulação ou expansão das populações pró-inflamatórias de células linfoides inatas poderá interferir diretamente na gravidez, resultando em perda da gravidez ou em resultados adversos.

Neste trabalho, propusemo-nos investigar o papel da progesterona no trabalho de parto pré-termo, pela sua ação nas células linfoides inatas. Analisámos as frequências relativas das células linfoides inatas, bem como os níveis plasmáticos de IL-4, IL-17, IL-22, e IFN-γ. Para esse efeito, incluímos um grupo de estudo de mulheres com trabalho de parto pré-termo atendidas no Departamento de Obstetrícia do Centro Hospitalar e Universitário de Coimbra (CHUC), e um grupo controlo composto por grávidas saudáveis seguidas em consulta pré-natal da mesma instituição. A administração de progesterona natural foi realizada com 200mg, via vaginal, uma vez ao dia (no grupo de estudo). As células linfoides inatas foram isoladas e caracterizadas a partir de amostras de sangue periférico materno, interface materno-fetal e sangue do cordão umbilical, utilizando a citometria de fluxo. As concentrações plasmáticas de citocinas foram determinadas em amostras de sangue do cordão e sangue periférico utilizando a técnica de ELISA, no momento do parto.

1. Introduction

The fact that there is a special connection between the mother and her offspring no one argues. Considering the latest advances in reproductive medicine, and that we are getting close to the announcement of an artificial womb[7], the debate regarding the biological nature of the relationship between mother and fetus gains a new momentum.

When looking at pregnancy, two distinct lines of reasoning can be addressed: on one hand the fetus can be considered as part of the mother (Parthood model); and on the other hand, the mother might be considered as a vessel and a provider for fetus development, which is an entity on its own right (Container model). Both views have enormous ethical philosophical implications. However, reducing such a complex biological event in two extreme views, fails to reflect the true nature of the process itself.

From the immunology point of view, we may first consider self-non-self-discrimination, since half the mother genetic heritage and half the father compose the newly developing being. In this sense, we may consider the fetus as a semi-allograft, a concept initially put forward in 1953, by *Sir Peter Medawar*[8]. In this view, taken from the knowledge attained in transplantation science, the fetus trophoblast, carrying paternal antigens, has to invade the mother uterine mucosa, in a process called implantation, while escaping immune defense mechanisms against alloantigens. This has become the first paradox in the biology of pregnancy. Indeed, the three major events in pregnancy, after the fertilization of the oocyte, involve an immunologic response. The implantation of the trophoblast, widely regarded as an inflammatory process on its own, then a protective anti-inflammatory milieu, needed throughout the whole development of the fetus, and labor itself, which is also regarded as an inflammatory event[9,4] . The mother immune system has to carry out two fundamental tasks during pregnancy. On one hand it has to

successfully protect the fetus and mother from pathogens, while at the same time achieving the necessary tolerance to a half genetically different organism.

A second key issue that has been puzzling gynecologists for long is the inflammation paradox or "good inflammation". As stated earlier the implantation process is achieved through an inflammatory response, which promotes tissue remodeling at the level of the uterine wall. It has been suggested by *Barash and colleagues, 2003*, that deliberate injuring of the endometrium, will yield better implantation outcomes[10], supporting the notion that a local induced inflammation would in some way aid the implementation of the trophoblast; however in a later study, *Liu W. and colleagues, 2017* have not come to the same conclusion[10]. The divergence in results regarding this approach, highlights our lack of knowledge of what is the real nature of this "good inflammation" that seems to prompt the implantation event. Beyond the implantation process, labor is in itself an inflammatory process, which when prompt prematurely may yield a premature baby, with enormous psychological and clinical implications. As so, in an attempt to enlighten scientific knowledge, we hope to shed some light on the possible underlying mechanism responsible for PTL, in light of a newly discovered set of immune cells generally referred to as innate lymphoid cells (ILC).

2. Innate lymphoid cells

ILC are a group of cells that share a common lymphoid progenitor. Initially these cells were classified in three distinct groups ILC1, ILC2 and ILC3 but later were grouped with the previous already classified natural killer (NK) cells and lymphoid tissue inducer (LTi). ILC are characterized by the absence of recombination activating gene (RAG)-dependent rearranged antigen receptors, lack of myeloid cell and dendritic cell phenotypical markers, hence denominated lineage negative (Lin^-). These cells share the expression of common γ chain, IL-7Rα (CD127). In addition, ILC2 are characterized by the expression IL-2Rα (CD25), a receptor that is also present in $CD56^{bright}$ NK cells but has lower expression in ILC1 and ILC3 [11,12].

ILC are functionally diverse and belong to the innate component of the immune system[11]. They were classified based on their relative cytokine profiles, centered on effector phenotypes that mirror T helper cells. Over the years, the classification of ILC has been subject of great debate, mainly due to their heterogeneity. ILC were initially classified as NK cells in 1975[13], afterwards, in 1997, another cell type was added, named lymphoid tissue inducer (LTi)[14]. While NK cells represent cytotoxic-ILC capable of killing virus-infected or tumor cells and release pro-inflammatory cytokines[15], LTi are critical for the development of secondary lymphoid organs during embryogenesis[16]. However, the nomenclature approved by the International Union of Immunological Societies (IUIS) considers five distinct groups: NK cells known to produce IFN-γ; Group 1 (ILC1) also known to produce IFN-γ, a Th1 like cytokine; Group 2 (ILC2), characterized by the expression of transcription factor Gata3 and the ability to produce Th2 like cytokines; Group 3 (ILC3), known to produce IL-22 and IL-17; and LTis, important in secondary lymphoid organ formation[12,17].

These cells play an essential role in tissue homeostasis, defense against infection, inflammation and tissue repair[17]. ILC are mainly tissue resident cells found in the mucosal surfaces [18], as well as in the decidua of pregnant women[19].

Moreover, it has become evident that ILC have great plasticity. Their effector characteristics are highly dependent on their microenvironment, mainly on the cytokines secreted by tissue resident cells, and other cells from the innate component of the immune system[20]. Due to ability of some ILC to produce pro- and anti-inflammatory cytokines and to the fact that ILC express Major Histocompatibility Complex Class II (MHC II) molecules, their importance in the regulation of labor is rational.

Immune tolerance and controlled inflammation are key processes in a successful pregnancy. Dysregulated inflammatory reactions often lead to complications such as spontaneous abortion, preterm labor, preeclampsia and intrauterine growth restriction[21,22].

2.1 Natural killer Cells and Group 1 innate lymphoid cells

The importance of NK cells in pregnancy is paramount, not only because these cells belong to the innate immune system, but also because NK cells play an important role in placentation, remodeling of the spinal arteries and control of trophoblast invasion [23–26]. Decidual NK (dNK) cells differ substantially from peripheral NK cells: peripheral NK cells are predominantly $CD56^{dim}$ $CD16^{+}$ instead, dNK cells are $CD56^{bright}$ $CD16^{-}$ [27,28]. This phenotype is accompanied by functional differences, since $CD56^{dim}$ $CD16^{+}$ have a strong cytolytic activity, while dNK cells are predominantly cytokine-producing cells.

Even though NK cells were discovered many years ago, it has only been more recently that these cells were included in the ILC group. Recent work by *Vento-Tormo et al. 2018* proposed three main dNK subsets: dNK1, dNK2 and dNK3 cells. This classification has

been further confirmed by *Huhn et al. 2020* [29,30]. Also, a previous work by *Yudanin et al. 2019,* conducted in different tissues other than uterine origin, highlights the overlapping characteristics of NK cells with ILC1, a fact also reported by *Huhn et al. 2020* which poses the question of dNK3 subsets may be in fact ILC1[31]. The nature and consequent nomenclature of the different dNK subsets and ILC1 is still a matter of great dispute. The implications of dNK cells being in fact ILC1 are enormous for the role of ILC in pregnancy; however, this discussion falls beyond the scope of this work.

Classically, uterine ILC1 are characterized by the expression of T-bet, eomesodermin (Eomes) and produce IFN-γ. ILC1 do not express perforin and have the inability to produce Th2 and Th17 type cytokines[19,32].

ILC1 can be further characterized by their surface markers $CD56^-$, $CD94^-$, $CD127^+$, $CD117^-$ [19], and have been identified low numbers in human decidua (< 3% of total ILC), suggesting a lesser role in pregnancy[33].

2.2. Group 2 innate lymphoid cells

Group 2 ILC are phenotypically characterized by the surface markers $CD56^-$, $CD127^+$, $CD161^+$ and chemoattractant receptor-homologous molecule expressed on T helper 2 cells (CRTH2) [34,19]. ILC2 are dependent on GATA binding protein 3 and transcription factor retinoic-related orphan receptor alpha (RORα) for their development [11,35]. ILC2 produce type 2 cytokines (IL-4, IL-5 and IL-13), under the control of IL-25 and IL-33, important in extracellular parasite infections and allergic responses[12]. The expression of CRTH2 is of great interest for labor, since it is a G protein–coupled receptor for prostaglandin D2, which promotes ILC2 differentiation and a type 2 pro-inflammatory responses[36]. Another important feature of ILC2 population, found in a study conducted in a mouse model, is the expression of MHC class II, as well as the co-stimulatory molecules CD80 and CD86[37]. In this study conducted *by* Oliphant *et al.2014*, it has been showed that ILC2 can not only perform endocytosis, but also process and present antigens[37]. These characteristics allow ILC2 to present antigens to T $CD4^+$ cells and induce proliferation towards a Th2 phenotype, in an IL-2 dependent manner [37]. These data, albeit conducted in mouse models, reinforces the notion of a crosstalk between the innate and the adaptive immune system[38–40].

2.3. Group 3 innate lymphoid cells

Group 3 ILC are characterized by the expression of the surface marker CD117 and the transcription factor RORγt. In a mouse model, it has been proven that ILC3 express MHC class II and have been shown to promote T cell mediated responses [41]. Two different studies suggest that ILC3 might promote neutrophil activation with pro-angiogenic abilities, contributing to the inflammatory phase needed for implantation [42,43]. ILC3 can be further divided based on the presence of the natural cytotoxic receptor (NCR) NKp44. ILC3 NCR^+ produce IL-22, while ILC3 NCR^- produce IL-17 [44,45]; both subsets have been found in human decidua[19]. NCR is also present in activated peripheral NK cells and in dNK. In NK cells, NCRs mediate cytotoxic responses[46] and antitumor responses[47]; however, when present in uterine NK cells, NCR receptors have an important role in placentation through the production of IL-8, VEGF, IP-10 and SDF-1[48].

The ability of ILC3 to act as pro-inflammatory agents, releasing IL-17, suggests a preponderant role in pregnancy, which both favours embryo implantation, and has an innate antimicrobial role. In fact, decidual ILC3 seem to be important to pregnancy maintenance through innate defences and tissue remodeling[19]. Nevertheless, the inappropriate release of pro-inflammatory cytokines during the quiescent phase of pregnancy may prompt complications, mainly the precocious activation of the normal mechanism of labor[49].

According to the above explanation, we can recognize that the classification of ILC is based on functional criteria. ILC functionally resemble adaptive lymphocytes, with the distinction that ILC lack antigen-specific receptors. Instead, ILC are known to exert their effects through the production of cytokines and cell surface molecules with important consequences for tissue homeostasis, inflammation, and disease. Dysregulation or expansion of pro-inflammatory ILC populations may directly promote disease through

production of pro-inflammatory cytokine, which seems to be important in the pathogenesis of PTL.

A summarized diagram of ILC ontogeny is presented in Figure 1, which highlights the similarities between ILC and T-helper cells (Th).

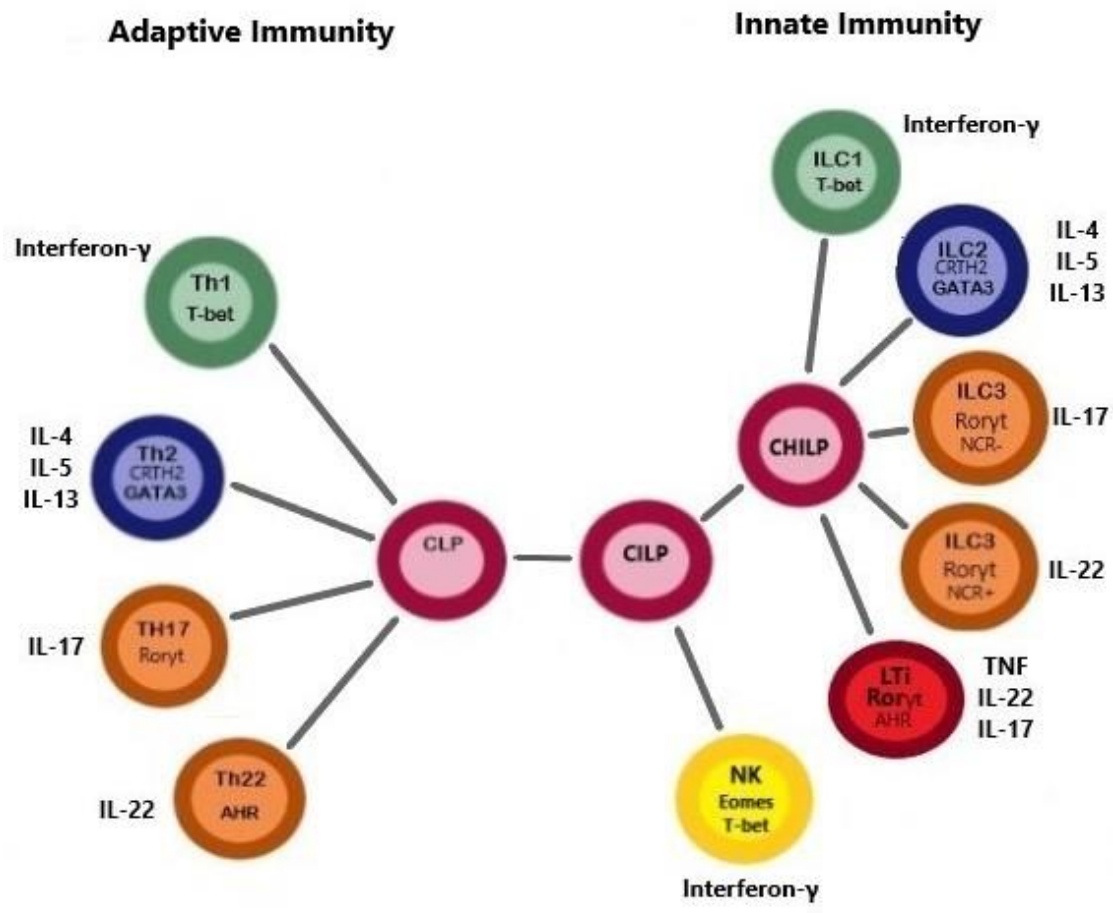

Figure 1- A common lymphoid progenitor (CLP), originated from a Hematopoietic Stem Cell (HSC) can give rise to adaptive and innate lymphocytes. Downstream of the CLP, a common ILC precursor (CILP) would then divide in: 1) a branch that differentiate into NK cells; 2) a branch that generate a common helper-ILC precursor (CHILP). The CHILP would further differentiate towards different branch of the ILC family namely ILC1, ILC2, ILC3 and generate LTi population.

Abbreviations: AHR: aryl hydrocarbon receptor; CRTH2: Chemoattractant receptor-homologous molecule expressed on TH2 cells; Eomes: eomesodermin; GATA3: GATA binding protein 3; IFNγ: interferon-γ; IL: interleukin; LTi: lymphoid tissue inducer; NCR: natural cytotoxicity receptor; NK: natural killer; ROR: Retinoic acid–related orphan receptor; T-bet: T-box transcription factor 21. (Figure credit: João Mendes)

3. Innate lymphoid cells in uterine and fetal compartments

Male et al. 2010 first made the distinction between uterine NK cells and ILC subsets in humans. In this work, ILC were first considered precursors of uterine NK cells, however these cells showed differences in function and phenotype through the expression of RAR Related Orphan Receptor C (RORC), Lymphotoxin α and IL2 genes[50], latter attributed to ILC3 and LTi subsets. Subsequent studies identified ILC1[51], ILC2[52] and ILC3[51,52] in human endometrium and decidua, based on evidence that ILC share a common lymphoid progenitor.

ILC1 can be found in the endometrium and decidua of pregnant women as early as 9–12 weeks of gestation[19], representing an important source of IFN-γ[51], implying a relevant role in the immune response against intracellular pathogens. In addition, the expression of CD103, an adhesion molecule that promotes the communication between lymphocytes and epithelial cells, suggests an epithelial localization of ILC1 in the endometrium and decidua[53].

Xu et al.2018 showed that, in term pregnancies, ILC2 is the most abundant population in human decidua, capable of producing Th2-type cytokines, such as IL-4, IL-5, and IL-13. In this study, the authors suggest that the pro-inflammatory properties of ILC2 might underlie the pathological process prompting PTL[33]. In fact, they also detected ILC3 in the decidua *parietalis*, capable of producing IL-17 and IL-22, suggesting that these cells may be responsible for inflammation-driven PTL.

ILC3 were also initially described as a subset of NK cells in human endometrium, expressing CD127, CD161, RORC and IL-22[50]. Later, work by *Vacca et al.* confirmed the ILC3 phenotype and their presence in human endometrium and decidua during pregnancy and further divided them into two subgroups ILC3 NKp44^{+} and ILC3 NKp44^{-}

[51,19]. It has been shown that, similarly to Th cells, ILC display some degree of plasticity in response to their microenvironment. Studies conducted in mouse models show that in response to IL-12 and IL-18, ILC3 reveal an increased expression of T-bet and decreased expression of RORγt, which results in IFN-γ production and loss of their capacity to produce IL-17 and IL-22 [54,55]. This data may explain in part the low numbers of ILC1 found by *Xu et al.2018* in late gestation, due to overlapping functions with ILC3 phenotypes. Whether the inflammatory response considered to take place in preterm birth is attributed to the actions of ILC1 or ILC3 remains to be elucidated.

Amniotic fluid surrounds the embryo and fetus, protecting it mechanically during development in the event the maternal abdomen is subject to trauma. Amniotic fluid also protects the fetus from infectious agents due to its inherent antibacterial properties [56] and provides the fetus with a reservoir of fluid, nutrients, and growth factors to allow normal development and growth of fetal organs[56]. The main population identified in amniotic fluid is ILC3 of fetal origin, expressing CD127, CD117, CD161, and CD56 [56]. Indeed, ILC3 are abundant in the amniotic fluid until the second trimester[56], when their numbers start to decay as gestation progresses[57]. In this context, the ability of ILC3 to produce IL17 suggests a role in regulating intra-amniotic infection[56].

Fetal ILC have been identified in the liver, secondary lymphoid organs (SLOs), intestine, lung and cord blood[58,59]. In the liver, ILC assume a preponderant role since it is in this organ that hematopoiesis takes place[60,61], and where ILC precursors (ILCPs) originate[,58] . In their work, Lim *et al.2017* have suggested that circulating ILCP can migrate to different tissues where they differentiate according to fetal development needs and organogenesis[58]. Moreover, studies from animal models suggest that the presence of LTi cells in the fetus is essential for the successful formation of SLOs such as the spleen, mesenteric lymph nodes, and Peyer's patches[62–65].

Previous work has demonstrated that NK, ILC1, ILC2, and ILC3 subsets can be readily identified in the human fetal intestine[44,56,66,67]. It has been shown that intestinal ILC2 produce IL-13 [66], while ILC3 and LTi-like cells produce IL-17A and IL-22 [44].

Mjösberg et al. 2011 report the presence of ILC2 in the fetal lung[66], while *Marquardt et al.2016* have detected increased numbers of ILC3 in the second trimester when compared to the first trimester[56].

Most of the information available regarding ILC comes from animal models. However, considering the great degree of similarity between mouse and human ILC ontology [12], we attempted a reasonable extrapolation to human biology.

The ubiquity of ILC presence in uterine and fetal compartment denotes the importance of innate immune system in pregnancy. Not only ILC take part in organ formation, but they also act as key mediators in protecting the fetus against infection and pathogens. The main findings that are the object of this work are summarized on table I.

Table I – Main findings in the literature regarding human ILC in uterine and fetal compartments.

Resident ILC population	Species	Tissue	Gestation	Main Findings	Reference
ILC1/ILC2/ILC3	Human	Liver	6 to 10 weeks	In this study the authors identified that fetal liver harbored almost exclusively NKp44− ILC3s, with ILC1s, ILC2s, and NKp44+ ILC3s being detectable only at later gestational age. Also, NKp44− ILC3s in the fetal liver were different from the corresponding population in the adult since fetal ILC3s expressed NRP1.	*Forkel M. et al. 2017*
ILC3	Human	Amniotic fluid Intestine Lung	15 to 16 Weeks	ILC3 are the main ILC population in the amniotic fluid, producing high levels of IL-17 and TNF. ILC3s are abundant in fetal intestine and lung.	*Marquard et al. 2016*
ILC1/ILC2/ILC3	Human	Umbilical cord blood, Fetal liver	14 to 20 weeks	Human ILCPs robustly generate all ILC subsets in vitro and in vivo. This study identified unipotent ILCPs. that could give rise to IFN-g$^+$ ILC1s, IL-13$^+$ ILC2s, or IL-17A$^+$ and/or IL-22$^+$ ILC3s.	*Lim et al. 2017*
ILC1/ILC2/ILC3	Human	Gut	16 to 22 weeks	The study applied mass cytometry to analyze ILC in the human fetal intestine, distinguished 34 distinct clusters and identified a previously unknown intermediate innate subset that can differentiate into ILC3 and NK cells.	*Li N. et al. 2018*
ILC1/ILC3	Human	Decidua	1st Trimester	Decidual ILC3 have a frequency comparable, if not higher, with that of tonsil ILC3. Results from this study indicate that NCR$^+$ ILC3 and LTi-like cells present in decidua can produce pro-inflammatory cytokines including IL-8, IL-22, IL-17A, TNF, and IFN-γ.	*Vacca et al. 2015*
ILC1/ILC2/ILC3	Mouse/ Human	Endometrium/ Decidua	1st Trimester	CD127$^+$ ILC1s are absent in human endometrium or decidua. ILC2s were found deep in the uterine wall and not in human or murine decidua, nor in human endometrium. NCR$^+$ ILC3s and LTi-like ILC3s are present in both human endometrium and decidua.	*Doisne et al. 2015*
ILC3	Human	Decidua	1st Trimester	NCR$^+$ ILC3 are present in decidual tissue where they produce CXCL8 and GM -CSF, suggesting that they may have a role in neutrophil recruitment and survival. NCR$^+$ ILC3-derived GM-CSF induces the expression of both Heparin-binding EGF-like growth factor and IL1ra in neutrophils, important in angiogenesis and trophoblast growth/invasion.	*Vacca et al. 2016*
ILC3	Human	Amniotic fluid (AF)/1st and 2nd trimester fetal tissue	1st Trimester	CD45+ cells in AF contained very low frequencies of T cells, B cells and monocytes. Fetal CD103+ ILC3s in AF are functional and produce high levels of IL-17 and TNF. Similar subset was identified in second trimester fetal gut and lung, suggesting that CD103+ ILC3s develop in fetal tissues and subsequently egress to the AF.	*Marquardt et al. 2016*
ILC2	Human	lung and gut	-	In fetal gut, ILC2 expressed IL-13 but not IL-17 or IL-22.	*Mjösberg et al. 2011*
ILC3	Human	PBMCs	3rd Trimester	Increased IL-17 levels observed in patients with preeclampsia, gestational diabetes and chronic diabetes are associated with innate lymphoid cells 3 (ILC3).	*Barnie et al. 2015*
ILC1/ILC2/ILC3	Human	Decidua	Term and Preterm Pregnancies	The proportion of total ILC was increased in the decidua *parietalis* of women with preterm labor. ILC1s were a minor subset of decidual ILC during preterm and term gestations; ILC2s were the most abundant ILC subset in the decidua during preterm and term gestations. The proportion of ILC2s was increased in the decidua *basalis* of women with preterm labor. The proportion of ILC3s was increased in the decidua *parietalis* of women with preterm labor; during preterm labor, ILC3s had higher expression of IL-22, IL-17A, IL-13, and IFN-γ compared to ILC2s in the decidua.	*Xu et al. 2018*

4. Innate Lymphoid cells and the induction of tolerance

In pregnancy, after a successful fertilization of the oocyte by the spermatozoa, and after reaching the phase of blastocyst, implantation in the uterine wall has to occur. At this point fetal-derived placental cells, denominated trophoblast, have to invade a modified layer of the maternal uterus, the decidua. These cells might follow two different pathways: some differentiate into the syncytiotrophoblast layer, representing the interface with maternal blood, to regulate oxygen and protein transport; while others follow an invasive pathway and differentiate into extravillous trophoblast (EVT) cells[68].

In order to escape the maternal immune system, trophoblast cells only express human leukocyte antigen (HLA) HLA-C, and the non-classical HLA-E, HLA-F and HLA-G molecules[69–72]. In pregnancy, one key mechanism regulating induction of tolerance is through the actions of HLA-G molecules. HLA-G gene is located at chromosome 6, within the class I gene cluster of MHC. HLA-G belongs to the non-classical HLA-class I (or class Ib) gene; it is expressed mainly in the fetal-maternal interface on the extravillous cytotrophoblast[73], amnion[74], thymus[75] and its soluble form can be detected in peripheral blood[76]. HLA-G exerts its effects by modulating antigen presenting cells [77]; suppressing proliferation of $CD4^+$ T lymphocytes[78,79] and inhibiting of NK cells' actions. In fact, HLA-G inhibits NK cells[76] cytolytic actions, upregulates NK inhibitory receptors[80] and is essential for implantation[81].

Also, in this perspective, there is evidence that progesterone, a key immunomodulatory steroid hormone, contributes to a pregnancy protective milieu by promoting HLA-G expression[82] and regulating NK activity[83,84].

Tolerance is widely regarded as an adaptive response. Accordingly, it is a process that involves antigen presentation, clonal expansion, and the formation of memory cells; the

expression of HLA class II molecules in ILC2 and ILC3 population suggests that these cells might also have a role in pregnancy by presenting paternal antigens to the mother's immune system. While ILC2 seem capable of eliciting T helper proliferation, *Hepworth et al. 2013* reported in animal models that ILC3 lack classical co-stimulatory molecules such as CD40, CD80 and CD86. If it is the case, ILC3 antigen presentation may, in fact, limit T cell responses by negatively regulating CD4+ T cell responses in vivo[38] through T cell anergy[85].

In respect to NK cells, it is suggested that the fetus evades dNK actions, due to the less polymorphic nature of the HLA-C genetic locus. In fact, in a review by *Su Liu et al. 2017*, it is highlighted that the dimorphic nature of HLA-C and the highly polymorphic nature of killer cell Ig-like receptors (KIR), might promote the interaction of paternal HLA-C antigens with uterine NK KIR receptors from the mother. Moreover, HLA-E, HLA-F, and HLA-G also have ability to bind dNK cells[86].

Three classes of different Nk receptors can bind HLA-I molecules, in the context of pregnancy: CD94/NKG2; KIR; and Ig-like transcripts (ILT) also called leukocyte immunoglobulin-like receptors (LILR)[86].

As put forward in his review *King A. et al (2000),* HLA-E will bind CD94/NKG2 receptor, and this signal inhibits cell lysis, due to the overexpression of the variant NKG2A. It is also mentioned that KIRD2 expressed on NK cells, have the ability to discriminate between two distinct HLA-C; one for self HLA-I, and other for non-self. This might be a preponderant feature in the evasion of EVT cells. As regard to HLA-G, it will bind to ILT receptors on NK cells, and depending on the type of receptors it binds (ILT2, ILT4) the signal might be inhibitory or activator[87].

In this context, there is evidence that progesterone, a key immunomodulatory steroid hormone, contributes to a pregnancy protective milieu by promoting HLA-G expression[82] and regulating NK activity.

Whether ILC are on the forefront in establishing tolerance towards the fetus is a matter that requires further research.

5. Innate Lymphoid cells in pregnancy related diseases

Studies in NK cell biology corroborate the involvement of the innate immune system in preterm birth, preeclampsia, fetal growth restriction, morbidly adherent placentation, as well as, spontaneous abortion[88–93]. Dysregulation or expansion of pro-inflammatory ILC populations may directly promote disease, through production of pro-inflammatory cytokines, namely IL-17, considered important in the pathogenesis of preeclampsia and preterm birth[94]. In addition, high levels of IL-18 and IFN-γ have been associated with preeclampsia[95]. Moreover, in preterm birth there is evidence for an inadequate inflammatory response[96].

Progesterone has been known to play an important role in reproductive health for the initiation and maintenance of pregnancy, with good results in the prevention of spontaneous abortion and recently in preterm labor[5,97,98]

The immunosuppressive effects of progesterone have been recognized for a long time. Despite its mode of action remaining largely unknown, progesterone has been widely adopted by clinicians around the world for prevention of preterm birth. Our group has already demonstrated that progesterone modulates human T regulatory cell population during pregnancy[2,3,97,99]. There is also evidence, conducted in a small sample of T cell clones, suggesting that progesterone favors Th2, while dampening Th1 and Th17

responses, and thus participates in the establishment of a favorable environment for pregnancy by its effects on T-cells[100]. Work from *Henderson et al. 2003* shows that NK cells do not express progesterone receptors[101]; however, the expression of CRTH2 in ILC2 suggests that ILC are subject to hormonal regulation. Also work done by *Gibson et al. 2020* shows that uterine NK (uNK) cells are regulated by membrane estradiol receptors (E46), highlighting the relevance of hormone regulation in NK activity during pregnancy.

6. Preterm birth with spontaneous preterm labor

Preterm Birth (PTB) is defined as delivery occurring before 37 completed weeks of gestation. Approximately 75% of preterm births (PTB) occur between 34 and 36 weeks. Although these late preterm infants experience significant morbidity, the great majority of perinatal mortality and most serious morbidity occur amongst the 16% of them whose birth occurred before 32 weeks[102].

PTB can result from a range of causes such as exposure to environmental triggers, maternal stress, fetal or maternal genetic abnormalities, and hormonal imbalance, amongst others.

In spite of the definitions chosen and the methods used to determine gestational age, the true incidence of PTB has increased in developed countries regardless of multiple strategies being carried out to avoid it. Hence, current estimate rates vary between 5% and 11% in developed countries and 18% in developing countries[103]. Notwithstanding the efforts to identify the main reasons for this increase, the biological mechanisms underlying PTB are still unknown. Therefore, the development of evidence-based management approaches for medical and obstetrical complications before 37 weeks gestational age is mandatory.

Preterm labor (PTL) falls into two broad categories, according to whether one or more steps of the parturitional process. The first group, often called *spontaneous preterm labor*, comprises preterm labor with intact membranes, preterm premature rupture of the membranes (pPROM), preterm cervical effacement or insufficiency, and, in some instances uterine bleeding of uncertain origin. The second group, entitled *indicated preterm labor*, comprises preterm labor that are medically initiated because of maternal or fetal compromise (preeclampsia, renal disease, diabetes *mellitus* with vascular disease, placenta *praevia* and intrauterine growth restriction). These categories are sometimes indistinguishable in clinical practice but are useful to systematize interventional strategies[104,105]. This research will focus only on spontaneous PTL, without pPROM nor preterm cervical insufficiency.

Amongst the risks that contribute to PTL are multiparous women below the age of 18 [106] and women with previous PTB[107]. A meta-analysis conducted by *Wendt et al. 2012*, concluded increased odds of PTL at inter-pregnancy intervals inferior to six or twelve months[108]. Moreover, short cervical length is associated with PTL; shorter lengths are associated with greater risk - usually the value 25mm is used as a cut-off[109,110]. Singletons and twins resulted from *in vitro* fertilization (IVF) have higher risk of PTL than spontaneously conceived singletons and twins pregnancies[111]. Other pregnancy complications that contribute to a higher risk of PTL are pre-eclampsia, pregestational and gestational diabetes, cervical incompetence, periodontal disease, maternal anemia, obesity, short stature, and low maternal vitamin D[109,112–119]. Placental, uterine, or fetal conditions such as placental *abruption*, placenta previa, polyhydramnios, uterine anomalies, leiomyoma, and fetal birth defects have also been associated with increased risk of PTL[120–125]. Smoking and the use of recreational or illicit drugs also contributes to an increased risk of PTL[126–129]. Even though many socio-demographic, obstetric, and

environmental factors have been attributed to PTL, its etiology remains largely unknown. One key aspect that assumes a preponderant role in this work is infection and inflammation. In fact, several infectious conditions have been associated to higher risk of PTL including human immunodeficiency virus, bacterial vaginosis, *Chlamydia trachomatis* infection, chorioamnionitis, urinary tract infections (particularly pyelonephritis), hepatitis C, malaria, and syphilis[130–137].

7. Progesterone

Pregnancy is considered to defy immunologic principles, since a semi-allogenic conceptus is tolerated rather than rejected. Local immune suppression of alloreactive responses to paternal antigens is essential for fetal development. Steroid hormones like progesterone and estradiol (E2) as well as gonadotropins, such as the human chorionic gonadotropin (hCG) are fundamentally involved in the regulation of the menstrual cycle and in the establishment and maintenance of pregnancy[138,139].

Progesterone is a steroid hormone primarily produced by the ovaries, placenta, and adrenal glands in humans. Progesterone was first identified by *Allen and collaborators* in 1933 and were the first to determine the molecular weight and partial molecular structure[140]. During the menstrual cycle, progesterone levels are relatively low during the pre-ovulatory phase, rise after ovulation, and are elevated during the luteal phase[141]. When pregnancy occurs, hCG initially maintains progesterone levels by inducing its production by the *corpus luteum*. After the luteal–placental shift, the placenta takes over progesterone production[142]. Progesterone is responsible for the appropriate preparation of the uterus for the implantation of the trophoblast, through the differentiation of stromal cells into decidual cells, in a process denominated decidualization. Moreover,

progesterone decreases the contractility of uterine smooth muscle cells[143,144]. Additionally, although placental progesterone production is maintained, some studies suggest that there is a functional decline in its activity that might be associated with the initiation of labor[145,146]. One of the major challenges in studying progesterone withdrawal in the initiation of labor, is the fact that the biochemical mechanisms involved in these processes are significantly different from species to species, making it very difficult to find a suited animal model for studying such process. In humans and primates, the placenta is the primary site of progesterone synthesis for most of pregnancy, but in most animal species, the *corpus luteum* is the site of progesterone synthesis throughout pregnancy[147].

There is evidence to support the immunomodulatory effects of progesterone in pregnancy[148]. Progesterone has been shown to inhibit NF-kB, COX-2 and PG synthesis; thrombin-induced IL-11 and IL-8 production[149,150]. Also, progesterone promotes T helper 2 cell differentiation and suppresses T helper 1 cells *in vitro*[151]. Moreover, progesterone promotes a downregulation of the pro-inflammatory cytokines IL-1β and IL-8 in peripheral blood leucocytes and a reduction in CD11b expression (required for transmigration) in circulating neutrophils[152]. Progesterone also contributes to myometrial quiescence and exhibits direct tocolytic effects[153]; increases cyclic AMP and reduces intracellular calcium, thereby reducing contractility [154].

7.1. Progesterone receptors

Progesterone signaling is accomplished via classic and non-classical pathways. The classical signaling pathway preconizes the ligation of progesterone to DNA binding sites called progesterone response elements (PREs). PRs exists in two isoforms PR-A and PR-B that differ only in that human PR-A transcript is 164 amino acids shorter than PR-B[155]. The immunomodulatory actions of progesterone are mainly attributed to PR-B, which is present for most of the pregnancy. PR-A expression increases markedly during the third trimester and is thought to inhibit PR-B actions suggesting a regulatory role[156]. The non-classical pathway is achieved by the ligation of progesterone to membrane receptors (mPR), belonging to the progestin and adipoQ receptor (PAQR) gene family, that bind progesterone at the cell surface and rapidly generate intracellular second messengers[157]; mPR receptors have been shown to be present in T regulatory cells[3]. Five genetically distinct forms of mPRs are known, namely mPR-α (PAQR7), mPR-β (PAQR8), mPR-γ (PAQR5), mPR-δ (PAQR6), and mPR-ε (PAQR9)[158]. While the actions of PRs are genomic and therefore slow, the progesterone signalling through mPRs are non-genomic and consequently faster. Work from our group, and others, have demonstrated that mPR-α is downregulated in preterm birth[3,159], while in term pregnancies there is a downregulation of mPR-α and mPR-β [159]. To date, there is no reference in the literature regarding the expression of progesterone receptors in ILC. The only relevant data comes from "The Immunological Genome Project" reporting presence of the progestin and adipoQ receptor family member 7 gene (PAQR7), a membrane progesterone receptor, as well as the nuclear progesterone receptor gene (PGR), in ILC1, ILC2 and ILC3 populations[160].

Studies conducted in rodents highlight the importance of progesterone in maintaining pregnancy because loss of systemic progesterone production, as a result of ovariectomy, or the use of the progesterone antagonist RU-486 induces cervical remodeling and leads to PTL[161]. In contrast, human labor is not preceded by a decrease in serum progesterone levels but instead results from a 'functional' withdrawal. Moreover, the progesterone antagonist RU-486 is widely used in humans to promote cervical ripening and readily induces abortion if given in early pregnancy[162], demonstrating the essential role of progesterone for maintenance of pregnancy. Despite the molecular and biological mechanisms of progesterone mode of action remain mostly unknown, some proposals are: 1) Modulation of the innate immune function (including inhibition of human neutrophil degranulation, suppression of potent type I interferon (IFN)-producing dendritic cells); 2) Modulation of the adaptive immune function (Treg proportions gradually fall during the course of pregnancy in women who were given progesterone treatment); 3) Progesterone dependent regulation of P4-Induced Blocking Factor (PIBF) expression (PIBF is a PR-regulated gene and potent immune-modulator capable of blocking cytotoxic activity and prostaglandin F2a (PGF2a) synthesis in lymphocytes); 4) Suppression of pro-inflammatory cytokines (promoting a Th2 dominant cytokine profile); 5) Anti-cytolytic activity (progesterone decrease the cytotoxic activity of decidual lymphocytes and block their perforin release in a concentration-dependent manner)[16]

7.2. Progesterone in Preterm Labor

Progesterone administration is commonly used in clinical practice to prevent PTB. Studies reporting its efficacy date over three decades ago[164] and the American College of Obstetricians and Gynecologists (ACOG) recognizes its use[165]. In an attempt to normalize protocols a review paper by *Tita et al. 2009* propose that the administration of progesterone for PTB prevention should follow specific guidelines and defines four essential point: "(1) for women with a prior spontaneous preterm birth (SPTB), weekly IM 17P (250 mg) initiated at 16-20 weeks, or daily vaginal natural progesterone (at least 100 mg) beginning before week 24 should be given; (2) for women with a short cervix (≤ 15 mm), 200 mg of vaginal natural progesterone suppositories; (3) for women with a twin pregnancy, progesterone is not routinely indicated, although its use may be prudent in the specific scenarios of a prior SPTB (250 mg 17P IM) or significantly (≤15mm) shortened cervix (200 mg suppository vaginally); and (4) for women with arrested preterm labor, progesterone (400 mg daily vaginal suppository or 341 mg 17P IM twice weekly) may be considered" [166]. In a systematic review and meta-analysis, *Romero et al. 2012* concluded that vaginal progesterone was associated with significant lower risk of preterm birth occurring from <28 weeks of gestation through <35 weeks of gestation, when comparing vaginal progesterone with placebo in women with a singleton gestation and a cervical length 25 mm[167]. Later, in a new study, *Romero et al. 2018* reaffirms his claims and in a systematic review and meta-analysis of individual patient data, concludes that vaginal progesterone decreases the risk of preterm birth and improves perinatal outcomes in singleton gestations with a short cervix, without any demonstrable deleterious effects on childhood neurodevelopment. These discrepancies in the literature highlight the necessity in the scientific and medical community for more clinical trials in order to assess the

benefits of progesterone use in the prevention of PTL. Moreover, more scientific studies are needed to clarify progesterone mode of action. In this sense, this investigation has a high applicability as PTL incidence is rising, despite multiple primary interventions being implemented to lower it. As such, the number of women worldwide who may benefit from this treatment is high. It is extremely important to point out that each extra day in uterus before term, conveys a significant reduction in children morbidity and mortality and hospital costs.

With this work, we intend to describe ILC1, ILC2 and ILC3 populations throughout pregnancy. In addition, by analyzing ILC relative frequencies before and after the administration of progesterone, we aim to study its effects on labor. Moreover, we will determine plasma concentrations of the most representative cytokines within samples of pregnant women in the moment of labor. In doing so, we aim to clarify the mechanisms underlying PTB, adding knowledge to the field of immunology. Moreover, with the increase incidence of PTB cases, this work aims to reduce children morbidity and mortality, as well as, hospital costs, through the development of new clinical protocols and discovery of new therapeutic targets.

8. Methods

The present study was approved by Coimbra Hospital and University Centre ethics committee. Signed informed consents were obtained from all patients whose blood samples, placentas and clinical data were used in this study. All methods involving human participants, human sera and human data were carried out in accordance with Declaration of Helsinki and approved by the Faculty of Medicine from Coimbra University, as well as The Ethics Committee for Health of the Centro Hospitalar e Universitário de Coimbra (document reference N. º 179/CES).

8.1. Population

Female patients who planned to deliver at the Obstetric Department of Coimbra Hospital and University Centre (CHUC) were invited to participate in the study.

The study inclusion criteria consisted of pregnant women monitored by normal prenatal appointments and women presenting to the emergency room in labor. Inclusion criteria for FTB comprised: healthy pregnant women attending normal prenatal appointments; full term singleton pregnancies, delivered after spontaneous labor; and first prenatal appointment before 14th week gestation. The inclusion criteria for the PTB group were as follows: admission to the Fetal Maternal Medicine Obstetric Department of CHUC with confirmed spontaneous PTL, singleton pregnancy, gestational age between 24 weeks + 0 days and 36 weeks + 6 days, intact membranes, cervical length ≤25 mm and the use of *Atosiban* (competitive oxytocin receptor antagonist) for tocolysis (for contraction cessation). Administration of natural progesterone was done after tocolysis with Atosiban, vaginally, once daily, in a 200mg dosage.

Exclusion criteria were the following: multiple gestation; preterm rupture of membranes; chorioamnionitis; placenta *preavia* or placental *abruption*; intrauterine growth restriction; and pre-existent maternal diseases, namely: hypertension, diabetes *mellitus*, autoimmune diseases, and allergies. Clinical chorioamnionitis was diagnosed based on histologic evaluation and clinical laboratorial parameters like fever, maternal tachycardia, fetal tachycardia, maternal leukocytosis, uterine tenderness, foul-smelling amniotic fluid; elevated maternal C-reactive protein and/or amniotic IL-6.

Women subjected to elective pre-labor *caesarean* section were not included as they had other medical pathologies, not focused on this work.

8.2. Isolation and characterization of innate lymphoid cells

In the FTB group peripheral blood was taken during routine blood analysis on 3 occasions: 1st, 2nd and 3rd trimester[1]. After delivery, peripheral blood, cord blood and placenta were collected. In PTB, peripheral blood samples were obtained on two occasions: before and 24 hours after treatment with vaginal progesterone. After delivery, peripheral blood, cord blood and placenta were collected.

Cells were isolated using a *Ficoll-paque*™ gradient and stained as further discussed for flow cytometry analysis.

In both PTB and FTB groups, the placenta was rinsed in phosphate buffered saline (PBS, Ca^{2+}- and Mg^{2+}-free) (Corning®, New York, USA), to wash cloths and superfluous blood. Decidual tissue was dissected while soaking in 1× PBS (Ca^{2+}- and Mg^{2+}-free). Lymphocytes were isolated from the decidua *basalis* and adjacent tissue (*villi*) as described by *Yi Xu et al.* 2015 [168]. Cells were counted on a *Beckman Coulter AcT Diff*

[1] 1st Trimester till 13 weeks plus six days; 2nd trimester between 14 and 28 weeks; 3rd over 28 weeks

automatic cell counter (Beckman Coulter, Brea, California, EUA), and a 100 µL cell suspension containing $1x10^6$ isolated lymphocytes was placed in a cytometry tube and labelled with primary antibodies (BD Biosciences, San Jose, USA). For lymphocyte discrimination, $CD45^+$ and $CD3^-$ were used. Lineage-negative (Lin^-) cells were labeled with CD1 (clone HI149), CD11c (clone B-ly6), CD34 (clone 581), CD123 (clone 7G3), TCRγδ (clone xB1), TCRαδ (clone T10B9), BDCA2 (clone 201A), FcER1 (clone AER-37), CD19 (clone HI149), CD14 (clone M5E2), and CD94 (clone HP-3D9) and discriminated against CD127 (clone A019D5). Cells expressing CD161 (clone HP-3G10) were then selected. To ascertain the different ILC populations, CD117 (clone 104D2), CRTH2 (clone BM16) and NKp44 (clone p44-8) antibodies were used as described *by Hazenberg et al. 2014* [34]. The same procedure was taken for isolated lymphocytes from blood samples and cord blood, in whole occasions. Gating strategy can be viewed in figure 2 and figure 3. Stained samples were acquired on a *BD FACS Canto II* flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with 3 lasers to allow multicolour detection with different fluorophores, using BD *FACSDiva v.6.1.3 software* (BD Biosciences, San Jose, USA). All samples were then analyzed with *FlowJo v.10.7 software* (Tree Star Inc., Ashland, OR, USA).

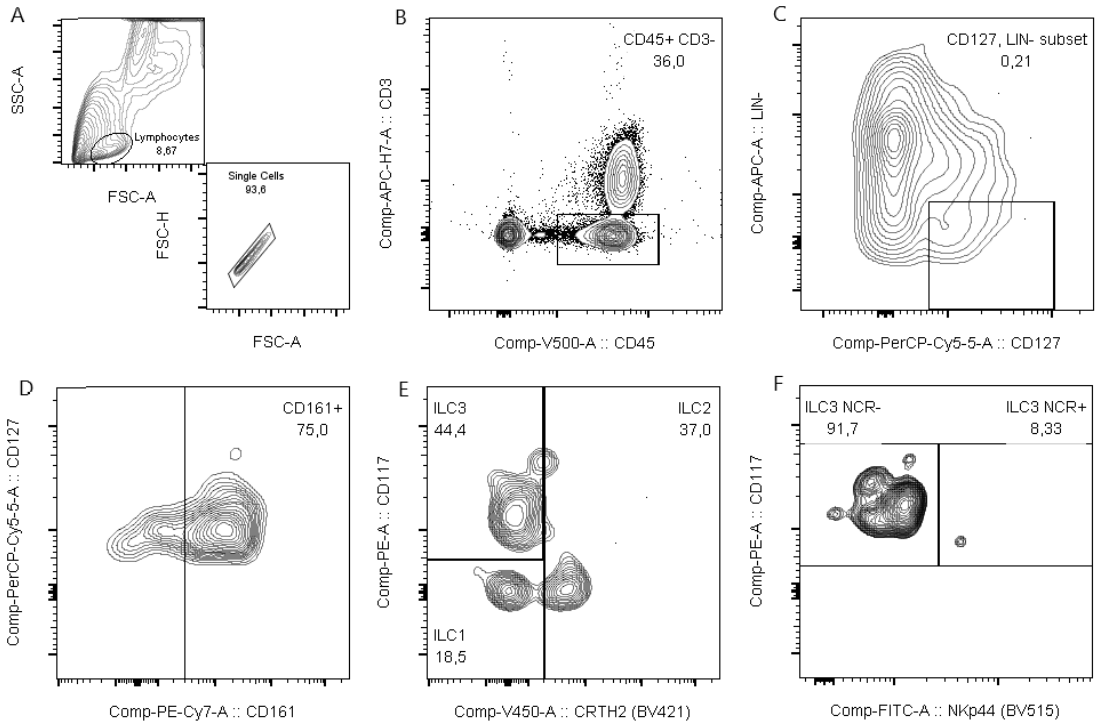

Figure. 2 – Gating strategy for identification of ILC3 subpopulations. **A.** Identification of lymphocyte population. **B.** Gating of $CD45^{+}CD3^{-}$cells. **C.** Selection $Lin^{-}CD127^{+}$ cells **D**. isolating $CD161^{+}$ cells. **E.** Gating ILC3 cells as $CRTH2^{-}$ $CD117^{+}$. **F**. Discrimination between ILC3 NCR^{+} and ILC3 NCR^{-} based on NKp44 expression (Data analyzed in FlowJo®).

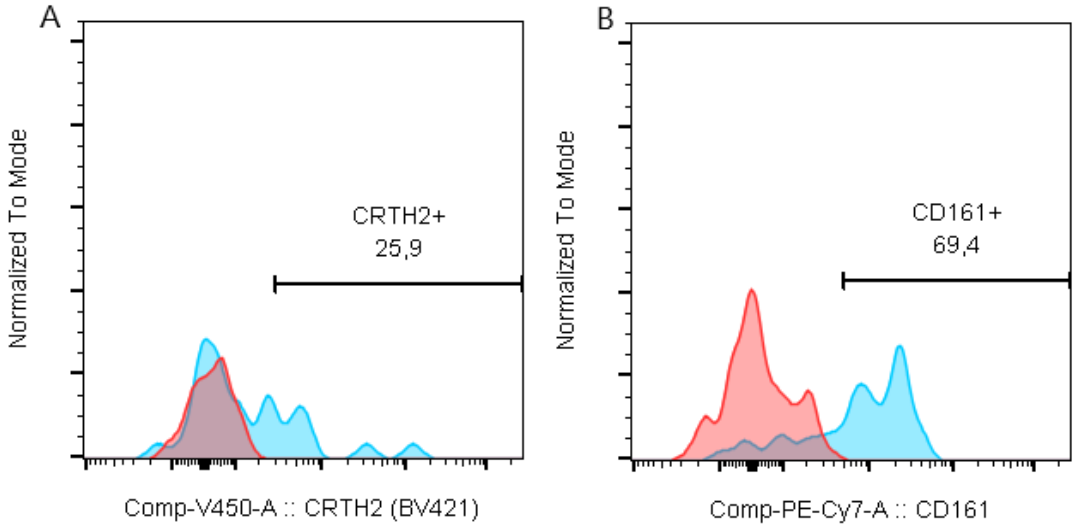

Figure. 3 - Full minus one (FMO) for CRTH2 (**A**) and FMO for CD161 (**B**)

8.3. Enzyme-Linked Immunosorbent Assay (ELISA)

Due to budget limitations, the most representative cytokines were chosen for each ILC population: IFN-γ for ILC1, IL-4 for ILC2; IL-17 for ILC3 NCR^- and IL-22 for ILC3 NCR^+. The choice of IL-4 and not IL-13 for the most representative cytokine of the ILC2 group is due to the fact that, despite belonging to the same family, and sharing the same receptors, IL-4 is regarded as a regulatory cytokine (responsible for a Th2 polarization) whereas IL-13 is regarded as an effector cytokine[169]. Due to ILC low relative frequencies, we chose to valorize a possible regulatory action from IL-4, in detriment to an effector role from IL-13.

For IFN-γ, IL-4, IL-17 and IL-22 ELISA determination we used *Biolegend Legend Max™ ELISA Kits*. Peripheral whole blood and cord blood were collected in the moment of labor to a 6 mL EDTA tube. To separate plasma from whole blood, tubes were centrifuged for 15 minutes at 1000g. Samples were stored in 200 µL aliquots at – 80º C to prevent repetitive freeze/thaw cycles. 100 µL of plasma was used in triplicate and absorbance was determined using a *Bio-Rad® model 600 microplate reader*, according to manufacturer protocols (Bio-Rad, Hercules, CA, USA). Average of triplicate readings was performed, and a standard curve was generated using a four-parameter logistic curve-fit to determine plasma concentrations in pg/mL.

8.4. Statistical analysis

Each data set was analyzed using student t-test analysis with a confidence interval of 95%. Statistical analysis was performed using *GraphPad Pris*m, version 7 (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered statistically significant at a *p* value of <0.05 and are annotated as follows: * $p<0.05$; ** $p<0.01$; *** $p<0.001$ and **** $p<0.0001$.

9. Results

In the current investigation, the full-term group comprises fifteen pregnant women and the preterm group comprises six preterm who delivered preterm. In the full-term labor group, the median maternal age was 34 years (33 < 95% CI< 37), the median gestational age at delivery was 40 weeks (39 < 95% CI< 40), the median birth weight was 3635 grams (2962 < 95% CI< 3755), and the median placental weight was 500.5 grams (448 < 95% CI< 603). In the preterm labor group, the median maternal age was 32 years (22 < 95% CI< 35), the median gestational age at delivery was 36 weeks (34 < 95% CI< 37), the median birth weight was 2505 grams (2130 < 95% CI< 3125) and the median placental weight 472 grams (413 < 95% CI< 731); these data are summarized in table II. All women in this study were non-smokers. In the clinical data we found a significant statistical difference in birth weight, which is lower in the PTB group ($p < 0.001$ t-test, 95% CI).

Initially, our study consisted of twenty-four pregnant women enrolled in the FTB group, but six failed the second trimester sample collection and three failed sample collection in the moment of labor. In both cases, these pregnant women opted not to continue with the study. Moreover, the PTB was initially comprised of nine pregnant women; however, three chose not to continue with the study (dropouts).

Table II – Descriptive statistics of full-term and preterm group. We found a significant statistical difference in birth weight, which is lower in the study group (* $p < 0.001$ t-test, 95% CI)

	Median Maternal Age (Years)	**Median Gestational Age (Weeks)**	**Median Birth Weight (Grams)***	**Median Placenta Weight (Grams)**
Full-term birth (n=15)	34 *(33 < 95% CI< 37)*	40 *(39 < 95% CI< 40)*	3635 *(2962 < 95% CI< 3755)*	500.5 *(448 < 95% CI< 603)*
Preterm birth (n=6)	32 *(22 < 95% CI<35)*	36 *(34 < 95% CI< 37)*	2505 *(2130 < 95% CI< 3125)*	472 *(413 < 95% CI< 731)*

9.1 Cytometry results:

9.1.1 Peripheral Blood

In our investigation, we found no differences in ILC1 populations' relative frequencies in peripheral blood in FTB group during the first, second or third trimester (Figure 4). Additionally, we found no differences in ILC2 populations' relative frequencies in peripheral blood in FTB group during the first, second or third trimester (Figure 5). Likewise, when comparing ILC3 populations' relative frequencies in peripheral blood in FTB group during the first, second or third trimester there weren't any differences (figure 6).

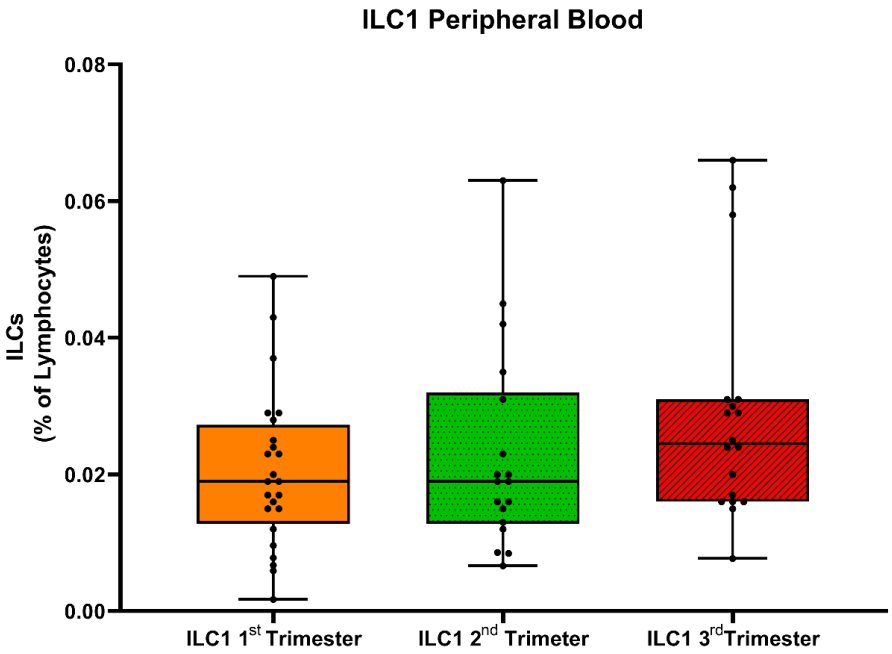

Figure 4 – Graphic displaying the relative percentage of the ILC1 populations, in relation to lymphocytes, in Peripheral Blood samples of the FTB in the 1st, 2nd and 3rd trimester.

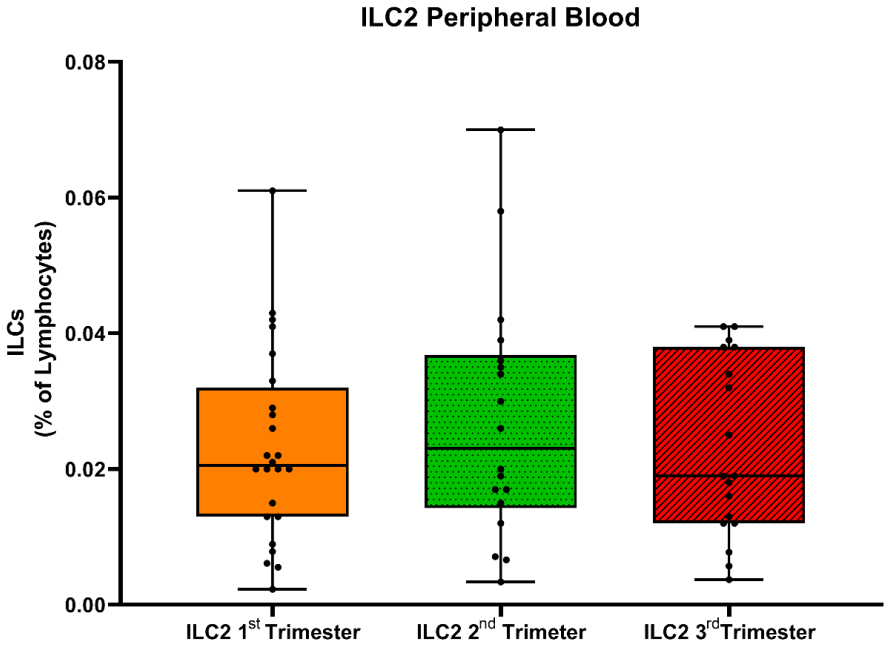

Figure 5 – Graphic displaying the relative percentage of the ILC2 populations, in relation to lymphocytes, in Peripheral Blood samples of the FTB in the 1st, 2nd and 3rd trimester.

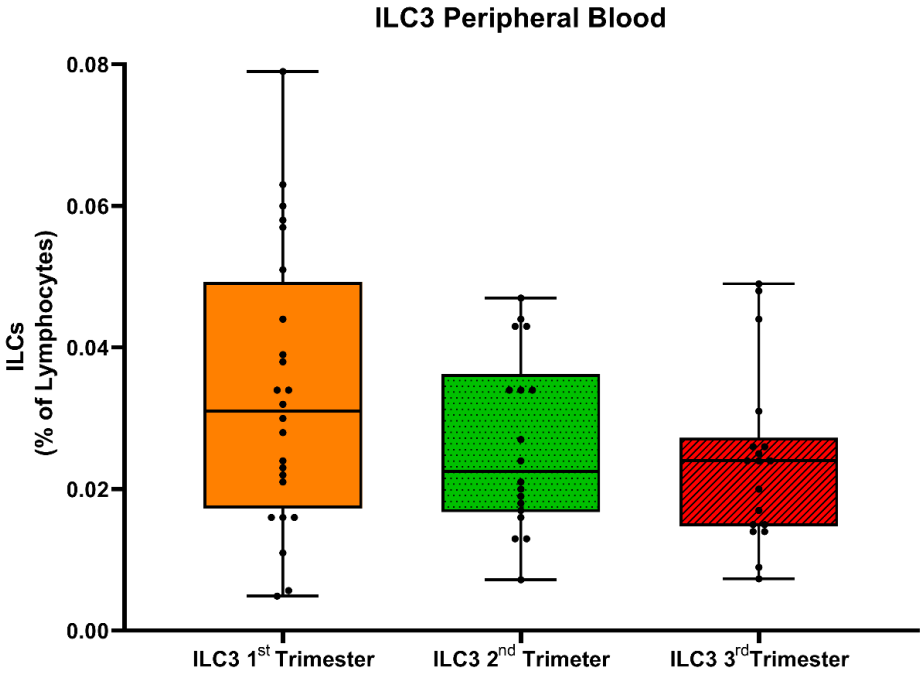

Figure 6 – Graphic displaying the relative percentage of the ILC3 populations, in relation to lymphocytes, in Peripheral Blood samples of the FTB in the 1st, 2nd and 3rd trimester.

9.1.2. Peripheral Blood from labor samples

Regarding our cytometry analysis, we found no differences when comparing peripheral blood samples from the moment of labor in the FTB group, when compared with peripheral blood samples from the PTB group (figure 7).

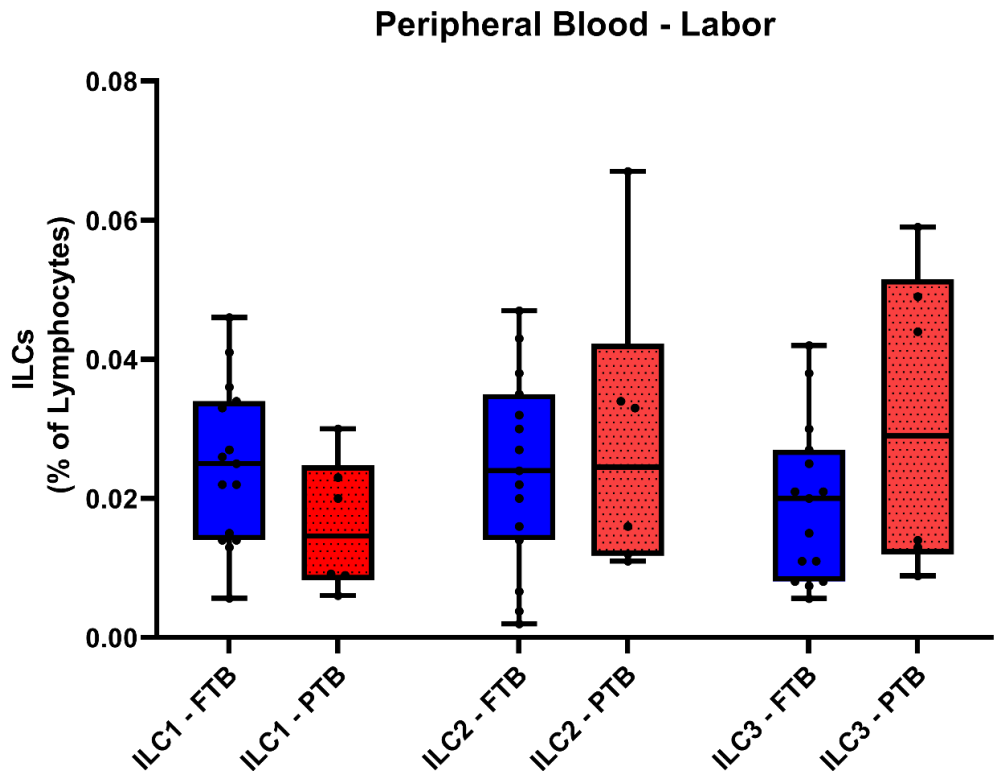

Figure 7 - Graphic displaying the relative percentage of the different ILC populations, in relation to lymphocytes, in Peripheral Blood samples in the moment of labor in full term birth (FTB) compared to preterm birth (PTB).

When observing the ILC3 subsets (NCR^- and NCR^+ cells) within the FTB and PTB group on labor, the ILC3 NCR^- population was clearly increased, representing the predominant ILC3 subset ($p< 0.001$, student t-test 95% CI) (Figure 8). The corresponding descriptive statistics are presented in table III.

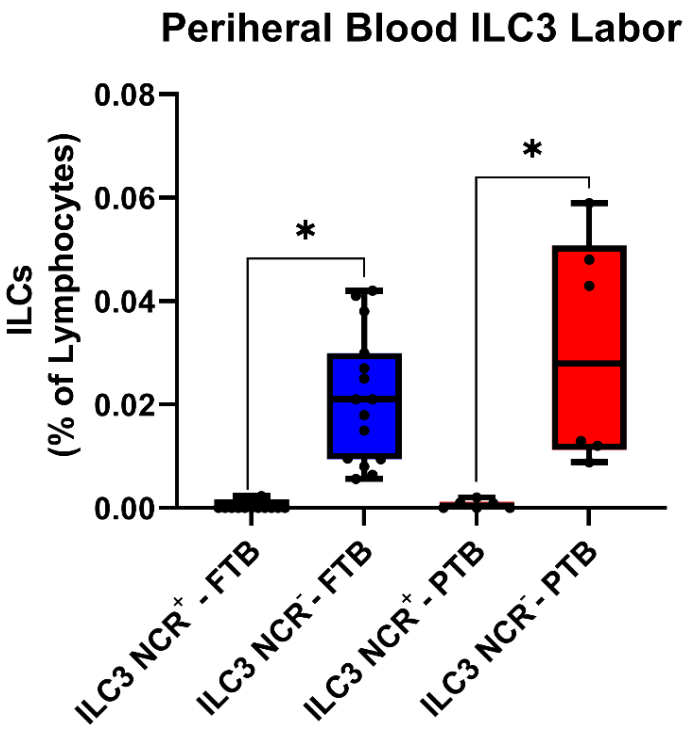

Figure 8 – ILC3 NCR Discrimination. Graphic displaying ILC3 subpopulations, in relation to lymphocytes, based on the presence of NCR receptor, determined in samples of peripheral blood from the moment of labor in the FTB and PTB groups. When analyzing ILC3 population within each group, it was possible to determine that NCR^- population was clearly increased, representing the predominant ILC3 subset (* p< 0.001, student t-test 95% CI).

Table III- Descriptive statistics of peripheral blood ILC3 from the moment of labor.

	ILC3		
	NCR^+ Median	**NCR^-** Median	**p Value** student t-test 95% CI
FTB	0,000 *0,000<95% CI <0,001610*	0,02100 *0,009420<95% CI <0,03000*	<0,0001
PTB	0,0004570 *0,000<95% CI <0,002000*	0,02800 *0,008860<95% CI <0,05900*	<0,0001

9.1.3. Peripheral Blood from preterm birth samples

When analyzing peripheral blood samples from women who underwent PTB, we found no statistical differences in ILC numbers before or after the administration of Progesterone in women who delivered PTB (Figure 9).

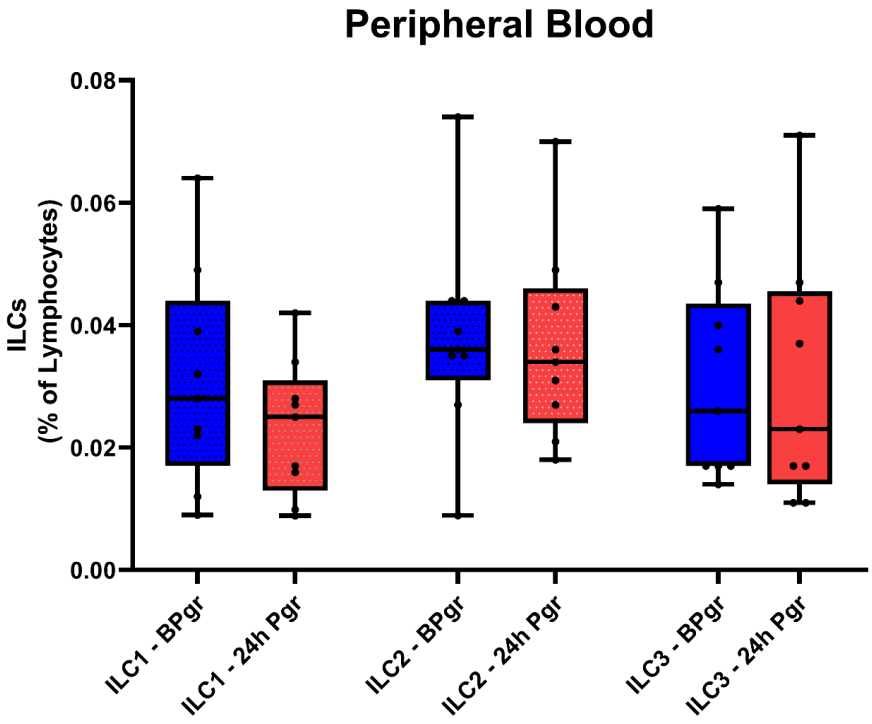

Figure 9 - Graphic displaying the relative percentage of the different ILC populations, in relation to lymphocytes, in Peripheral Blood samples before (BPgr) or 24h after the administration of Progesterone (24h Pgr) in the PTB group.

9.1.4. Decidual samples

Regarding the decidua, there was significant higher relative frequencies of ILC2 and ILC3 in the decidua of women from the PTB group when compared with women belonging to the FTB ($p<0,05$ t-test, 95% CI and $p<0.01$ t-test, 95% CI respectively) (Figure 10). Moreover, in decidual samples, we found ILC3 relative frequencies higher than those of ILC2 population, both in FTB and PTB groups ($p< 0.01$, student t-test 95% CI), representing the dominant population.

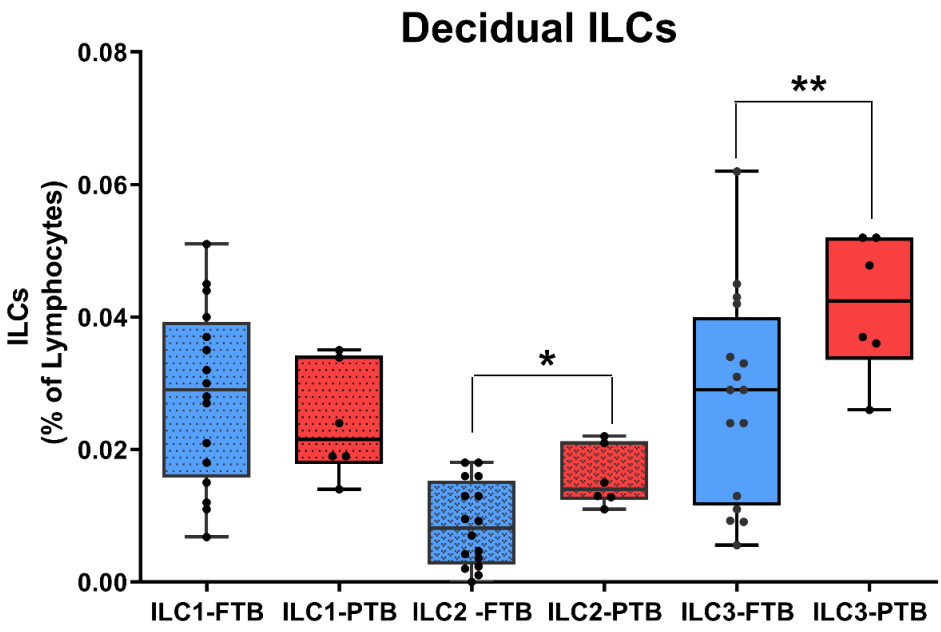

Figure 10 - Decidual ILC in FTB and PTB. Graphic displaying the relative percentage of the different ILC populations, in relation to lymphocytes, in full term birth (FTB) compared to preterm birth (PTB), in human decidua. Multiple t-student tests were used for statistical analysis with a 95% confidence interval, p-value * $p<0.05$; ** $p<0.01$ (two tailed).

The same result was obtained when analyzing ILC3 subsets (NCR^- and NCR^+ cells) within the FTB and PTB group; ILC3 NCR^- population was the predominant ILC3

subset (p< 0.001, student t-test 95% CI) (Figure 11). The corresponding descriptive statistics are presented in table IV.

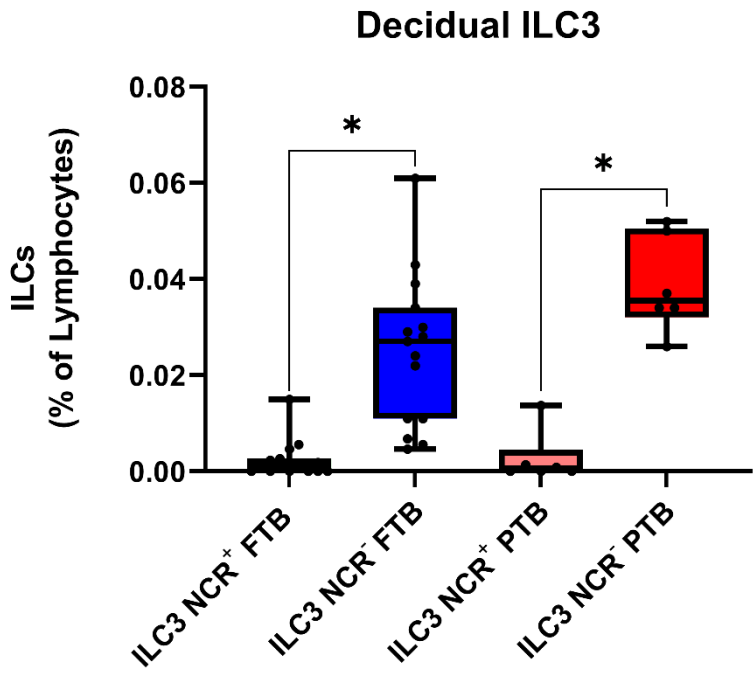

Figure 11 – ILC3 NCR Discrimination. Graphic displaying ILC3 subpopulations relative percentage, in relation to lymphocytes, based on the presence of NCR receptor, determined in samples of decidua from the FTB and PTB groups. When analyzing ILC3 population within each group, it was possible to determine that NCR^- population was clearly increased, representing the predominant ILC3 subset (* p< 0.001, student t-test 95% CI).

Table IV- Descriptive statistics of decidual ILC3 samples

	ILC3		
	NCR^+ Median	**NCR^-** Median	**p Value** student t-test 95% CI
FTB	0,001050 *0,000<95% CI < 0,002610*	0,02700 *0,01100<95% CI< 0,03400*	<0,0001
PTB	0,0004155 *0,000<95% CI < 0,01370*	0,03550 *0,02600<95% CI < 0,05200*	<0,0001

9.1.5. Cord blood samples

In our cytometry analysis of cord blood samples we verified higher ILC2 frequencies also in PTB group (p<0, 05 t-test, 95% CI) (Figure 12).

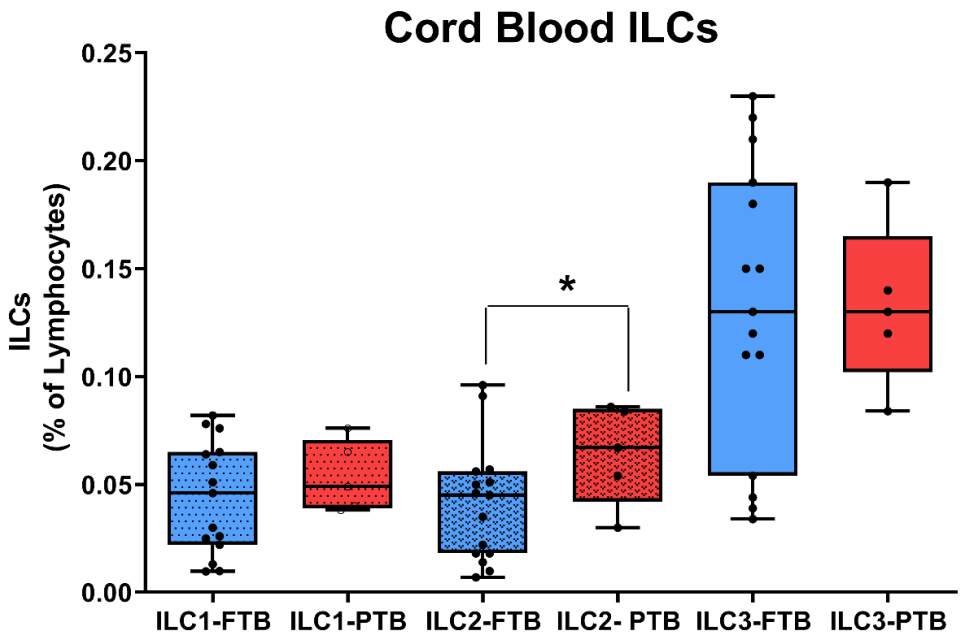

Figure 12 - Cord blood ILC in FTB and PTB. Graphic displaying the relative percentage of the different ILC populations, in relation to lymphocytes, in FTB compared to PTB in cord blood samples. Multiple t-student tests were used for statistical analysis with a 95% confidence interval, p-value * p<0.05; (two tailed).

Regarding the distribution of the relative frequencies of the different ILC3 populations, the same result was observed, when comparing ILC3 NCR^- and ILC3 NCR^+ cells within the FTB and PTB group. The ILC3 NCR^- population was the dominant ILC3 subset (p< 0.001, student t-test 95% CI) (Figure 13). The corresponding descriptive statistics are presented in table V.

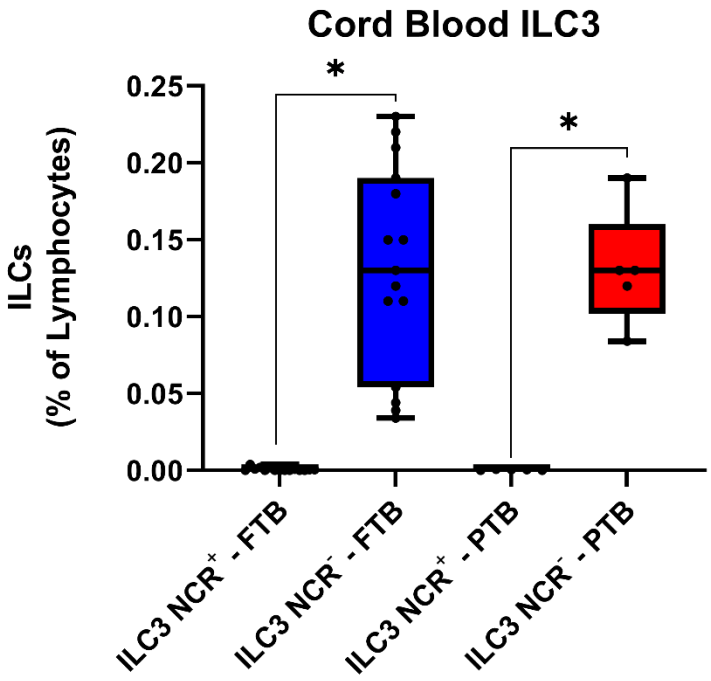

Figure 13 – ILC3 NCR Discrimination. Graphic displaying ILC3 subpopulations relative percentage, in relation to lymphocytes, based on the presence of NCR receptor, determined in samples of cord blood from the FTB and PTB groups. When analyzing ILC3 population within each group, it was possible to determine that NCR^- population was clearly increased, representing the predominant ILC3 subset (* $p < 0.001$, student t-test 95% CI).

Table V- Descriptive statistics of cord blood ILC3 samples

	ILC3		
	NCR^+ *Median*	**NCR^-** Median	**p Value** student t-test 95% CI
FTB	0,000 *0,000 <95% CI < 0,0009450*	0,1300 *0,05400 <95% CI< 0,1900*	<0,0001
PTB	0,0004320 *0,000 <95% CI < 0,0007170*	0,1300 *0,08400 <95% CI < 0,1900*	<0,0001

The same result was observed in cord blood samples as we found ILC3 relative frequencies higher than ILC2 population, both in FTB and PTB groups ($p < 0.01$, student t-test 95% CI), representing the dominant population. A complete and comprehensive description of the statistics found on the different samples is presented in table VI.

Table VI - Descriptive statistics found on the different samples studied.

ILC Population	Median in peripheral blood samples 1st trimester	Median in peripheral blood samples 2nd trimester	Median in peripheral blood samples 3rd trimester	Median in peripheral blood samples labor	Median in cord blood samples labor	Median in decidual samples labor	ILC Population	Median in peripheral blood samples
ILC1-FTB	0,0190 *0,0150<95% CI <0,0250*	0,0190 *0,0130<95% CI <0,0310*	0,0245 *0,0160<95% CI <0,0310*	0,0250 *0,0140<95% CI <0,0340*	0,0460 *0,0220<95% CI <0,0650*	0,0068 *0,0150<95% CI <0,0400*	**ILC1 BPgr**	0,0280 *0,01737<95% CI <0,04441*
ILC1– PTB	-	-	-	0,01460 *0,00603<95% CI <0,0300*	0,0490 *0,0380<95% CI <0,0760*	0,0140 *0,0140<95% CI <0,0350*	**ILC1 24h Pgr**	0,0250 *0,01456<95% CI <0,03161*
ILC2-FTB	0,0205 0,0130<95% CI <0,0290	0,0230 0,0150<95% CI <0,0360	0,0190 0,0120<95% CI <0,0380	0,0240 0,0140<95% CI <0,0350	0,0450 0,0180<95% CI <0,0560	0,0027 0,00234<95% CI <0,0160	**ILC2 BPgr**	0,0360 0,02491<95% CI <0,05130
ILC2-PTB	-	-	-	0,0245 *0,0110<95% CI <0,0670*	0,0670 *0,0300<95% CI <0,0860*	0,0124 *0,0110<95% CI <0,0220*	**ILC2 24h Pgr**	0,0340 *0,02431<95% CI <0,04880*
ILC3-FTB	0,0310 *0,0210<95% CI <0,0440*	0,0225 *0,0170<95% CI <0,0340*	0,0240 *0,0150<95% CI <0,0260*	0,0200 *0,00806<95% CI <0,0270*	0,1300 *0,0540<95% CI <0,1900*	0,0290 *0,0110<95% CI <0,0420*	**ILC3 BPgr**	0,0260 *0,01805<95% CI <0,04262*
ILC3-PTB	-	-	-	0,0290 *0,00886<95% CI <0,0590*	0,1300 *0,0840<95% CI <0,1900*	0,0424 *0,0260<95% CI <0,0520*	**ILC3 24h Pgr**	0,0230 *0,01524<95% CI< <0,04654*

9.2. Enzyme-Linked Immunosorbent Assay results: Cord blood samples

In our ELISA determination of IL-17, IL-22 and IL-4 plasma levels in peripheral blood and cord blood samples, there were no differences between FTB and PTB groups (Figure 14, 15 and 16 respectively).

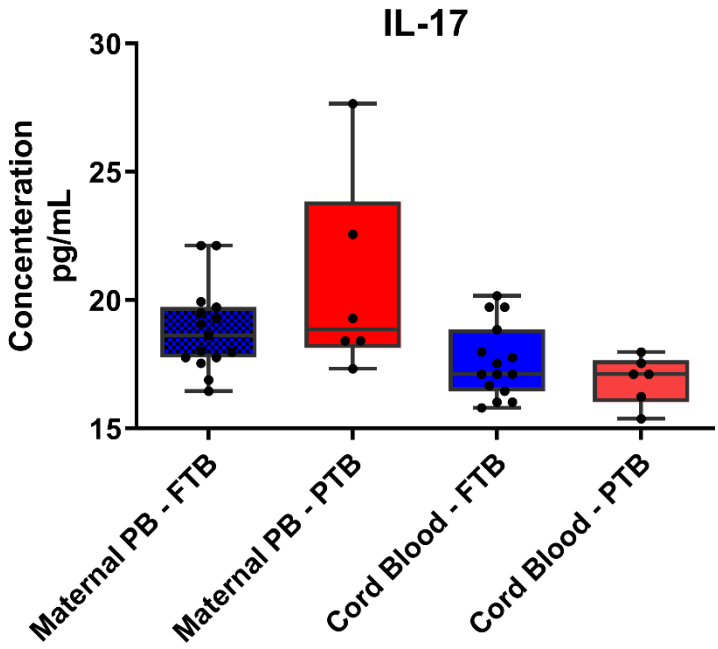

Figure 14 - Enzyme-Linked Immunosorbent Assay (ELISA) in maternal peripheral blood and cord blood in FTB and PTB. Graphic displaying IL-17 plasmatic concentrations plasmatic concentrations of IL-17 in maternal Peripheral Blood (PB) as well as, in cord blood FTB and PTB. Student's t-tests were used for statistical analysis with a 95% confidence interval.

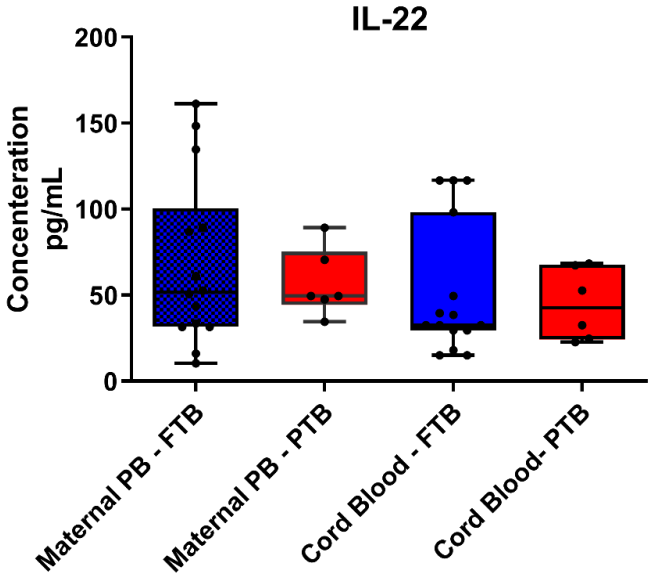

Figure 15 - Enzyme-Linked Immunosorbent Assay (ELISA) in maternal peripheral blood and cord blood in FTB and PTB. Graphic displaying IL-22 concentrations of IL-22 in maternal Peripheral Blood (PB) as well as, in cord blood FTB and PTB. Student's t-tests were used for statistical analysis with a 95% confidence interval.

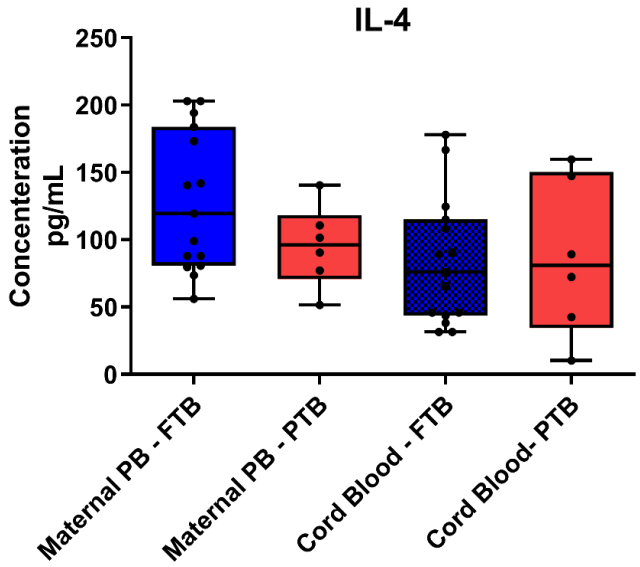

Figure 16 - Enzyme-Linked Immunosorbent Assay (ELISA) in maternal peripheral blood and cord blood in FTB and PTB. Graphic displaying IL-4 plasmatic concentrations in maternal Peripheral Blood (PB) as well as, in cord blood FTB and PTB. Student's t-tests were used for statistical analysis with a 95% confidence interval.

Moreover, we found reduced levels of IFN-γ in peripheral blood samples of women who delivered PTB (t-student test with a 95% confidence interval, p-value * $p<0.05$; two tailed) (Figure 17). The main findings of the current work are summarized in table VII and table VIII.

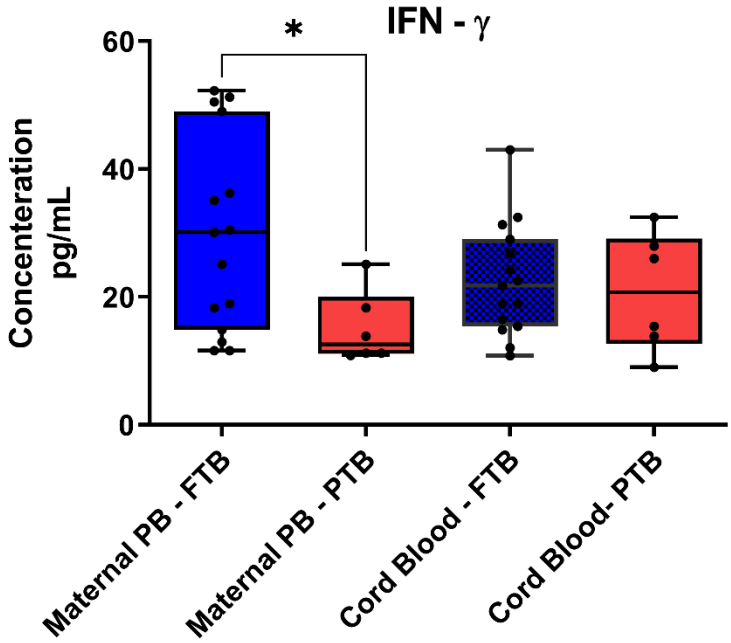

Figure 17 - Enzyme-Linked Immunosorbent Assay (ELISA) in maternal peripheral blood and cord blood in FTB and PTB. Graphic displaying IFN-γ plasmatic concentrations in maternal Peripheral Blood (PB) as well as, in cord blood FTB and PTB. A statistically significant decrease in IFN-γ plasma concentration was found, in peripheral blood samples in women with PTB (t-student test with a 95% confidence interval, p-value * $p<0.05$; (two tailed)).

Table VII – Main findings of the present work regarding ILC populations

	Peripheral Blood (1st, 2nd, 3rd trimester)	**Peripheral Blood Labor**	**Peripheral Blood Pg Administration**	**Decidual Cells**	**Cord Blood**
ILC1	No significant differences	No significant differences	No significant differences	No significant differences	No significant differences
ILC2	No significant differences	No significant differences	No significant differences	**Higher levels in PTB ($p<0.05$)**	**Higher levels in PTB ($p<0.05$)**
ILC3	No significant differences	No significant differences	No significant differences	**Higher levels in PTB ($p<0.05$)**	No significant differences

Table VIII – Main findings of the present work regarding cytokine plasmatic concentrations.

	Cord Blood FTB	**Cord Blood PTB**	**Maternal Peripheral Blood FTB**	**Maternal Peripheral Blood PTB**
IL-17	No significant differences	No significant differences	No significant differences	No significant differences
IL-22	No significant differences	No significant differences	No significant differences	No significant differences
IFN-γ	No significant differences	No significant differences	No significant differences	**Lower levels in PTB ($p<0.05$)**
IL-4	No significant differences	No significant differences	No significant differences	No significant differences

10. Discussion

Human ILC are mainly tissue resident cells important in mediating inflammation. ILC1 are important in promoting immunity to intracellular pathogens and are associated with inflammatory bowel disease[170]. ILC2 support anti-parasite immunity and play an important role in airway inflammation[171]. ILC3 are essential in immunity against extracellular pathogens, skin inflammation[172] and mediating graft-*versus*-host disease (GvHD) in allogeneic bone marrow transplantation[173]. Moreover, it is feasible to consider that ILC might contribute to the mechanism of immune tolerance during pregnancy. Tolerance is widely regarded as an adaptive response, a process that involves antigen presentation, clonal expansion, and the formation of memory cells; the expression of HLA class II molecules in ILC2 and ILC3 population suggests that these cells might also have a role in pregnancy by presenting paternal antigens to the mother's immune system. While ILC2 seem capable of eliciting T helper proliferation, *Hepworth et al. 2013* reported that ILC3 lack classical co-stimulatory molecules such as CD40, CD80 and CD86. If it is the case, ILC3 antigen presentation may, in fact, limit T cell responses by negatively regulating CD4+ T cell responses *in vivo*[38] through T cell anergy[85].

The data thus far published in the literature shows that it is becoming increasingly evident that ILC have a preponderant role in mediating immune function. Despite apparent overlapping characteristics with Th cells, they are capable of locally secrete a vast diversity of cytokines that consent them to maintain tissue homeostasis and immune surveillance.

These preliminary results demonstrate that ILC are present in the decidua of both FTB and PTB pregnant women, since we clearly detected them at the maternal-fetal interface, as well as in cord blood samples (Figure 10 and Figure 12 respectively). Moreover, with

this work we can also state that ILC are present in peripheral blood of pregnant women, with relative frequency values like those found in decidual samples. In addition, when analyzing the relative ILC frequencies in the different samples, we can observe that these are higher in cord blood samples, and that ILC3 relative frequencies present the highest numbers in both decidua and cord blood samples. Even though data collected from cord blood samples are representative of the fetus immune system, a work conducted by *Kanold et al. 2019* has shown that there are maternal immune cells circulating in the cord blood[174]. This work suggests a bidirectional effect, in which maternal immune cells may be orchestrating the fetal immune response. Nonetheless, these effects are poorly understood and require additional studies.

One of the central goals of this work was to investigate the effect of progesterone administration on ILC relative frequencies. As such, we found no correlation between progesterone administration and ILC numbers. This latter result may be explained in part by progesterone exerting its effects only locally[6] and by the fact that ILC (namely NK cells) have shown functional and phenotypic differences according to tissue location[27,28]. ILC, specifically the ILC2 and ILC3 populations, have been described to play a preponderant role in pregnancy[33,51]. Additionally, as mentioned by *Mjösberg and Spits 2016* [45], ILC3 NCR^+ cells produce mainly IL-22, while NCR^- cells produce IL-17 [45], which is intriguing since IL-22 is considered a homeostatic cytokine that contributes to tissue and organ integrity. On the other hand, IL-17 mostly functions as a proinflammatory cytokine [175]. Herein, we found ILC3 NCR^- to be the dominant ILC3 population in peripheral blood, decidua, and cord blood, which is in accordance with the findings highlighted by *Vacca et al. 2015* [19]. Undeniably, ILC3 population can produce proinflammatory cytokines in *deciduas* isolated from women in their first trimester of pregnancy, highlighting a potentially important role for ILC3 cells in the first stages of

pregnancy. Moreover, *Zaretsky et al. 2004* showed that, *in vitro*, IL-6 can cross the placenta, suggesting a bidirectional transfer between mother and fetus of inflammatory mediators[176]. These facts support our results showing elevated ILC2 and ILC3 frequencies in decidua of women that underwent PTB, as well as IL-17 concentration found in our ELISA experiments. Moreover, the concentrations on cord blood and maternal PB determined on labor day in the FTB group were comparable to those found in the PTB group, denoting an inflammatory role of IL-17 in the instigation of labor (figure 14) . Regarding IL-22 levels, a work carried out by *Perfetto et al. 2015* [177] suggests that IL-22 may play a key role in maintaining decidual homeostasis and help to constrain inflammation by contracting the effect of IL-17. These authors found lower levels of IL-22 in *deciduas* of women with unexplained recurrent pregnancy loss. Again, the fact that we found comparable values of IL-22 plasma concentrations in PTB and FTB groups (Figure 15), may reflect a loss of the protective role of IL-22 and the prevalence of the pro-inflammatory effect of IL-17 in the instigation/ beginning of labor.

The discovery of reduced plasma levels of IFN-γ in peripheral blood samples of women who delivered PTB is noteworthy (Figure 17; t-student test with a 95% confidence interval, p-value * $p<0.05$; (two tailed)). A study by *Hanna N et al. 2004* demonstrated that IFN-γ represses ciclo-oxigenase-2 (COX-2) expression and prostaglandin E2 (PGE2) production in human placental samples from both term and preterm labor deliveries, suggesting that functional withdrawal of IFN-γ may be involved in the onset of term or preterm labor [173]. Since our results are from peripheral blood and cord samples, further work needs to focus on decidual samples to identify IFN-γ producing ILC, namely ILC1 or NK cells. Nonetheless, we cannot reject the assumption that an exacerbated Th2-type response in PTB may be orchestrated by ILC2 that also have been shown to promote tissue homeostasis[178,179,180]. However, studies

conducted in animal models have shown that ILC2 can also display proinflammatory functions in the context of asthma[181,182]. Moreover, *Huang et al. 2015* and *Wallrapp et al. 2018*, suggest that IL-25, a member of the IL-17 family, can induce ILC2 to an inflammatory phenotype[183,176]. The similar results of IL-4 plasma levels of cord blood samples found in the FTB and PTB groups (Figure 16) support this hypothesis. In fact, in a mouse model, IL-4 has been shown to have inflammatory proprieties by activating eosinophils[184] which are reported to have an important role in preterm birth[185]. A possible mechanism proposed by this study that needs further investigation, is the passage of IL-17 through fetal membranes to the *fetus*, inducing ILC2 to an inflammatory phenotype accompanied by reduced levels of INF-γ. Moreover, ILC2 are capable of presenting antigens to T $CD4^+$ cells and induce proliferation towards a Th2 phenotype in an IL-2 dependent manner [37]. This is a remarkable finding since it proposes a crosstalk between the innate and adaptive immune systems[38–40]. However, ILC3 have been shown to express MHC class II molecules, promoting a T cell-mediated response[41]. This fact may elucidate the role of ILC in mounting an adaptive immune response, potentially representing a tolerance mechanism towards the *fetus*, which is of substantial interest. A strong point of this study is its corroboration with existing hypothesis that an inadequate inflammation triggered immunological response prompts PTB, suggesting a key role of ILC in this process; unfortunately, numerous mechanisms underlying ILC actions in pregnancy remain to be ascertained.

The data herein presented suggest that in PTB group there is an inflammatory response orchestrated by an elevation of ILC2 and ILC3 in decidual samples. However, this group is being treated with progesterone, which is manly an anti-inflammatory hormone. To explain this discrepancy, further work is needed to ascertain the expression of progesterone receptors in the different ILC populations. In fact, previous work conducted

by *Areia et al. 2016* demonstrated a decline in Tregs positive for membrane progesterone receptor ($mPR^{\alpha+}$), theorizing a reduction in progesterone anti-inflammatory actions through Treg $mPR^{\alpha+}$ cells[186].

Our future work will address the possibility of sterile inflammation. Sterile intra-amniotic inflammation is commonly observed in patients with spontaneous preterm labor[96]; it occurs in response to a variety of triggers, and it is through pattern recognition receptors (PRRs) and damage-associated molecular patterns (DAMP), that the innate immune system mounts an inflammatory response to non-microbial signals. It has been suggested by *Horton et al. 2015* that NCR in NK cells may act as PRRs, or DAMPs regulating NK function[48]. We consider this finding relevant since ILC3 also express NCR receptors.

Our study does present some limitations that must be considered. Because our small sample size, the results should be interpreted with caution. To address this issue, future studies should include a larger number of women, with particular attention to a comprehensive analysis of cytokine release throughout pregnancy. Moreover, to determine which cytokines are being produced by the different ILC population, cell sorting and intracellular assays, as well as expression assays, should be conducted. Additionally, sorted cells should be analyzed for the expression of membrane and nuclear progesterone receptors, and in vitro assay could be conducted in order to assess ILC responsiveness to progesterone.

11. Conclusion

The identification of specific prenatal therapies and their efficacy to improve maternal and neonatal outcomes have been a mainstay in Maternal-Fetal Medicine investigation. As such, in the present work, we sought to study the role of progesterone in spontaneous PTL, through the actions of ILC. Moreover, we aimed to expand the current knowledge on the immunology of pregnancy by focusing on ILC fluctuations during pregnancy and meticulous analysis of cytokine profile.

Progesterone has been known to play an important role in the prevention of spontaneous abortion and recently in preterm labor; however, in this study, we found no effect of progesterone administration in ILC populations. We cannot exclude that such effect exists at the maternal fetal interface, since ILC show differences in phenotypes and function according to tissues location. This later fact may also explain the reason we found no differences in the relative frequencies of ILC populations in peripheral blood samples of pregnant women in the control group during the first, second and third trimester.

Nevertheless, we found a statistically significant increase of the relative frequencies of ILC2 and ILC3 populations in decidua samples in the PTB group when compared to the FTB. Moreover, we found also a statistically significant increase of the relative frequencies of the ILC2 population of cord blood samples in the PTB group, when compared to the FTB. Regarding the plasmatic concentrations of cytokines, there was a statistically significant decrease of IFN-γ in maternal peripheral blood samples in the PTB group, when compared to the FTB. All these findings suggest that labor might be characterized by decreased tissue remodeling and repair functions, accompanied by a

marked inflammatory response, due to high levels of ILC2 and ILC3. In addition, our ELISA experiments propose that labor might be characterized by a functional INF-γ withdrawal. Consequently, our results are encouraging, as they suggest a role of ILC in the regulation of labor.

Further research in this field is fundamental to public health because PTB is increasing and represents a major public cost. Additional studies could, therefore, diminish patient suffering and relieve economic burden. For this reason, clarifying ILC actions in labor may have a significant impact in clinical practice.

Future work focusing on endocrine and environmental factors influencing ILC phenotype, as well as cytokine release patterns, will contribute to answer unsolved questions in clinical practice, and allow the elaboration of new clinical protocols that will significantly contribute to the reduction of children morbidity and mortality.

Research conducted in ILC biology and their role in pregnancy is still scarce. As such, an effort should be made to raise awareness in the different obstetric centers, in order to expand this work to a broader population.

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