Ana Luísa Fialho Amaral de Areia

Progesterone in Preterm Birth: Role of regulatory T-cells

Tese de doutoramento no Programa de Doutoramento em Ciências da Saúde, ramo de Medicina, orientada por Professora Doutora Anabela Mota Pinto e Professor Doutor José Paulo Achando Silva Moura e apresentada à Faculdade de Medicina da Universidade de Coimbra

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On the front cover:

Photographic composition of a premature baby resting on a T regulatory cell.

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no âmbito do Programa Doutoral em Ciências da Saúde,

para candidatura ao grau de Doutor em Ciências da Saúde, ramo de Medicina,

realizada sob a orientação científica da

Professora Doutora Anabela Mota Pinto e do

Professor Doutor José Paulo Achando Silva Moura

Title

Progesterone in Preterm Birth: Role of regulatory T-cells

Progesterona no Parto pré-termo: Papel das células T reguladoras

Keywords

Preterm Birth; Treg cells; Regulatory T-Cells; progesterone; mPRα; pregnancy; IL-10; TGF-β; cytokines; labour; obstetrics

Table of Contents

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My sincere thankfulness to all friends who helped me to accomplish this project.

A word of recognition to all pregnant women for their unconstrained and chivalrous participation in this research.

Finally, I would like to dedicate this Thesis to my family:

- To my parents, whose principles, example, devotion and unconditional support have carried me throughout my life.
- Words will not be sufficient to express my endearment to Miguel, my beloved husband. His relentless and endless love, inspiration, motivation and guidance led me through this long journey.
- To Beatriz, Afonso and Guilherme, my dearly loved children, for being the sunshine of my life. For all the moments, but especially for those when I doubted myself and they sat beside me and with their unrestricted love, helped me perceive the way out.

Preamble

The focus for the research for the present Thesis was to establish the mechanism through which progesterone' therapy effects are accomplished in preterm labour.

In light of this, our main aim was to establish the role of regulatory T lymphocytes in progesterone administration in preterm labour.

The study was designed for a single tertiary care obstetric unit since threatened preterm deliveries with gestational age below 34 weeks are referred to these units of perinatal differentiated care.

The knowledge attained with this project is of utmost importance for Obstetricians, answering some unsolved questions in clinical practice (which will sustain the use of progesterone in these circumstances), besides allowing the elaboration of a clinical protocol based on unequivocal scientific evidence.

The novelty of this research is that human studies are inexistent on the subject proposed, even though there are several investigations worldwide concerning preterm birth.

Additionally, this investigation has a high applicability as PTL incidence is rising, despite multiple primary interventions being implemented in an effort 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.

Along with the specific studies necessary for the present Thesis, the author also endorsed a previous investigation demonstrating that progesterone conferred a longer median latency period until delivery, after successful tocolysis, albeit by an undisclosed mechanism (Areia, Fonseca *et al.*, 2013). This prompted the intellectual challenge of roust around the quest for progesterone's mechanism in preterm labour.

Publications

1: **Areia A**, Vale-Pereira S, Alves V, Rodrigues-Santos P, Moura P, Mota-Pinto A.

Membrane progesterone receptors in human regulatory T cells: a reality in pregnancy.

BJOG. 2015 Feb 2. [Epub ahead of print]. PubMed PMID: 25639501.

Impact factor: 3.862 (2013 Journal Citation Reports® – Thomson Reuters)

Rank 6 in 78, Quartil 1, in Obstetrics and Gynecology

2: **Areia A**, Fonseca E, Moura P.

Progesterone use after successful treatment of threatened pre-term delivery.

J Obstet Gynaecol. 2013 Oct;33(7):678-81. PubMed PMID: 24127952.

Impact factor: 0.604 (2013 Journal Citation Reports® – Thomson Reuters)

Rank 72 in 78, Quartil 4, in Obstetrics and Gynecology

3. **Areia A**, Vale-Pereira S, Alves V, Rodrigues-Santos P, Moura P, Mota-Pinto A.

Does progesterone administration in preterm labor influence Treg cells?

Under review

4. **Areia A**, Vale-Pereira S, Alves V, Rodrigues-Santos P, Moura P, Mota-Pinto A.

Can membrane progesterone receptor α on T regulatory cells explain the ensuing of Human Labour?

Submitted

Acronyms

A

APCs: Antigen presenting cells

C

CA: Chorioamnionitis

$CTLA_4$: Cytotoxic T-lymphocyte associate protein 4

D

DC: Dendritic cells

E

ELISA: Enzyme linked immunosorbent assay

F

FIRS: Fetal inflammatory response syndrome

Foxp3: Transcription factor forkhead box P3

FSC: Forward light scatter

H

HLA: Human leukocyte antigens

I

IL-2: Interleukin-2

IL-10: Interleukin-10

M

Min: Minutes

N

NF-κB: Nuclear factor kappa light chain of activated B cells

NK: Natural-killer

P

PBMCs: Peripheral blood mononuclear cells

PG: Prostaglandins

PGDH: Hydroxyprostaglandin dehydrogenase

PGHS: Prostaglandin synthase

PPROM: Premature rupture of the membranes

PR: Progesterone receptors

mPR: Membrane progesterone receptors

nPR: Nuclear progesterone receptors

PTL: Preterm labour

R

RT-PCR: Real Time Polymerase Chain Reaction

S

SSC: Side light scatter

T

TGF-β: Transforming growth factor-β

Th: T helper cells

TLRs: Toll-like receptors

Treg: Regulatory T-cells

iTreg: Induced/adaptive Treg cells also named *peripherally derived* Treg cells

nTreg: Natural Treg cells also named *thymus derived* Treg cells

Outline of Thesis

In *Chapter I*, the background contextualizing the theme chosen for the present Thesis is presented. A description of the functional unit comprising mother and fetus is given, along with a review of the actual knowledge of the immunology of pregnancy and the relevance of the immunoinflammatory phenomenon in pregnancy and labour. Afterwards, a preamble to preterm labour and progesterone is offered, together with an evaluation of the existent literature about the theme and clinical guidelines recommendations by the time of the Thesis origin. Finally, existent gaps are mentioned and the hypothesis of this Thesis is postulated.

In *Chapter II*, the key research aims that will be addressed in this Thesis are presented.

In *Chapter III*, Materials and Methods are thoroughly explained, including the population studied (with clarification of inclusion and exclusion criteria), explanation of flow cytometry methodology, Enzyme-Linked Immunosorbent Assay (*ELISA), Western blot* and Real-time polymerase chain reaction (*RT-PCR*) techniques, and the rationale for *in vitro* studies.

Throughout *Chapter IV* the results of this investigation are described, categorized by the different procedures applied.

In *Chapter V*, the discussion of the results of our research is presented. Each result is considered in comparison with the available evidence and with previous similar reports in terms of strength of evidence and dissimilarities.

In *Chapter VI*, our Thesis' conclusions are presented and the benefits of intervention explained.

Finally, in *Chapter VII* future research lines are proposed.

Resumo

O Parto pré-termo (PTL) é uma das principais causas de morbilidade e mortalidade neonatais, sendo responsável por 11% de todos os partos, ocorrendo espontaneamente na maioria dos casos. Mesmo as crianças que sobrevivem a um PTL têm maior incidência de sequelas a longo prazo, abrangendo défices de desenvolvimento psico-motor, patologia neurológica (como a paralisia cerebral) e um aumento do risco de doenças da vida adulta.

As mulheres com ameaça de parto pré-termo revertida com tocólise, mantêm um risco elevado de episódios recorrentes. A terapia tocolítica de manutenção é ainda controversa mas o uso de progesterona nestes casos, tem-se revelado promissora. No entanto, a sua utilização não está ainda bem protocolada, para o que contribui a ausência de explicação científica clara para o seu modo de atuação.

Assim, na gestação, a progesterona atua como um imuno-esteróide, contribuindo para o estabelecimento de um meio protetor.

Durante a gravidez, a função primordial das células T reguladoras (Treg) circulantes parece ser a proteção da unidade feto-trofoblástica da rejeição pelo sistema imunológico materno, sob a influência da progesterona.

As células Treg medeiam a sua função protetora através do contacto célula a célula ou pela secreção de citocinas imunossupressoras. Vários estudos demonstraram que a supressão da função dos leucócitos ativados pelas células Treg é obtida através da produção de IL-10 e TGF-β. Por outro lado, a progesterona apresenta-se como um regulador crítico das células Treg durante a gravidez mas através dum mecanismo ainda desconhecido.

Recentemente foi encontrado no sistema imunológico humano um recetor membranar α de progesterona (mPRα), que contribui para os mecanismos não genómicos (rápidos) desta hormona. Este recetor poderá mediar as ações da progesterona nas células Treg e subsequentemente no PTL.

Estudos em humanos são inexistentes nesta temática e esta pesquisa propõe uma inovação na compreensão e tratamento do PTL.

O nosso estudo incluiu 14 mulheres grávidas com PTL (grupo de estudo) e 20 grávidas normais (grupo controlo), de forma a proporcionar alguns resultados preliminares, tendo em conta os restritos critérios de inclusão definidos e a incidência da patologia em questão.

A colheita de sangue periférico foi efetuada em 3 ocasiões em mulheres grávidas normais, para caracterização e quantificação de células Treg, determinação dos níveis plasmáticos de IL-10 e de TGF-β, e a expressão de mPRα. As mesmas determinações foram realizadas em mulheres internadas com PTL, também em 3 momentos (após tocólise, 24 horas após progesterona e no dia do parto).

O estudo das células Treg e do mPRα em células Treg foi efetuado por citometria de fluxo, recorrendo à expressão de múltiplos anticorpos monoclonais que caracterizam estas populações. A nossa população de células T reguladoras foi definida como a portadora das seguintes características: $CD4^{+}CD25^{high}CD127^{low}Foxp3^{+}$.

As concentrações plasmáticas de citocinas (IL-10 e TGF-β) foram determinadas utilizando a técnica de *Enzyme-linked immunosorbent assay* (*ELISA*), de modo a explorar a influência da regulação destas citocinas nos dois grupos de mulheres.

Os transcritos de mRNA de Foxp3, mPRα, IL-10 e TGF-β foram determinados utilizando *Real-time polymerase chain reaction* (*RT-PCR*) e para confirmar a presença das respetivas proteínas foi realizado *Western Blot*. Após o parto, utilizando a metodologia

imuno-histoquímica, foi estudada a percentagem de células Treg, os níveis de IL-10 e TGF-β e a existência de mPRα nas placentas de ambos os grupos.

Por último, utilizando amostras de sangue obtidas do grupo PTL, foram executados estudos *in vitro*, de forma a determinar se o mecanismo subjacente às ações da progesterona em células Treg no PTL seria resultante da sua interação com o mPRα.

Este estudo demonstrou um aumento no *pool* de células Treg após a terapêutica com progesterona de 38.3% para 52%, indicando um possível mecanismo através do qual o seu papel benéfico no PTL é alcançado. Além disso, a administração de progesterona traduziu-se num aumento de todas as populações celulares estudadas.

Foi ainda possível corroborar a importância das citocinas imunossupressoras IL-10 e TGF-β na contenção do fenómeno imunoinflamatório, que se considera desencadear o PTL, ao demonstrar níveis mais elevados no grupo de estudo.

Superando as nossas expectativas iniciais, esta investigação comprovou a existência de mPRα no *pool* de células Treg durante a gravidez humana (segundo trimestre, terceiro trimestre e dia do parto) e, adicionalmente, após tratamento com progesterona, foi possível constatar uma diminuição de células Treg $mPR\alpha^{+}$ de 32.6% para 13.8%,

Este projeto estudou a capacidade da progesterona reduzir o número de PTL por modulação da percentagem das células Treg através do mPRα.

Em conclusão, estes resultados permitem fundamentar cientificamente a forma de atuação da progesterona, de forma a justificar a sua prescrição preventiva e terapêutica no PTL, permitindo uma diminuição das consequências maternas e neonatais, com a subsequente redução das sequelas inerentes à prematuridade.

Summary

Preterm labour (PTL) is one of the major causes of neonatal morbidity and mortality worldwide, accounting for 11% of all deliveries, most of them occurring spontaneously. Even those children who survive a PTL have an increased incidence of long-term *sequelae*, including neurodevelopmental deficits (as cerebral palsy) and an increased risk of a spectrum of diseases in adulthood.

Women with PTL arrested with tocolytic therapy remain at increased risk of recurrent preterm labour and, although maintenance tocolytic therapy is still controversial, progesterone use seems promising. Nevertheless, it is not universally used due to the lack of a scientific explanation to support its mode of action.

Progesterone acts as an immunosteroid by contributing to the establishment of a pregnancy protective milieu.

During pregnancy, the primordial function of circulating T regulatory (Treg) cells seems to be the protection of the fetal-trophoblastic unit from rejection by the maternal immune system, under the influence of progesterone.

Moreover, Treg cells mediate its protective function via cell to cell contact or by the secretion of immunosuppressive cytokines. Several studies have shown that suppression of the function of activated leukocytes by Treg cells is achieved by the production of IL-10 and TGF-β. Furthermore, progesterone is thought to be the critical regulator of Treg cells during pregnancy, but the mechanism remains unclear.

Membrane progesterone receptor α (mPRα) was recently found on human immune system and seems responsible for the rapid non-genomic actions of progesterone. This receptor could mediate progesterone actions on Treg cells and was demonstrated to be involved in PTL.

Human studies are inexistent in this field and this research proposes an innovation in the understanding of preterm birth and on its treatment.

Our study included 14 pregnant women with PTL (study group) and 20 normal pregnant women (control group), as these numbers would provide preliminary results, taking into account the restricted inclusion criteria and the incidence of the disease.

Characterization and quantification of Treg cells, IL-10 and TGF-β plasma levels and the existence of mPRα was performed in peripheral blood on 3 occasions in normal pregnant women. The same determinations were conducted in women admitted with PTL, also on 3 occasions (after tocolysis, 24 hours after progesterone treatment and on delivery day) in peripheral blood.

Treg cells and mPRα studies were done by flow cytometry analysis, using multiple monoclonal antibody expression that characterizes these cell populations. We defined our Treg cell pool as the one characterized by $CD4^{+}CD25^{high}CD127^{low}Foxp3^{+}$.

Cytokine plasmatic concentrations (IL-10 and TGF-β) were measured using "*Enzyme-linked immunosorbent assay*" (*ELISA*) technique to explore the influence of these suppressor cytokines in the two groups of women.

The mRNA transcripts of Foxp3, mPRα, IL-10 and TGF-β were determined using *real-time polymerase chain reaction* (*RT-PCR*). To validate the presence of the respective proteins *Western blot* was performed.

After birth, placentas of both groups were analyzed to determine Treg cells percentage, IL-10 and TGF-β levels and the existence of mPRα in both groups, using immunohistochemistry methodology.

Using the same blood samples retrieved from PTL group, *in vitro* studies were done, in order to prove that the mechanism behind Progesterone actions on Treg cells is linked to mPRα.

This research demonstrated a significant increase in the Treg cell pool after progesterone treatment, from 38.3% to 52%, indicating a possible mechanism by which its beneficial role in PTL is achieved. Moreover, progesterone administration resulted in an enhancement of all blood populations being studied.

Also, it attested the importance of the immunosuppressive cytokines IL-10 and TGF-β in the containment of the immunoinflammatory phenomenon, thought to prompt PTL, demonstrating higher levels in the study group.

Exceeding our primary presumptions, we also attested the existence of mPRα in Treg cell pool during human pregnancy (second trimester, third trimester and delivery day), with surprising results revealing a decrease in $mPR\alpha^{+}$ Treg cells after progesterone treatment, from 32.6% to 13.8%.

This exploratory research studied if progesterone's ability to reduce PTL is achieved through modulation of Treg cells frequencies by means of mPRα.

Finally, this study comes up to a scientific explanation to support the role of progesterone in PTL, allowing its preventive and therapeutic prescription, which will result in the reduction of pregnant women's internments and of children's intensive care requirements, with a subsequent reduction in children's handicaps and hospital costs.

Chapter 1 - Background

1. The Fetal/Mother Border

Amnion, chorion and decidua as a functional unit

Fetal membranes development is a complex process that starts with the formation of amniotic and chorionic cavities. The rapid growth of the amniotic cavity leads to the disappearance of the exocoelomic cavity and juxtaposes the amnion to the chorion, which in turn is in close anatomical and functional contact with the decidua (Pasquier & Doret, 2008).

Thus, the commonly designed "fetal membranes", that delimitate an extensive portion of the border between the fetal and the maternal environments, are composed of three layers: amnion and chorion, of ovular origin, and the decidua, of maternal origin (Pasquier & Doret, 2008).

Chorionic villi in contact with the decidua basalis proliferate to form the *chorion frondosum* which is the fetal component of the placenta; the avascular fetal membrane that abuts the decidua is the *chorion laeve* (Cunningham, Leveno *et al.*, 2009).

Human decidua contains abundant immune cells during gestation, with more than 30% of stromal cells in the 1st trimester expressing the leukocyte common antigen CD45 (Bulmer, Morrison *et al.*, 1991; Chen, Liu *et al.*, 2012).

Decidual population of Natural-Killer (NK) cells, macrophages, decidual stromal cells and T helper cells ($CD3^+CD4^+$) constitute 30 to 40% of decidual cells (Bulmer, Morrison *et al.*, 1991), but B cells are absent (Hanssens, Salzet *et al.*, 2012). There are four major populations of decidual leukocytes present in early pregnancy: uterine NK, macrophages, Dendritic cells (DC) and T-cells (Chen, Liu *et al.*, 2012). The precocious

elevation of lymphocyte number suggests that the influx and the proliferation of these cells are under hormonal influence. Special techniques like electron microscopy and immunohistochemistry underline the intimate contact between the trophoblast and these immune cells (Hanssens, Salzet *et al.*, 2012; von Rango, 2008).

The major cellular component of the decidua is decidual stromal cells. These cells exert different immune activities that have emerged as relevant to the immunologic interaction between mother and fetus and may lead to either a normal pregnancy or abortion (Chen, Liu *et al.*, 2012).

In human hemochorial placenta, fetal trophoblast cells appear to be in extremely close contact with the maternal immune cells (Chen, Liu *et al.*, 2012). Thus, immunologic interactions between mother and fetus during pregnancy are thought to occur in the placenta (Bulmer, Morrison *et al.*, 1991; von Rango, 2008). Moreover, it seems that there are two maternal-fetal interfaces: one made of an immunologically neutral population (in contact with the maternal immune system), and another, immunologically active population of trophoblast cells migrating to the decidua (Mor & Abrahams, 2008).

Recent evidence points out to the existence of a bidirectional trafficking across the maternal-fetal interface. Fetal cells have the potential to infiltrate maternal tissues and to differentiate into different types of cells (liver, muscle, skin), transforming the mother to a chimera. Also, these fetal cells play a special role in repairing maternal tissues that are damaged by a pathologic process (Mor & Abrahams, 2008).

The amniochorion is recognized as a leaky structure, with an extremely low transepithelial potential and high conductance. However, there are marked differences between the amniotic layer, which appears to be a more diffusional barrier, and the

underlying chorionic layer. In addition, inflammatory mediators appear to weaken the amniotic membrane barrier via disruption of tight junctions (Ross, 2011).

Apoptosis, or programmed cell death, is an active mechanism through which superfluous or non-functional cells are eliminated in order to maintain tissue normality. During pregnancy, apoptosis plays an important role in the induction of maternal tolerance and in trophoblast differentiation and turnover (Hanssens, Salzet *et al.*, 2012). Destruction of activated T and B cells through apoptosis induces a specific tolerance (Vinatier, Dufour *et al.*, 1996).

2. Immunology of Pregnancy

I. Local immune regulation

Medawar propositions of 1953 explained the maternal immunological acceptance of the conceptus considering the fetal-maternal relationship as an allograft. The success of pregnancy could then possibly result from fetal antigenic neutrality or immaturity, absence of contact between fetal cells and the mother's immune system or maternal decrease of immunological activity throughout gestation (Medawar, 1953). They established a theoretical framework for scientific research and clinical reasoning for the next three decades.

None of them was to be confirmed in its original form but, nevertheless, they all contained some inherent truth and insight as the development of immunology has highlighted in recent years: the fetus is not antigenic neutral, but the trophoblastic cells that contact maternal tissues have scant antigenic expression; there is control, although selective, of cell trafficking at the placental circulation; there is no maternal immunological suppression, but the modulation of systemic and local immune responses facilitates the survival, progression, differentiation and growth control of the trophoblast.

The fetus naturally expresses an antigenic identity that is in part of paternal origin and, thus, different from its mother. In fact, in cases of assisted reproduction technology recurring to oocyte donation or surrogate motherhood, it may be totally allogenic.

In any case, an immune rejection response would be inevitable. However, the fact is that it is not the fetus but rather the trophoblast, at the placental and membrane interfaces, that contacts the mother's immune system – and these trophoblastic cells have a remarkably neutral antigenic identity, expressing only sufficient Human leukocyte antigens (HLA) and apoptosis-inducing ligands to escape lysis by non-specific immune NK cells or to suppress their activity (Veenstra van Nieuwenhoven, Heineman *et al.*, 2003).

The HLA system is the locus of genes that encode for proteins on the surface of cells that are responsible for regulation of the immune system in humans. Its main function is to bind to peptide fragments derived from pathogens and display them on the cell surface for recognition by the appropriate T-cells. HLAs corresponding to MHC class I (A, B, and C) present peptides from inside the cell and attract cytotoxic T-cells ($CD8^+$), which destroy cells.

HLAs corresponding to MHC class II (DP, DM, DOA, DOB, DQ, and DR) present antigens from outside of the cell to T-lymphocytes. These particular antigens stimulate the multiplication of T helper cells ($CD3^+CD4^+$), which in turn stimulate antibody-producing B-cells to produce antibodies to that specific antigen (*The Immune System in Health and Disease*, 2001).

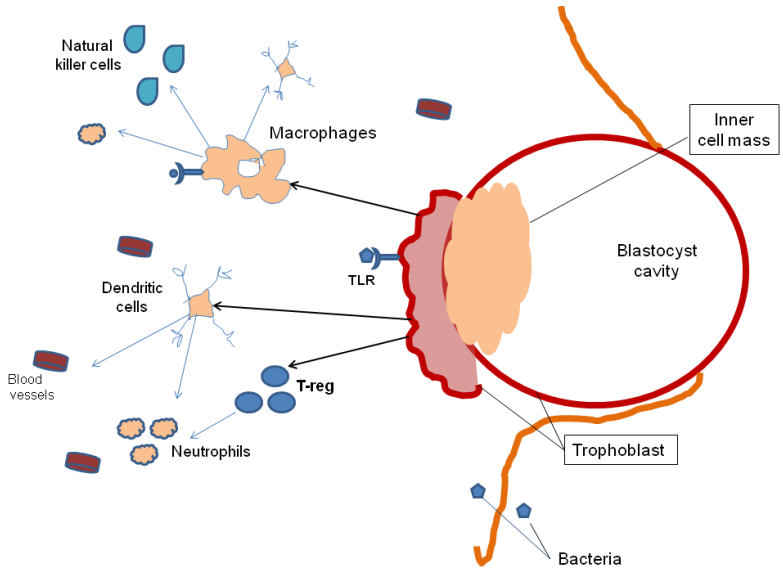
In reality, the amniotic membrane seems to be an immune privileged tissue and to contain some immunoregulatory factors. In fact, a major histocompatibility complex class Ib gene (HLA-G molecule) appears to be an important immunosuppressive factor during pregnancy. Expression of HLA-G in amniotic membrane may influence the host immune system in two ways: first it may play the role of a tolerogenic peptide and the host lymphocyte or DC may be inactivated by HLA-G's binding to inhibitory receptors;

secondly, HLA-G may be recognized by some T-cells and then provide an activation of $CD8^+$ T-cells (because CD8 can bind to HLA-G), and these cells may have a suppressor function (Kubo, Sonoda *et al.*, 2001).

So, the theoretical paradigm as evolved from one of maternal-fetal tolerance to that of a decidual-trophoblastic-amniotic tolerance and active cooperation (Mor & Abrahams, 2008): a successful pregnancy is the consequence of numerous complex interactions between the receptive uterus and the mature blastocyst, under immune humoral control (Chen, Liu *et al.*, 2012).

In reality, mother and fetus do not relate as host-allograft, but their complex relations are more similar to parasite and host, or tumour and host, consisting of not only support and nourishment, but also working together against common external dangers (Figure 1).

Figure 1. Trophoblast-immune interaction



Legend: Adapted from (Mor & Abrahams, 2008). Treg: T regulatory cells; TLR: Toll like receptor.

Results of recent studies suggest that the trophoblast functions like the conductor of a symphony where the musicians are the cells of the maternal immune system. Consequently, the success of pregnancy depends on how well the trophoblast communicates with each immune cell type and then how all of them work together (Mor & Abrahams, 2008).

Different immunological participants present in the decidua benefit from instructions sent by the trophoblast, which refines, educates, activates and sometimes neutralizes and even uses them, to develop in harmony. On the other hand, the presence of an active local immune response is essential to trophoblastic development, as many cytokines act as growth factors and, ultimately, force differentiation and stop invasion of the decidua (Hanssens, Salzet *et al.*, 2012; Medawar, 1953).

Since the beginning of pregnancy, immunity cells are present in the decidua, place of contact between the mother and the fetal-placental unit: these cells will suffer differentiation and specific education. The embryo expresses histocompatibility antigens since the 2-cell stadium but he won´t be in direct contact with the mother, even in the fetal stage, as the trophoblast and the amnion separate them at the placental and membrane interfaces. Villositary trophoblast is characterized by the absence of HLA antigens, class I and II (Hanssens, Salzet *et al.*, 2012).

Endometrial decidualization affects all cellular populations: decidual stromal cells, glandular cells and immune system cells. Decidual stromal cells help the immunological phenomenon in various ways, secreting immunosuppressive substances, producing cytokines, phagocytizing immune cells, presenting antigens and regulating macrophage activation (Hanssens, Salzet *et al.*, 2012).

The placenta, for a long time considered as a protective fetal barrier reinforced by the presence of sialic acid, mucopolysaccharides and the effects of hormones like human chorionic gonadotropin and human placental lactogen, is in reality very porous (Medawar, 1953). In fact, there are 5 zones of interaction at the fetal-maternal level. During the 1st trimester, the contact between immune maternal cells and fetal cells is limited to the decidua. At the beginning of the 2nd trimester, maternal blood initiates perfusion of the intervillous space; syncytiotrophoblast microparticles can detach into the mother's circulation, enlarging immunity contacts to the entire maternal organism (Hanssens, Salzet *et al.*, 2012).

II. Regulatory T-cells

T-cells are known to play an essential role in immune regulation and immune stimulation (Saito, Nakashima *et al.*, 2010).

T helper (Th) cells, expressing $CD3^+CD4^+$, can be divided in 2 subsets, taking into account the different cytokine production, as Th_1 cells, which produce interleukin 2 (IL-2) and interferon γ, implicated in cellular immunity; and Th_2 cells, which produce IL-4, IL-5 and IL-13, involved in humoral immunity (Saito, Nakashima *et al.*, 2010).

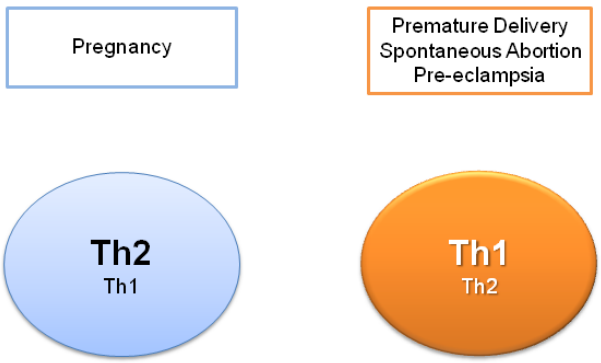
Maternal tolerance toward fetal alloantigens was explained, in the past, by a major Th_2-type immunity during pregnancy, which took primacy over Th_1-type immunity, protecting the fetus from maternal Th_1-cell attack (Saito, Nakashima *et al.*, 2010). However, the Th_1/Th_2 paradigm is now insufficient to explain the mechanism by which the fetus is not rejected by maternal immune cells (Saito, Nakashima *et al.*, 2010). This

Th_1/Th_2 paradigm has been expanded to include $CD4^+$ regulatory T-cells and T helper 17 cells, since it became evident that some studies were not fitting into the original theory (Polese, Gridelet *et al.*, 2014).

Pregnancy is a Th_2 phenomenon (Figure 2); the shift away from type 1 cytokines production during pregnancy is beneficial for pregnancy, as these types of cytokines (like interferon α and tumour necrosis factor β) are harmful by inhibiting embryonic and fetal development. Type 2 cytokines on the other hand, stimulate trophoblast outgrowth and invasion (Veenstra van Nieuwenhoven, Heineman *et al.*, 2003).

Many studies have reported during pregnancy a predominant Th_2-type immunity and suppressed Th_1-type immunity (Saito, Nakashima *et al.*, 2010), which is induced at the fetal-maternal interface by both Th_2 cell migration and Th_2 cell differentiation (Saito, Nakashima *et al.*, 2010). This could be explained by the fact that albeit inflammation is necessary for successful implantation, excessive inflammation can cause embryo impairment (Saito, Nakashima *et al.*, 2010).

Figure 2. Th_1/Th_2 balance in physiological pregnancy and in gestational diseases



Legend: Adapted from (Challis, Lockwood *et al.*, 2009).

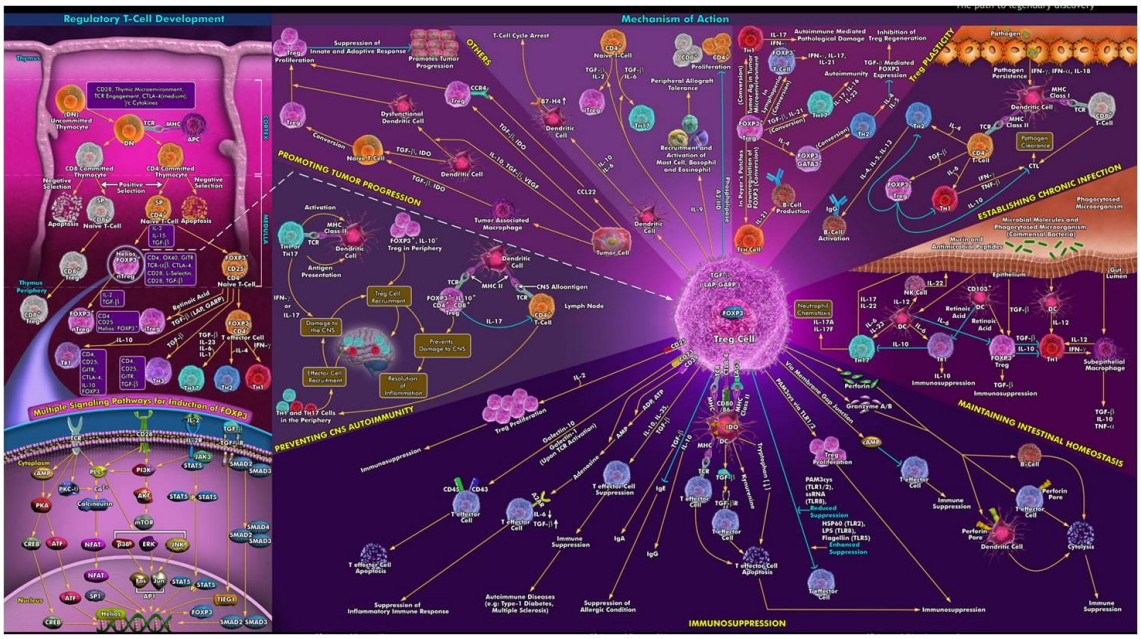
Th_{17} cells, which produce the proinflammatory cytokine IL-17, play a significant role in the induction of inflammation and have been proposed as a pathogenic mechanism in autoimmune diseases and acute transplant rejection (Saito, Nakashima *et al.*, 2010).

Inversely, regulatory T (Treg) cells are recognized as a major cell subset at peripheral immune tolerance, having a suppressive effect on inflammatory responses and being one of the candidates to explain maternal tolerance (Leber, Teles *et al.*, 2010). As so, Treg cells play a primordial role in immune regulation including autoimmunity, induction of tolerance, anti-infectious immunity and cancer (Guerin, Prins *et al.*, 2009; Saito, Nakashima *et al.*, 2010). These cells constitute about 5% of the peripheral $CD4^+$ T-cell population and play a crucial role in the maintenance of immune homeostasis against self antigens (Lastovicka, 2013).

Treg cells are defined as a cellular subset on the basis not only of their surface phenotype but also by their functional characteristics. Despite the unique suppressive properties of Treg cells compared with other lymphocytes, their cellular features are less distinct (Guerin, Prins *et al.*, 2009). Treg cells are generally identified on the basis of their constitutive expression of surface markers including the IL-2 receptor CD25, glucocorticoid-induced tumour necrosis factor receptor, Cytotoxic T-lymphocyte associate protein 4 ($CTLA_4$), together with low expression of CD45RB and CD127 (Guerin, Prins *et al.*, 2009). Nevertheless, as each of these markers can also be expressed on the surface of other cell populations, there has been difficult to identify a definitive surface marker that distinguishes Treg cells from related T-cells (Guerin, Prins *et al.*, 2009).

Moreover, Treg cells produce soluble Transforming growth factor-β (TGF-β) and Interleukin-10 (IL-10) (Leber, Teles *et al.*, 2010).

Figure 3. Regulatory T-cell Pathway



Legend: With permission from (BioLegend).

$CD4^+CD25^+$ regulatory T-cells were first described as a specialized T-lymphocyte population responsible for the maintenance of immunological self-tolerance by actively suppressing self-reactive lymphocytes (Guerin, Prins *et al.*, 2009; Leber, Teles *et al.*, 2010). These cells are strong suppressors of inflammatory immune responses, being essential in the prevention of destructive immunity in the body (Guerin, Prins *et al.*, 2009). $CD4^+CD25^+$ Treg cells are important cells for the maintenance of peripheral tolerance (Saito, Nakashima *et al.*, 2010) and for regulation of effector T-cells, such as Th_1, Th_2 and Th_{17} cells (Saito, Nakashima *et al.*, 2010).

Treg cells act not only to control T-cells that react with self antigens that have escaped thymus negative selection, but also they limit the extent and duration of responses exerted by effector T-cells. Therefore, Treg cells can be viewed as sentinels of tissue integrity, preventing damage that might be caused by aberrant or uncontrolled immune responses (Guerin, Prins *et al.*, 2009).

The master gene for the differentiation to Treg cells is transcription factor Forkhead box P3 (Foxp3) (Saito, Nakashima *et al.*, 2010). $Foxp3^+$ Treg cells can be divided into two subpopulations based on the expression of inducible T-cell costimulator (Lastovicka, 2013). These subpopulations are both anergic and suppressive, but exert different molecular mechanisms for suppression. While inducible T-cell $costimulator^-Foxp3^+$ Treg cells mediate their suppressive activity via TGF-β, inducible T-cell $costimulator^+Foxp3^+$ Treg cells additionally secrete IL-10 (Lastovicka, 2013).

Treg cells are recognized to inhibit proliferation and cytokine production in both $CD4^+$ and $CD8^+$ T-cells, immunoglobulin production by B cells, cytotoxic activity of NK cells, and maturation of monocytes and dendritic cells, thus ensuing tolerance induction (Lastovicka, 2013; Saito, Nakashima *et al.*, 2010). As so, Treg cells can suppress their proliferation directly without the presence of Antigen presenting cells (APCs). There are evidences for either contact-dependent suppression, suppression via immunosuppressive cytokines or other factors. One efficient way to suppress immune responses is by direct killing of $CD4^+$ effector cells, which was described by Grossman *et al.* (Lastovicka, 2013).

Nevertheless, the main mechanism of suppression of Treg cells consists in influencing the activation status of APCs. In this regard the key molecule is $CTLA_4$,

which competitively inhibits the binding of CD28 to its ligands CD80 and CD86 and thus inhibits co-stimulation of effector T-cells (Lastovicka, 2013). $CTLA_4$, together with the adhesion molecule lymphocyte function associated antigen 1, also down regulates the expression of CD80 and CD86 on APCs (Lastovicka, 2013). Treg cells do not only reduce antigen presenting activity of APCs, but also support an immunosuppressive cytokine milieu by reducing IL-6 while increasing IL-10 production by DCs; this cytokine exerts immunosuppressive effects on various cell types (Lastovicka, 2013). Moreover, Treg cells express high levels of CD25 and consecutively deprive the environment of IL-2, which can also affect the survival of effector T-cells (Lastovicka, 2013).

Functional studies revealed that Treg cells might regulate immune cell responses directly at the maternal-fetal interface by altering the function of several immune cell subtypes. It has been verified that Treg cells generate a tolerant microenvironment both by interacting with other immune cells like DCs and NK cells, but also by inducing the expression of immune regulatory molecules such as TGF-β, directly at the interface between fetus and mother (Leber, Teles *et al.*, 2010).

As so, their unique properties and behaviour confer Treg cells the capacity to perform unique functions in the events of reproduction and pregnancy (Guerin, Prins *et al.*, 2009).

First considered to be a homogeneous population, Treg cells are now recognized to have two distinct pathways of generation distinguished by having different antigen specificities and T-cell receptor signal strength and co-stimulatory requirements. The two main groups of Treg cells represent natural Treg cells (nTreg) and induced/adaptive Treg cells (iTreg) (Lastovicka, 2013). nTreg are generated in the

thymus, in a continuous mode by a selection process, express high levels of CD25 and Foxp3, which is inevitable for their development and function. iTreg cells arise in the periphery, emerging from naive T-cells after exposure to antigens in the peripheral lymphoid organs (Leber, Teles *et al.*, 2010; Polese, Gridelet *et al.*, 2014); under the influence of suppressive cytokines and antigen-specific activation they develop into $Foxp3^+$ Treg cells (Lastovicka, 2013).

Moreover, at least three subsets of iTreg with distinct suppressive mechanisms are distinguished by their phenotype, cytokine secretion and tissue origin. (Guerin, Prins *et al.*, 2009). $CD8^+$ T-cells with regulatory properties have also been described, but less is known about their ontogeny, regulation and function (Guerin, Prins *et al.*, 2009).

In spite of that, many authors support a simplification of Treg cell's nomenclature, recommending that *thymus-derived* Treg cell (*t*Treg cell) should be used instead of nTreg and *peripherally derived* Treg cell (*p*Treg cell) should be used instead of iTreg cells (Abbas, Benoist *et al.*, 2013).

Over the years, the careful study of Treg cells gave rise to the question of what subtype of Treg cells is acting in pregnancy (Polese, Gridelet *et al.*, 2014).

Zenclussen *et al.* showed that nTreg are important for mouse pregnancy establishment while iTreg act at later pregnancy stages (Polese, Gridelet *et al.*, 2014). Nevertheless, in humans the question remains.

In 2004 appeared the first report implicating Treg cells in pregnancy (Guerin, Prins *et al.*, 2009), with a systemic expansion of Treg at very early stages of human pregnancy (Leber, Teles *et al.*, 2010).

Striking evidence indicates that Treg cells are specific to paternally derived cells, which highlights the probable function of protection of paternally derived cells from immune rejection by the mother's immune system (Leber, Teles *et al.*, 2010). Besides, it is unquestionable that Treg cells expand in the periphery in human pregnancy and are present in important numbers at the decidual-trophoblastic interface, preferentially in the maternal decidua (Leber, Teles *et al.*, 2010). Although the presence of Treg cells has been confirmed at the fetal–maternal interface near the beginning of pregnancy, the mechanisms of Treg cells' migration have not been identified yet (Leber, Teles *et al.*, 2010). One promising explanation is that Treg cells are attracted by human chorionic gonadotropin at the maternal-fetal interface (Schumacher, Heinze *et al.*, 2013).

Some studies investigated the dynamics of lymphocyte subpopulations during pregnancy but without considering markers definitively identifying Treg cells (Guerin, Prins *et al.*, 2009). Some reports demonstrated an increase in circulating $CD4^{+}CD25^{+}$ cells during early pregnancy with a peak phase at the second trimester and a decline post-partum, to levels slightly higher than pre-pregnancy levels (Guerin, Prins *et al.*, 2009). This elevation during the first and second trimesters has been confirmed in other investigations identifying more precisely Treg cells as $CD25^{high}$ cells, with a clear decline in $CD4^{+}CD25^{high}$ Treg cells occurring during the weeks prior to delivery (Guerin, Prins *et al.*, 2009). This implies a potential role for Treg cells in the immunological changes preceding labour, and prompts speculation that their decline might be a causal factor in the inception of labour (Guerin, Prins *et al.*, 2009). However, studies using more specific markers and animal models are needed to address the possibility of any active role of Treg cells in parturition (Guerin, Prins *et al.*, 2009).

Notwithstanding, today the most widely accepted phenotype for Treg cells is the coexpression of CD4, CD25 and Foxp3 (Lastovicka, 2013).

How Treg cells are generated and expand during pregnancy is still under dispute. (Leber, Teles *et al.*, 2010). Antigenic presentation of paternal structures in reproductive tissues may be responsible for Treg cells' increase at the beginning of pregnancy and their later expansion (Leber, Teles *et al.*, 2010).

As already stated before, Treg cells in general mediate their protective function either directly by cell to cell contact or via secretion of immunosuppressive cytokines.

In regard to immunosuppressive cytokines, Treg have been shown to secrete IL-10 and TGF-β and thereby suppress the effector functions of activated leukocytes (Leber, Teles *et al.*, 2010).

IL-10 is thought to be the foremost cytokine for pregnancy maintenance. It might play a role in dampening the inflammatory response and may possibly have therapeutic value (Romero & Lockwood, 2008). Accordingly, IL-10 has been recognised as a key factor in modulating or promoting resolution of the inflammatory process associated with term labour and with intrauterine infection-associated preterm labour (Pineda-Torres, Flores-Espinosa *et al.*, 2014).

Several studies have demonstrated that there were no changes in IL-10 expression across normal pregnancy in peripheral blood samples. Nevertheless, lower IL-10 levels have been associated with increased risk of preterm birth, which may be expected as IL-10 is anti-inflammatory; yet, other studies have reported null associations (Ferguson, McElrath *et al.*, 2014).

TGF-β has long been known to reveal immunosuppressive and anti-inflammatory properties (Mesdag, Salzet *et al.*, 2014), besides its capacity to preferentially induce Treg cell differentiation (Teles, Thuere *et al.*, 2013). Moreover, at local uterine level, TGF-β blocks differentiation of Th_1 and Th_2 cells (Gargano, Holzman *et al.*, 2008), promoting Treg cell responses (Teles, Thuere *et al.*, 2013).

The $CD4^+CD25^+$ T-cells comprising the expanded pool in pregnancy are highly enriched for Foxp3 and exert suppressive function in vitro (Guerin, Prins *et al.*, 2009). Furthermore, cells expressing the Treg cell activation marker $CTLA_4$ are more prevalent in peripheral blood and term decidua of normal healthy pregnant women compared with non-pregnant women (Guerin, Prins *et al.*, 2009). Besides, studies reported that Treg cells accumulate in decidual tissue at densities greater than in peripheral blood (Guerin, Prins *et al.*, 2009).

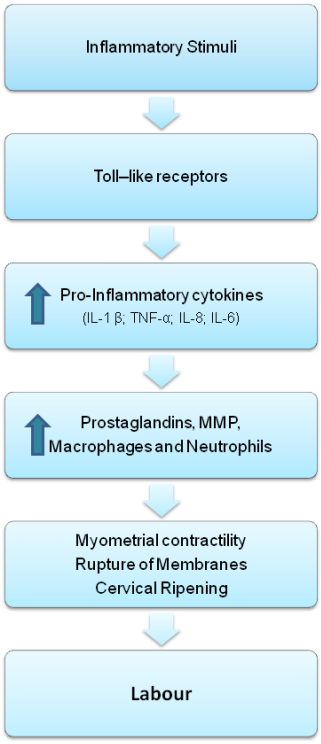
Current research hypothesis propose that the potential of trophoblastic antigens to induce a natural and tolerogenic maternal response engages cytokines, chemokines, indoleamine 2,3-dioxygenase and galectin-1 derived from the fetal-placental unit, which suggests a possible strategy to treat some forms of pregnancy pathologies via immune regulation (Chen, Liu *et al.*, 2012).

3. Inflammation: its role in pregnancy and labour

The pregnant uterus is enriched with specialized immune cells primed to play roles in implantation, placentation and parturition. The major cell types comprise uterine NK cells, DC, T lymphocytes and macrophages (Thaxton, Nevers *et al.*, 2010), as explained previously.

Implantation and parturition are specifically characterized by mechanisms of local inflammatory activity (Norman, Bollapragada *et al.*, 2007; Thaxton, Nevers *et al.*, 2010; van Mourik, Macklon *et al.*, 2009). In fact, proinflammatory cytokines, matrix degrading proteins, altered transcriptional factors, rapid hormonal changes and immune cell activity, are paramount for uterine activation and the onset of labour (Mendelson, 2009) (Figure 4).

Figure 4. Inflammation and Parturition



Legend: Adapted from (Challis, Lockwood *et al.*, 2009). MMP: Matrix Metalloproteinases.

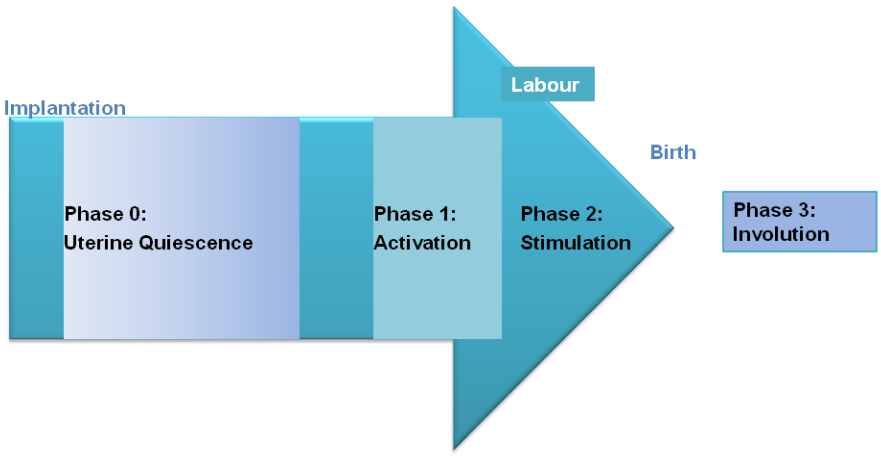
Decidual macrophages also contribute to parturition, given their potential to express proinflammatory mediators. Furthermore, both term and preterm labour have been associated with the selective accumulation of those cells (Erlebacher, 2013).

The safety of the gestational period, comprised of decidualization, placentation and fetal development, requires uterine quiescence guided by high levels of progesterone and the production of anti-inflammatory cytokines (Challis, Lockwood *et al.*, 2009; Kelly, King *et al.*, 2001).

Consequently, parturition is characterized by an influx of immune cells into the myometrium to promote the recrudescence of an inflammatory process. This proinflammatory environment promotes the contraction of the uterus, cervix modifications and the ultimate liberation of the infant and the placenta (Greene, Creasy *et al.*, 2008).

The physiological process of normal parturition at term can be divided in 4 phases (Figure 5).

Figure 5. Stages of Parturition



Legend: Adapted from (Behrman & Butler, 2007).

During pregnancy the uterus remains quiescent, passively accepting distension in order to accommodate the increasing volume of gestational components and this corresponds to phase 0 (*quiescence*); phase 1 (*activation*) involves a level of uterine stretch that determines hypothalamic-pituitary-adrenal activation. Phase 2 (*stimulation*) refers to stimulation of the activated uterus by various hormones, including corticotrophin-releasing hormone, locally produced Prostaglandins (PG) and oxytocin. These sequential processes lead to a common pathway of parturition involving increased uterine contractility, cervical ripening and decidual and amnio-chorionic activation. Phase 3 (involution) corresponds to postpartum uterus' return to the pre-pregnant status (Behrman & Butler, 2007; Challis, Lockwood *et al.*, 2009).

Temporal increase in inflammatory signals initiates labour. Inflammatory cytokines such as Tumour necrosis factor α (TNF-α) and IL-1 β and chemokines such as IL-8, increase in the decidual microenvironment including amniotic fluid and fetal membranes. This induces signals for innate immune cells to become activated (Protonotariou, Chrelias *et al.*, 2010; Romero, Brody *et al.*, 1989).

Upon initiation of a proinflammatory cascade, including activation of Nuclear factor kappa light chain of activated B cells (NF-κB), uterine inflammatory cells produce chemokines and cytokines. Increased uterine activation of transcription by NF-κB, leads directly to high levels of cyclooxigenase 2, PGE_2, gap-junction protein connexin 43 and up-regulation of oxytocin receptors (Lindstrom & Bennett, 2005).

The role PG, namely PGE_2 and $PGF_{2\alpha}$, is essential in the commencement of labour, as they promote the proliferation of gap-junctions at myometrial level (which allows rapid and generalized membrane depolarization and uterine global contractions) and

modifications of the extracellular matrix in the cervix (that permits passive dilatation). In human amnion and decidua there is increased production of PG during parturition. Even more, it seems that there is an increase of PG (PGE_2 and $PGF_{2\alpha}$) in amniotic fluid and in maternal plasma after labour has started (Gibb, 1998).

The amnion and the chorion are important local sources of arachidonic acid, and the intracellular activation of the enzymatic pathways of prostaglandin synthesis in the fetal membrane, with extension to the decidua, is an essential step in the process of labour. It is uncertain if the first stimulus goes from the decidua to the fetal membranes, or if it works the other way around (Gibb, 1998).

Arachidonic acid is stored in various membrane phospholipids within the cell and is released via various phospholipases. The arachidonic acid thus formed is then converted to prostaglandin H via the enzyme prostaglandin synthase (PGHS); primary PG is inactivated by hydroxyprostaglandin dehydrogenase (PGDH).

Recent studies have clearly shown that, in both the amnion and chorion *laeve*, there is a marked increase in PGHS activity during labour and that this is due to increased expression of the PGHS-2 isomer in fetal tissues. But it seems that PGHS-2 expression in the decidua is absent, indicating that in this maternally derived tissue, PGHS-1 isoenzyme may be of more significance in relation to PG formation than the PGHS-2 isoenzyme (Gibb, 1998).

The chorion *laeve* possesses a very active PGDH and acts as a barrier between PG formed in the amnion and the chorion itself, and their transfer to the decidua and hence to the myometrium (Gibb, 1998).

Challis *et al*. suggested that in the chorion, PGDH might be important in regulation of PG availability at the uterus. Indeed, subsequent studies demonstrated that there is a decrease in PGDH expression in fetal membranes in the lower uterine segment covering the cervix, suggesting that this may allow PG from this area of the membranes to access the cervix and result in cervical ripening. Changes may then occur with a reduction in PGDH activity in the fundal portion of the uterus as labour progresses, allowing active PG to reach the myometrium and create conditions for coordinate and global contractions (Gibb, 1998).

In addition, glucocorticoids may have a dual role increasing PG formation via stimulation of PGHS-2 expression and, at the same time, decreasing PG metabolism by inhibiting PGDH expression. Besides, other studies also revealed that progesterone produced locally within the chorion *laeve* is responsible for maintaining PGDH activity (Gibb, 1998).

Or is it the fetus that ultimately controls the moment of birth in normal circumstances? It seems that Hippocrates considered that the baby ruptured the membranes and forced labour in search for the nourishment that the mother's womb could no longer adequately provide. Seemingly naive, or almost of magic nature, the explanation has found more modern formulations through the notion that fetus has direct control of amniotic fluid' composition (and thus indirectly of local PG synthesis), which could account for the essential chronological coordination between fetal maturity and birth.

4. Preterm Labour

I. Introduction

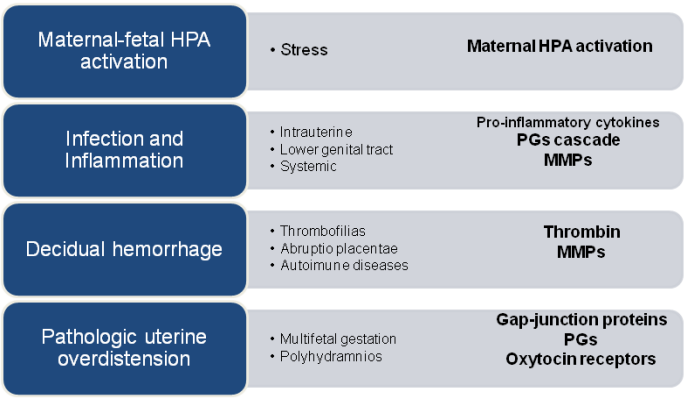
Preterm labour (PTL) is defined as delivery occurring before 37 completed weeks of gestation. Approximately 75% of preterm births occur between 34 and 36 weeks. Although these late preterm infants experience significant morbidity, the great majority of perinatal mortality and most serious morbidity occurs amongst the 16% of them whose birth occurred before 32 weeks (J.D. Iams, Romero *et al.*, 2008).

In spite of the definitions chosen and the methods used to determine gestational age, the true incidence of preterm birth has increased in developed countries regardless of multiple strategies being carried out to avoid it (J.D. Iams, Romero *et al.*, 2008). Hence, current estimate rates vary between 5 and 11% in developed countries and 18% in developing countries (Borna & Sahabi, 2008; How & Sibai, 2009; Martin, Hamilton *et al.*, 2013; Rai, Rajaram *et al.*, 2009; Tita & Rouse, 2009). Nevertheless, European 2010 data suggest that some countries managed to block that increase ("Euro-Peristat project with SCPE and Eurocat. European Perinatal health report. The health of pregnant women and babies in Europe in 2010 ", 2013).

There are numerous possible reasons for this increase, the two most important being the increased number of late preterm births (between 34 and 36 weeks) and the increased number of multifetal gestations that result from fertility therapies (J.D. Iams, Romero *et al.*, 2008). In addition to the rise attributable to improved gestational dating by ultrasound, there has been a true increase in the number of preterm births between 34 and 36 weeks ensuing from a resolution to terminate pregnancy for medical or obstetric reasons (J.D. Iams, Romero *et al.*, 2008).

Preterm births are the foremost unsolved problem in perinatal medicine and are preceded by numerous clinical conditions that fall into two broad categories, according to whether one or more steps of the parturition process (cervical ripening, membrane and decidual activation, and coordinated uterine contractility) has or has not been instigated (J.D. Iams, Romero *et al.*, 2008). The first group, often called *spontaneous preterm births,* comprises preterm labour 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 births,* comprises preterm births 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 (J.D. Iams, Romero *et al.*, 2008; Mao, Wang *et al.*, 2010).

Figure 6. Etiologies and Pathways leading to Spontaneous Preterm Birth



Legend: Adapted from (Behrman & Butler, 2007). HPA: hypothalamic-pituitary-adrenal; PG: prostaglandins; MMP: matrix metalloproteinases.

PTL accounts for 70% of all perinatal mortality; moreover, approximately 65-95% of neonatal deaths can be attributed to prematurity complications (Howson, Kinney *et al.*, 2013) (Borna & Sahabi, 2008; How & Sibai, 2009).

Even those children who survive a PTL have an increased incidence of sequels that diminish their quality of life, not only in the short term (intraventricular haemorrhage, necrotizing enterocolitis, respiratory distress syndrome, bronchopulmonary dysplasia and jaundice) but also in the long term (asthma, deafness, cerebral palsy, retinopathy and psychomotor retardation). (How & Sibai, 2009; Rai, Rajaram *et al.*, 2009; Tita & Rouse, 2009).

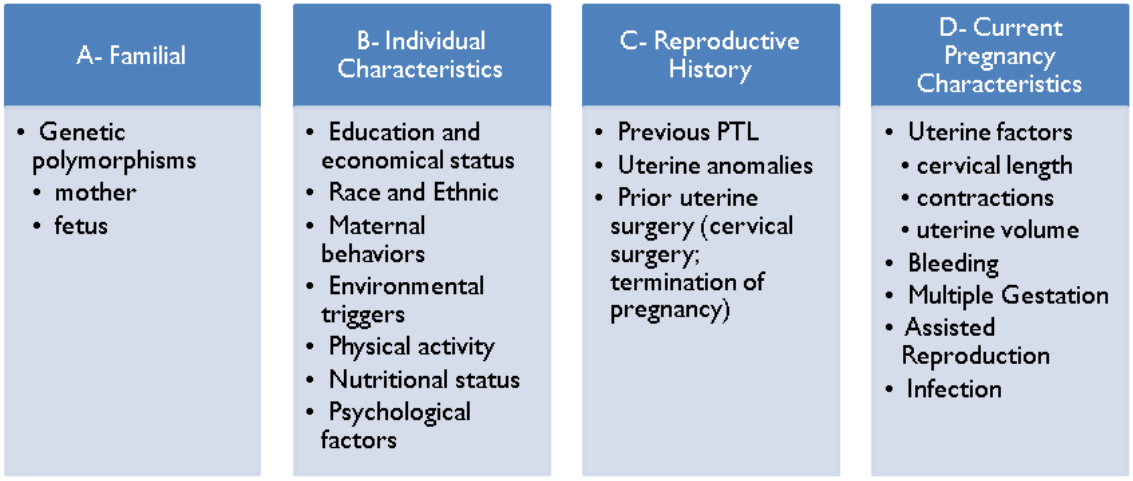
The diagnosis of preterm labour is generally based upon clinical criteria of regular painful uterine contractions accompanied by cervical change (dilation and/or effacement). The presence of vaginal bleeding and/or ruptured membranes increases diagnostic certainty. Because the clinical criteria for preterm labour are poorly predictive of the diagnosis until labour is well established, over-diagnosis is common (J. D. Iams, 2003).

Ultrasound measurement of cervical length and laboratory analysis of cervicovaginal fetal fibronectin level help to support or exclude the diagnosis (Melamed, Hiersch *et al.*, 2013).

II. Risk Factors

PTL 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 (Figure 7).

Figure 7. PTL Risk Factors (Adapted from Creasy and Resnik's Maternal-Fetal Medicine: Principles and Practice, 2008)



It seems that gene-environment interactions play a significant role in determining the risk of PTL. Polymorphisms of certain critical genes may be responsible for a harmful inflammatory response in those that possess them. Accordingly, polymorphisms that increase the magnitude or the duration of inflammatory response (TNF2 allele; IL-1 RA2) were associated with an increased risk of PTL (Holst & Garnier, 2008).

However, infection is one of the most heralded causes of PTL due to the drastic link between underlying infectious agents and their ability to promote inflammatory responses (Thaxton, Nevers *et al.*, 2010).

Data from human studies provide information consistent with bacterial infection resulting in spontaneous PTL. Moreover, preterm deliveries (in the larger group of spontaneous PTL) and PPROM are often associated with intra-uterine inflammation or Chorioamnionitis (CA) (Choi, Jung *et al.*, 2012; Gantert, Been *et al.*, 2010).

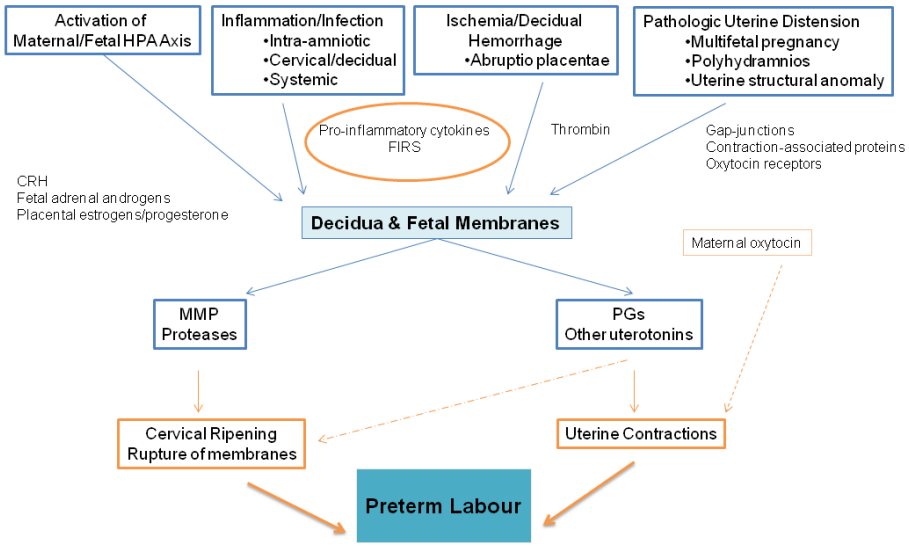
While the evidence for infection mediated PTL is substantial, the underlying mechanisms that induce early birth, due to pathogenic presence, remain unclear. Investigation into the mechanisms that lead to PTL in response to pathogenic agents ought to take into consideration several factors: the route of entry, which determines where the agent will subsist and what pathways will be activated (as the same pathogen delivered by alternative routes can lead to differential inflammatory responses); and the fact that different pathogens may elicit varied inflammatory responses (Thaxton, Nevers *et al.*, 2010).

III. From Inflammation to Preterm Birth

It is commonly accepted that the act of giving birth is the final step in a proinflammatory signaling cascade that is orchestrated by an intrauterine milieu coupled to hormonal cues (Thaxton, Nevers *et al.*, 2010). Consequently, the inflammatory process plays a pivotal role during the pathogenesis of human labour, both in term and preterm deliveries. Very likely, it is the immune response of the host

that presumably leads to the inflammatory response and preterm birth (Thaxton, Nevers *et al.*, 2010). Thus, the immunoinflammatory response, particularly cytokine production during pregnancy, is a field that might be explored in the understanding of the molecular mechanisms behind PTL.

Figure 8. Pathways to Preterm birth



Legend: Adapted from (Behrman & Butler, 2007). HPA: hypothalamic-pituitary-adrenal; PG: prostaglandins; MMP: matrix metalloproteinases; CRH: corticotrophin-releasing hormone; FIRS: fetal inflammatory response syndrome.

Despite numerous studies on the role of circulating Treg cells, the mechanisms that underlie their function are not so clear; these cells may be flexible to switch between tolerance and antimicrobial activity. As so, the paradox of pregnancy, fetal-trophoblastic tolerance versus protection of the mother, may possibly be explained by a higher flexibility and plasticity of Treg cells (Ernerudh, Berg *et al.*, 2011).

There have been ambiguities in the literature regarding systemic Treg cells frequencies in normal pregnancy; nonetheless, Treg cells function or frequency deviation could be involved in pregnancy complications (Ernerudh, Berg *et al.*, 2011; Mao, Wang *et al.*, 2010).

During human pregnancy there is an enrichment of Treg cells in the decidua, presumably recruited from blood, which show a stable and highly suppressive phenotype that might be important to fetal tolerance. This is accomplished by direct cell-to-cell contact or by immunosuppressive cytokine production such as IL-10 and TGF-β (Leber, Teles *et al.*, 2010).

If, as stated above, normal parturition at term results from the activation of inflammatory mechanisms and prostaglandin synthesis by the amnio-chorio-decidual unit, this process may be abnormally initiated out of time by any interference having this proinflammatory potential, as membrane mechanical rupture or infectious agents (Romero & Lockwood, 2008).

The Common Pathway

The conventional view, which has dominated the study of preterm parturition, is that term and preterm labour are based on the same mechanisms, albeit occurring at different gestational ages and probably instigated by different stimuli.

Indeed, they do share a common pathway, which includes increased uterine contractility, cervical ripening and membrane rupture. Subsequently, it has been proposed that the essential distinction between term and preterm labour is that the former results from "physiologic activation" of this common pathway, whereas

preterm labour results from "pathologic activation", due to a disease process (Romero & Lockwood, 2008).

Accordingly, the *common pathway of parturition* is defined as the anatomic, biochemical, immunoinflammatory, endocrinologic, and clinical events that occur in the mother and fetus in both term and preterm labour. Much clinical importance has been placed on the uterine components of the pathway (myometrial contractility, cervical ripening, and membrane rupture) (Romero & Lockwood, 2008).

In addition, activation of the uterine components of the common pathway of parturition may be synchronous or asynchronous. Synchronous activation results in clinical spontaneous preterm labour whereas asynchronous activation results in a different phenotype. For instance, predominant activation of the membranes leads to PPROM, that of the cervix to cervical insufficiency, and that of myometrium to preterm uterine contractions (Romero & Lockwood, 2008).

Spontaneous Preterm Parturition as a "Syndrome"

Due to the immediacy of the onset of labour and the necessary shift from anti to proinflammatory signalling cascades, it is not surprising that unscheduled parturition is one of the most menacing complications of pregnancy, with the resultant adverse perinatal outcomes (Thaxton, Nevers *et al.*, 2010).

Additionally, upon infection, as it is rarely the foreign organism that directly causes preterm birth; rather, it is the immunoinflammatory response of the host evoked by the pathogen that leads to aberrant pregnancy outcomes (Thaxton, Nevers *et al.*, 2010).

There are data that support the hypothesis of an inflammation-triggered inadequate immunologic response with a consecutively increased risk of PTL (Holst & Garnier, 2008).

Throughout the literature, bacteria show a stronger correlation with increased incidence of PTL compared to virus; this may be due to the differential sites of infection. Usually bacteria are found in mucosal membranes that surround the amniotic sac or those lining the intrauterine canal; on the other hand, viruses, needing the host cell machinery for replication, tend to infect trophoblast cells of the placenta, as these cells possess specific receptors needed for viral particle entry (Arechavaleta-Velasco, Koi *et al.*, 2002; Parry, Holder *et al.*, 1997).

A very plausible explanation for that commencement of those distinct immune pathways is probably by the activation of Toll-like receptors (TLRs) (Thaxton, Nevers *et al.*, 2010). TLRs are a diverse group of innate immune sentinel receptors evolutionarily conserved, with each TLRs (1 to 10) being specific for a different pathogen associated molecular pattern. Importantly, TLRs are expressed on trophoblast and uterine immune cells.

So, it is likely that differential uterine immune responses occur due to the multiplicity of pathogens that ensue activation of any one of these TLRs, ultimately leading to deleterious inflammation and PTL (Thaxton, Nevers *et al.*, 2010).

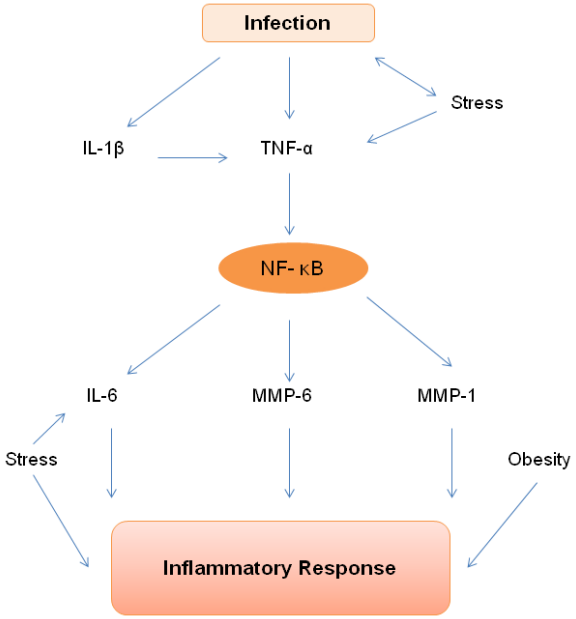
Accordingly, evidence demonstrates that the activated TLR pathways and the route of pathogenic entry (intrauterine ascension versus systemic infection) may determine the immunological cascade of deviant cellular and cytokine activity that lead to PTL. The majority of these pathways lead to an increase in the NF-κB activity that

allows the production of inflammatory cytokines and chemokines (Lindstrom & Bennett, 2005).

Inflammation and its mediators, chemokines, proinflammatory cytokines (IL-1β, TNF-α), and other mediators are critical to preterm parturition induced by infection. In addition, the redundancy of the cytokine network implicated in parturition is such that blockage of a single cytokine is insufficient to prevent preterm delivery in the circumstance of infection (Romero & Lockwood, 2008).

Current literature advocates that systemic inflammatory responses might induce TNF-α through NF-κB pathway to activate events leading to PTL. In contrast, in the local intra-uterine setting, the mode of action is mostly likely TNF-α independent (Thaxton, Nevers *et al.*, 2010) (Figure 9).

Figure 9. Pathophysiology mechanisms leading to the induction of labour and dilatation of the cervix, in the presence of ascending infection



Legend: Adapted from (Holst & Garnier, 2008). MMP: matrix metalloproteinases; NF-κB: nuclear factor kappa light chain of activated B cells.

Intra-uterine Infection

Ascending genital infection is the most frequent mechanism of intra-uterine infection and represents an important risk factor for preterm labour, PPROM and preterm delivery before 32 weeks of gestation. It occurs when pathogenic bacteria pass the cervical barrier causing decidual and chorioamniotic inflammation, characterized by bacterial infection of the amniotic fluid (Gomez, Ghezzi *et al.*, 1995).

Interestingly, as stated before, several reports refer that bacterial agents are rarely found at the placental level, in contrast to viral pathogens. Evidence suggests that viral entry into trophoblast cells induces trophoblast apoptosis and the resultant inflammatory events can lead to PTL (Goldenberg, Hauth *et al.*, 2000; Romero, Sirtori *et al.*, 1989).

The presence of infectious agents in the chorioamnion engenders a maternal and fetal inflammatory response characterized by the release of a combination of proinflammatory and inhibitory cytokines and chemokines in the maternal and fetal compartments (Tita & Andrews, 2010).

When infection is limited to the decidua or the amniochorion space (localized inflammation confined to chorion-decidua), the inflammatory process detected within the membranes is of maternal origin and is denoted **amnionitis**. The next stage is microbial invasion of the amniotic cavity through the amnion (inflammation in amnion or chorionic plate without funisitis), being named **chorioamnionitis** (CA); this intra-amniotic inflammatory process appears to be of fetal rather than maternal origin (Thaxton, Nevers *et al.*, 2010). CA alone induces maternal systemic inflammatory

reaction which clinically presents as amnion infection syndrome causing pyrexia and elevated inflammatory markers in the mother; unfortunately, early detection is difficult (Holst & Garnier, 2008).

In the final stage, **funisitis,** there is fetal invasion by microorganisms that elicits fetal inflammatory response characterized by infection or inflammation of the umbilical cord (Park, Moon *et al.*, 2009; Thaxton, Nevers *et al.*, 2010; Tita & Andrews, 2010). This is referred as fetal inflammatory response syndrome (**FIRS**) which can be proven histologically by the presence of funisitis or biochemically by the detection of elevated IL-6 serum levels during the perinatal period. In the presence of FIRS, there is a dramatic increase of fetal and neonatal morbidity compared to CA alone (Holst & Garnier, 2008).

The presence of bacteria induces the release of proinflammatory cytokines (IL-1, IL-6, TNF-α) by macrophages, amnion, decidua and myometrium. These cytokines, together with endotoxins released by Gram-negative bacteria, induce an increase in the production of PG, endothelin and corticotrophin-releasing hormone in decidual, chorionic and amniotic cells that will further provoke uterine contractions (Holst & Garnier, 2008; Romero, Mazor *et al.*, 1992; Romero, Mazor *et al.*, 1992).

Also, IL-1 and TNF-α can trigger the secretion of matrix metalloproteinases from chorionic and cervical cells which induce the degradation of extracellular matrix of the lower uterine segment and cervix (Holst & Garnier, 2008).

IL-1 in addition, mediates the release of IL-8 from decidual, chorionic, amniotic and cervical cells, which lead to the activation and recruitment of elastase-producing granulocytes, further contributing to the modification of cervical extracellular matrix

(Lockwood, 1994; Rechberger & Woessner, 1993). In the end, through contractions and cervical dilatation, labour will progress.

Notwithstanding, host defense mechanisms preventing intra-amniotic infection remain poorly elucidated (Tita & Andrews, 2010).

Fetal involvement

The proportion of preterm infants exposed to CA increases with decreasing gestational age, to up to 80% below 28 weeks gestational age (Lahra, Beeby *et al.*, 2009).

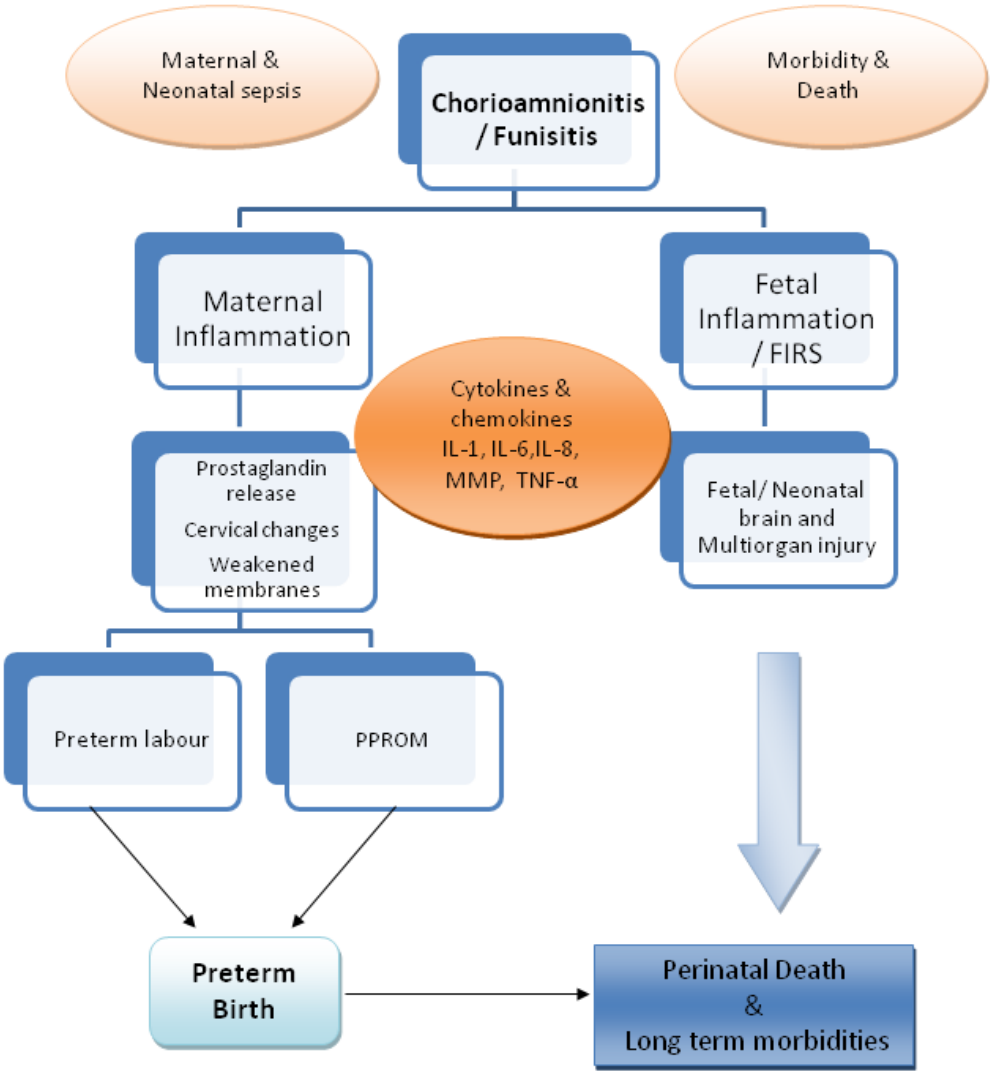
When intrauterine inflammation is present, the fetus may be exposed through direct contact with amniotic fluid or through the fetal-placental circulation. The consequent response to CA has been referred as FIRS (Gotsch, Romero *et al.*, 2007), as previously stated. Fetal exposure to infection may lead to perinatal death, neonatal sepsis and other postnatal complications (Lahra, Beeby *et al.*, 2009).

Numerous reports corroborate the neurotoxic effect of bacterial endotoxins and proinflammatory cytokines on the fetal brain. Moreover, infection is associated with a reduced cardiovascular regulation, which contributes significantly to perinatal morbidity (Holst & Garnier, 2008).

Although earlier studies focused mainly on neurological and respiratory outcomes, additional *sequelae* of CA related FIRS have recently been described in several other areas of the fetal organism, turning it into a multi-organ disease of the fetus (Choi, Jung *et al.*, 2012).

Therefore, evidence is increasing that the effects of CA/FIRS on health and disease may extend beyond the neonatal period (Gantert, Been *et al.*, 2010), hassling the importance of infection derived PTL (Figure 10).

Figure 10. Perinatal morbidity and mortality associated with inadequate inflammatory response



Legend: Adapted from (Tita & Andrews, 2010). MMP: matrix metalloproteinases; FIRS: fetal inflammatory response syndrome; PPROM: preterm premature rupture of membranes

IV. Prevention

Efforts to prevent preterm birth morbidity and mortality may be categorized as *primary* (directed to women before or during pregnancy to prevent and reduce the risk), *secondary* (aimed at eradicating or reducing risk in women with known risk factors), or *tertiary* (initiated after the process of parturition has begun, with the purpose of preventing delivery or improving outcomes) (J.D. Iams, Romero *et al.*, 2008).

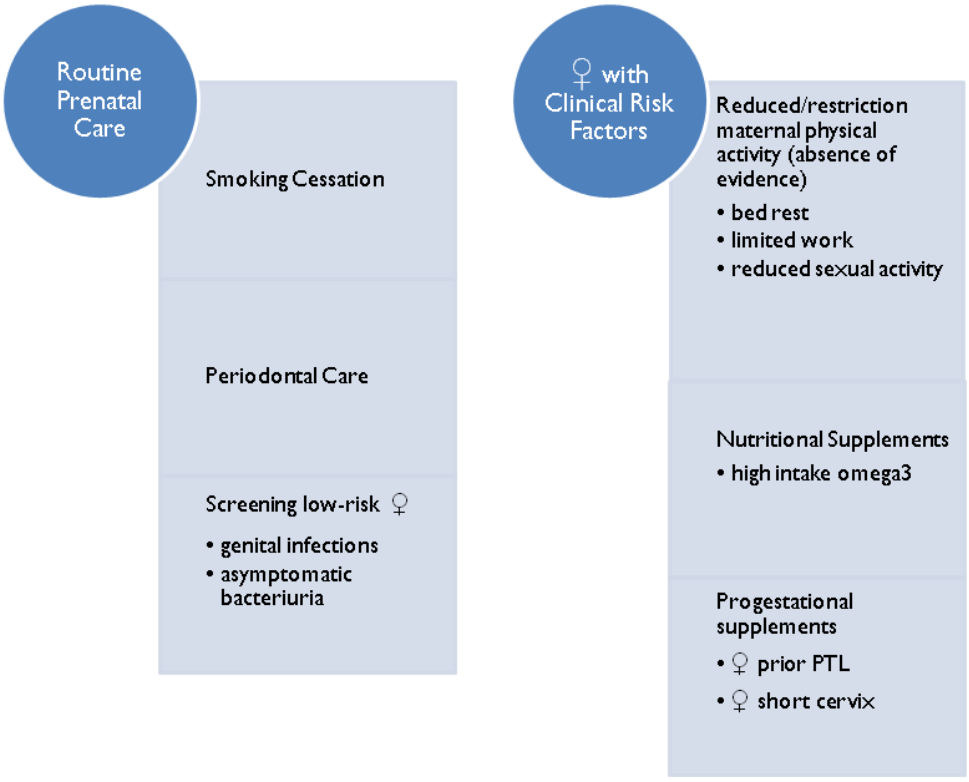
Most obstetric care for preterm birth has been focused on tertiary interventions such as differentiated perinatal care, tocolysis, antenatal corticosteroids, and optimal timing of indicated preterm birth. These procedures are intended to reduce the burden of prematurity-related illness and unfortunately, have minimal effect on the incidence of preterm birth (J.D. Iams, Romero *et al.*, 2008).

Primary prevention of the morbidity and mortality of PTL is an increasingly incontestable strategy as the limitations of tertiary care are recognized. Secondary care for women at risk is a strategy limited to removal rather than avoidance of risk.

Until the multiple pathways that contribute to PTL are better understood, attempts during pregnancy (secondary and tertiary prevention) must restrain the possibility that prolongation of pregnancy intended to promote fetal maturation may, in some cases, allow unremitting exposure to a suboptimal or even perilous intrauterine environment. Indeed, PTL is not a health outcome but instead a surrogate end point for optimal fetal, infant, and long-term health (J.D. Iams, Romero *et al.*, 2008).

More than 50% of preterm births occur in pregnancies without obvious risk. Thus, prevention of these PTL might be dealt by incorporating preventive procedures into routine prenatal care or by screening apparently low-risk women for specific risk factors, or both (J.D. Iams, Romero *et al.*, 2008).

Figure 11. Prematurity Prevention



Legend: Adapted from Creasy and Resnik's Maternal-Fetal Medicine: Principles and Practice, 2008). PTL: Preterm labour

V. Treatment

Treatment of symptomatic PTL is aimed at arresting labour long enough to transfer the mother to the appropriate hospital for delivery and to allow administration of corticosteroids; these two interventions have consistently been shown to reduce perinatal mortality and morbidity. Other interventions directed at reducing neonatal and infant morbidity and mortality include pre-delivery antibiotics (in cases of PPROM) and neuroprotectants (J.D. Iams, Romero *et al.*, 2008).

Common treatment of PTL

Antenatal corticosteroids

Current guidelines support a single course of antenatal steroids (betamethasone or dexamethasone) for women at risk of preterm birth (J.D. Iams, Romero *et al.*, 2008).

Tocolytic therapy

Tocolytic agents hamper uterine muscle contractions after the parturitional process is established and therefore have restricted opportunity in preterm birth prevention. As so, the goal of tocolysis is to reduce neonatal morbidity and mortality postponing delivery long enough to allow administration of corticosteroids and

maternal *in utero* transport to a differentiated perinatal hospital with adequate Neonatal Intensive Care Units ((J.D. Iams, Romero *et al.*, 2008).

There are several types of tocolytics available, whose actions diverge between: reducing intracellular ionized calcium levels (ritodrine, terbutalin); blocking calcium chanels (nifedipine); acting as calcium antagonist (ionic magnesium); inhibiting PG (indomethacin); relaxing the smooth-muscle (nitroglycerin) or by antagonistic actions on the oxytocin receptor. The use of these agents intends to inhibit myometrial contractility with the aim of hampering the process of preterm labour (cease contractions) (Cunningham, Leveno *et al.*, 2009).

Oxytocin stimulates contractions in labour at term causing release of calcium into the cytoplasm. Oxytocin receptor antagonists compete with oxytocin by binding to receptors in the myometrium and decidua, preventing or reducing calcium release.

Among the available tocolytic agents, the oxytocin receptor antagonist atosiban inhibits spontaneous and oxytocin-induced contractions, although not influencing prostaglandin-induced contractions. Maternal side effects are uncommon, because oxytocin receptors are located only in the uterus and breast (J.D. Iams, Romero *et al.*, 2008). So, it may be hypothesized to be the preeminent choice in cases of PTL treatment.

Neuroprotectants

Antenatal maternal treatment with magnesium sulfate has been inconsistently associated with reduced rates of intraventricular hemorrhage, cerebral palsy, and

perinatal mortality in premature infants born before 32 weeks' gestation (J.D. Iams, Romero *et al.*, 2008).

Maintenance therapy

Meta-analysis published on PTL do not support the idea of using the available tocolytic agents as maintenance tocolytic therapy (Borna & Sahabi, 2008). However, due to the high economical and health burden associated with prematurity, the finding of a pharmacological agent that would unquestionably be efficacious in those cases is required.

Progesterone, an agent with proven ability to act at the level of uterine quiescence, seems a promising therapy in the prevention and treatment of PTL, as well as a maintenance therapy after tocolysis. Nevertheless, as the mechanisms involved in preterm labour are complex and multifactorial, a tocolytic agent such as progesterone may not be effective for all patients.

5. Progesterone

I. Pharmacological characteristics

Biochemical structure

Progesterone is a crucial hormone in the female reproductive tract, secreted by the ovarian *corpus luteum* and the placenta, after 12 weeks of pregnancy (Polese, Gridelet *et al.*, 2014).

The name progesterone in itself combines not only functional properties but also its biochemical structure, seeing that it is the combination of: *Pro* - in favour; *gest* – gestation; (*st*) er (*ol*) – sterol; and *one* - ketone group.

Progesterone has a major role in pregnancy maintenance and evidence demonstrates its secretion in the amnion, chorion and decidua of the human species (Chibbar, Wong *et al.*, 1995; Oh, Kim *et al.*, 2005).

Progestogens can be classified as natural or synthetic. Natural compounds are those whose chemical composition is comparable to those produced by live organisms. Quite the reverse, synthetic progestogens (or progestins) are those created in the laboratory, whose constitution has been adapted and do not match up to the naturally occurring steroid. Accordingly, progesterone is a natural progestogen whereas 17α-hydroxyprogesterone caproate is a synthetic one (Romero & Stanczyk, 2013).

Progestins are available in natural or synthetic formulations for oral, intramuscular or vaginal administration. Natural micronized progesterone is an accurate replication of the progesterone produced in the *corpus luteum* and placenta. For that reason it is easily metabolized in the body, with negligible side effects. The peak plasma

concentration will depend on the dose and route of administration: vaginal and rectal routes take about 4 hours; and intramuscular route takes about 2–8 hours (How & Sibai, 2009).

Transvaginal administration of progesterone evades first-pass hepatic metabolism and is linked to rapid absorption, high bioavailability, and additional local endometrial effects. Besides, it provides superior and more sustained progesterone concentrations, being the ideal route of administration in numerous occasions. Despite the fact that this route has fewer side effects and absence of local pain, it is allied to inconsistent blood concentrations (How & Sibai, 2009).

Administration of progesterone rather than its derivatives has the advantage of ensuring that all pathways activated by endogenous progesterone synthesis will be activated. Levels of progesterone in the body are tightly regulated and thus exogenous progesterone is subject to rapid metabolism by the liver and target tissues, rationale to administer it by the vaginal route (Byrns, 2014).

Type of receptors

Nuclear receptors

Nuclear Progesterone receptors (nPR) belong to the superfamily of nuclear receptors. They are encoded by a gene located on chromosome 11, having at least 3 different isoforms studied so far (Polese, Gridelet *et al.*, 2014).

nPR-A has 164 amino acids truncated from the N-terminus, whilst nPR-B is encoded by the full length transcript (Ernerudh, Berg *et al.*, 2011). During the foremost part of pregnancy, expression of nPR-B predominates and it is this isoform that is

responsible for the effects traditionally ascribed to progesterone during pregnancy, including suppression of the immune system. However, during the third trimester, expression of nPR-A increases markedly, probably being responsible for the activation of the inflammatory mechanisms that culminate in delivery. Likewise, nPR-A directly represses the activation of some nPR-B dependent genes. So, the pathways activated by these two receptors are frequently in direct opposition to one another, particularly concerning genes involved in inflammation and thus initiation of parturition.

A further truncated form, nPR-C, has also been proposed to be up-regulated late in gestation and stimulated by inflammatory cytokines (Byrns, 2014).

Membrane receptors

While long theorized, membrane localized progesterone receptors only recently have been identified and characterized (Byrns, 2014). There are two different families of membrane progesterone receptors: progesterone receptor membrane component 1 and 2 and membrane associated progesterone receptor α, β, γ, δ and ε (Byrns, 2014; Zachariades, Mparmpakas *et al.*, 2012).

Although progesterone receptor membrane components have been primarily studied in the context of breast and ovarian function, lately it has been investigated the connection of progesterone receptor membrane component 1 and delivery (Byrns, 2014).

The membrane associated progesterone receptors (mPR) belong to the PAQR family of proteins (Kowalik, Rekawiecki *et al.*, 2013) and are putative G-protein coupled receptors that induce signal transduction cascades in response to binding of

progesterone and other ligands (Dressing, Goldberg *et al.*, 2011; Karteris, Zervou *et al.*, 2006).

Two of the mPR isoforms, mPRα and mPR β, are downregulated in the uterus during term labour, while mPRα is also downregulated during preterm labour (Fernandes, Pierron *et al.*, 2005). T lymphocytes appear to respond to progesterone through mPRs, suggesting a role for the mPRs in immune system's regulation, and therefore parturition; moreover, all three isoforms of mPR (α, β, γ) are expressed in high levels in T lymphocytes (Ndiaye, Poole *et al.*, 2012).

The immunosuppressive effects of progesterone have been well described and several non-genomic actions of progesterone on lymphocytes have been reported. Rapid effects of progesterone on T lymphocytes include increased intracellular calcium, decreased pH and suppression of antigen-induced calcium increase (Ndiaye, Poole *et al.*, 2012).

Mode of action

Progesterone actions may be accomplished through genomic and non-genomic mechanisms. The genomic mechanism is a slow process (taking hours to days); involves DNA transcription and protein synthesis and employs nPR. The non-genomic pathway is rapid, taking only minutes, and is mediated through mPR and progesterone receptor membrane component (Polese, Gridelet *et al.*, 2014).

II. Role in pregnancy

A successful pregnancy is dependent on the coordinated development of the placenta and on its ability to act as a steroidogenic organ. Progesterone plays a central role in the establishment and maintenance of human pregnancy, exerting its effects by binding and activating progesterone receptors (Zachariades, Mparmpakas *et al.*, 2012).

Progesterone mediates the structural remodeling that occurs early in pregnancy: soon after implantation, progesterone acts in the uterine walls to induce differentiation of stromal cells into decidual cells. Furthermore, progesterone also stimulates the morphological changes of the cervix and other tissues that help pregnancy maintenance (Byrns, 2014). Progesterone's essential role in maintaining pregnancy is primarily through assuring uterine quiescence. This is achieved by way of calcium-calmodulin-myosin light chain kinase system suppression, but also by reducing calcium influx and altering smooth muscle resting potential (Dodd & Crowther, 2010).

Parturition is accepted to be an inflammatory event, as it is predominantly driven by inflammatory cytokine and prostaglandin signaling (Byrns, 2014). Inflammatory pathways stimulate multiple events that lead to parturition, such as cervical ripening, rupture of membranes and uterine contractions. Furthermore, it is progesterone responsibility to refrain these inflammatory events until term. Moreover, progesterone inhibits uterine contractions not only by inhibition of prostaglandin production, but also by reducing smooth muscle cell's contractility, as explained previously.

There are several unanswered questions surrounding progesterone' role in pregnancy. What molecular mechanisms support and enhance progesterone during

pregnancy and what molecular changes occur that turn off progesterone signaling and allow parturition, are the most dazzling.

In human species, contrary to other mammals, serum progesterone levels steadily increase throughout pregnancy, pointing out a different mechanism to determine human labour timing. Some of the mechanisms that have been proposed include changes in progesterone metabolism within target tissues and changes in progesterone receptors isoforms (Byrns, 2014).

III. Immunoinflammatory interactions

Accordingly, progesterone is an immunomodulatory critical hormone in the regulation of human T-cell population during pregnancy, since it leads to a series of functional events in numerous immune cell types (Dressing, Goldberg *et al.*, 2011).This immunosuppression prevents the maternal immune system from rejecting the fetus and assures that pregnancy proceeds until term (Byrns, 2014).

The immunosuppressive effects of Progesterone have been known for a long time (Polese, Gridelet *et al.*, 2014). Throughout pregnancy, progesterone inhibits the immune system, which is, at the present time believed to be its most important role (Byrns, 2014; Mendelson, 2009). In 1995, Piccinni *et al.* demonstrated that progesterone favours the development of Th_2 $CD4^+$ cells and suggested that progesterone could be partly responsible for Th_2 predominance during pregnancy (Piccinni, Giudizi *et al.*, 1995; Polese, Gridelet *et al.*, 2014).

The association between Treg cells and progesterone levels was confirmed in humans (Weinberg, Enomoto *et al.*, 2011). Mjosberg *et al.* pointed out progesterone regulatory role on Treg cells during human pregnancy (Mjosberg, Svensson *et al.*, 2009) and both in vivo and in vitro models indicate that progesterone not only increases Treg cells proportion, but also their suppressive capacity (Mao, Wang *et al.*, 2010).

The emergence of the $Th_1/Th_2/Treg/Th_{17}$ paradigm led to the study of progesterone effects on those cells. Studies suggest that progesterone favours Th_2 and Treg cells, while dampening Th_1 and Th_{17}, thus participating in the establishment of a favourable environment for pregnancy by its effects on T-cells (Polese, Gridelet *et al.*, 2014).

Treg cells are recruited before implantation to induce a favourable environment for embryo nidation and afterwards are essential for pregnancy preservation (Polese, Gridelet *et al.*, 2014). What's more, in human T-cells progesterone inhibits differentiation of Th_{17} and decreases associated factors like RAR-related orphan receptor C and IL-17 (Xu, Dong *et al.*, 2013). In animal models it has been attested that progesterone increases the proportion of Treg cells, TGF-β and IL-10 expression, enhancing Treg cells' suppressive function (Mao, Wang *et al.*, 2010). However, the exact mechanism of the immunomodulatory role of progesterone is still unknown.

Since 1980, some groups have tried to identify expression of progesterone receptors during pregnancy, notwithstanding with contradicting results (Mansour, Reznikoff-Etievant *et al.*, 1994; Szekeres-Bartho, Csernus *et al.*, 1983). Nevertheless, the gathering of scientific data enabled not only to verify the presence of lymphocytic progesterone receptors (Szekeres-Bartho, Reznikoff-Etievant *et al.*, 1989), but also to validate the existence of progesterone induced blocking factor and its role in

pregnancy (Szekeres-Bartho & Polgar, 2010). Recently, some authors have attempted to demonstrate that the actions of progesterone on T lymphocytes were mediated by one or more putative membrane receptors, but all experiments were done in non-pregnant animal models (Ndiaye, Poole *et al.*, 2012).

How progesterone acts on T-cells is still under dispute, with some researchers defending both nuclear and non-nuclear receptor's contribution (Lee, Lydon *et al.*, 2012).

Recently membrane progesterone receptors (mPR) were found in human peripheral blood T lymphocytes and their levels seem to change in preterm labour (Dressing, Goldberg *et al.*, 2011). These receptors may be responsible for the rapid non-genomic actions of cellular activation made by progesterone (Dressing, Goldberg *et al.*, 2011; Zhu, Hanna *et al.*, 2008) and progesterone interaction with the immune system (Larsen & Hwang, 2011).

One of these receptors, mPRα, is the receptor whose function has been better characterized and has been shown to be localized in human placental syncytiotrophoblast (Dosiou, Hamilton *et al.*, 2008). Its actions are accomplished through stimulation of mitogen-activated protein kinases cascade and 3',5'-cyclic adenosine monophosphate inhibition, using inhibitory pathways linked to G proteins (Zhu, Hanna *et al.*, 2008). Accordingly, it constitutes an appropriate target to explain progesterone connection to T-cells or even to Treg cells, and their subsequent actions on PTL.

6. Progesterone and preterm delivery

I. State of the art

Progesterone supplementation for women at risk for preterm birth has been investigated based on several plausible mechanisms of action, including reduced gap junction formation and oxytocin antagonism leading to relaxation of smooth muscle, maintenance of cervical integrity, and anti-inflammatory effects (J.D. Iams, Romero *et al.*, 2008). Although the exact mechanism by which progesterone can exert the effect of uterine relaxation is still unknown, it is assumed that this is achieved through various actions, including: 1) blockage of Prostaglandin F2 α and α-adrenergic receptors, 2) deletion of genes necessary for contractility; 3) decrease in myometrial oxytocin receptors; 4) stimulation of myometrial relaxation systems (such as nitric oxide), and 5) blockage of the appearance of inter-cellular junctions (gap–junctions) (Rai, Rajaram *et al.*, 2009).

Moreover, some authors defend that uterine quiescence until labour is due in part to anti-inflammatory actions, by the modulation of myometrial expression of a number of genes belonging to micro RNA 200 family and their targets, that ultimately regulate inhibition of the expression of contractility related genes in the myometrium (Norwitz & Caughey, 2011; Renthal, Chen *et al.*, 2010).

Likewise, the use of a progesterone antagonist *in vitro* seems to increase the expression of genes that result in PTL (Larsen & Hwang, 2011).

The beneficial effect of supplemental progesterone compounds is not universally observed in women with a prior preterm birth, indicating that some paths to recurrent preterm birth are not influenced by this therapy (J.D. Iams, Romero *et al.*, 2008). The absence of effect in twin pregnancy, together with decline in preterm births among women with historical risk and short cervix, suggests that the effect may be related to modulation of inflammation or cervical ripening more than an effect on uterine contractility (J.D. Iams, Romero *et al.*, 2008).

During the 1980s and 1990s, progesterone was used to prevent preterm birth (mainly in France), until some published cases of cholestasis unexpectedly halted its prescription (Fuchs, Audibert *et al.*, 2014). Since then, some randomized controlled trials appeared and verified treatment's efficiency and safety at low doses (Fuchs, Audibert *et al.*, 2014).

Most studies and meta-analysis of progesterone and PTL focus on the issue of prophylactic administration of progesterone by the vaginal (da Fonseca, Bittar *et al.*, 2003; Dodd, Flenady *et al.*, 2008; Fonseca, Celik *et al.*, 2007; O'Brien, Adair *et al.*, 2007), intramuscular (Dodd, Crowther *et al.*, 2005; Dodd, Flenady *et al.*, 2006; Facchinetti, Paganelli *et al.*, 2007; Mackenzie, Walker *et al.*, 2006; Meis, Klebanoff *et al.*, 2003; Rouse, Caritis *et al.*, 2007; Sanchez-Ramos, Kaunitz *et al.*, 2005) or oral routes (Erny, Pigne *et al.*, 1986), in the presence of a previous preterm delivery or short cervix. Nevertheless, further studies and more clinical trials are needed to achieve reliable results regarding the use of progesterone in women with PTL being treated with tocolytic therapy (Coomarasamy, Thangaratinam *et al.*, 2006; Erny, Pigne *et al.*, 1986; Rai, Rajaram *et al.*, 2009).

In the literature there are only three trials and one meta-analysis on the use of progesterone after an effective treatment of PTL. The study by Facchinetti *et al.*, published in 2007, pregnant women with PTL were randomized to receive intramuscular progesterone (17α-hydroxyprogesterone caproate) or an expectant attitude; their results were suggestive of the efficiency of progesterone in combination with tocolytic therapy (RR 0.43, 95% CI 0.12 to 1.5) (Facchinetti, Paganelli *et al.*, 2007). These results were promising and pointed to the advantage of progesterone usage.

In another randomized study published in 2008, *Borna and Sahabi* assessed the efficacy of tocolytic maintenance therapy with vaginal progesterone (400 mg daily) after PTL arrest *versus* no treatment. In this study, treatment group had a latency period until delivery 12 days longer than the control group (36 versus 24 days, P=0.04) and a lower incidence of recurrent PTL (35 versus 58%, P=0.09) (Borna & Sahabi, 2008).

The third randomized study published, accomplished by the author of this Thesis, pointed out to the benefit of maintenance tocolytic therapy with vaginal progesterone, after a successful tocolysis for preterm labour. That study, despite the small number of cases, acknowledged that vaginal progesterone could be regarded as a first-line therapy after successful tocolysis for preterm labour (Areia, Fonseca *et al.*, 2013).

In the only published meta-analysis, Dodd *et al.* showed that the relative risk (RR) of PTL was significantly reduced below both 34 weeks of gestation (RR 0.15, 95% CI: 0.04 - 0.64) and 37 weeks (RR 0.65, 95% CI 0.54 - 0.79) (Dodd, Flenady *et al.*, 2008).

II. Clinical guidelines

The use of progesterone is associated with benefits in infant health following administration in women considered to be at increased risk of preterm birth due either to a prior preterm birth or where a short cervix has been identified on ultrasound examination. In the Cochrane database, progesterone *versus* no treatment for women following presentation with threatened preterm labour was associated with a statistically significant reduction in the risk of infant birthweight less than 2500 g (one study; 70 infants; RR 0.52, 95% CI 0.28 to 0.98). However, the authors concluded that further trials were required to assess the optimal timing, mode of administration and dose of administration of progesterone therapy when given to women considered to be at increased risk of early birth (Dodd, Jones *et al.*, 2013).

As for combination tocolytic therapy including progesterone for PTL, there is only one trial published, which compared ritodrine plus vaginal progesterone *versus* ritodrine alone (one trial, 83 women); there were no significant differences between groups for most outcomes reported, although the latency period (time from recruitment to delivery) was increased in the group receiving the combination of tocolytics. As so, the conclusion was that further trials were needed before specific conclusions on the use of combination tocolytic therapy for preterm labour can be driven (Vogel, Nardin *et al.*, 2014).

French authors, in a recent review article, stated that literature data corroborate the value of progesterone administration in PTL prevention in singleton pregnancies in specific situations. Accordingly, its prescription is recommended with duration and

type of administration (vaginal and/or intramuscular) depending on previous PTL, gestational age and cervical length (Fuchs, Audibert *et al.*, 2014).

ACOG committee stated recently that vaginal progesterone should be used for the prevention of preterm birth in women with a short cervix (with or without a history of preterm birth), leaving 17α-hydroxyprogesterone caproate for the prevention of PTL in women with a singleton gestation and a history of previous PTL. Moreover, evidence also demonstrated a reduction in neonatal morbidity/mortality with both interventions (Romero & Stanczyk, 2013).

The Society for Maternal-Fetal Medicine stated that the conclusion of published randomized trials indicates that in women with singleton gestations, no prior PTL, and cervical length ≤ 20 mm at ≤ 24 weeks, vaginal progesterone is related with reduction in PTL and perinatal morbidity and mortality, and can be offered in these cases (Society for Maternal-Fetal Medicine Publications Committee, 2012).

Nonetheless, universal cervical length screening in singleton gestations without prior PTL for the prevention of PTL remains a point of debate (Society for Maternal-Fetal Medicine Publications Committee, 2012).

Regrettably, there are no international recommendations concerning progesterone usage after successful arrest of threatened preterm labour.

7. Gap

- Treg cells have important roles in immune regulation but how Treg cells are generated and expand during pregnancy is still under dispute. As so exact role of Treg cells in pregnancy is still unknown
- Moreover, recent investigation revealed contradicting results concerning Treg cells numbers throughout pregnancy. Hence, we ought to determine the normal variation of Treg cells in the 2nd and 3rd trimesters and on delivery day.
- Progesterone is used in preterm labour but the mechanism by which its actions are accomplished is unknown. We propose that there is a connection between Treg cells and progesterone.
- T lymphocytes appear to respond to progesterone through mPRs, suggesting a role for the mPRs in immune system's regulation and therefore parturition. mPRα is the receptor whose function has been more investigated, and as so, was further included in our investigation in order to determine if progesterone rapid actions on Treg cells in PTL, are obtained through this receptor.

Human studies are inexistent in this field and this research proposes an innovation in the understanding of preterm birth and on its treatment.

8. Hypothesis

Are Progesterone beneficial effects on PTL mediated through Treg cells?

Chapter II - Aims

Primary Endpoint

Determine progesterone effects on regulatory T-cells in women with PTL in peripheral blood

Secondary Endpoints

1) Establish Treg cells variation during normal pregnancy in peripheral blood;

2) Role of mPRα on Treg cells during normal pregnancy;

3) Establish the effects of progesterone treatment on mPRα in PTL pregnancies, in peripheral blood;

4) Cytokine studies in women during normal pregnancy - evaluation in peripheral blood and establish the effects of progesterone in the cytokine levels produced by Treg in PTL;

5) Confirmation of the results by different techniques (*WB* and *PCR*);

6) *In vitro* progesterone studies on Treg cells through mPRα (determine that progesterone effects on Tregs are accomplished through mPRα).

7) Determine the presence and difference of Treg cells, IL-10, TGF-β and mPRα in the maternal-fetal interface (placenta) in both groups.

Chapter III - Materials and Methods

1. Population

We undertook an exploratory study consisting of a cohort of 34 women, divided in two groups: Control group comprising 20 normal pregnant women attending prenatal appointments; and Study group, composed of 14 pregnant women with threatened preterm labour, both recruited at our Obstetrics Unit, from December 2013 to December 2014.

Inclusion criteria for control group comprised: healthy pregnant women attending normal prenatal appointments at our unit without pre-existing diseases; singleton pregnancies and first prenatal appointment before 14th week gestation.

For the study group, inclusion criteria consisted of admission in the Obstetric Unit of Coimbra University Hospital Centre with confirmed preterm labour; singleton pregnancy; gestational age between 24 weeks$^{+\ 0\ days}$ and 33 weeks$^{+\ 6\ days}$; intact amniotic membranes; cervical length ≤ 25 mm and use of atosiban (competitive antagonist of oxytocin receptors) for tocolysis.

Exclusion criteria for normal pregnancy group consisted of multiple gestation, pre-existing diseases, placenta *praevia* and non-compliance with the scheduled prenatal appointments.

Regarding our study group, exclusion criteria consisted of multiple gestation, pre-existing diseases, preterm rupture of membranes, chorioamnionitis, placenta *praevia*, placental abruption, clinical signs of infection (maternal temperature ≥ 37.5°C, white blood cells ≥ 15.000 cells/mm^3 in maternal blood), or usage of hormone therapies, within 3 months before enrolment (with the exception of corticoids for lung maturation).

The presence of risk factors for both groups was defined as one of the following: existence of a previous PTL, tobacco use, low social-economic status, extreme physical activity or/and risk behaviours (drug abuse, several sexual partners).

Gestational age was assessed by ultrasound.

Administration of natural progesterone was done after tocolysis with atosiban; 200 mg of natural progesterone was given vaginally, once daily.

Administration of progesterone, rather than one of its derivatives, has the advantage of ensuring that all the pathways activated by endogenous progesterone synthesis would be activated. Levels of progesterone in the body are tightly regulated, and thus exogenous progesterone is subject to rapid metabolism by both liver and target tissues. As so, progesterone vaginal administration ensures higher bioavailability, fewer secondary effects, rapid absorption and additional local effects on the endometrium. Due to its rapid metabolism, progesterone must be administered frequently, so once daily was preferred (Byrns, 2014).

Peripheral blood samples were obtained on three occasions in both groups.

In the control group they were collected:

- 2nd trimester (14-28 weeks);
- 3rd trimester (> 28 weeks);
- Day of delivery (immediately before labour).

In the study group specimens were taken:

- After tocolysis with atosiban, previously to progesterone first administration;
- 24 hours after treatment with 200 mg daily vaginal natural progesterone;
- Day of delivery (immediately before labour).

The Ethical Committees of Coimbra University Faculty of Medicine (registration number CE-151/2011; issued in 31/01/2012) and Coimbra University Hospital (registration number CHUC-008-12; issued in 02/05/2013) approved the investigation and informed consent was obtained from each participant.

Specimen Collection

Peripheral venous blood samples were obtained and collected in lithium heparin tubes. Samples were kept in a cool environment until they were processed, within 1 hour of collection, whenever possible.

2. Flow cytometry

In brief, 100 µL of whole blood containing 0.5-1×10^6 white blood cells were placed in a appropriate test tube and stained to localize the mPRα receptor on the cell surface, using the N-terminal mPRα antibody as described by Thomas *et al.* (Thomas, Pang *et al.*, 2007). Cells were first incubated in a blocking solution (0.5% bovine serum albumin in phosphate buffered saline solution) for 30-60 minutes (min) and then incubated with the mPRα antibody (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) at room temperature for a further 30-60 min. Cells were washed with phosphate buffered saline 0.5% bovine serum albumin and incubated for 30 min with Cruz Fluor 488 goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), at room temperature in the dark. Cells were washed with phosphate buffered saline 0.5% bovine serum albumin solution, and the surface was stained with PB conjugated anti-CD4, PE-Cy7 conjugated anti-CD25 and PerCP-Cy 5.5 conjugated anti-CD127 (Biolegend, San Diego, CA, USA).

Subsequently, to perform the intracellular staining for Foxp3 detection, we used the staining set (eBioscience, San Diego, CA, USA) and AF647-labeled anti-human Foxp3 (Biolegend, San Diego, CA, USA), following the manufacturer's instructions.

Stained samples were acquired on a FACS Canto II instrument (BD Biosciences, San Jose, USA) equipped with 3 lasers to allow multicolour detection with different fluorophores, using FACS DIVA software (BD Biosciences, San Jose, USA).

Lymphocyte populations were selected according to the scattering signal in forward angle (FSC-A) and side light scatter (SSC-A), and at least 50,000 gated lymphocyte cells were detected for each sample. Dead cells were excluded by forward

and side scatter characteristics and a FSC-A *vs*. FSC-H dot plot was used to discriminate doublets, detecting disparity between cell size *vs*. cell signal.

Isotype control antibodies were used to help assess the level of background staining, as well as samples without staining and single stain, for each fluorochrome.

Treg Analysis and mPRα expression

We defined our regulatory T-cell population as being $CD4^{+}CD25^{high}CD127^{low}Foxp3^{+}$, although the literature varies when considering markers for the exact phenotype of a Treg cell population.

For the isolation of the specific lymphocyte population, $CD4^{+}$ T-cells were first gated and CD25 and CD127 expression was analyzed. Therefore, gating strategies were employed to evaluate the percentage of $CD4^{+}CD25^{high}CD127^{low}$ cells, the percentage of Treg cells, the mean fluorescence intensity (MFI) of Foxp3 in the $CD4^{+}CD25^{high}CD127^{low}Foxp3^{+}$population and the percentage of Treg cells that express mPRα and respective MFI.

Our gating strategy for identifying the Treg population was based on a total lymphocyte gate based on FSC/ SSC dot plot followed by doublet discrimination with an FSC-A *vs*. FSC-H dot-plot. Accordingly, CD4 positive cells were gated over SSC characteristics; depending on CD25 and CD127 expression, $CD4^{+}$ cells were gated based on the expression of $CD25^{high}$ and $CD127^{low}$ markers and, therefore $CD4^{+}CD25^{high}CD127^{low}$ population was detected.

We moved on to the $CD4^{+}CD25^{high}CD127^{low}$ population, and also searched for $Foxp3^{+}$cells. In the regulatory T-cell population, the $mPR\alpha^{+}$ subset was identified and characterized by percentage and MFI.

FlowJo software (Tree Star Data Analysis Software, Ashland, OR, USA) was used for flow cytometry analysis. We also performed absolute count on each population based on lymphocyte number present in complete blood count.

3. Enzyme linked immunosorbent assay (ELISA)

In order to detect and quantify proteins typically secreted or released from cells, *ELISA* technique is frequently used.

Accordingly, plasma was obtained from blood samples collected in heparin tubes after centrifugation and was stored in heparin tubes at - 80°C, in order to measure IL-10 and TGF-β levels, to explore the influence of these suppressor cytokines in our groups of study. Plasma supernatants were collected from each group (normal and preterm pregnancies) and IL-10 and TGF-β concentrations were quantified with cytokine-specific ELISA kits (Human IL-10 ELISA Ready-SET-Go® and Human/Mouse TGF beta 1 Ready-SET-Go®, Biosciences, San Diego, CA, USA), following the manufacturer's instructions. The TGF beta 1 ELISA kit has a minimum detection rate of 8 pg/mL and the IL-10 ELISA kit has a minimum detection rate of 2 pg/mL.

Immobilizing a target-specific capture antibody onto a high protein binding capacity ELISA plate enables capture of the target protein that is then detected by a protein-specific biotinylated antibody. Afterwards, the target protein is quantified using a colorimetric reaction based on activity of avidin-horseradish peroxidase (bound to the biotinylated detection antibody) on a specific substrate. The optical density of the end-product is measured using a spectrophotometer.

In brief, the experimental procedure for both kits consisted in: diluting the aforementioned capture antibodies in a concentration of 1-4 µg/mL and placing 100 µL/well; the plates were left at 4°C overnight. Wells were then aspirated and washed with appropriate buffer and plates were inverted on absorbent paper to remove any residual buffer; 100 µL/well of our standard proteins and plasma samples

were added to the appropriate wells, as well as 100 µL/well of detection antibody; ultimately, 100 µL of tetramethylbenzidine solution was added to each well and incubated at room temperature. Finally, 50 µL of Stop Solution was inserted.

Following manufacturer's instructions and according to the amount of IL-10 or TGF-β bound in the initial step, colour developed proportionately.

Colour intensity was measured using an absorbance spectrophotometer (Bio-Rad Laboratories, CA, USA) within 10 min after adding the Stop Solution, at 450 nm; the lowest and highest standard concentrations used for each cytokine were adjusted according to the standard curve fitting of the standard concentrations, after mathematical interpolation.

All trials were repeated twice.

4. Western Blot

As the blood population being studied (Treg cells and their mPRα^{+} subset) are scarce in peripheral blood, in order to try to obtain a greater number of cells that could accomplish our investigational goals, we used not only our pregnant women samples [peripheral blood mononuclear cells (PBMCs) and sorted Treg Cells], but also buffy coats of women's healthy donors.

The buffy coat is the fraction of an anticoagulated blood sample that contains most of the white blood cells and platelets following density gradient centrifugation of the blood. It is collected in the interface of plasma/red blood cells and constitutes a perfect enriched sample for assays.

For assessment by *Western blot*, blood samples (buffy coats, normal pregnancy group and preterm group) were previously submitted to Ficoll-hypaque density gradient centrifugation in order to obtain PBMCs.

After flow cytometry staining, as described previously, blood samples from normal pregnancy group and preterm group were submitted to FACS-sorting performed on a FACSAria III cell sorter (BD Biosciences, San Jose, USA) in order to collect sorted Treg populations within best conditions, to ensure high purity samples. Part of the samples were lysed with RealTime ready Cell Lysis Kit (Roche Diagnostics, Mannheim, Germany) and frozen at - 80°C until RNA extraction for the *RT-PCR* analysis and the rest of the sample was used for Western blot analysis.

All *Western Blot* assays were performed adapted from protocols formerly described by Ndiaye *et al.* (Ndiaye, Poole *et al.*, 2012), Thomas *et al.* (Thomas, Pang *et al.*, 2007) and Dosiou *et al.* (Dosiou, Hamilton *et al.*, 2008).

To prepare samples for running on a gel, cells were lysed to release the proteins of interest (since this disrupts the cell membrane and solubilizes intracellular proteins so they can migrate individually through the separating gel).

The sorted cells (Treg cells and not-Treg cells) and PBMCs from normal and preterm pregnancies were lysed and extracted with RIPA buffer, supplemented with protease inhibitor cocktail (Roche Diagnostics Mannheim, Germany). The samples were sonicated 3 times for 10-15 seconds, to complete cell lyses and to reduce samples viscosity. Then each cell lysate was boiled at 95°C for 10 min and spined at 13000 rpm in a microcentrifuge for 30 seconds.

Cell lysates (15µL) were separated by electrophoresis on 10% Sodium dodecyl sulfate-polyacrylamide gel and transferred to a membrane (Merck Millipore, Darmstadt, Germany). The blots were blocked with a mixture of Tris-Buffered Saline and Tween 20 containing 2.5% non-fat dry milk and then incubated with primary antibody solution at 4°C overnight, under a constant voltage of 40 Volts. After washing with 0.5% Tris-Buffered Saline and Tween 20 Milk, the membranes were incubated with secondary antibody for 2 hours at 4°C. After another cleanse, the bands were detected with Western chemiluminescent substrate (GE Healthcare, Uppsala, Sweden) for 5 min and VersaDoc imaging system (Bio-Rad Laboratories, California, USA). All the procedures were done at room temperature and roughly about 20µg/protein /lane were loaded.

These membranes were re-incubated other times with different primary and secondary antibodies, using a stripping procedure, to remove previous bands labelled. For this purpose, membranes were placed in distilled water for 5 min, then 5 min in 0.2M sodium hydroxide and finally in water again for another 5 min.

As an internal control for quantity and degradation levels of protein in each sample, immunoblots were normalized by labelling the membranes to β-actin diluted in 0.5% milk in Tris-Buffered Saline and Tween 20 (1:5,000).

Antibodies used for western blotting were rabbit IgG mPRα (1:2,000; Sta Cruz Biotechnology, Dallas, TX, USA), rabbit IgG Foxp3 (1µL/mL, Thermo-scientific, Rockford, IL, USA), Anti-Rabbit IgG Alkaline Phosphatase (1:20000, GE Healthcare, Uppsala, Sweden), rabbit IgG IL-10 (1:5,000; Thermo-scientific, Rockford, IL, USA), β-actin mouse and anti-β-actin mouse (1:5,000 and 1:20,000, respectively; Sigma-Aldrich St. Louis, MO, USA).

All experiments were repeated twice.

5. Real Time Polymerase Chain Reaction (RT-PCR)

For assessment by *RT-PCR*, blood samples from pregnant women were previously submitted to flow cytometry staining and FACS-sorting, as previously described.

RNA extraction, cDNA synthesis & RT-PCR

Total RNA was extracted from PBMCs using a RNA extraction kit (High Pure RNA Isolation Kit; Roche Diagnostics, Mannheim, Germany), according the manufacturer's instructions. Sorted fractions (Treg and not-Treg cells) were isolated using a RealTime ready Cell Lysis Kit (Roche Diagnostics, Mannheim, Germany).

RNA concentration was determined by spectrophotometric analysis (NanoDrop; Thermo Scientific, UK). RNA (200 ng from blood tissue and 500 ng from cell lysates) was reverse-transcribed into cDNA using Transcriptor Universal cDNA Master (Roche Diagnostics, Mannheim, Germany), using oligo (dT) plus random hexamers according to the manufacturer's instructions.

Real time *PCR* used an mPRα specific assay based on short hydrolysis locked nucleic acid substitute probe, together with a RealTime ready DNA Probes Master (Roche Diagnostics Mannheim, Germany), according to the manufacturer's instructions.

The following conditions were used: one step of denaturation at 95°C followed by 45 cycles of amplification at 95°C for 10 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 1 second; this was followed by one step of extra cooling at 40°C

for 30 seconds. A control sample that was not reverse transcribed was used to confirm that the products obtained were not amplified from genomic DNA.

Ikaros family zinc finger 1 and 2 (IKZF1 and IKZF2) were used as Housekeeping genes, as they have been shown to be adequate normalisers and displayed minimal variation between all samples (≤1 control value).

Primers for mPRα (Sense: 5_-CTGGAAGCCGTATATCTACGT-3; Antisense: 5_-TGTAATGCCAGAACTCGGAC-3) were designed from public sequence databases, with an annealing temperature of 58°C (GenBank accession number for mPRα AF313620).

Real-time PCR results were analyzed using the LightCycler®480 2.0 Instruments (Roche Diagnostics Mannheim, Germany).

6. *In vitro* studies

To conduct the planned *in vitro* experiments, we had to test not only the stimulus for our population, but also the ideal progesterone concentration to use.

In order to perform the required stimulus, Dynabeads® (Invitrogen, Carlsbad, CA, USA) were used, in eight normal pregnancy samples. Dynabeads®, Human T-Activator CD3/CD28, are uniform 4.5 µm, para-magnetic polymer beads coated with an optimized mixture of monoclonal antibodies against the CD3 and CD28 cell surface molecules of human T-cells. The CD3 antibody is specific for the epsilon chain of human CD3, which is considered to be a subunit of the T-cell receptor complex. The CD28 antibody is specific for the human CD28 co-stimulatory molecule (which is the receptor for CD80 (B7-1) and CD86 (B7-2) molecules that are present in antigen presenting cells). Both antibodies are mouse anti-human IgG coupled to the same bead, mimicking *in vivo* stimulation by antigen presenting cells. Both the bead size and the covalent antibody coupling technology are critical parameters to allow the simultaneous presentation of optimal stimulatory signals to the T-cells in culture, thus allowing their full activation and expansion ("Dynabeads® Human T-Activator CD3/CD28,") (www.lifetechnologies.com/cellisolation).

In short, 1×10^6 PBMCs were placed in 100–200 µL medium in a 96-well tissue culture plate and 25 µL of Dynabeads® were added to obtain a bead-to-cell ratio of 1:1 and 30 U/ml of recombinant IL-2 were supplied. The samples were then incubated during 24 hours at 37°C, 5% CO_2 and 95% humidity conditions. After the incubation period, tubes were placed on a magnet for 1–2 min to separate the beads from the solution and the supernatant containing the cells was transferred to a new tube. After

this step, flow cytometry analysis of cell's suspensions and *ELISA* measurements of culture supernatants were done, as previously described.

As for progesterone concentration, the chosen concentrations of 0.06, 0.6 and 6 μM were the most often seen in the literature and eight normal pregnancy samples were used. Progesterone (4-Pregnene-3,20-dione), powder bioReagent suitable for cell culture was used, according to the manufacturer's instructions (P8783; Sigma-Aldrich, St Louis, MO, USA). PBMCs with the appropriate amount of progesterone for those conditions were then incubated during 24 hours at 37°C, 5% CO_2 and 95% humidity. After the incubation period, flow cytometry analysis of the different blood populations and *ELISA* measurements of culture supernatants were performed using the methodologies formerly depicted.

After assuring the affordable best conditions, we moved on to the intended functionality assays.

As so, in vitro studies were carried out with peripheral blood samples from preterm birth group before progesterone treatment.

For that, PBMCs were isolated and stimulated in vitro with the appropriate medium, with CD3/CD28 and IL2 during 24h, 37°C and 5% CO_2. In these assays multiple conditions were tested in multiwell microplates in order to verify the mechanisms behind Progesterone actions on Treg cells (progesterone linking to mPRα). Those different circumstances were: progesterone in a 0.6 μM concentration; progesterone antagonist in a 0.5 μM concentration; progesterone agonist in a 0.5 μM concentration and a mixture of those.

Moreover, past 24hours, flow cytometry evaluation of the different blood populations and of IL-10 and TGF-β levels in culture supernatants was performed, in those tested conditions (Progesterone/Antagonist/Agonist/Combination), as described previously in Flow cytometry and *ELISA* sections.

The progesterone used was specific for cell culture, within the concentration which achieved better results in our prior essays.

As a progesterone antagonist we used Mifepristone (RU-486), which is a synthetic, steroidal anti-progestogen. The anti-progestational activity of mifepristone results from competitive interaction with progesterone at progesterone-receptor sites. Mifepristone produces mixed agonist/antagonist effects on immune cells compared with progesterone but is antagonistic to the rapid membrane progesterone receptor-mediated non-genomic responses (Chien, Lai *et al.*, 2009).

Nandrolone (19-Nortestosterone) is a synthetic androgen which demonstrated intrinsic progestational effects in vitro (Beri, Kumar *et al.*, 1998). Moreover, its binding to progesterone receptors leads to progestational activity similar to progesterone in rabbits (Beri, Kumar *et al.*, 1998). Owing to these proprieties and its availability in our Laboratory, we assumed its usage as a progesterone agonist. This has to do with the economical burden associated with the only specific agonist for mPRα studied so far, which we could not afford (Org OD 02; NV Organon, Oss; The Netherlands) (Ndiaye, Poole *et al.*, 2012; Zachariades, Mparmpakas *et al.*, 2012).

7. Maternal-fetal Interface - Placenta

The maternal-fetal interface is the place where it is assumed that all pregnancy alterations will be highlighted in terms of cytokines and Treg cells. So, it is assumed that the assumptions made in peripheral blood will be even more relevant in the placenta. For these purposes immunohistochemical methodology was used.

Placental tissues were obtained from each patient included in the study on delivery day (term and preterm), treated and evaluated afterwards.

Sections of each formalin-fixed paraffin-embedded placenta were analyzed for the presence of Treg cells, IL-10, TGF-β and mPRα by this technique.

Placental tissues were obtained from the maternal side of the placenta; tissue samples were taken from the centre of the cotyledons, evenly across the placenta, with size of approximately 0.2 and 0.5 cm^3. The tissues were dissected to remove any visible connective tissue and calcium deposits.

The following protocol was basically applied to all antibodies: paraffin sections (3 μm thick) were placed on coated slides and allowed to dry overnight for 37°C and after deparaffinization and rehydration, antigen unmasking was performed.

Endogenous peroxidase activity was quenched using incubation in 3% diluted hydrogen peroxide. For blocking nonspecific binding of primary antibody, Ultra V Block (Ultra Vision Kit; TP-015-HL; LabVision) was applied to the sections and then the sections were incubated at room temperature with the primary antibodies referred in flow cytometry methodology.

After washing with phosphate-buffered saline, slides were incubated with

biotin-labelled secondary antibody. Primary antibody binding was localized in tissues using peroxidise conjugated streptavidin; 3,3-diaminobenzidine tetrahydrochloride was used as the chromogen, according to manufacturers' instructions. The slides were counterstained with haematoxylin, dehydrated and mounted; in parallel, known positive and negative controls were used. Immunohistochemical staining antibody was scored according to Hirsch's intensity and percentage of positive cells. When possible, the Broder's adapted grading was also used, that functions to evaluate the expression based on the percentage of cells with specific biding to the chosen antibodies.

The results of this technique were still being processed at the time this Thesis was written.

8. Bibliographic search

A systematic literature review of several databases including PubMed, Cochrane Controlled Trials Register, EMBASE, Scopus and ISI Web of Knowledge was undertaken up to February 2015, using the MeSH keywords "(Regulatory T-Cells OR Treg cells OR cytokines OR TGF-β OR IL-10 OR progesterone OR mPRα) AND (Preterm Birth OR obstetrics OR labour)" as search terms. The only limit in all searches was for studies in other languages than Portuguese, English or French. There were no limits on type of study, year of publication or human versus animal.

In addition to database searches, the author also used references in the retrieved articles to identify additional articles of interest, if applicable.

9. Statistical analysis

Data were analyzed by IBM® SPSS statistics 21 software (IBM® Corporation, New York, USA) and quantitative results following a normal distribution were expressed as mean ± standard deviation (SD), while median and interquartile range (IQR) values were used for skewed distributions.

For the comparative analysis among groups, Student t-tests were used for unpaired and paired comparisons among normal distributions, while Anova One-way analysis of variance was performed for comparison between 3 groups, with Bonferroni correction.

For qualitative results, proportions were used and comparisons were done using the Qui-square test or Fisher exact test when appropriate, while for multiple groups' comparisons, nonparametric Mann-Whitney U tests were performed.

Statistical significance was considered for a two-sided p value < 0.05.

All missing values were excluded from the analysis.

Chapter IV - Results

1. Flow Cytometry

In the control group (n= 20), with a mean age of 28.8 ± 5.3 years, a total of 60 peripheral venous blood samples were collected. The samples were equally retrieved in the 2nd trimester (mean gestational age of 20.6 ± 1.5 weeks), 3rd trimester (mean gestational age of 31.8 ± 2.2 weeks) and delivery day (mean gestational age of 39.2 ± 1.97 weeks). The majority of women were nulliparas (70%) and only 15% (3/20) presented risk factors. These clinical data are shown in Table 1.

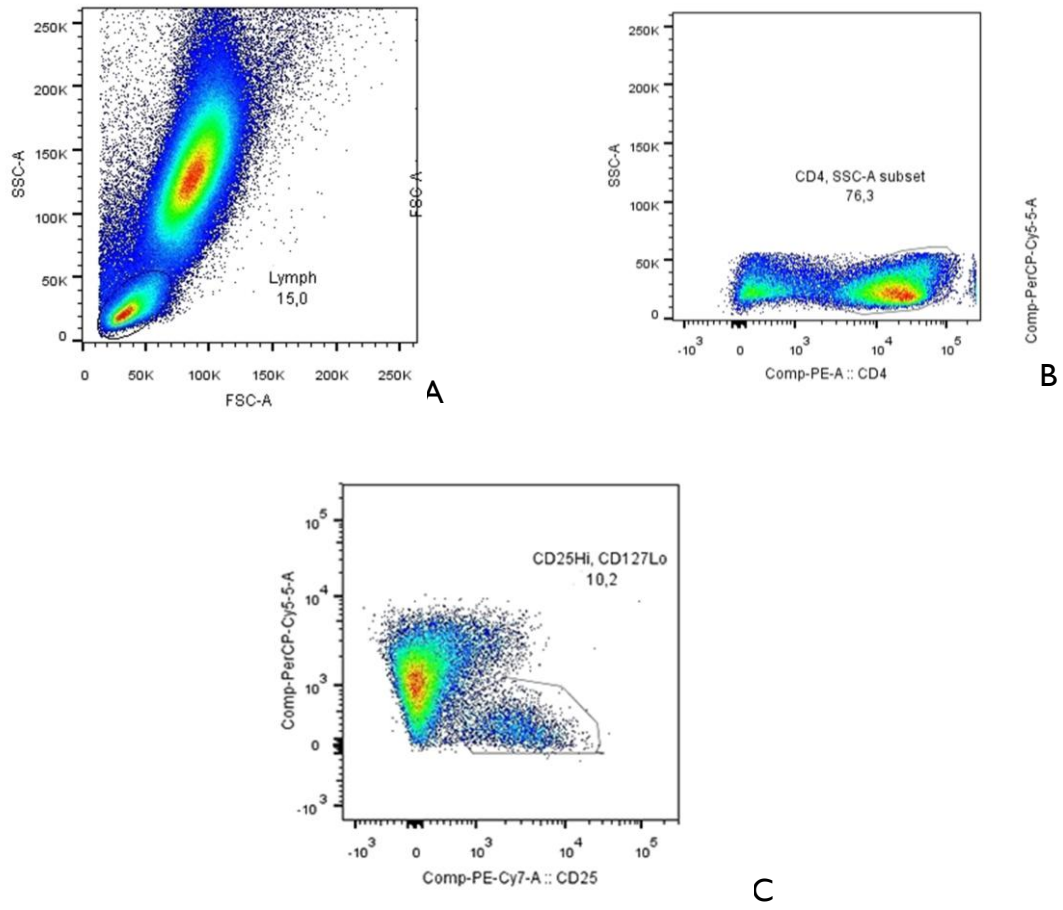
Table 1. Clinical data from Normal Pregnancy group

Variable	Value
Age (Years)	
Mean ± standard deviation	28.8 ± 5.3
(min-max)	(20-37)
Gestational age (weeks)	
Mean ± standard deviation	
• 2nd Trimester	20.6 ± 1.5
• 3rd Trimester	31.8 ± 2.2
• Delivery	39.2 ± 1.97
Baby Birthweight (grams)	
Mean ± standard deviation	3253 ± 456.7
(min-max)	(2020-4000)
Nullipara (n, proportion)	14 (70 %)
Risk factors* (n, proportion)	3 (15 %)

Legend: * Risk factors were considered as one of the following: existence of a previous PTL, tobacco use, low social-economic status, extreme physical activity or/and risk behaviours.

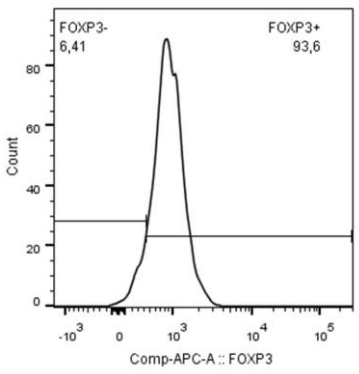
Figure 12 shows our flow cytometry gating strategy for the $CD4^{+}CD25^{high}CD127^{low}Foxp3^{+}$ population (regulatory T-cell population) in peripheral blood.

Figure 12. Flow cytometry gating strategy for $CD4^{+}CD25^{high}CD127^{low}Foxp3^{+}$Tregs analysis in peripheral blood: dot plots

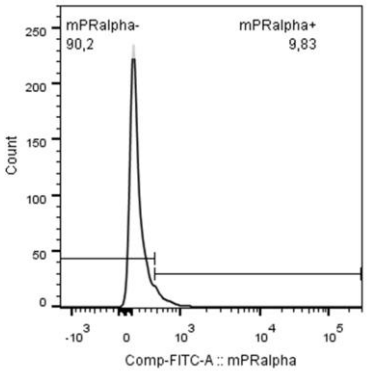


Legend: Figure A: FSC *vs.* SSC: Lymphocyte gate; Figure B: SSC-A *vs.* PE CD4: $CD4^{+}$ population; Figure C: PerCP-Cy5.5 CD127 *vs.* PE-Cy7: $CD4^{+}CD25^{high}CD127^{low}$ population.

Figure 13. Flow cytometry gating strategy for $CD4^{+}CD25^{high}CD127^{low}Foxp3^{+}$Tregs analysis in peripheral blood: histograms



D



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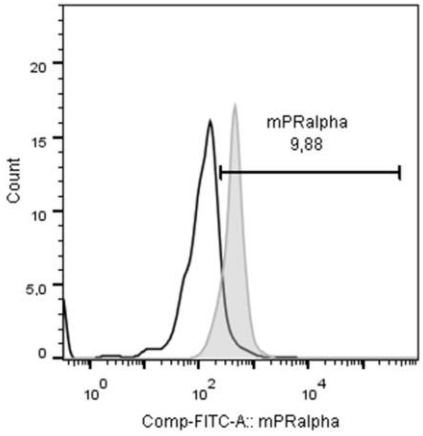


F

Legend: Figure D: APC Foxp3: Foxp3 expression in $CD4^{+}CD25^{high}CD127^{low}$ population; Figure E: FITC anti-mPRα: percentage of $mPR\alpha^{+}$ subset within the total $CD4^{+}CD25^{high}CD127^{low}Foxp3^{+}$Treg cell pool; Figure F: Isotype control for mPRα.

Table 2 shows the absolute number and percentage of the different populations studied, in the normal course of pregnancy. To ascertain the existing variation of those different blood populations throughout pregnancy, multiple comparisons were made using ANOVA with Bonferroni correction.

Table 2. Analysis of different blood T-cells populations among Normal Pregnancy group

	2nd Trimester	3rd Trimester	Delivery	*p*
$CD4^+$				
• percentage	44.8 ± 9.7*	32.7 ± 18.3*	44.1 ± 16.0	**0.04***
• absolute number	940 ± 624	643 ± 545	1009 ± 815	**ns**
$CD4^+CD25^{high}CD127^{low}$				
• percentage	4.8 ± 4.8*	6.8 ± 2.8	9.7 ± 3.4*	**0.001***
• absolute number	51.3 ± 59.6.2	45.4 ± 43.8	89 ± 75.1	**ns**
Tregs				
• percentage	31.2 ± 24.3*	56.8 ± 28.6*#	25.7 ± 25.1#	**0.01*** **0.002**#
• absolute number	23.2 ± 53.7.1	21.2 ± 22	19 ± 17.4	**ns**
MFI $Foxp3^+$	779 ± 116	991 ± 331	766 ± 262	**ns**
Tregs $mPR\alpha^+$				
• percentage	7.9 ± 10.1*	36.3 ± 41.1*	19.6 ± 32.3	**0.02***
• absolute number	2 ± 5.4	2.7 ± 3.76	2.3 ± 4	**ns**
MFI $mPR\alpha^+$	1342 ± 2230	3867 ± 6651	3680 ± 4968	**ns**

Legend: All data are presented as mean and standard deviation. Absolute Number: number cells/µl blood. Tregs: $CD4^+CD25^{high}CD127^{low}$ $Foxp3^+$; MFI: Mean fluorescence intensity; ns: non significant; *#: differences between the marked groups; * Significant mean difference between two groups at the 0.05 level using ANOVA (multiple comparisons using Bonferroni correction).

As the results show, the percentage and absolute number of $CD4^+$ in the total lymphocytes were higher on the second trimester and on delivery day, with a statistical significant decrease in the 3rd trimester in comparison with the 2nd trimester (32.7 *vs.* 44.8; p=0.04).

On the contrary, the percentage of $CD4^{+}CD25^{high}CD127^{low}$ cells showed a steady increase throughout pregnancy, reaching their upper value on delivery day, with a significant statistical increase in relation to the 2nd trimester (4.8 *vs.* 9.7; p=0.001).

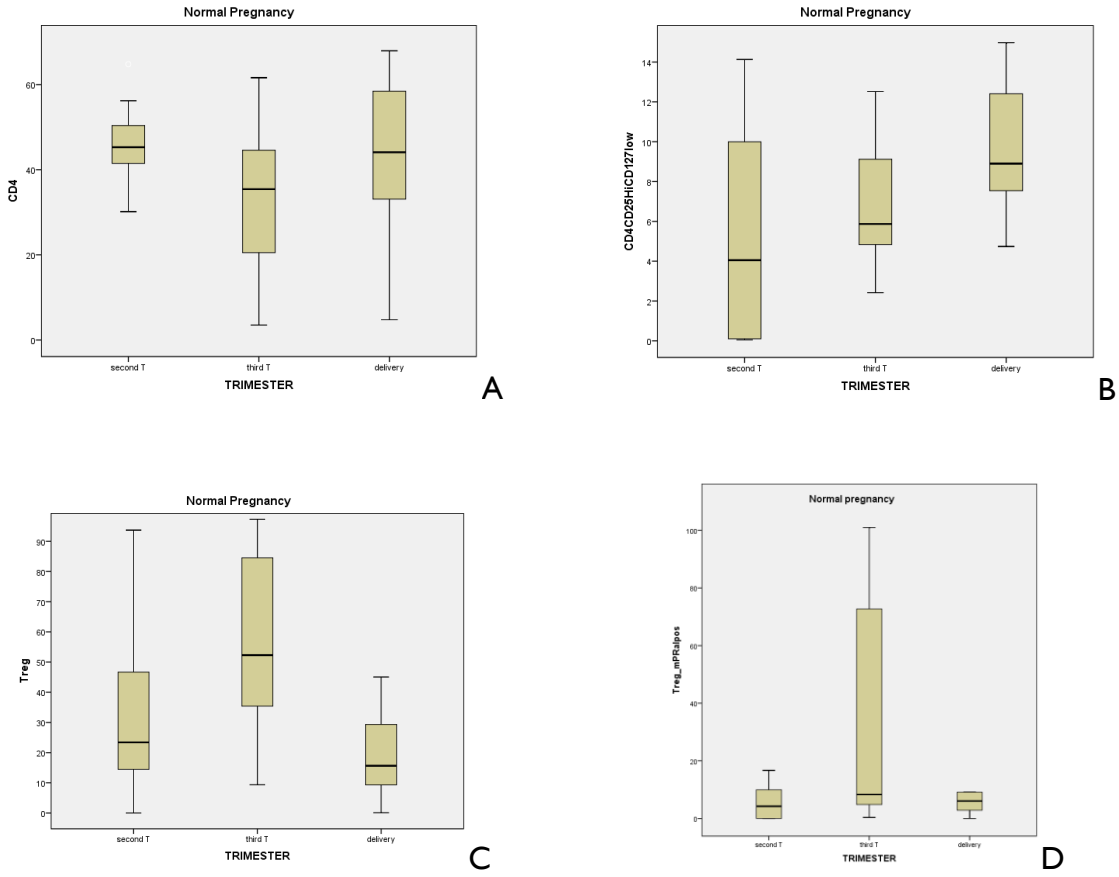
As for the regulatory T-cell pool, we could verify the utmost levels of their percentage in the 3rd trimester (56.8 ± 28.6), with statistical significant lower values both in the 2nd trimester (31.2 *vs.* 56.8; p=0.001), as on delivery day (25.7 *vs.* 56.8; p=0.002).

Moreover, we were able to validate the expression of mPRα in those three specific times of pregnancy, with a peak of 36.3 ± 41.1 % of those Treg cells positive for this marker in the third trimester, revealing a significant statistical increase in relation to the 2nd trimester (36.3 *vs.* 7.9; p=0.02).

In short, we could verify the following statistical significant differences:

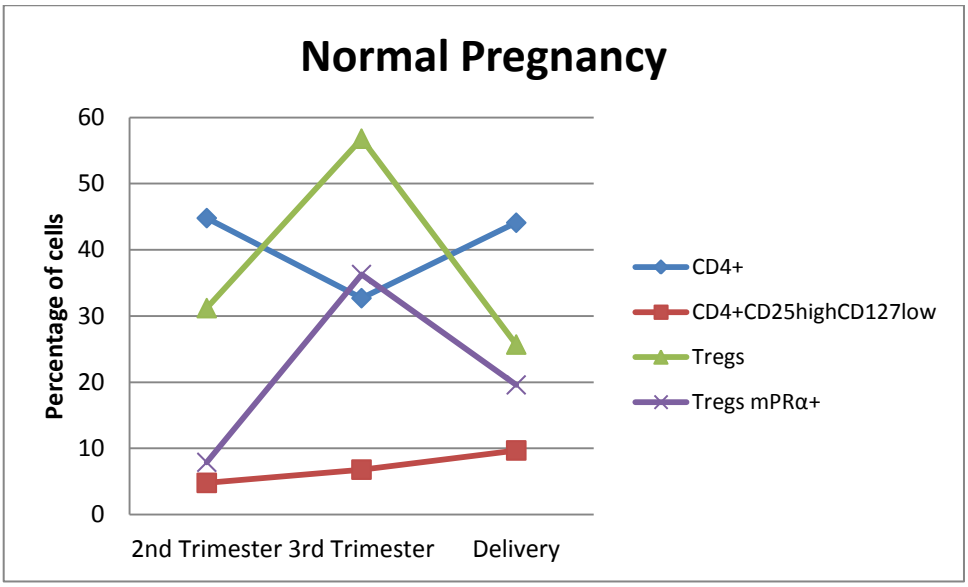
- $CD4^{+}$percentage higher in the second than the third trimester (p=0.04);
- $CD4^{+}CD25^{high}CD127^{low}$ percentage lower in the second trimester than on delivery day (p=0.001);
- Treg cells percentage both between the second and third trimesters (higher in the third trimester; p=0.01) and between the third trimester and delivery day (higher in the third trimester; p=0.002);
- $mPR\alpha^{+}$ Treg cells percentage higher in the third than the second trimester (p=0.02).

Figure 14. Box-plots representative of the percentage variation of the different blood populations studied in Normal Pregnancy



Legend: Figure A: $CD4^+$ percentage; Figure B: $CD4^+CD25^{high}CD127^{low}$ percentage; Figure C: Treg cells percentage; Figure D: $mPR\alpha^+$ Treg cells percentage.

Figure 15. Evolution of the different blood populations studied in Normal Pregnancy



In the Study Group, a total of 14 women presenting with PTL were enrolled, with peripheral venous blood samples obtained before progesterone treatment, after progesterone treatment and on delivery day (within a total of 42 peripheral venous blood samples).

There were three out of 42 missing data from blood samples in our research, due to sample inadequacy.

Clinical information of preterm group is presented in Table 3.

Table 3. Clinical Data from Preterm Pregnancy group

Variable	Value
Age (Years)	
Mean ± standard deviation	29.9 ± 6.3
(min-max)	(16-37)
Gestational age admission (weeks)	
Mean ± standard deviation	30.3 ± 2.5
(min-max)	(25-34)
Cervical length (millimeters)	
Mean ± standard deviation	17.4 ± 4.8
(min-max)	(11-25)
Gestational age delivery (weeks)	
Mean ± standard deviation	36.3 ± 3.0
(min-max)	(30-40)
Baby Birthweight (grams)	
Mean ± standard deviation	2510.7 ± 550.8
(min-max)	(1490-3180)
Nullipara (n, proportion)	7 (50 %)
Presence of risk factors* (n, proportion)	9 (64 %)

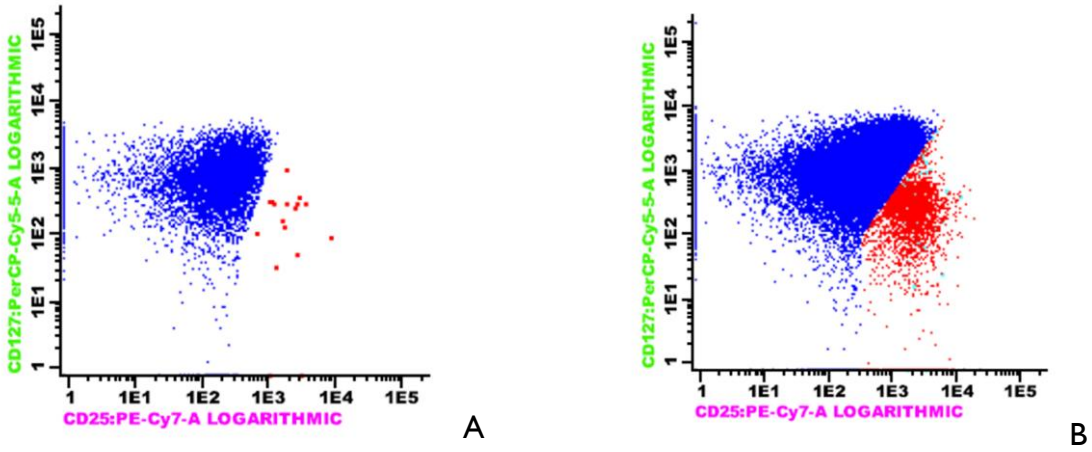
Legend: * Risk factors were considered as one of the following: existence of a previous PTL, tobacco use, low social-economic status, extreme physical activity or/and risk behaviours.

Their mean age was 29.9 ± 6.3 years, with a mean gestational age at admission of 30.3 ± 2.5 weeks and a mean cervical length at admission of 17.4 ± 4.8mm. These data reflect the strict inclusion criteria used in the study.

Risk factors for PTL were present in 64% of those women.

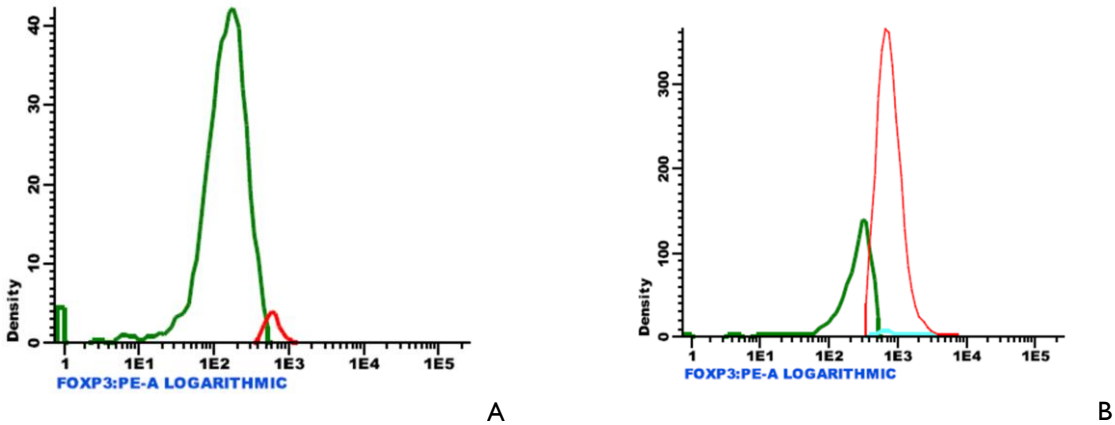
Figure 16 shows an example of the $CD4^{+}CD25^{high}CD127^{low}Foxp3^{+}$ population (regulatory T-cell population) in peripheral blood in preterm pregnancy, before and after progesterone treatment.

Figure 16. Regulatory T-cell population ($CD4^{+}CD25^{high}CD127^{low}Foxp3^{+}$) in peripheral blood in Preterm Pregnancy group: Dot-plots



Legend: Dot-plots representing the different lymphocyte population, with regulatory T-cell marked in red. A: before progesterone treatment; B: after progesterone treatment.

Figure 17. Regulatory T-cell population ($CD4^{+}CD25^{high}CD127^{low}Foxp3^{+}$) in peripheral blood in Preterm Pregnancy group: histograms



Legend: Histograms representing the regulatory T-cell population marked in red and the $CD4^{+}CD25^{high}CD127^{low}Foxp3^{-}$ population in green. A: before progesterone treatment; B: after progesterone treatment.

Table 4 shows the comparison between T-cell populations in blood before and after progesterone treatment, and all results represent the paired samples for each patient.

Table 4. Comparison between T-cell populations in blood according to progesterone treatment in Preterm Pregnancy group

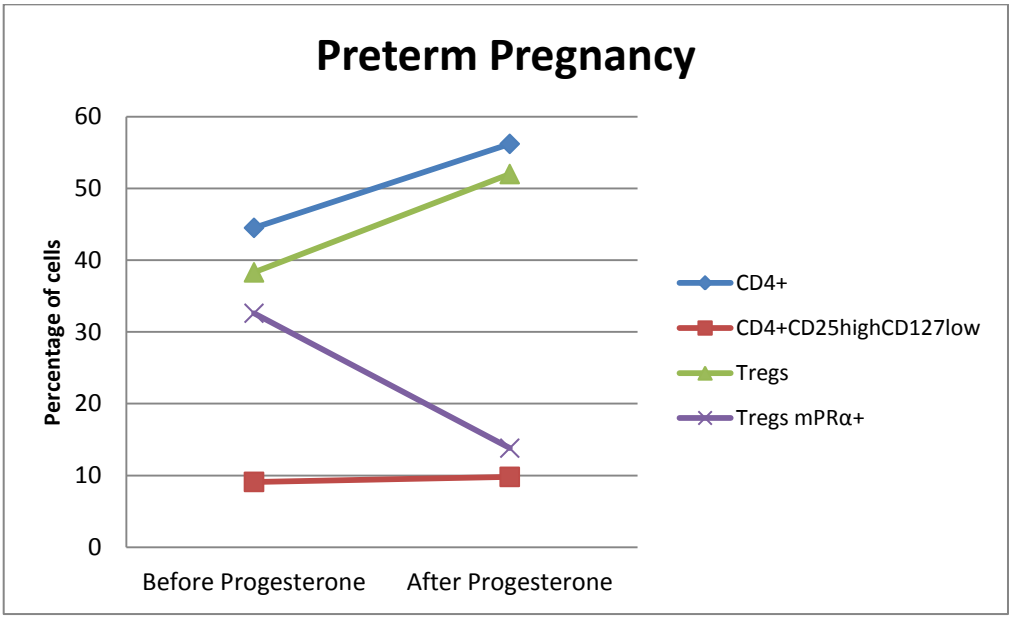
	Progesterone treatment			
Lymphocyte population	**Before**	**After**	***Mean difference***	***p****
$CD4^+$				
Percentage	44.5 ± 16.6	56.2 ± 14.9	*11.7 ± 16.3*	**0.023**
Absolute Number [a]	1512 ±1978	1748 ± 1744	*236 ± 626*	0.2
$CD4^+CD25^{high}CD127^{low}$				
Percentage	9.1 ± 4.2	9.8 ± 3.2	*0.7 ± 5.7*	0.67
Absolute Number [a]	170 ± 270	172 ± 208	*2.1 ± 136*	0.96
Tregs [b]				
Percentage	38.3 ± 29.1	52 ± 26.3	*13.7 ± 25*	0.07
Absolute Number [a]	51 ± 85.8	79.9 ± 68.9	*28.9 ± 85.7*	0.25
MFI $Foxp3^+$	959 ± 579	1075 ± 1062	*1116 ± 602*	0.5
Tregs $mPR\alpha^+$ [b]				
Percentage	32.6 ± 40.2	13.8 ± 25.2	*18.8 ± 34.8*	0.07
Absolute Number [a]	7.5 ± 9.9	6.8 ± 19.7	*0.7 ± 12.5*	0.8
MFI $mPR\alpha^+$	5911 ± 7734	3849 ± 4168	*2062 ± 6012*	0.24

Legend: *Paired sample Student t test. All data are presented as mean ± standard deviation (SD), with a significance level of 0.05; [a] number cells/µl blood; [b] Tregs: $CD4^+CD25^{high}CD127^{low}$ $Foxp3^+$; MFI: Mean fluorescence intensity.

As is shown, there was a statistical significant increase after progesterone treatment in the percentage of $CD4^+$ T-cells, with a mean difference in their proportion of 11.7 ± 16.3 (p= 0.023). An enhancement after progesterone administration could also be verified in both the percentage and absolute number of $CD4^+CD25^{high}CD127^{low}$ population and of Treg cell population (those cells expressing the intracellular marker Foxp3), although not reaching statistical significance.

Opposite results could be perceived in the percentage of $mPR\alpha^+$ Treg cells, were there was a decrease after progesterone treatment, even though without statistical significance (mean difference in their proportion of 18.8 ± 34.8; p= 0.07).

Figure 18. Evolution of T-cell populations in blood according to progesterone treatment in Preterm Pregnancy group



Additionally, to ascertain whether these different T-cell populations in blood differed on delivery day between normal and preterm pregnancy groups, a subsequent subgroup analysis was made (Tables 5 and 6).

Table 5. Delivery day: Comparison of clinical data between Normal and Preterm groups

Variable	**Value**	
	Normal	**Preterm**
Age (Years)		
Mean ± standard deviation	28.8 ± 5.3	29.9 ± 6.3
(min-max)	(20-37)	(16-37)
Gestational age (weeks)		
Mean ± standard deviation	39.2 ± 1.97	36.3 ± 3.0
	(37-41)	(30-40)
Baby Birthweight (grams)		
Mean ± standard deviation	3253 ± 456.7	2510.7 ± 550.8
(min-max)	(2020-4000)	(1490-3180)
Nullipara (n, proportion)	14 (70 %)	7 (50 %)
Risk factors* (n, proportion)	3 (15 %)	9 (64 %)

Legend: * Risk factors were considered as one of the following: existence of a previous PTL, tobacco use, low social-economic status, extreme physical activity or/and risk behaviours.

Table 6. Delivery day: Comparison of different blood populations between normal and preterm pregnancies

Blood Populations	Groups		
	Normal	**Preterm**	**p**
$CD4^+$			
• percentage	44.1 ± 16	55.5 ± 8	0.09
• absolute number	1009 ± 815	2977 ± 5333	0.4
$CD4^+CD25^{high}CD127^{low}$			
• percentage	9.7 ± 3.4	9.8 ± 3.7	0.9
• absolute number	89 ± 75	387 ± 810	0.4
Tregs			
• percentage	25.7 ± 25.1	52 ± 23.3	**0.03**
• absolute number	19 ± 17.4	108 ± 192	0.3
MFI $Foxp3^+$	766 ± 262	933 ± 228	0.2
Tregs $mPR\alpha^+$			
• percentage	19.6 ± 32.3	12.2 ± 19.9	0.6
• absolute number	2.3 ± 3.6	11.2 ± 16.2	0.2
MFI mPRα+	3680 ± 4968	4482 ± 5382	0.7

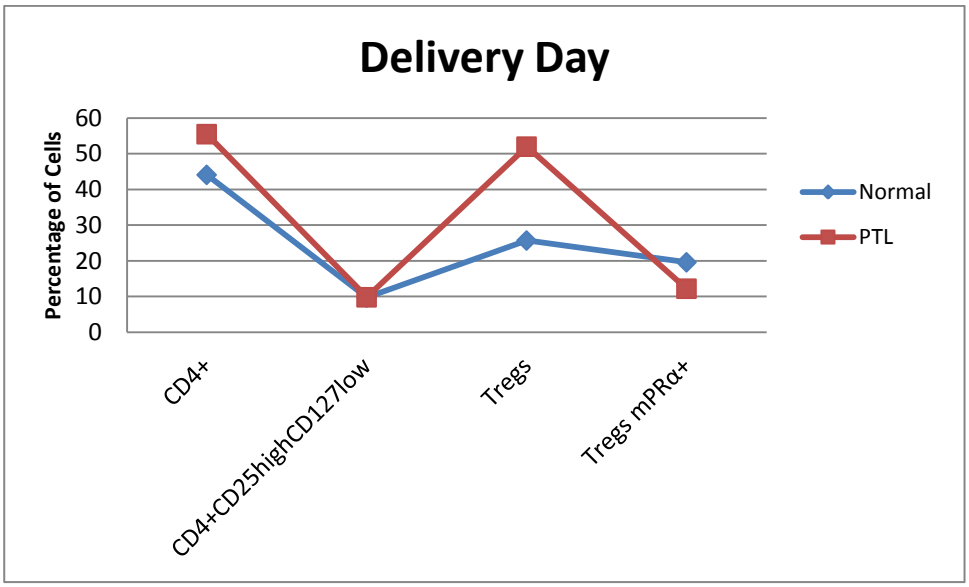
Legend: All data are presented as mean and standard deviation. Absolute Number: number cells/µl blood. Tregs: $CD4^+CD25^{high}CD127^{low}$ $Foxp3^+$; * Significant mean difference between two groups at the 0.05 level using independent samples T-test.

In control group, delivery day occurred at a mean gestational age of 39.2 ± 1.97 weeks, whereas in the preterm birth group it took place at a mean gestational age of 36.3 ± 3 weeks, giving rise to newborn babies with 3253 ± 457 and 2511 ± 551 grams, respectively.

The analysis revealed in the preterm group higher percentages and absolute numbers of the blood populations being studied, with a statistical significant increase in the Treg cell pool percentage (25.7 *vs.* 52; p= 0.03).

On the contrary, among mPRα+ Treg cells of the preterm group discrepant results were perceived, with a decline in percentage and a rise in their absolute number.

Figure 19. Delivery day: Different blood populations in normal and preterm pregnancies

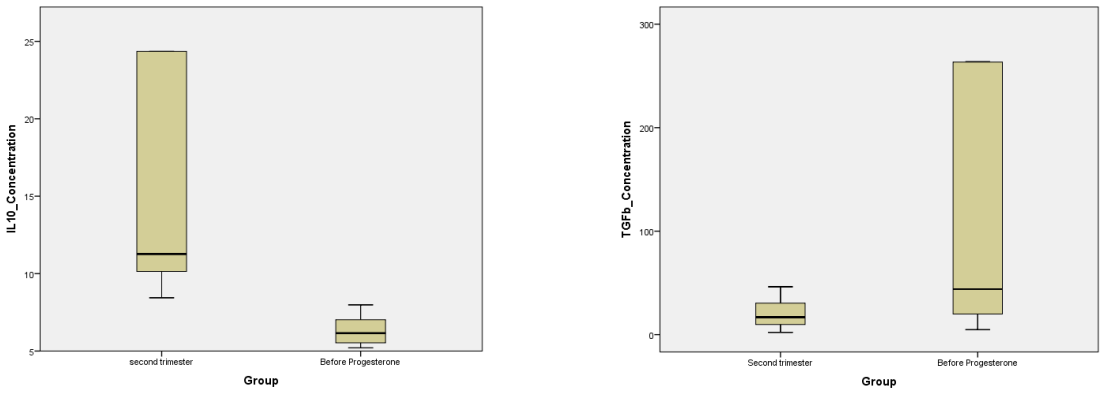


2. *ELISA* Assays: Cytokine Results

To establish if plasma cytokine levels were influenced by the existence of a threatened preterm delivery or by progesterone treatment, peripheral blood samples already taken from the 2 groups previously described (normal pregnancy and preterm pregnancy) were evaluated for IL-10 and TGF-β concentrations.

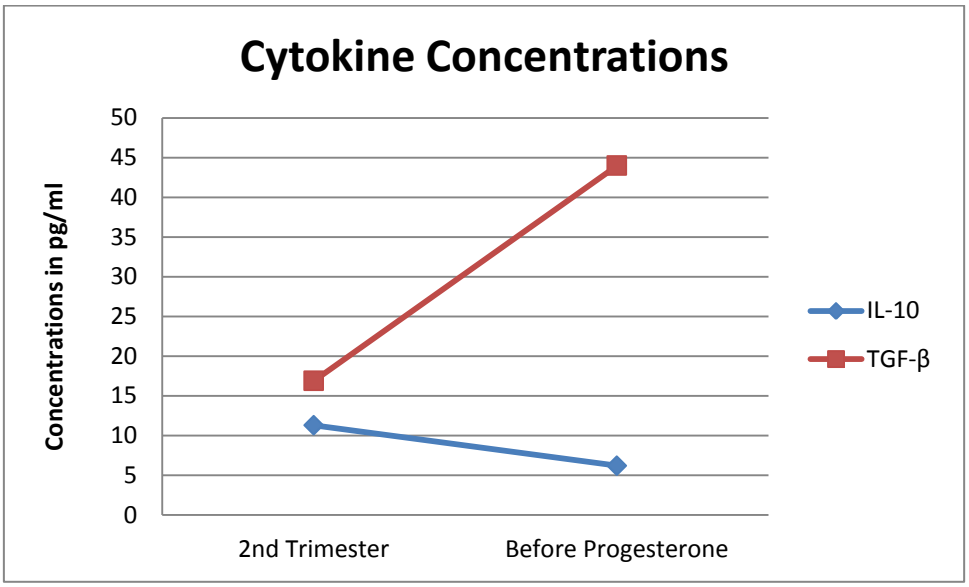
Initially, we investigated if the presence of threatened preterm delivery altered those cytokine concentrations in comparison to normal second trimester pregnancy (Figures 20 and 21).

Figure 20. Comparison of plasma cytokine concentrations between normal second trimester pregnancy and preterm groups



Legend: Concentrations in pg/ml.

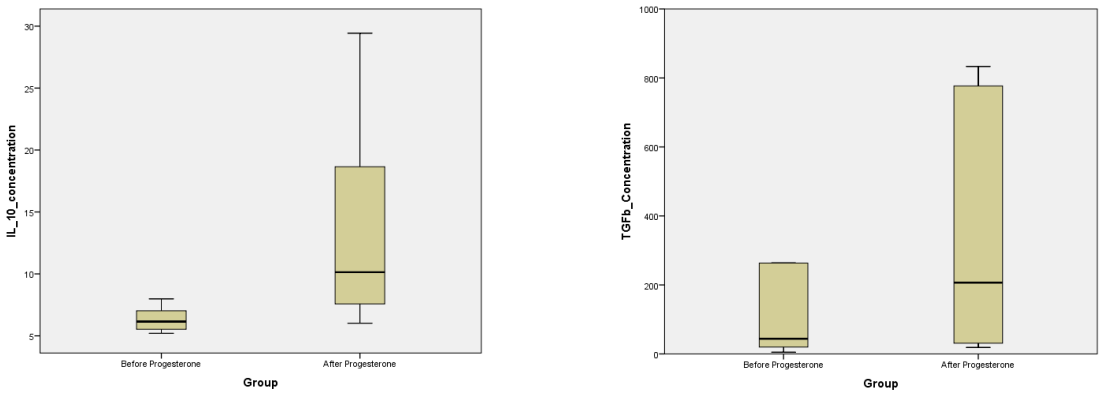
Figure 21. Evolution of plasma cytokine concentrations between normal second trimester pregnancy and preterm groups



Results revealed that IL-10 concentration was significantly lower in the preterm group (6.2 *vs.* 11.3 pg/ml; p=0.01); on the contrary, TGF-β concentration was significantly higher in that group (44 *vs.* 16.9 pg/ml; p=0.01).

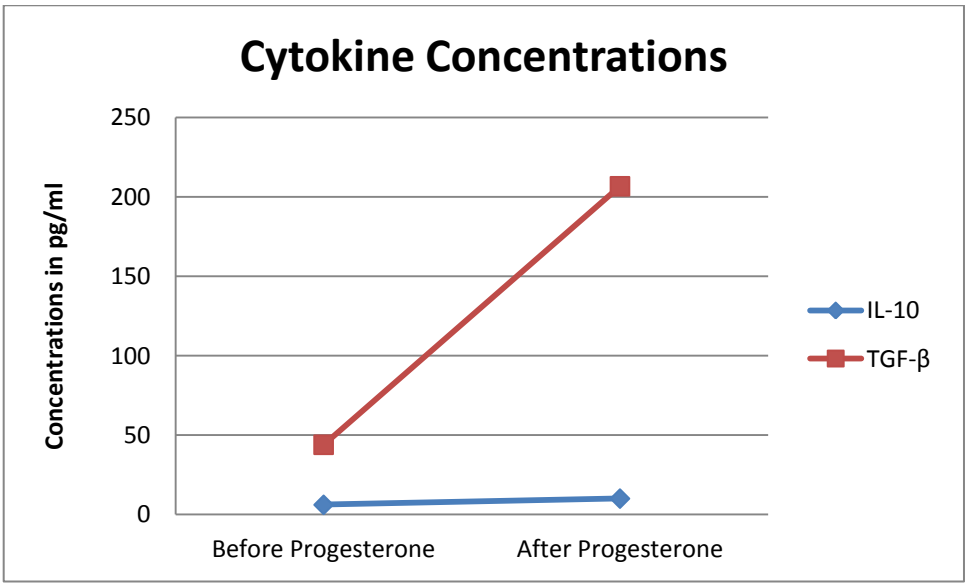
Subsequently, comparisons were undertaken involving progesterone treatment in the preterm group.

Figure 22. Variation of plasma cytokine concentrations with Progesterone administration in preterm group



Legend: Concentrations in pg/ml.

Figure 23. Evolution of plasma cytokine concentrations with Progesterone administration in preterm group



In these circumstances, there was a statistical significant increase in IL-10 concentration after progesterone treatment, from 6.2 to 10.1 pg/ml, p= 0.001. Furthermore, TGF-β concentration augmented after progesterone treatment (44 *vs.* 206.7 pg/ml), albeit no statistical significance.

All results of plasma cytokine levels are summarized in table 7.

Table 7. Plasma Cytokine Concentrations

	Normal pregnancy	**Preterm Pregnancy**			
	Second Trimester	***Before Progesterone***	***After Progesterone***	***p****	***p*§***
IL-10					
• Median	11.3*	6.2*§	10.1§	<0.001	0.01
• IQR	36.8	1.6	16.2		
TGF-β					
• Median	16.9*	44*§	206.7§	0.016	0.17
• IQR	23.4	244	771.3		

Legend: Concentrations in pg/ml. All data are presented as median and Interquartile range (IQR).

*§ Non-parametric tests (Mann-Whitney U-Test) between marked groups.

3. Western Blot

In order to confirm the previous discoveries, different samples were tested for the existence of the Foxp3, mPRα and IL-10 proteins in different groups: Buffy-coats of healthy donors; PBMC; sorted Treg cells and not-Treg cells fractions (normal and preterm pregnancies).

All experiments were repeated twice and negative results were repeated after a stripping technique. A summa of all experiments is reproduced in Table 8.

Table 8. Western Blot results of different samples tested

	Buffy - Coats	**PBMC**		**Treg cells**		**Not-Treg Cells**	
Pregnancy		***Normal***	***Preterm***	***Normal***	***Preterm***	***Normal***	***Preterm***
Foxp3	+	-	-	+	-	+	-
β-actin	+	+	+	-	-	+	+
mPRα	+	-	-	-	-	-	-
β-actin	+	na	na	na	na	na	na
IL-10	+	-	-	-	-	-	-
β-actin	+	-	-	-	-	-	-

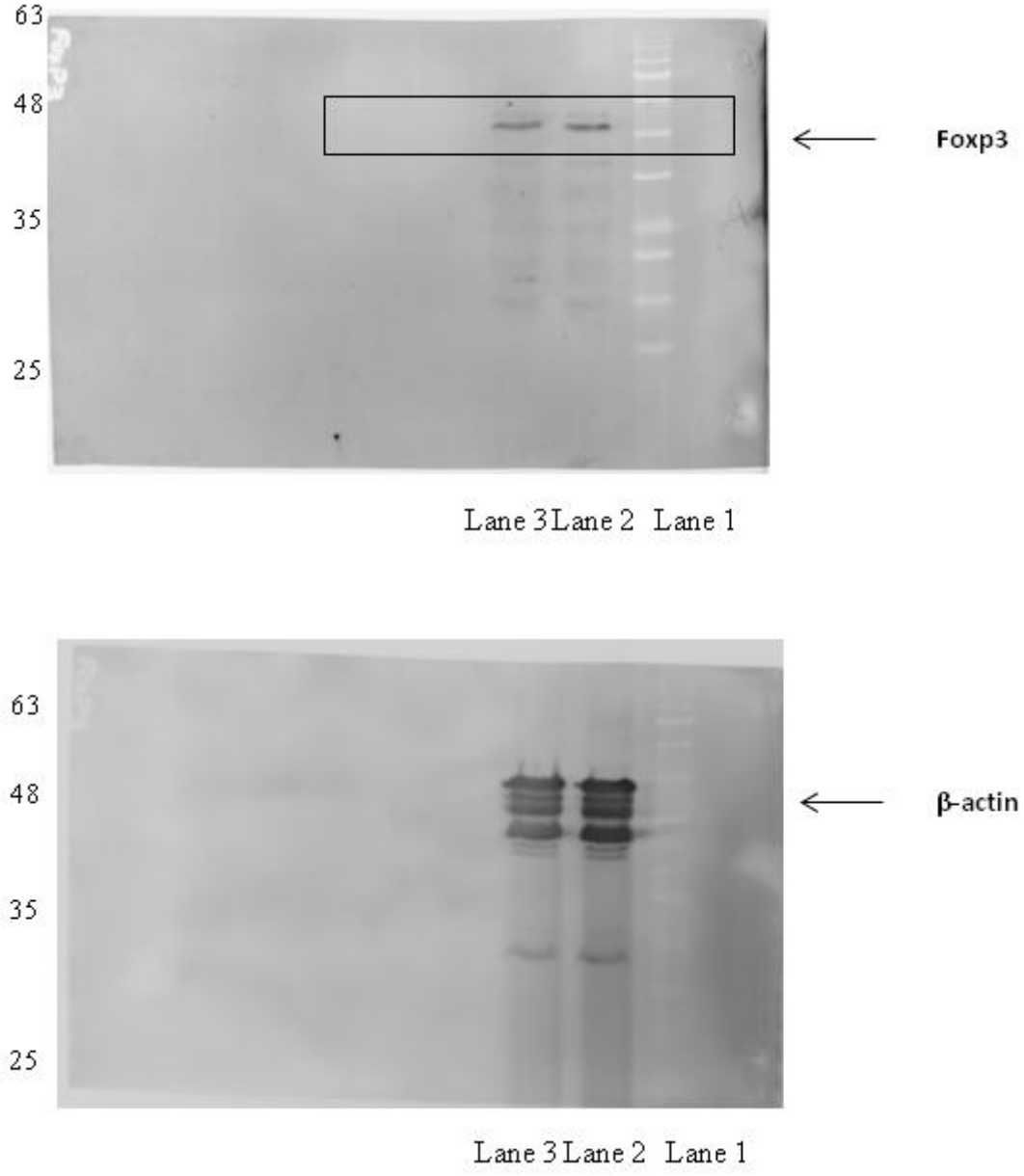
Legend: PBMC: Peripheral blood mononuclear cells; Foxp3: 47,25 KDa; mPRα: 40 KDa; IL-10: 18 KDa; β-actin: endogenous control; (+): Positive (Presence of the protein); (-): Negative (Protein could not be detected); na: non-applicable.

The presence of Foxp3 protein could be corroborated in Buffy-coats and normal pregnancy (Treg cells and not-Treg cells) samples, whereas the presence of mPRα and IL-10 proteins could only be validated in Buffy-coats.

In all other trials the presence of the referred proteins could not be detected.

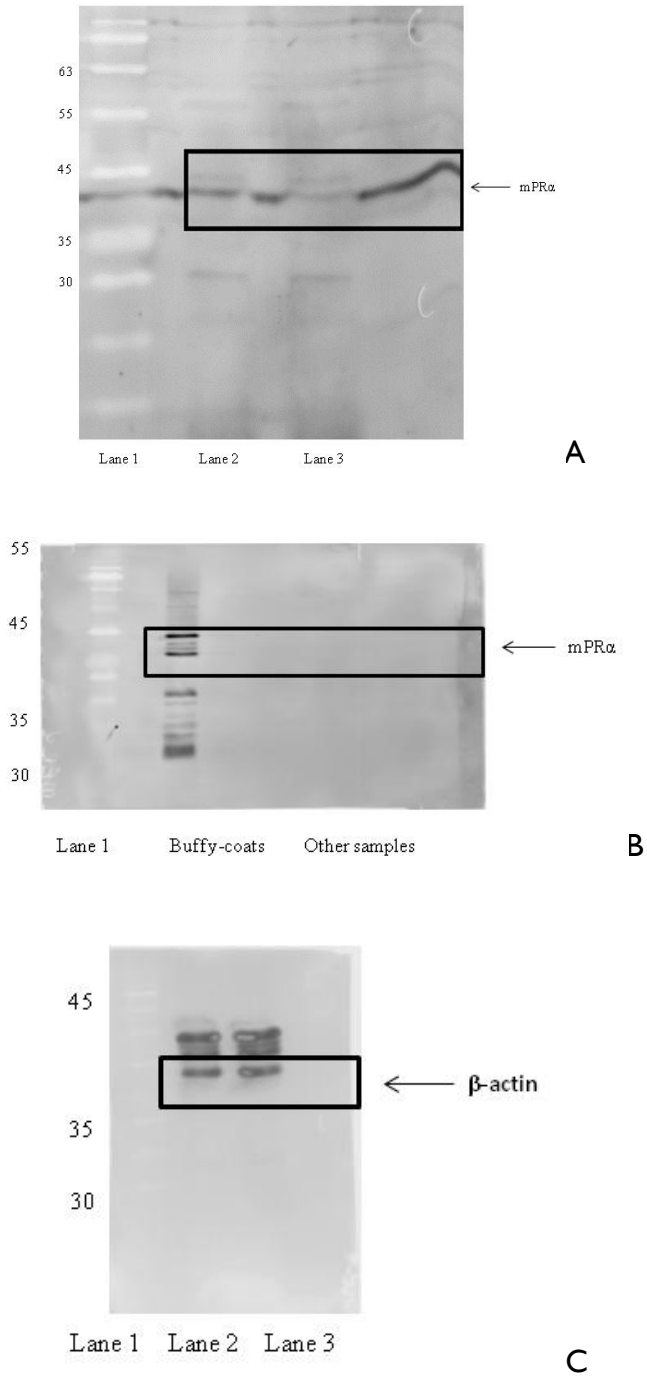
Images of our positive results are depicted in Figures 24, 25 and 26.

Figure 24. Western Blot analysis of Foxp3 protein (47.25kDa) and β-actin in Buffy-Coats of healthy donors.



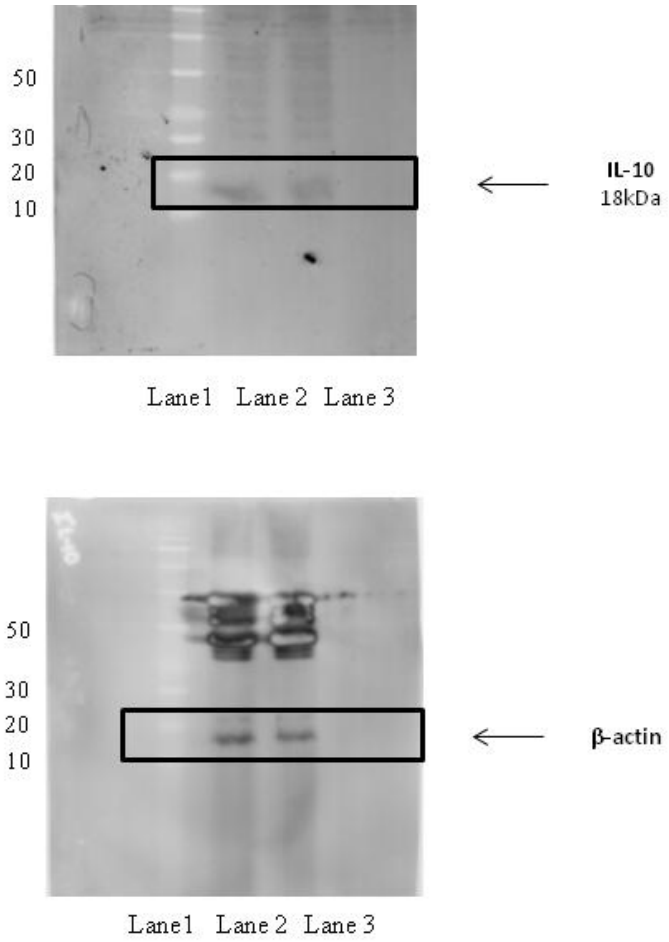
Legend: Western Blot analysis of Foxp3 expression; 20µg/protein/lane; Foxp3 antibody concentration (1:2000); Lane 1: molecular weight marker, kDa; Lanes 2 and 3: Foxp3.

Figure 25. Western Blot analysis of mPRα protein (40 kDa) and β-actin in Buffy-Coats of healthy donors.



Legend: Western Blot analysis of mPRα expression in Buffy-Coats (A), Buffy-Coats and preterm labour samples (B) and β-actin (C); 20µg/protein/lane; mPRα antibody concentration (1:2000); Lane 1: molecular weight marker, kDa; Lanes 2 and 3: mPRα.

Figure 26. Western Blot analysis of IL-10 protein (18kDa) and β-actin in Buffy-Coats of healthy donors



Legend: Western Blot analysis of IL-10 expression; 20μg/protein /lane; IL-10 antibody concentration (1:2000); Lane 1: molecular weight marker, kDa, Lanes 2 and 3: IL-10.

4. Real Time Polymerase Chain Reaction (RT-PCR)

Afterwards, we explored the presence of Foxp3, mPRα, TGF-β and IL-10 genes in pregnancy using PBMC (normal and preterm pregnancies) and sorted Treg cells and not-Treg cells fractions (normal and preterm pregnancies). All tests were carried out twice.

Table 9. Real time *PCR* results of different samples tested from pregnant women.

	Genes	Foxp3	mPRα	TGF-β	IL-10	IKZF1	IKZF2
Samples							
PBMC Normal pregnancy		+	+	+	+	+	+
PBMC Preterm labour		+	+	+	+	+	+
Normal Pregnancy Group	Treg	—	—	—	—	—	—
	Not-Treg	—	—	+	—	+	—
	Treg	+	—	+	—	+	+
	Not-Treg	+	—	+	—	+	—
	Treg	+	—	+	—	+	+
	Not-Treg	—	—	+	—	+	—
	Treg	—	—	—	—	—	—
	Not-Treg	—	—	—	—	—	—
	Treg	—	—	+	—	—	—
	Not-Treg	—	—	—	—	—	—
Preterm Group Before Progesterone	Treg	—	—	+	—	—	—
	Not-Treg	—	—	—	—	—	—
	Treg	+	—	—	—	—	—
	Not-Treg	—	—	—	—	—	—
	Treg	—	—	—	—	—	—
	Not-Treg	—	—	—	—	—	—
Preterm Group After Progesterone	Treg	—	—	—	—	—	—
	Not-Treg	—	—	—	—	—	—
	Treg	—	—	—	—	—	—
	Not-Treg	—	—	—	—	—	—
	Treg	—	—	—	—	—	—
	Not-Treg	—	—	—	—	—	—

Legend: PBMC: Peripheral blood mononuclear cells; IKZF1 and IKZF2: Ikaros family zinc finger 1 and 2 (Housekeeping genes); (+) Amplification: Gene Present; (-) No sample amplification: Insufficient quantity of the gene or gene absent.

As demonstrated in Table 9, there was amplification of Foxp3 gene in the two PBMC samples; in 2 out of 4 normal pregnancy Treg cells samples; and in 1 out of 6 preterm pregnancy Treg cells samples (before Progesterone).

Concerning mPRα gene, there was amplification only in the two PBMC samples.

As for TGF-β gene, amplification could be perceived in the two PBMC samples; in 3 out of 4 normal pregnancy (Treg and not-Treg cells) samples; and in 1 out of 6 preterm pregnancy Treg cells sample.

Regarding IL-10 gene, amplification occurred merely in the two PBMC samples.

There was no gene amplification in all other cases.

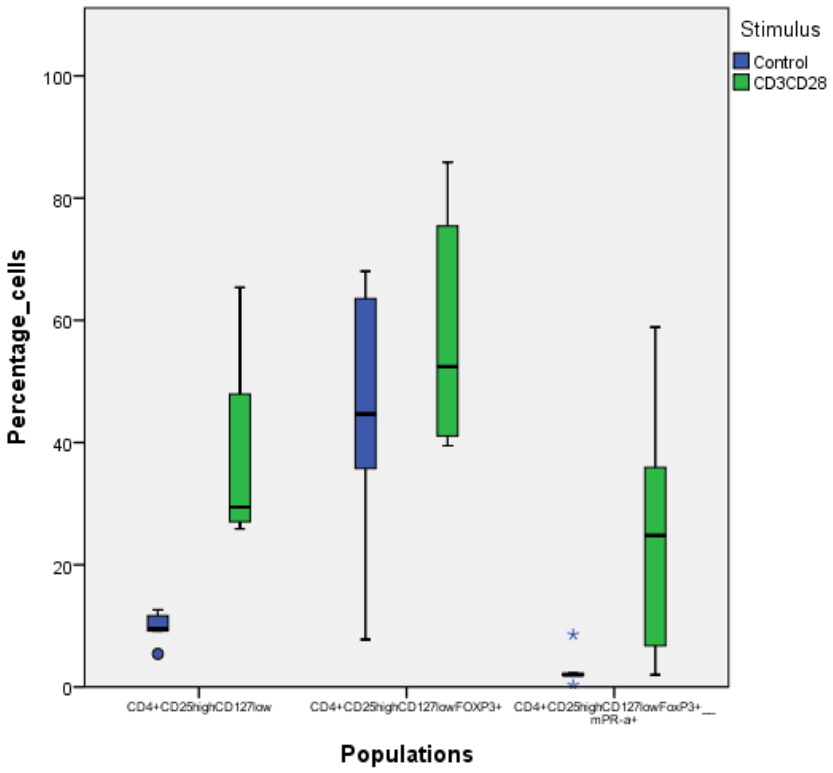
5. *In Vitro* Studies

To conduct the planned *in vitro* studies, as there were no similar data published, initially we had to test which would be the best stimulus for our sorted population and the ideal progesterone concentration to use.

Eight samples from group A were used for the stimulus and for progesterone concentration alternatives.

As for the best stimulus, the use of CD3 and CD28 revealed an increase in the population of $CD4^{+}CD25^{high}CD127^{low}$, regulatory T-cells and $mPR\alpha^{+}$ regulatory T-cells (Figure 27).

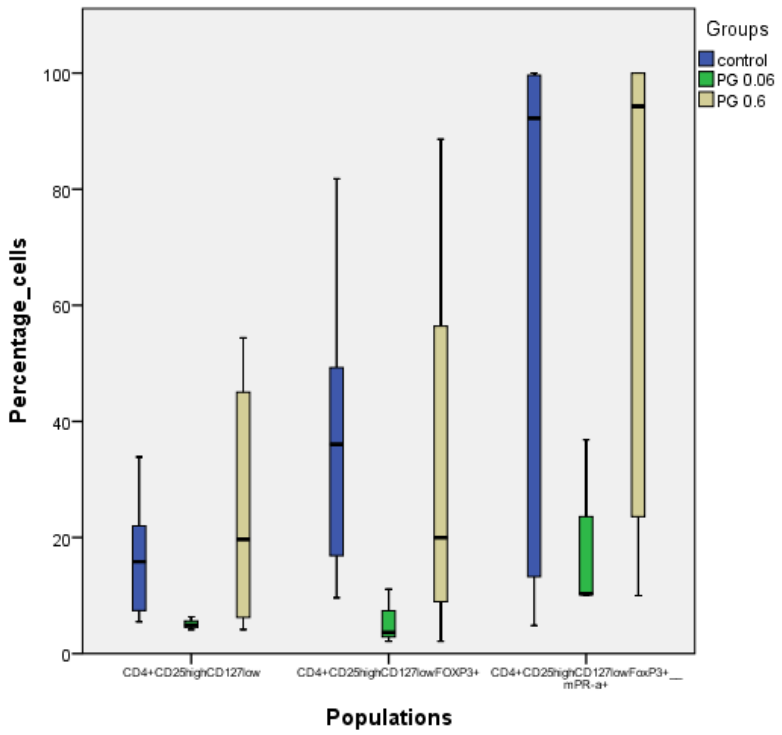
Figure 27. Variation of the different blood populations with stimulus



With respect to progesterone concentrations, we tested 0.06, 0.6 and 6 µM; the use of Progesterone at a 6 µM concentration resulted in cell death in all samples.

Applying progesterone at a dose of 0.6 µM achieved an increase in the intended blood populations, reason for its selection for the subsequent experimentations (Figure 28).

Figure 28. Variation of the different blood populations with diverse progesterone concentrations



Legend: PG: progesterone.

After those preliminary approaches, using blood samples from Preterm Group before progesterone administration, we aimed at determining the variation of $CD4^+$, $CD4^+CD25^{high}CD127^{low}$, regulatory T-cells and $mPR\alpha^+$ regulatory T-cells, and TGF-β and IL-10 concentrations, with different *in vitro* conditions.

Table 10. *In vitro* results of T-cell populations after different conditions using blood samples from preterm group

	Different *in vitro* conditions					
	Control	***P4 0,6***	***Mif 0,5***	***Nand 0,5***	***P4+Mif***	***P4+Nand***
Lymphocyte population [a]						
$CD4^+$	49.6 ± 16.8	43.9 ± 18.1	88.3 ± 18.1	83.8 ± 30.1	84 ± 29.7	98.7 ± 1.6
$CD4^+CD25^{high}CD127^{low}$	7.4 ± 5.0	29.9 ± 25.9	18.7 ± 10	13.8 ± 4	17.3 ± 5.4	19.2 ± 4.2
Tregs[b]	3.8 ± 2.7	57.9 ± 31.1	88 ± 17.5	92.7 ± 12.6	58.6 ± 29.4	95.6 ± 6.2
Tregs $mPR\alpha^+$ [b]	70.8 ± 34	99.9 ± 0.06	95.7 ± 8.1	93.5 ± 15.8	81.3 ± 34	99.9 ± 0.07

Legend: All data are presented as mean ± standard deviation (SD). *P4- Progesterone; Mif- Mifepristone; Nand- Nandrolone;* [a] Percentage of cells/μl blood; [b]Tregs: $CD4^+CD25^{high}CD127^{low}$ $Foxp3^+$.

Figure 29. Evolution of the different blood populations under multiple *in vitro* conditions

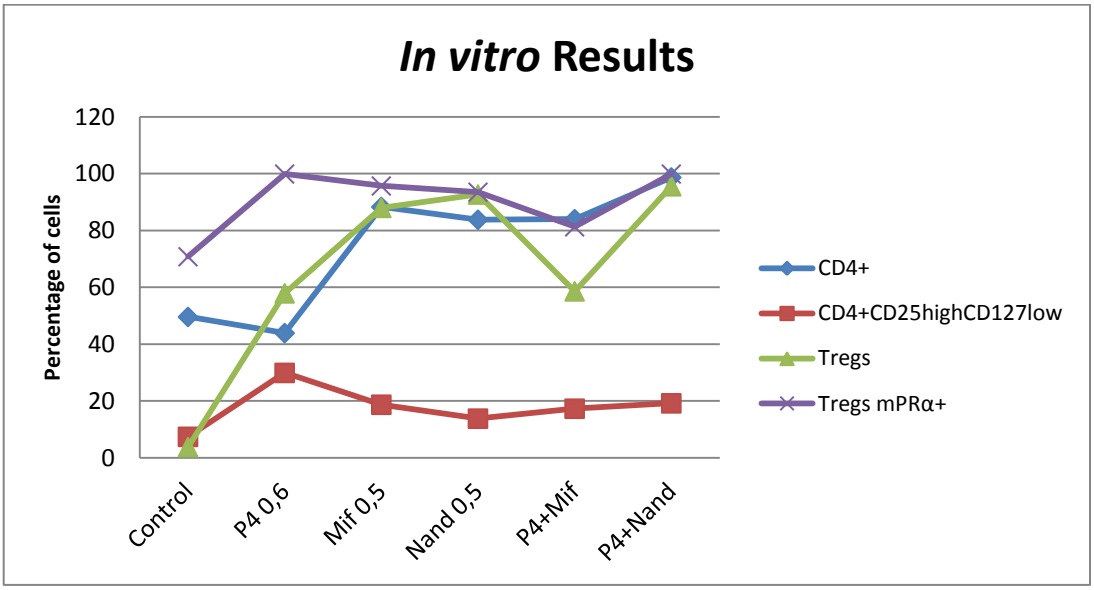


Table 11. *In vitro* results of plasma cytokine levels after different conditions using blood samples from preterm group

	Different *in vitro* conditions					
	Control	**P4 0,6**	**Mif 0,5**	**Nand 0,5**	**P4+Mif**	**P4+Nand**
IL-10 [a]	76.4 ± 64.2	12.4 ± 11.2	36.6 ± 35.5	27.3 ± 18.6	9.0 ± 5.0	24.8 ± 26.3
TGF-β [a]	453 ± 334	461 ± 112	193 ± 209	450 ± 516	535 ± 756	187 ± 162

Legend: All data are presented as mean ± standard deviation (SD). *P4- Progesterone; Mif- Mifepristone; Nand- Nandrolone;* [a] *Concentration in pg/ml.*

Resuming Tables 10 and 11, the diverse conditions created in the laboratory gave rise to the following results in relation to control:

- ✓ Progesterone at a 0.6 μM concentration
    i. Increases $CD4^{+}CD25^{high}CD127^{low}$, Treg cells and Treg cells expressing mPRα and TGF-β concentration
    ii. Decreases $CD4^{+}$ cells and IL-10 concentration
- ✓ Antagonist use (Mifepristone at a 0.5 μM concentration)
    i. Increases $CD4^{+}$, $CD4^{+}CD25^{high}CD127^{low}$, Treg cells and Treg cells expressing mPRα
    ii. Decreases TGF-β and IL-10 concentrations
- ✓ Agonist use (Nandrolone at a 0.5 μM concentration)
    i. Increases $CD4^{+}$, $CD4^{+}CD25^{high}CD127^{low}$, Treg cells and Treg cells expressing mPRα
    ii. Decreases TGF-β and IL-10 concentrations
- ✓ Progesterone + Antagonist
    i. Increases $CD4^{+}$, $CD4^{+}CD25^{high}CD127^{low}$, Treg cells and Treg cells expressing mPRα; and TGF-β concentration
    ii. Decreases IL-10 concentration
- ✓ Progesterone + Agonist
    i. Increases $CD4^{+}$, CD4+$CD25^{high}CD127^{low}$, Treg cells and Treg cells expressing mPRα
    ii. Decreases TGF-β and IL-10 concentrations
- ✓ Agonist + Antagonist
    i. Inconclusive as only one sample's cells survived for evaluation

In conclusion, comparing with the control, the **highest** values for each population were obtained with the conditions:

- ✓ **$CD4^+$cells**: Progesterone + Agonist
- ✓ **$CD4^+CD25^{high}CD127^{low}$ cells**: Progesterone
- ✓ **Treg cells**: Progesterone + Agonist
- ✓ **Treg cells expressing mPRα**: Progesterone

In the opposite, the **lowest cytokine concentrations** were obtained:

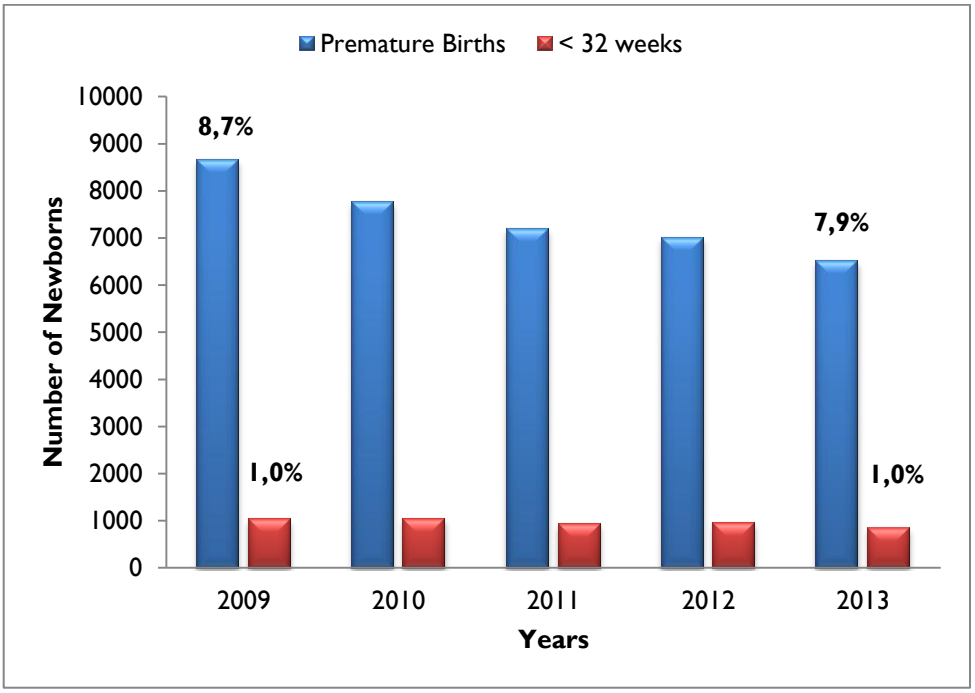
- ✓ **TGF- β:** Progesterone + Agonist
- ✓ **IL-10:** Progesterone + Antagonist

Chapter V - Discussion

According to the World Health Organization, there are 13 million preterm babies born each year worldwide. Despite the various measures to reduce this rub, the incidence of preterm labour has increased in almost all countries, continuing to be a relatively common pathology ("Euro-Peristat project with SCPE and Eurocat. European Perinatal health report. The health of pregnant women and babies in Europe in 2010 ", 2013). Current estimate rates of PTL vary between 5 and 11% in developed countries and 18% in developing countries. (Blencowe, Cousens *et al.*, 2012; Borna & Sahabi, 2008; How & Sibai, 2009; Rai, Rajaram *et al.*, 2009; Tita & Rouse, 2009)

In Portugal the incidence of preterm births has varied from 6.5% in 1995 to 7.9% in 2013. In the last five years the incidence has fluctuated as depicted in the following figure:

Figure 30. Prematurity data from Portugal (2009 to 2013)



Source: *Grupo do Registo Nacional do Recém-nascido de Muito Baixo Peso em Portugal* and ("Demographic statistics- Birth and Mortality indicators. Statistics Portugal - Instituto Nacional de Estatística (INE) [online database],").

Preterm birth is the leading direct cause of neonatal death (death in the first 28 days of life), with over one million deaths annually being attributable to prematurity. Moreover, preterm birth is the second most common cause of death in children younger than 5 years (Blencowe, Cousens *et al.*, 2012).

The risk of neonatal mortality decreases as gestational age at birth increases. Even for the survivals, preterm birth continues to be a major determinant of short and long-term morbidity in infants and children (Martin, Hamilton *et al.*, 2013). Moreover, prematurity not only affects the newborn directly, but can also result in a vicious intergenerational (mother and child) cycle of risk (Howson, Kinney *et al.*, 2013).

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 labour. Nonetheless, progesterone mediated responses are complex because they are mediated by multiple types of receptors (Zachariades, Mparmpakas *et al.*, 2012).

Undoubtedly this steroid is able to prevent the maternal immune system from activating effector T-cells capable of attacking trophoblastic cells, resulting in a T-cell tolerance during pregnancy (Chien, Lai *et al.*, 2009). Recent data suggests that progesterone may be important in maintaining uterine quiescence in the latter half of pregnancy by limiting the production of stimulatory prostaglandins and inhibiting the expression of contraction-associated protein genes within the endometrium (Norwitz & Caughey, 2011). However, the exact mechanism through which this is accomplished is still under research.

Regulatory T-cells were shown to expand during human pregnancy, with functional studies finding that they create a tolerant microenvironment through regulation of immune cell responses at the fetal-maternal interface (Leber, Teles *et al.*, 2010).

$CD4^{+}CD25^{high}CD127^{low}$ isolated Treg cells appear to be the best reached Treg population regarding purity, function, stability and in vitro expansion capacity, consenting isolation of pure Treg populations with high suppressive activity (Hartigan-O'Connor, Poon *et al.*, 2007). However, the most widely accepted phenotype for Treg cells is the co-expression of CD4, CD25 (α-chain of the IL-2 receptor) and Foxp3 (Lastovicka, 2013).

Foxp3 is regarded as a lineage molecule for Treg cells and it is an intracellular marker. Consequently, it is very susceptible to degradation within a short time, the detection of which is difficult and not really usable in large sample series. Moreover, Foxp3+ T-cells are phenotypically and functionally heterogeneous and involve both suppressive and non-suppressive T-cells (Lastovicka, 2013).

Furthermore, CD127 was for a long period seen as an efficient tool to determine the phenotype and functional activity of Treg cells. Yet, there has been increasing controversy in comparisons with $CD4^{+}CD25^{high}Foxp3^{+}$ cells, particularly in the context of chronic infections, as it appears that the existence of an underlying disease can cause an intense CD127 down modulation on formerly $CD127^{+}$ T effector cells (Lastovicka, 2013). However, no attention was given to it regarding pregnancy.

As such, it is currently accepted that CD127 expression inversely correlates with the Foxp3 expression and suppressive activity of Treg cells (Lastovicka, 2013). We therefore assumed the $CD4^{+}CD25^{high}CD127^{low}$ $Foxp3^{+}$ as the phenotype of the Treg cell *pool* in our research, making some comparisons feasible.

Since 1980, some groups have tried to identify expression of progesterone receptors during pregnancy, notwithstanding with contradicting results (Mansour, Reznikoff-Etievant *et al.*, 1994; Szekeres-Bartho, Csernus *et al.*, 1983).

Nevertheless, the gathering of scientific data enabled not only to verify the presence of lymphocytic nuclear progesterone receptors (Szekeres-Bartho, Reznikoff-Etievant *et al.*, 1989), but also to validate the existence of progesterone induced blocking factor and its role in pregnancy (Szekeres-Bartho & Polgar, 2010). Recently, some authors have attempted to demonstrate that the actions of progesterone on T lymphocytes were mediated by one or more putative membrane receptors, but all experiments were done in non-pregnant animal models (Ndiaye, Poole *et al.*, 2012). Moreover, although receptors for estrogens have been confirmed in Treg cells (Mjosberg, Svensson *et al.*, 2009), to the best of our knowledge progesterone receptors have not been studied in this subset of human cells.

In the quest for a novel agent in PTL treatment, progesterone emerges as a good candidate due to its immunomodulatory action, supposedly acting as the critical regulator of Treg cells during pregnancy, by an unknown mechanism.

Consequently, this is the first investigation focusing not only on the role of progesterone administration on Treg cells in cases of PTL, but also on the existence of mPRα in human pregnancy Treg cells (Areia, Vale-Pereira *et al.*, 2015).

This research thus postulates a primordial role for Treg cells in the intertwining between mPRα and progesterone's action in human pregnancy.

Main Findings and Interpretation

The variation of the number of Treg cells during the 3 trimesters of normal human pregnancy is still under debate, with some authors reporting a rise in the 1st trimester with a peak in the 2nd trimester (Xiong, Zhou *et al.*, 2010), whilst others say there is a reduction in the 2nd trimester (Saito, Nakashima *et al.*, 2010; Teles, Thuere *et al.*, 2013).

In normal pregnancy, our results demonstrated higher percentages of Treg cells in the third trimester (56.8 ± 28.6), upholding their protective role against maternal immune reactions and labour ensuing, as depicted in the literature (Xiong, Zhou *et al.*, 2010).

Moreover, the lowest value of Treg cells percentage could be verified on delivery date (25.7 ± 25.1), confirming recent data that indicated a significant decrease of Treg cells expressing Foxp3 in women in labour at term (Schober, Radnai *et al.*, 2012).

On the opposite side, $CD4^{+}CD25^{high}CD127^{low}$ cells presented rising percentages all the way through pregnancy, with their uppermost percentage on delivery day (9.7 ± 3.4), suggesting the likely importance of activation of this population in the recrudescence of the immunoinflammatory phenomenon nowadays believed to be behind the initiation of labour.

Some authors have indicated a significant decrease in $CD4^{+}$T-cells within the total leukocyte *pool* in spontaneous labour, which could indicate that a strong immune stimulation and subsequent apoptosis of the activated $CD4^{+}$ T-cells may occur during that specific time (Schober, Radnai *et al.*, 2012). When comparing our results with those published in the literature, this population showed an increase on delivery day in relation to the 3rd trimester (to percentages of 44.1 ± 16.0), in opposition with what is

published; this could be explained probably by the fact that our measurements where undertaken before the aforementioned apoptosis occurred. These results underline the importance of further investigation to define the exact role of this population in order to labour's progress.

Progesterone withdrawal is a key event in parturition process in all mammals studied so far (Mesiano, Wang *et al.*, 2011). The human species is exclusive since progesterone levels remain sustained during pregnancy and labour, which prompted several investigators to delve into an elucidation for the triggering of human labour.

Effectively, progesterone levels in the maternal and fetal circulations and in the amniotic fluid are relatively high throughout pregnancy and even during labour and delivery, decreasing only after delivery of the placenta. Thus, the prevailing theory postulates that human parturition involves a functional, rather than systemic, progesterone withdrawal (Mesiano, Wang *et al.*, 2011).

Overwhelming our expectations, we were able to prove the existence of mPRα in Treg cell pool all throughout human pregnancy (second trimester, third trimester and delivery day) (Areia, Vale-Pereira *et al.*, 2015), with the highest percentage obtained in the 3rd trimester (36.3 ± 41.1), and a decline on delivery day (19.6 ± 32.3). This may give a possible clarification for the reduction in progesterone's anti-inflammatory function (functional withdrawal) with normal systemic progesterone levels that prompts labour.

Differing from our findings, preceding works had revealed that mPRα messenger RNA had a peak in middle pregnancy, with a reduction at the end of pregnancy (Dressing, Goldberg *et al.*, 2011), prompting the challenge of further investigation in order to elucidate this theme.

In the literature there are 3 articles which intended to study Treg cells in pregnant women with PTL (Kisielewicz, Schaier *et al.*, 2010; Schober, Radnai *et al.*, 2012; Xiong, Zhou *et al.*, 2010), although none of them studied the influence of progesterone treatment on Treg cell population. Moreover, aims, inclusion criteria, T-cell population studied, and conclusions are different among the 3 studies.

The article of Xiong *et al*. was the first to demonstrate changes in circulating $CD4^{+}CD25^{high}Foxp3^{+}$ Tregs in PTL (Xiong, Zhou *et al.*, 2010), and their work evidenced a lower proportion of that particular T-cell population in PTL. Although including only one sample of 31 women, the definition of Treg cell population was different, not including the CD127 marker. Nevertheless, these results make our study even more consistent, highlighting the importance of progesterone administration in increasing Treg cell pool in these women.

Kisielewicz et al. (Kisielewicz, Schaier *et al.*, 2010) studied the parallelism between PTL and organ transplant rejection. The results showed that in PTL, Treg cells show a reduced suppressive activity of their circulating $CD4^{+}CD25^{high}$ $CD127^{low}$ cells and a decrease in the level of $HLADR^{+}$ expression. However, of the 21 PTL women included (already excluding PPROM and cervical incompetency), there were no excluding criteria and there was only one sample for each patient.

Schober et al. considered the suppressive activity and changes in the composition of the regulatory T-cell pool (Schober, Radnai *et al.*, 2012). They included 46 PTL women, but no exclusion criteria were used and the definition of PTL cases included PPROM and cervical incompetency. Their results inferred a decrease in the percentage of $HLADR^{+}$Treg cells, in HLADR expression and a decrease in the suppressive activity of Treg cells.

As previously stated, this is an innovative research focusing on the role of progesterone administration on Treg cells (defined as $CD4^{+}CD25^{high}CD127^{low}$ $Foxp3^{+}$ cells) in cases of PTL.

As a result, our investigation demonstrated that there was a considerable increase after progesterone treatment in PTL group among the percentage of $CD4^{+}$ T-cells (from 44.5 to 56.2; p=0.023) and in Treg cell pool percentage (from 38.3 to 52; p=0.07). This vindicates the knowledge that progesterone conduces to the establishment of a favourable environment for inhibiting labour (Polese, Gridelet *et al.*, 2014) and to the enhancement of Treg cell function *in vitro* (Mao, Wang *et al.*, 2010).

The data reported in the literature concerning the effect of pregnancy-specific hormones on Foxp3 expression by Treg cells are contradictory (Schober, Radnai *et al.*, 2012). The withdrawal of hormones at the end of pregnancy may affect Foxp3 expression by Treg cells, which was shown to be enhanced by progesterone in human studies (Mao, Wang *et al.*, 2010). On the opposite side, other authors postulate that progesterone, whose maximum levels are seen at the end of pregnancy, has the capacity to reduce Foxp3 expression by Treg cells *in vitro* (Mjosberg, Svensson *et al.*, 2009).

Our results are in accordance with the hypothesis of *Mao et al.*, as $CD4^{+}CD25^{high}CD127^{low}$ $Foxp3^{+}$ cells (Treg cell pool) demonstrated an increase in their percentage (from 38.3 to 52; p=0.07) and number (from 51 to 79.9; p=0.25) after progesterone administration.

Stunning results were the ones that revealed a decrease in $mPR\alpha^{+}$ Treg cells after progesterone treatment both in percentage (from 32.6 to 13.8; p=0.07), as in number (from 7.5 to 6.8; p=0.8). This raises the possibility of receptor's unavailability due to the methodology used (a receptor formerly engaged by progesterone may not be

available for specific antibody linking), which needs further corroboration.

Nevertheless, some authors had already stated that mPRα^{+} is downregulated during preterm labour (Byrns, 2014; Fernandes, Pierron *et al.*, 2005), although these experiments resulted from endometrial biopsies and not from peripheral blood samples; in addition, mPRα transcripts seem to decline upon PTL (Zachariades, Mparmpakas *et al.*, 2012).

Concerning the comparisons on delivery day, the smaller gestational age at delivery and birthweight in PTL group compared to normal pregnancy, can be merely explained by the preterm condition itself.

Notwithstanding, the finding of all blood populations higher in PTL group compared with the control group on delivery day, with a statistical higher percentage of Treg cells (52 *vs.* 25.7; p=0.03), reinforces the fact that an inflammation-triggered immunologic response prompts PTL (Holst & Garnier, 2008). This contradicts the data published by *Schober et al.* wherein Treg cell pool was not different on delivery day between normal 3rd trimester women and preterm women (Schober, Radnai *et al.*, 2012). Yet, this could be explained by the fact that in our research, pregnant women from PTL group had been previously given progesterone, which has undoubtedly immunomodulatory effects.

The process of normal term parturition is characterized by leukocyte infiltration and secretion of proinflammatory mediators into the intrauterine environment (Schober, Radnai *et al.*, 2012). Withal, inflammation at the maternal–fetal interface is one of most well established causes of preterm birth.

That being so, excessive levels of proinflammatory cytokines or reduced levels of anti-inflammatory cytokines may give an explanation for the succeeding events.

Because of their well-known involvement in this mechanism, a number of studies have measured cytokines in pregnant women in an attempt to identify predictive clinical markers of premature birth (Ferguson, McElrath *et al.*, 2014).

As previously elucidated, iTregs represent a rather heterogeneous family of Tregs, with two main subsets: type 1 regulatory T-cells (which are induced by IL-10), and T helper 3 regulatory T-cells, which are induced by TGF-β. TGF-β and IL-10 are thus the primary cytokines involved in iTreg formation (Kisielewicz, Schaier *et al.*, 2010).

As induced Treg cells mostly act by the production of immunosuppressive cytokines, we determined cytokine levels in normal pregnancy, choosing the 2nd trimester, as it was the period most referred in the literature as having the highest values of Treg cells. There are no data in the literature concerning this question, and so, these calculations were important to be able to compare with the ones obtained from the preterm group.

Scientific evidence demonstrated that progesterone not only directly alters cytokine production to induce Th2 cytokines, but also promotes dendritic cell's production of IL-10 (Dressing, Goldberg *et al.*, 2011). As so, it would be logical to compare immunosuppressive cytokine levels before and after progesterone administration.

Interleukin-10 is a cytokine that has been recognized as a key factor in modulating or promoting resolution of the inflammatory process associated with term labour and with intrauterine infection-associated preterm labour (Pineda-Torres, Flores-Espinosa *et al.*, 2014).

Several studies have demonstrated that there were no changes in IL-10 expression across normal pregnancy in peripheral blood samples (Denney, Nelson *et al.*, 2011).

Nevertheless, lower IL-10 levels have been associated with increased risk of preterm birth, which may be expected as IL-10 is anti-inflammatory; yet, other studies have reported null associations (Ferguson, McElrath *et al.*, 2014).

Moreover, recent data revealed decreased IL-10 levels in LPS-stimulated peripheral blood mononuclear leukocyte across the second trimester in women destined to deliver preterm compared to term (Harper, Li *et al.*, 2013).

In our initial approach, levels of IL-10 were compared between normal 2nd trimester pregnancy *vs.* PTL group. Consistent with the latest investigation (Ferguson, McElrath *et al.*, 2014), we could ascertain that IL-10 levels were lower in PTL group (6.2 *vs.* 11.3 pg/ml; $p<0.001$), evidencing that downregulation of IL-10 favours an inflammatory state that promotes the mechanism of labour.

TGF-β has long been known to reveal immunosuppressive and anti-inflammatory properties (Mesdag, Salzet *et al.*, 2014), besides its capacity to preferentially induce Treg cell differentiation (Teles, Thuere *et al.*, 2013). Moreover, at local uterine level, TGF-β blocks differentiation of Th_1 and Th_2 cells (Gargano, Holzman *et al.*, 2008), promoting Treg cell responses (Teles, Thuere *et al.*, 2013).

Although some works revealed that lower TGF-β levels clustered with higher intensity of inflammatory mediators in preterm labour placentas (Faupel-Badger, Fichorova *et al.*, 2011), other investigations prompted that higher levels of TGF-β in midpregnancy were associated with increased odds of PTL (Gargano, Holzman *et al.*, 2008).

In conformity with the later, when comparing between normal 2nd trimester pregnancy *vs.* PTL group, TGF-β demonstrated higher values in PTL group (16.9 *vs.* 44 pg/ml; $p=0.016$). This could be explained by the fact that TGF-β is believed to be the

most relevant inductor of Treg cells lineage (Kisielewicz, Schaier *et al.*, 2010), being not just important to induce Treg cells, but also being a product of their suppressive capacity (Gargano, Holzman *et al.*, 2008).

Subsequently, we proceeded to determine the effect of progesterone treatment on cytokine levels produced in PTL.

Both IL-10 and TGF-B augmented after progesterone treatment in PTL, with statistical significance for IL-10 (from 6.27 to 10.1; p=0.001). This not only corroborates our flow cytometry results (which revealed higher levels of Treg cells after progesterone treatment), but also substantiates the importance of these cytokines as the means by which Treg cells achieve suppression of the immunoinflammatory phenomenon, thought to prompt PTL.

In the quest for our results' elucidation, *Western Blot* and *RT-PCR* techniques were performed, nonetheless with underwhelming results.

As for *Western Blot*, only on buffy-coats' samples the presence of Foxp3, mPRα and IL-10 proteins could be relentlessly confirmed.

Expected results would be that Foxp3 and IL-10 proteins could be detected in all preterm samples, after progesterone administration, translating the higher proportion of Tregs cells postulated by our investigation; on the contrary, mPRα protein might not be detected after progesterone treatment, as our results detected small percentage of this receptor. Nevertheless, this did not occur in our research.

One could argue that the inconsistent detection of each of these proteins in the different samples studied (term, preterm, Treg cells or not), not only depends on the available quantity of the protein (being produced by a minor blood population), but also has several specific technical procedures that can influence the final results.

Several attempts were made to surpass the initial results: pregnant women's PBMC use (in the urge to assemble a higher number of cells); the merge of several samples before sorting; and the application of the striping technique on the different membranes obtained, regrettably all without success.

In regard to *RT-PCR*, we accomplished to demonstrate Foxp3, mPRα, TGF-β and IL-10 genes in our pregnant women's PBMC (term and preterm), which authenticates our discoveries. As so, the novelty of mPRα in Treg cells in human pregnancy is undoubtable.

In spite of that, these promising results could not be ascertained in all blood samples, irrespective of gestational age or progesterone treatment. The unsystematic presence of the genes in question may possibly be justified by an insufficient quantity of the former (supported by their presence in PBMC samples) or could be the consequence of the exquisiteness of the intended determinations. Several replication of the procedures were undertaken, namely with preterm birth samples, albeit no different results attained.

We moved on to the *in vitro* studies where we aimed to determine that progesterone effects on Tregs were accomplished through mPRα.

Corroborating our *in vivo* results, administration of progesterone resulted in an increase of almost all blood populations studied, namely Treg cell pool and $mPR\alpha^{+}$ Treg cells, consubstantiating our discoveries.

The use of the antagonist ought to reduce all blood populations; exactly the opposite results were perceived, inferring that this is not a specific mPRα antagonist. Nevertheless, our results corroborate the fact that in the presence of progesterone, mifepristone acts as a competitive progesterone receptor antagonist, whereas in the

absence of progesterone, mifepristone acts as a partial agonist, as depicted by some authors (Chien, Lai *et al.*, 2009).

Conversely, despite all our drawbacks in relation to the agonist used, it managed to amplify all blood populations, astonishingly even more than progesterone alone (regarding $CD4^+$ cells and Treg cell pool).

Our revelation that the highest values obtained for $CD4^+$ cells and Treg cell pool were related to the use of the association of progesterone and the agonist, are somewhat expected, since our preliminary results demonstrated that progesterone in that concentration achieved a rise in the intended blood populations; the merging of an agonist should increase even more that levels, which occurred.

Meanwhile, the fact that the utmost percentages of $CD4^+CD25^{high}CD127^{low}$ cells and of $mPR\alpha^+$ Treg cells were attained with the use of Progesterone alone, discloses the fact that Nandrolone is not a specific agonist for mPRα, as we desired.

As for cytokine levels produced by the blood populations *in vitro*, surprisingly all conditions dampened IL-10 levels, with the lowest values with the association of progesterone and antagonist. Even though at first sight this seems a peculiar result (even more if we consider our data on PTL, that revealed in increase in IL-10 values after progesterone administration), there are important facts that need to be taken into account: first, the progesterone used *in vivo* was not the same nor in formulation nor in dosage as the one used for the *in vitro* essays; second, some authors advocate that suppressive' Treg cells activity (that we can argue that is accomplished also by IL-10 production) is reduced in PTL (Schober, Radnai *et al.*, 2012), and all our samples were from PTL women; third, the association of progesterone and antagonist, resulted in an increment of the inhibitory action achieved by the antagonist on its own, raising

the possibility of a synergy between progesterone and mifepristone actions on this matter; finally, and probably more important, cells depend on their environmental milieu to fulfil their in vivo functions (as much as we want to mimic those conditions in vitro, selfsame is not always accomplished).

Concerning TGF-β, progesterone augmented its levels, as did the association of progesterone and antagonist. The later, raises again issues concerning the effects of progesterone on mifepristone actions.

The dubious results of attaining the lowest TGF-β values with the association of progesterone and agonist can be explained probably by the non-specificity of the agonist used, aside from the arguments used previously for IL-10, namely the reduced suppressive activity of Treg cells in PTL (Schober, Radnai *et al.*, 2012) and the different environment characteristics.

To summarize, even though an enormous effort was made towards obtaining the best conditions to perform our *in vitro* experiments, it was not possible to afford the best formulations desired for these essays: not only the accessible agonist was not specific for mPRα, but also the obtainable antagonist seems to have partial agonistic effects.

Strengths

This is the primary research concerning the variation of specific T-cell subsets in three precise obstetrical times (2^{nd}, 3^{rd} trimesters and delivery day), whose results were subsequently correlated not only with cytokine production, but also submitted to validation by other Laboratory techniques.

From this initial approach, the fact that on delivery day all blood populations presented values completely different from the ones noted on 3^{rd} trimester ones, empowers the theory of the existence of an immune triggering that inducts human labour.

In addition to being the first human investigation concerning Treg cell changes after progesterone in PTL, our study has strict inclusion and exclusion criteria, bringing together women with similar clinical characteristics, subjected to the same tocolytic treatment with atosiban (and without confounding pathologies), making our results reliable. Also, it is an original study since the same determinations were made for each patient before and after treatment with progesterone. Moreover, as the samples were taken 24 hours after progesterone administration, we ascertained that the alterations in the Treg cell *pool* are due to that treatment, and not to normal evolutionary changes in pregnancy.

Therewithal, another strong point of our research lies in the fact that this is an innovative report on humans regarding the existence of mPRα in Treg cells, which is of utmost importance since it opens up a promising field in immunology, as it may help to explain more exactly progesterone's actions on PTL, allowing its more rational, widespread and effective usage.

Furthermore, as all methods are thoroughly described, the results are open to reproducibility studies by other research groups.

Limitations

Nevertheless, there are limitations in our study that need to be considered.

The different antibody panel chosen to characterize our populations is controversial (as discussed previously) and the small number of samples means our results should be inferred with caution.

Initially $CTLA_4$ and HLA DR were also taken into account as recent research established their link to the immunosuppressive phenotype of induced activated Treg cells. Unfortunately, due to limited budget we could not meet the expense of applying them in our investigation.

Moreover, the functionality of the Treg cell pool of those patients should be analyzed.

Our attempt to validate the outcomes by using various procedures was inconsistent, prompting the urge for further research.

Chapter VI - Conclusion and Benefits of intervention

Preterm labour is one of the major causes of neonatal morbidity and mortality worldwide.

In Portugal, of the 82797 live born children in 2013, 7.9% of them were premature, which corresponds to 6476 babies .

In the quest for a novel agent in PTL treatment, progesterone emerges as a good candidate in clinical studies so far, due to its immune modulator action, supposedly acting as the critical regulator of Treg cells during pregnancy, by an unknown mechanism.

This research demonstrated a significant increase in the Treg cell *pool* after progesterone treatment, indicating a possible mechanism by which its beneficial role in PTL is achieved. Also, it confirmed the importance of the immunosuppressive cytokines IL-10 and TGF-β in attaining containment of the immunoinflammatory phenomenon, thought to prompt PTL.

Exceeding our primary presumptions, we also attested the existence of mPRα in Treg cell pool during human pregnancy, with staggering results revealing a decrease in $mPR\alpha^{+}$ Treg cells after progesterone treatment.

This knowledge clarifies our understanding of therapeutic clinical outcomes of progesterone, enabling obstetricians to apprehend its role on labour, providing validation for the clinical investigation and establishment of secure and safe clinical protocols worldwide in PTL.

Additionally, it prompts a new strategy in PTL treatment, conveying the benefit of the combination of tocolytic therapy with progesterone.

This will ultimately allow universal obstetrical progesterone prescription in PTL,

both in prevention and in treatment.

In due course, this strategy will hopefully allow the reduction of pregnant women's internments and of children's intensive care requirements, with a subsequent reduction in children's handicaps and health costs.

Chapter VII - Future Research

In a near future it would be desirable to authenticate our results using *Western Blot* and *RT-PCR*, surpassing the constraint of minor blood populations, possibly by obtaining blood samples in superior amounts and from a higher number of preterm labour women. Moreover, meticulous methods are being studied and tested in our laboratory in order to achieve these intended purposes.

Following all that was stated regarding our in vitro experiments, it would be imperative to use a specific mPRα agonist and antagonist, so as to verify the functionality of this receptor in Treg cells from pregnant women.

Finally, exceeding the capacity of this study, but being already drawn as a future strategy, we will verify the mechanisms more likely behind progesterone actions on preterm birth using microRNA and gene expression profiling on cell sorted enriched suspensions of Tregs obtained from in vitro experiments, corroborating their intervention in vivo.

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Membrane progesterone receptors in human regulatory T cells: a reality in pregnancy

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Objective To provide evidence of the existence of membrane progesterone receptor alpha (mPRα) on regulatory T cells (Treg) in peripheral blood during pregnancy, postulating a possible explanation for the effect of progesterone on preterm birth.

Design Cross-sectional study.

Setting Tertiary Obstetric Department in a University Hospital.

Population Healthy pregnant women.

Methods Treg cells from peripheral blood samples were studied by flow cytometry using multiple monoclonal antibody expression.

Main outcome measures Evaluate the number and percentage of $CD4^+CD25^{high}CD127^{low}$, the number and percentage of Treg cells among the total $CD4^+$ T cells, and the percentage and mean fluorescence intensity (MFI) of mPRα in that population, using several gating strategies.

Results 43 peripheral blood samples were collected from healthy women during pregnancy, whose median gestational age was 28.7 ± 7.1 (16–40) weeks. The percentage of $CD4^+$ in the total lymphocytes was 43% (32–51) and the percentage of $CD4^+CD25^{high}CD127^{low}$ was 4.8% (1.6–5.9), with only 45% (16–72) of those cells expressing the intracellular marker FoxP3 (Treg cell pool). We confirmed the existence of mPRα in that specific population because 8.0% (2.02–33) of the Treg cells were marked with the specific monoclonal antibody, with an $mPR\alpha^+$ MFI of 719 (590–1471).

Conclusions This research shows that Treg cells express mPRα during pregnancy, which might play an important role in immune modulation by progesterone.

Keywords Pregnancy, progesterone receptor, T regulatory cells.

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Introduction

It is commonly accepted that the act of giving birth is the final step in a pro-inflammatory signalling cascade. Consequently, the inflammatory process plays a pivotal role in the triggering of human labour both in term and in preterm birth (PTB).[1]

Maternal acceptance of the fetus during pregnancy results from T-cell tolerance rather than immunosuppression. However, there is strong evidence that maternal T cells are not exposed to fetal alloantigens and that changes in the production of progesterone play a major role in modulating local immunosuppression.[2]

The abundance or modulation of systemic regulatory T cells (Treg) could be involved in pregnancy complications.[3,4] However, it is not known whether the Treg suppressive mechanism is specific to PTB or if it is also involved in spontaneous normal term birth.[5] Progesterone has a major role in pregnancy maintenance and its secretion has been demonstrated in the amnion, chorion and decidua in humans.[6,7]

In the 1st trimester, progesterone is critical to pregnancy preservation until the placenta takes over this function. In later pregnancy, however, its function is less clear.[8] Although progesterone levels in the maternal circulation do not change significantly in the weeks or days preceding labour, the onset of labour is associated with a functional withdrawal of progesterone activity.[8,9]

There are several unanswered questions surrounding the role of progesterone in human pregnancy. Of these, the questions of what molecular mechanisms support progesterone action during pregnancy and what molecular

changes turn off progesterone signalling and allow parturition, are the most intriguing.[10] In the quest for a novel agent in PTB treatment, progesterone emerges as a good candidate due to its immunomodulatory action.[11] Although the exact mechanism of its immunomodulatory role is still unknown, reports demonstrate its rapid effects on human T cells.[2,12]

The extranuclear activity of progestins was identified to be mediated by an alternative membrane-localised progesterone receptor (mPR), which may be responsible for the rapid cell activation prompted by progesterone[11,13] and progesterone interaction with the immune system.[14]

The function of one of these receptors, mPRα, has been investigated[15] but its expression on specific subsets of immune cells has hardly been demonstrated.[12,16]

It is thus tempting to infer that mPRα is the mechanism by which progesterone regulates Treg cells, explaining progesterone actions during pregnancy and PTB. The aim of this investigation is to ascertain whether mPRα is present on Treg cells in peripheral blood during pregnancy.

Methods

We undertook a cross-sectional study of healthy women attending normal prenatal appointments at our Obstetrics Unit between December 2013 and May 2014. Exclusion criteria consisted of multiple gestation, pre-existing disease, preterm rupture of membranes, chorioamnionitis, placenta *praevia*, placental abruption, clinical signs of infection (maternal temperature ≥37.5°C, white blood cells ≥15 000 cells/mm^3 in maternal blood) or use of hormone therapies within 3 months before enrolment.

Gestational age was assessed by date of last menstrual period or by ultrasound performed in the first trimester.

The investigation was approved by the Ethical Committees of Coimbra University and Coimbra University Hospital and informed consent was obtained from each participant.

Specimen collection

Peripheral venous blood samples were obtained and collected in lithium heparin tubes. Samples were kept in a cool environment until being processed within 1 hour of collection.

Flow cytometry staining

In brief, 100 ml of whole blood containing 0.5–1 × 10^6 white blood cells was placed in a clean test tube and stained to localise the mPRα receptor on the cell surface, using the N-terminal mPRα antibody described by Thomas et al.[17] Cells were first incubated in a blocking solution [0.5% bovine serum albumin {BSA}], in phosphate-buffered saline (PBS) for 30 minutes to 1 hour and then incubated with the mPRα antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for a further 30 minutes to 1 hour. Cells were washed with PBS 0.5% BSA and incubated for 30 minutes with Cruz Fluor 488 goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Inc.) at room temperature in the dark. Cells were washed with the blocking solution, and the surface was stained with PB conjugated anti-CD4, PE-Cy7 conjugated anti-CD25, and PerCP-Cy 5.5 conjugated anti-CD127.

Subsequently, intracellular staining for detection of FoxP3 was performed using AF647 labelled anti-human FoxP3 (Biolegend, San Diego, CA, USA) and the staining set (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Flow cytometry data were acquired on a FACS Canto II instrument (BD Biosciences, San Jose, CA, USA) equipped with three lasers to allow multicolour detection with different fluorophors, using FACS DIVA software (BD Biosciences).

Lymphocyte populations were selected according to the forward angle (FSC-A) and side angle (FSC-H) scattering signal, and at least 50 000 gated lymphocyte cells were detected for each sample. Dead cells were excluded by forward and side scatter characteristics and an FSC-A versus FSC-H dot plot was used to discriminate doublets, detecting disparity between cell size versus cell signal.

Isotype control antibodies were used to help assess the level of background staining, as well as samples without staining and single stain, for each antibody.

Treg analysis and mPRα expression

Gating strategies were employed to evaluate the percentage of $CD4^+CD25^{high}CD127^{low}$ cells, the percentage of Treg cells in total $CD4^+$ T cells, and the percentage and mean fluorescence intensity (MFI) of mPRα in that population.

Our gating strategy for identifying the Treg population was based on a total lymphocyte gate based on a FSC/Side light scatter (SSC) dot plot followed by doublet discrimination with an FSC-A versus FSC-H dot plot. Accordingly, CD4-positive cells were gated over SSC characteristics; depending on CD25 and CD127 expression, $CD4^+$ cells were gated based on the expression of $CD25^{high}$ and $CD127^{low}$ markers, and the $CD4^+CD25^{high}CD127^{low}$ population was detected. As the literature varies as to the markers for the exact phenotype for a Treg cell population, we moved on to the $CD4^+CD25^{high}CD127^{low}$ population, and also searched for $FoxP3^+$ cells. In the $CD4^+CD25^{high}CD127^{low}FoxP3^+$ (regulatory T-cell population), the $mPR\alpha^+$ subset was identified and characterised by percentage and mean fluorescence intensity (MFI).

The statistical analysis was based on at least 15 000–20 000 gated $CD4^+$ cells. FLOWJO software (Tree Star Data Analysis Software, Ashland, OR, USA) was used for the flow cytometry analysis.

Real time PCR and Western blot analysis

For mPRα assessment by RT-PCR and Western blot, blood samples were submitted to Ficoll-hypaque density gradient centrifugation to obtain peripheral blood mononuclear cells (PBMCs). PBMCs were then collected under optimal conditions to ensure high purity samples. Part of the PBMCs were lysed in RNeasy RLT lysing buffer (Qiagen, Austin, TX, USA) and frozen at −80°C until RNA extraction. The rest of the cells were treated with RIPA buffer and completed with protease inhibitors, which enables rapid and efficient cell lysis and solubilisation of proteins until subsequent Western blot assays. Both techniques were performed based on protocols previously described by Ndiaye et al.,[18] Thomas et al.[17] and Dosiou et al.[16] RT-PCR results were analysed in a Light Cycler 480 (Roche Instruments).

Statistical analysis

Data were analysed by IBM® SPSS 21 Statistics software (IBM Corporation, Armonk, NY, USA) and data are expressed as mean ± standard deviation (SD) or median and interquartile range (IQR) values, as appropriate for the type of distribution.

Using the nonparametric Mann–Whitney *U*-test, statistical comparison were made between groups of the total number and percentages of $CD4^+CD25^{high}CD127^{low}$ of $CD4^+$ T cells, the total number and percentages of the Treg cell subset within the total $CD4^+CD25^{high}CD127^{low}$ population, and the total number and percentages of $mPR\alpha^+$ Treg cells, and the MFI between the different women's characteristics (parity and gestational age) was determined. Statistical significance was considered for a P value <0.05. There were no missing data in our population sample.

Results

A total of 43 peripheral venous blood samples were extracted from healthy pregnant women with a median gestational age of 28.7 ± 7.1 (16–40) weeks, divided between 2nd trimester (42%; $n = 18$) and 3rd trimester (58%; $n = 25$). Clinical data of the population are shown in Table 1.

First, $CD4^+$ T cells were gated and analysed for the expression of CD25 and CD127; subsequently, the number and percentage of $CD4^+$ $CD25^{high}$ $CD127^{low}$ cells were estimated for all participants. Afterwards, Treg cells were characterised by the expression of FoxP3 and mPRα to estimate both the percentage and absolute number of Treg cells and the $mPR\alpha^+$ expression on those Treg cells. The MFI of $mPR\alpha^+$ on Treg cells was estimated for all participants.

Figure 1 (A,B in the main article, Figure S1a–d) shows our flow cytometric gating strategy for the $CD4^+CD25^{high}CD127^{low}FoxP3^+$ population (regulatory T-cell population) in peripheral blood.

Table 1. Clinical data

Variable	Value
Age (Years)	
Mean ± SD (min–max)	30 ± 4.8 (21–37)
Gestational age (weeks)	
Mean ± SD (min–max)	28.7 ± 7.1 (16–40)
$n = 43$	
Nullipara (n, proportion)	29 (67%)
2nd Trimester (n, proportion)	18 (42%)
3rd Trimester (n, proportion)	17 (39%)
Delivery (n, proportion)	8 (19%)

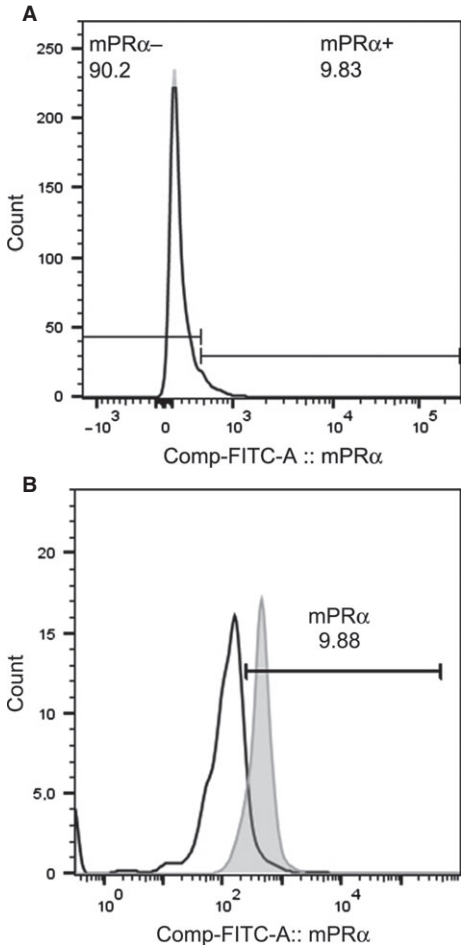

Figure 1. Flow cytometric gating strategy for $CD4^+CD25^{high}CD127^{low}FoxP3^+$ Treg analysis in peripheral blood. Peripheral blood lymphocytes were stained with FITC-labeled anti-mPRα, APC-labelled anti-FoxP3, PE-Cy7-labeled anti-CD25, PerCP-Cy 5.5-labeled anti-CD127 and PE-labelled anti-human CD4 antibodies. A, FITC anti-mPRα histogram: percentage of $mPR\alpha^+$ subset within the total $CD4^+CD25^{high}CD127^{low}FoxP3^+$Treg cell pool. B, Isotype control for mPRα.

Table 2 shows the absolute number and percentage of the different populations studied, in the normal course of pregnancy.

As the results show, the percentage of $CD4^+$ in the total lymphocytes was 43% (32–51) and the percentage of $CD4^+CD25^{high}CD127^{low}$ was 4.8% (1.6–5.9), with only 45% (16–72) of those cells expressing the intracellular marker FoxP3 (Treg cell pool).

We were able to verify the expression of mPRα in that specific population, as 8.0% (2.0–33) of those Treg cells were positive for this marker, with an $mPR\alpha^+$ MFI of 719 (590–1471).

To ascertain whether the number or percentage of $CD4^+$ cells, $CD4^+$ $CD25^{high}$ $CD127^{low}$, Treg cells and $mPR\alpha^+$ Treg cells varied with different clinical characteristics, a subgroup analysis was done, as shown in Supporting Information Table S1. The clinical characteristics analysed compared with others were as follows: parity (nullipara if it was the first pregnancy); 2nd trimester (14–27 weeks); 3rd trimester (≥28 weeks); delivery date.

The percentage and absolute number of $CD4^+CD25^{high}CD127^{low}$ was elevated in women in the 3rd trimester or at delivery date ($P = 0.001$), with the highest levels at delivery date ($P = 0.04$ and $P = 0.007$, respectively).

The percentage and absolute number of Treg cells were higher in women in the 3rd trimester, with the strongest difference shown in the percentage of Treg cells ($P = 0.02$).

Finally, the percentage of $mPR\alpha^+$ Treg cells was higher in the nulliparas ($P = 0.026$) and there was an increase in the absolute number of $mPR\alpha^+$ Treg cells from the 2nd to the 3rd trimester of pregnancy, although this was not statistically significant (0.25 versus 1.22, $P = 0.08$, respectively). No other comparisons between groups had statistical significance, although a trend towards a higher number of $CD4^+$ cells could be perceived in the date of delivery ($P = 0.058$).

Western blot experiments designed to examine mPRα protein expression showed the presence of a protein about 40 kD in size in our PBMC samples. Representative results of two independent experiments are shown in Figure 2.

Moreover, expression of mPRα mRNAs was detected by RT-PCR using mPRα-specific primers.

Table 2. Absolute number and percentage of the different populations studied

n = 43	$CD4^+$ (total lymphocytes)	$CD4^+$ $CD25^{high}$ $CD127^{low}$ (in $CD4^+$ T lymphocytes)	Treg cells (in $CD4^+$ $CD25^{high}$ $CD127^{low}$)	$CD4^+$ $CD25^{high}$ $CD127^{low}$ $FoxP3^+$ $mPR\alpha^+$
% Cells				
Median	43	4.8	45	8.0
IQR	(32–51)	(1.6–5.9)	(16–72)	(2.0–33)
Absolute number*				
Median	959.9	42.91	11.5	0.98
IQR	(302.1–1517.4)	(3.23–86.2)	(1.07–36.5)	(0.08–2.55)
MFI				
Median	–	–	–	719
IQR				(590–1471)

IQR, interquartile range; MFI, mean fluorescence intensity.
*number cells/μl blood.

Discussion

Progesterone has been known to play an important role in the reproductive tract for the initiation and continuation of pregnancy, with good results in the prevention of spontaneous abortion and recently in preterm labour. Nonetheless, progesterone-mediated responses are complex because they are mediated by multiple types of receptors.[19]

Undoubtedly this steroid is able to prevent the maternal immune system from activating effector T-cells capable of attacking fetal cells, resulting in a T-cell tolerance during pregnancy.[2] Recent data suggest that progesterone may be important in maintaining uterine quiescence in the latter half of pregnancy by limiting the production of stimulatory prostaglandins and inhibiting the expression of contraction-associated protein genes within the endometrium.[8] However, the exact route by which this is accomplished is still being researched.

Regulatory T cells were shown to expand during human pregnancy, with functional studies finding that they create a tolerant microenvironment through regulation of immune cell responses at the fetal–maternal interface.[20]

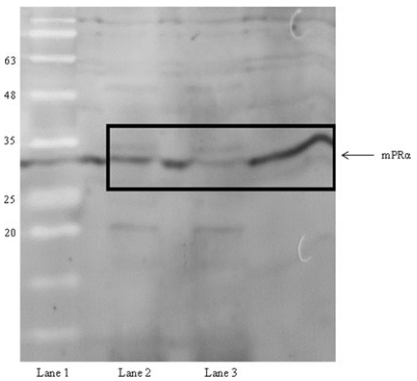

Figure 2. Representative results of Western blot analysis. Western blot analysis of mPRα expression in peripheral blood mononuclear cells (PBMCs): 20 μg/protein/lane; mPRα antibody concentration (1 : 2000). Lane 1 – molecular weight marker; kD. Lanes 2 and 3 – mPRα in PBMCs.

Since 1980, some groups have tried to identify expression of progesterone receptors during pregnancy, although with contradictory results.[21,22] Nevertheless, the gathering of scientific effort has enabled not only the presence of lymphocytic progesterone receptors[23] to be verified, but also validation of the existence of progesterone-induced blocking factor and its role in pregnancy.[24] Recently, some authors have attempted to demonstrate that the actions of progesterone on T lymphocytes are mediated by one or more putative membrane receptors, but all experiments were done in non-pregnant animal models.[18] Moreover, although receptors for oestrogens have been confirmed in Treg cells,[25] to the best of our knowledge progesterone receptors have not been studied in this subset of human cells.

Main findings

This research postulates a primordial role for mPRα in the intertwining between Treg cells and progesterone in human pregnancy.

In our work, we have shown the existence of mPRα in the Treg cell pool, with 8.0% of Treg cells being $mPR\alpha^+$, with an MFI of 719.

Some authors have indicated a significant decrease in $CD4^+$ T cells within the total leucocyte pool in spontaneous labour, which could indicate that a strong immune stimulation and subsequent apoptosis of the activated $CD4^+$ T cells may occur during labour.[5] When comparing our results with those published in the literature, this population remained almost unchanged throughout the whole pregnancy, with a slight increase on delivery day, which contrasts with published literature.

$CD4^+CD25^{high}CD127^{low}$ isolated Treg cells appear to be the best Treg population achieved regarding purity, function, stability and *in vitro* expansion capacity, promising isolation of pure Treg populations with high suppressive activity.[26] Our results were similar to published ones, with $CD4^+CD25^{high}CD127^{low}$ cells making up 4.8% (1.6–5.9) of the $CD4^+$ T-cell population.

However, the most widely accepted phenotype for Treg cells is the co-expression of CD4, CD25 [α-chain of the interleukin (IL)-2 receptor] and FoxP3.[27] We therefore assumed that the $CD4^+CD25^{high}CD127^{low}$ $FoxP3^+$ is the phenotype of the Treg cell pool.

FoxP3 is regarded as a lineage molecule for Treg cells and it is an intracellular marker. Consequently, it is very susceptible to degradation within a short space of time, and it is difficult to detetct and not really usable in large sample series. Moreover, $FoxP3^+$ T cells are phenotypically and functionally heterogeneous and involve both suppressive and non-suppressive T cells.[27]

Furthermore, CD127 was for a long period seen as an efficient tool to determine the phenotype and functional activity of Treg cells. Yet, there has been increasing controversy in comparisons with $CD4^+CD25^{high}FoxP3^+$ cells, particularly in the context of chronic infections.[27] However, no attention was given to it in the context of pregnancy. As such, it is currently accepted that CD127 expression inversely correlates with FoxP3 expression and suppressive activity of Treg cells .[27]

The data reported in the literature concerning the effect of pregnancy-specific hormones on FoxP3 expression by Treg cells are very contradictory.[5] The withdrawal of hormones at the end of pregnancy may affect FoxP3 expression by Treg cells, which was shown to be enhanced by progesterone in human studies.[3] Other authors postulate that progesterone, whose maximum levels are seen at the end of pregnancy, has the capacity to reduce FoxP3 expression by Treg cells *in vitro*.[25] Moreover, recent data indicate a significant decrease of Treg cells expressing FoxP3 in the 3rd trimester and women in labour at term.[5]

We therefore also determined FoxP3 expression in our research, making some comparisons feasible. Within $CD4^+CD25^{high}CD127^{low}$ cells, only 45% were Treg cells, corroborating the idea that Tregs in pregnant women have a reduced expression of FoxP3.[25]

Strengths

The strengths of our research lie in the fact that this is the first report on humans regarding the existence of mPRα in Treg cells, which is of utmost importance as it opens up a promising field in immunology and may help to explain more exactly the effects of progesterone on PTB, thereby allowing its more rational, widespread and effective use.

Moreover, as all methods are thoroughly described, the results are open to reproducibility studies by research groups.

Limitations

Nevertheless, there are limitations in our study that need to be taken into account. The different antibody panel chosen to characterise our populations is controversial (as discussed previously) and the small number of samples means our results should be considered with caution.

Interpretation

Controversies still persist that could not be solved by our results. The variation of the number of Treg cells during the three trimesters of human pregnancy is still under debate, with some authors reporting a rise in the 1st trimester with a peak in the 2nd trimester, and others reporting a reduction in the 2nd trimester.[28] In our study, the percentage and absolute number of $CD4^+CD25^{high}CD127^{low}$ cells was highest at delivery, suggesting the likely importance of activation of this population in the recrudescence of the inflammatory phenomenon nowadays believed to be labour. The results for Treg cells, with the highest

levels in the 3rd trimester, make this hypothesis even more reasonable.

Finally, the higher percentage of mPRα^{+}Treg cells in the nulliparas could be explained by the different physiological characteristics that differentiate circulating immune cells in pregnant women who have had a previous delivery.

Conclusions

We demonstrated the existence of mPRα in the circulating Treg cell pool. More information regarding its existence in the maternal–fetal interface (decidua) is necessary.

Further investigation should determine the functionality of this receptor and the mechanisms by which Treg cells modulate fetal protection and labour. Membrane progesterone receptor α might emerge as an instrument by which progesterone regulates Treg cells, allowing a rational recommendation for progesterone usage in PTB.

Disclosure of interests

The authors report no conflict of interest

Contribution to authorship

ALA and PM were responsible for recruiting the pregnant women and for data collection, evaluation and manuscript preparation. The other authors (SV-P, VA, PR-S, AM-P) were responsible for flow cytometry analysis, data collection, evaluation and manuscript revision.

Details of ethics approval

This is an original clinical research approved by the Ethical Committees of Coimbra University (2011) and Coimbra University Hospital (2013) and informed consent was obtained from each participant.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. (a) FSC versus SSC dot plot: lymphocyte gate. (b) SSC-A versus PE CD4 dot plot: $CD4^{+}$ population. (c) PerCP-Cy5.5 CD127 versus PE-Cy7 dot plot: $CD4^{+}CD25^{high}CD127^{low}$ population. (d) APC FoxP3 histogram to determine FoxP3 expression in $CD4^{+}CD25^{high}CD127^{low}$ population.

Table S1. Subgroup analysis of different blood populations in T cells among pregnant women. ■

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OBSTETRICS

Progesterone use after successful treatment of threatened pre-term delivery

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Pre-term delivery is the leading cause of neonatal morbidity, mortality and long-term sequels. This is an open label randomised controlled trial with women with confirmed threatened pre-term labour (TPTL) after efficient tocolytic therapy with atosiban. The main outcome measure of this study was the latency period until delivery and secondary outcomes were the number of recurrent episodes of TPTL and fetal and maternal morbidity. Patients were assigned to treatment or control groups using a computer generated randomisation table. The treatment group received 200 mg vaginal progesterone daily until delivery and the control group received no therapy or placebo. The study cohort comprised 52 pregnant women, 26 in each arm, showing similar characteristics; the treatment group had a longer latency period until delivery and this was statistically significant (55 vs 38 days, $p = 0.024$). This study points to the benefits of the vaginal administration of progesterone, especially in prolonging latency period until delivery.

Keywords: Atosiban, pre-term delivery, progesterone, randomised controlled trial

Introduction

Pre-term delivery (PTD) incidence (defined as delivery occurring before 37 complete weeks of gestation) has increased, despite multiple strategies being carried out to prevent it. Current estimates vary between 7% and 12% in developed countries and 22% and 26% in developing countries (Borna and Sahabi 2008; How and Sibai 2009; Rai et al. 2009; Tita and Rouse 2009).

Approximately 65–95% of neonatal deaths can be attributed to prematurity complications (Borna and Sahabi 2008; Rai et al. 2009). Even those children who survive a PTD have an increased incidence of sequelae, both short term (intraventricular haemorrhage, necrotising enterocolitis, respiratory distress syndrome, bronchopulmonary dysplasia and jaundice) and long term (asthma, deafness, cerebral palsy, retinopathy and psychomotor retardation) (Borna and Sahabi 2008; How and Sibai 2009; Tita and Rouse 2009).

Tocolysis is currently used in cases of PTD, merely to enable administration of two doses of betamethasone with a 24-h gap for lung maturation (complete steroid cycle), which undoubtedly modifies perinatal outcome (Borna and Sahabi 2008; How and Sibai 2009; Rai et al. 2009; Tita and Rouse 2009).

Pregnant women with threatened pre-term labour (TPTL) and successfully treated with tocolytic therapy are at an increased risk of new episodes of PTD (relapses) (Borna and Sahabi 2008). However, maintenance tocolytic therapy, after an effective tocolysis is of questionable efficacy, not only for prolonging pregnancy but also for affecting pregnancy results, regardless of the agent used (Borna and Sahabi 2008).

A meta-analysis published on PTD does not support the idea of using tocolytic maintenance therapy after the effective treatment of an episode of TPTL (Thornton 2005). However, progesterone, an agent with a proven ability to act to maintain uterine quiescence, seems a promising therapy (Borna and Sahabi 2008).

Most studies and meta-analyses of progesterone and PTD focus on its prophylactic administration by the vaginal (da Fonseca et al. 2003; Dodd et al. 2008; Fonseca et al. 2007; O'Brien et al. 2007), intramuscular (Dodd et al. 2005; Dodd et al. 2006; Mackenzie et al. 2006; Meis et al. 2003; Rouse et al. 2007; Sanchez-Ramos et al. 2005) or oral routes (Erny et al. 1986), in the presence of a previous pre-term delivery or short cervix, but further studies and more clinical trials are needed to achieve reliable results on the use of progesterone in women with PTD being treated with tocolytic therapy (Coomarasamy et al. 2006; Erny et al. 1986; Facchinetti et al. 2007; Tita and Rouse 2009).

The aim of this study was to determine if progesterone administration after successful tocolysis can prolong the latency period until delivery, reduce recurrence of TPTL and reduce fetal and maternal morbidity.

Materials and methods

A prospective, randomised controlled trial (treatment vs no treatment), was conducted with women with a singleton pregnancy between 24 and 34 weeks' gestation, who had a proven PTD arrested successfully by tocolytic therapy with atosiban.

This study was performed in the Obstetrics Department of Coimbra University Hospital between January 2008 and October 2010, after approval by the Ethics Committee of the Faculty of Medicine. To ensure ethical and deontological principles, the patients proposed for inclusion were asked for their informed consent. An interim analysis was conducted in 2011, to find some preliminary results because the inclusion rate was too slow.

Patient inclusion criteria were pre-term delivery successfully arrested with atosiban as the tocolytic agent, treated in our

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hospital's fetal–maternal medicine ward for PTD; a single fetus pregnancy; gestational age between 24 weeks + 6 days and 33 weeks + 6 days; intact membranes and a cervical length ≤ 25 mm. Pre-term delivery was defined as the existence of regular contractions (four contractions in 20 min or eight in 60 min), changes in cervical length (≤ 25 mm) or a positive fibronectin test.

Cervical length was measured by transvaginal ultrasound, with the bladder empty. The canal was measured in a straight line from the internal to the external os and at least three measurements were obtained, with the shortest recorded.

Exclusion criteria were: multiple pregnancies; cervical cerclage; pre-term premature rupture of membranes; suspected chorioamnionitis and the presence of placenta praevia.

On admission, all patients were submitted to an atosiban protocol (for 48 h) and a cycle of steroids for lung maturation (two intramuscular administrations of 12 mg betamethasone 24 h apart). The pregnant women underwent analytical studies that included a complete blood count, biochemistry with renal and liver function, C-reactive protein, urine culture and a vaginal swab. Also, an obstetric ultrasound was performed to evaluate cervical length and estimate fetal weight. All these tests were repeated each week, while the patients remained in the hospital. The chosen cervical length (values ≤ 25 mm) was based on data from a study of pre-term delivery prediction, in which this was the value for the 10th percentile in a low-risk population (Iams et al. 1996).

Simultaneously, any adjuvant measures deemed necessary were implemented such as bed rest, intravenous hydration, administration of lactulose, administration of low molecular weight heparin (for the increased thromboembolic risk caused by prolonged immobilisation) and passive physical therapy.

After diagnosing a PTD episode, patients were informed of the ongoing study and those who agreed to participate were randomly selected through a computer-generated randomisation table. Odds (progesterone) and evens (control) defined treatment allocation and the principal investigator managed the list; there was no allocation concealment.

Patients who were randomised to the treatment group received 200 mg of vaginal progesterone daily, starting on the day immediately after terminating atosiban and continued until the day of delivery. Patients randomised to the control group received expectant treatment.

The 200 mg progesterone dosage was chosen because it was believed that pregnant women with TPTL had a particularly high risk of recurrent episodes. The vaginal route was deemed to offer the most advantages for the study. During hospitalisation, progesterone was given by the hospital, and after discharge, it was purchased by the patients.

Monitoring of all patients evaluated was guaranteed both during hospitalisation and after discharge at antenatal appointments in our institution, at intervals suited to each clinical situation and according to the gestational age at the time of admission.

The primary outcome measure was the time until delivery (latency period), defined as the number of days between completing atosiban therapy and the day of delivery. The secondary outcome measures evaluated were the incidence of recurrent episodes of TPTL, and fetal and maternal morbidity. Recurrence of pre-term labour was defined as recurrence of contractions within 48 h of discontinuing atosiban intravenous treatment and arrest of contractions. Arrested pre-term labour was defined as a 12 h contraction-free period after intravenous therapy had been discontinued. Fetal morbidity was defined as the existence of intraventricular haemorrhage, necrotising enterocolitis and respiratory distress syndrome (RDS). A low birth weight infant was defined as weighing less than 2,500 g. Maternal morbidity was defined as the existence of haemorrhage (with subsequent anaemia) or infection.

To calculate the sample size, we used the results of the study by Borna and Sahabi (2008), where the mean latency period in days was 36.1 in the progesterone group and 24.5 in the control group. To obtain similar results with a power of 80% and a significance of 0.05, we would need 38 pregnant women per arm.

Categorical data were tested for significance with the χ^2- or Fisher's exact tests and continuous data were tested for significance with the Student's *t*-test or Mann–Whitney *U* test, according to their distribution, with a 95% confidence interval (CI). Statistical significance was defined as $p < 0.05$. Continuous data measures are reported as means and standard deviation (SD) or medians and interquartile range (IQR); categorical data are reported as total number and percentages.

All randomised patients were included in an intention-to-treat analysis.

Results

In total, 52 women admitted to our department met all the inclusion criteria and agreed to participate in the study. They were then randomly assigned to receive 200 mg of vaginal progesterone administration (26 women) or an expectant management (26 women). All pregnant women were compliant and none of the patients was lost to follow-up (Figure 1); this was achieved by thorough counselling and frequent contact with the participants.

The two groups were similar with respect to maternal age, gestational age at admission, PTD risk factors, cervical length on admission and abnormal vaginal swab or analytical tests; baseline characteristics of the patients are shown in Table I.

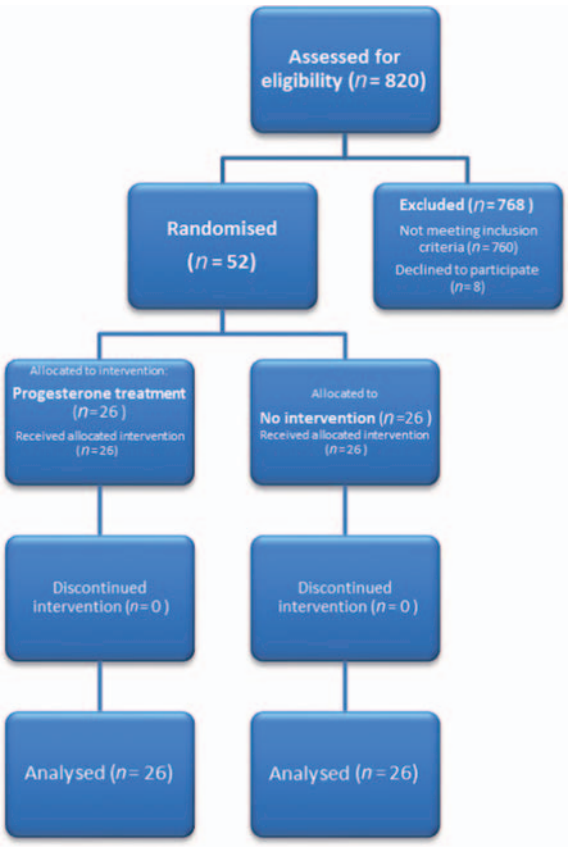

Figure 1. CONSORT statement 2010 flow diagram.

Table I. Maternal demographic and clinical characteristics at randomisation.

	Progesterone ($n = 26$)		Control ($n = 26$)		
Groups	*n*	(%)	*n*	(%)	*p* value
Age (years) (mean ± SD)	30.1 ± 4.5		28.38 ± 5.8		0.24[§]
Gestational age at admission (mean ± SD)					
Weeks	28.33 ± 2.78		29.41 ± 2.29		0.1[§]
Days	198.4 ± 19.5		206.4 ± 16.1		0.11[§]
Risk group*	9	34.6	9	34.6	1[#]
Cervical canal length (mm) (median, IQR)	18.31 (16–22)		18.46 (14–23)		0.8[¶]
Abnormal analytical tests[†]	6	23	8	31	0.5[#]
Abnormal vaginal swab[‡]	4	15.4	1	3.8	0.1[#]

SD, standard deviation; IQR, interquartile range. *Risk group: maternal age < 18 years; multiparity; smoking; low body mass index; occupation; assisted reproduction techniques; low socioeconomic status; previous pre-term delivery. [†]Leukocytosis > 15,000/mm^3 or C-reactive protein > 1.5 mg/dl. [‡]Positive culture. [§]Student's *t*-test, # χ^2 or Fisher's exact tests, [¶]Mann–Whitney *U* test.

The primary and secondary outcome measures in women with pre-term delivery are presented in Table II. In an intention-to-treat analysis and for the main outcome measure, the progesterone group demonstrated a statistically significant longer median latency period until delivery: 55.0 vs 38.0 days, $p = 0.02$.

For the secondary outcomes there was a non-statistically significant difference between the progesterone and the control groups in recurrent pre-term labour (7.7 vs 30.8%, $p = 0.07$) and no significant differences were found in fetal morbidity (19 vs 24%, $p = 0.67$). Neonates with respiratory distress syndrome occurred in two cases in each group (7.6%) and there were no cases of neonatal necrotising enterocolitis, congenital malformations or intraventricular haemorrhage.

There was a nearly statistically significant difference between the progesterone and the control groups in the gestational age at delivery (37.8 vs 36.6 weeks, $p = 0.07$).

No significant differences were found for low birth weight (38.5% vs 42.3%, $p = 0.78$), birth weight (2628 vs 2547 g, $p = 0.7$) and maternal morbidity (8% vs 8%, $p = 1.0$) between the progesterone and control groups, respectively.

There were no adverse effects related to progesterone treatment.

Discussion

Our study demonstrated that progesterone conferred a longer median latency period until delivery, after successful tocolysis.

Although the exact mechanism by which progesterone can exert the effect of uterine relaxation is still unknown, it is assumed that it is achieved through actions that include: (1) blockage of progesterone, prostaglandin F2α and α-adrenergic receptors; (2) deletion of genes necessary for contractility; (3) decrease in myometrial oxytocin receptors; (4) stimulation of myometrial relaxation systems (such as nitric oxide) and (5) blockage of the appearance of intercellular junctions (gap-junctions) (Rai et al. 2009).

Premature labour can be classified as spontaneous (40–50% of cases), after pre-term premature rupture of membranes (25–40% of cases) or iatrogenic, due to maternal, fetal or placental complications (pre-eclampsia, renal disease, diabetes mellitus with vascular disease, placenta praevia and intrauterine growth restriction) (How and Sibai 2009).

As the mechanisms involved in pre-term labour are complex and multifactorial, a tocolytic agent such as progesterone may not be effective for all patients.

In the literature there are only two trials and one meta-analysis on the use of progesterone after effective treatment of PTD.

In a study by Facchinetti and co-workers, published in 2007, pregnant women with PTD were randomised to receive intramuscular progesterone (17α-hydroxyprogesterone caproate) or an expectant management; their results suggest the efficiency of progesterone in combination with tocolytic therapy (RR 0.43, 95% CI 0.12–1.5). These results were promising and indicate the advantage of progesterone usage, as in our study, although the progesterone formulation and administration method differ.

Table II. Primary and secondary outcome measures in women with pre-term delivery.

	Progesterone ($n = 26$)		Control ($n = 26$)				
Groups	*n*	(%)	*n*	(%)	*p* value	RR	95% CI
Latency period (days) (median, IQR)	55 (40.5–71.2)		38 (13–58)		0.02[§]		
Recurrence of pre-term labour	2	7.7	8	30.8	0.07	0.25	0.06–1.07
Gestational age at delivery (mean ± SD)							
Weeks	37.8 ± 1.1		36.6 ± 2.4		0.07[¶]		
Days	254 ± 24.7		246 ± 24.6		0.20[¶]		
Birth weight (g) (mean ± SD)	2,547 ± 642		2,628 ± 829		0.70[¶]		
Low birth weight	10	38.5	11	42.3	1	0.91	0.47–1.76
Respiratory distress syndrome	2	7.7	2	7.7	1	1.00	0.15–6.57
Fetal morbidity	5	19.2	6	24	0.67	0.83	0.29–2.39
Maternal morbidity	2	7.7	2	7.7	1	1.00	0.15–6.57

SD, standard deviation; IQR, interquartile range; RR, relative risk. [§]Mann–Whitney *U* test, χ^2 or Fisher's exact tests, [¶]Student's *t*-test.

In another randomised study, published in 2008, Borna and Sahabi assessed the efficacy of tocolytic maintenance therapy with vaginal progesterone (400 mg daily) after PTD arrest vs no treatment. In their study, as in ours, the treatment group had a latency period until delivery 12 days longer than the control group (36 vs 24 days, $p = 0.04$) and a lower PTD recurrence (35 vs 58%, $p = 0.09$).

Our study also demonstrated that the progesterone group had a longer mean latency period until delivery (55 vs 38 days). Moreover, our results were better in terms of prolongation of pregnancy (a further 5 days) and very similar regarding recurrent pre-term labour and gestational age at delivery, despite the absence of statistical significance.

Nevertheless, there are several differences between our trial and the previous studies. In the first place, progesterone dosage was lower in our trial (200 mg vs 400 mg), demonstrating that the same benefits can be obtained with a lower dosage. The vaginal route was also preferred in our study because of the enhanced bioavailability (since it avoids the hepatic first pass effect); fewer side-effects (including sleep, fatigue, headache, swelling and nausea); more rapid absorption (taking about 4 h to reach peak plasma concentration of progesterone) and the additional benefit of progesterone's local effects on the endometrium (higher concentrations achieved, relaxation of myometrial smooth muscle, blockage of oxytocin action, inhibition of gap-junction formation). Additionally, it can be administered by the patient, both in the hospital and after discharge. It should be noted that the tocolytic agent used in our patients was atosiban, making this the first clinical trial where this agent was used consistently. Atosiban is an oxytocin antagonist and is a novel tocolytic agent with a high safety profile and better tolerance. Finally, no prophylactic antibiotics were prescribed, avoiding bias.

In the only published meta-analysis, Dodd et al. (2008) showed that the relative risk (RR) of PTD was significantly reduced below 37 weeks (one study, 60 women, RR 0.29, 95% CI 0.12 to –0.69).

The strength of our study is that the findings were obtained in patients after tocolysis was achieved with atosiban, using a progesterone dosage and formulation that is simple to administer and without undesirable side-effects. Also, this trial has a high applicability as PTD incidence is rising, despite multiple primary interventions being implemented in an effort to lower it; as such, the number of women worldwide who may benefit from this treatment is high. This progesterone therapy is also undemanding and cheap. Although we could demonstrate pregnancy prolongation in the treatment group, no other meaningful clinical outcome was achieved due to the small sample size.

Our trial does have some limitations that need to be mentioned. First, it was not double-blinded, as it was not possible to create a placebo similar to progesterone in our country; second, the sample size was small and the power to detect clinically important outcomes was reduced, since the study would need to last for several years in order to recruit the number of patients initially calculated.

It is extremely important to point out that each extra day in uterus before term conveys a significant reduction in morbidity, mortality and cost, both in the neonatal intensive care units and in the long term (Dodd and Crowther 2010).

In conclusion, this study indicates the benefit of maintenance tocolytic therapy with vaginal progesterone after successful tocolysis for pre-term labour, to be confirmed in a randomised clinical trial with a large enough sample size to achieve statistical significance.

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