

Th17/Treg balance in high-risk pregnancies

António José Costa Lima da Cunha Braga

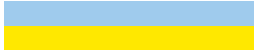


António Braga: Th17/Treg balance in high-risk pregnancies



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CICLO DE ESTUDOS
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List of international publications made within the scope of the thesis

Braga A, Barros T, Faria R, Marinho A, Carvalheira G, Rocha G, Farinha F, Neves E, Vasconcelos C, Braga J. **Systemic lupus erythematosus and pregnancy: A retrospective single-center study of 215 pregnancies from Portugal.** Lupus. 2021 Nov;30(13):2165-2175. doi: 10.1177/09612033211050340

Braga A, Barros T, Faria R, Marinho A, Rocha G, Farinha F, Neves E, Vasconcelos C, Braga J. **Systemic Lupus Erythematosus and Pregnancy: a Portuguese Case-Control Study.** Clin Rev Allergy Immunol. 2022 Apr;62(2):324-332. doi: 10.1007/s12016-021-08893-y

Braga A, Neves E, Guimarães J, Braga J, Vasconcelos C. Th17/Regulatory T cells ratio evolution: **A prospective study in a group of healthy pregnant women.** J Reprod Immunol. 2022 Feb;149:103468. doi: 10.1016/j.jri.2021.103468

Braga A, Neves E, Guimarães J, Braga J, Vasconcelos C. **The dynamics of Th17 / Treg ratio in SLE patients during pregnancy.** J Reprod Immunol. 2022 Jun;151:103622. doi: 10.1016/j.jri.2022.103622

Braga A, Neves E, Guimarães J, Braga J, Vasconcelos C. **Th17 / Treg ratio: A prospective study in a group of pregnant women with preeclampsia and fetal growth restriction.** J Reprod Immunol. 2023 Sep;159:104122. doi: 10.1016/j.jri.2023.104122

Abstract

Pregnancy is a transitory state where there are profound physiological changes that aim to sustain foetal growth. These changes are transversal to all maternal physiological systems, so the immune system is no exception. From nidation, trophoblastic and embryo-foetal growth requires a dynamic and complex maternal immune response that allows the mother to tolerate the development of a "transplant" full of paternal antigens. This immunological paradox, first described in 1953 by Medawar, is still shrouded in mystery, but the establishment of tolerance mechanisms seems to be vital to the development of a healthy pregnancy. The failure to establish a uterine environment that promotes immune tolerance has been associated with the development of obstetric pathology. Of the multiple mechanisms involved and already described, one of the most important seems to be linked to maternal T regulatory (Treg) and Th17 cells. In fact, a preponderance of the pro-inflammatory response mediated by Th17 cells has been associated with the establishment of obstetric complications such as miscarriage, preterm birth, or the development of hypertensive disorders of pregnancy. These findings are also partly found in patients with various autoimmune diseases. Systemic Lupus Erythematosus (SLE) is one of these pathologies. This syndrome, which is characterised by a multisystemic involvement that predominantly affects women of childbearing age, seems to have in its etiology the presence of an exaggerated pro-inflammatory response mediated by Th17 cells. Thus, since SLE frequently intersects with pregnancy, it was used as a study model in this thesis. The main objective of this thesis was to study Treg and Th17 cells populations in healthy pregnant women and in pregnant women with previous immunological pathology, in this case using SLE as a model. The relative frequency of these lymphocyte populations in pregnant women with obstetric pathology was also studied. To meet these objectives, the characterization of pregnant women in the cohort of patients with Lupus at our center was initially carried out. This study allowed us to review our reality as well as to describe the real risk of obstetric complications associated to SLE in Portuguese patients.

It was found that, even in the presence of controlled disease, the risk of developing obstetric complications is significantly higher in these patients. To allow the characterization of circulating T cells in pregnant women with autoimmune or obstetric pathology, the analysis of these cell populations was initially carried out in healthy pregnant women throughout pregnancy. We published the first prospective study with the quantification of Th17 and Treg lymphocyte populations throughout pregnancy and postpartum in healthy pregnant women. This study allowed us to identify the establishment of a probable pro-inflammatory state with a relative increase in the number of Th17 cells at the end of pregnancy and in the

postpartum period. These findings may help to explain the higher risk of developing inflammatory or immunological pathology in late pregnancy and in the puerperium, as well as may be important in the initiation of parturition mechanisms.

The evolution of the circulating number of these cell populations in pregnant women with SLE was also evaluated, throughout pregnancy to postpartum. This study allowed us to verify that the findings observed in healthy pregnant women were not observed in pregnant women with Lupus. We found that, in these patients, there was a pro-inflammatory state from the beginning of pregnancy. This fact may be justified by an intrinsic immune dysregulation present in patients with Lupus, or possibly by the influence of immunomodulatory therapy used by these patients. In pregnant women with no pathological history who developed pre-eclampsia and foetal growth restriction, we found that the diagnosis of these obstetric complications was associated with a relative increase in the number of Th17 cells, especially in the presence of maternal systemic involvement as in pre-eclampsia.

With our studies, it was found that pregnancy in SLE patients is associated with a high risk of adverse obstetric outcomes, even in the presence of controlled disease. A relative increase in Th17 cells count is associated with the development of obstetric pathology such as preeclampsia and foetal growth restriction. This thesis brings together a set of original publications, highlighting the first Portuguese case-control study published with pregnant women with Lupus, the first prospective study published with an evaluation of the variation in the number of Th17 and T reg cells in circulation in healthy pregnant women throughout pregnancy until postpartum, and the first published study with an evaluation of the number of these cells in pregnant women with foetal growth restriction.

Obstetrics still hides one of the greatest secrets of modern medicine, which is related to the lack of etiological knowledge of preeclampsia and placental insufficiency. The differences found in the Treg and Th17 lymphocyte populations described in this thesis point to a possible path to be explored, possibly with therapeutic implications that could aim to improve obstetric outcomes in pregnant women with previous autoimmune disorders or who develop obstetric pathology.

Keywords

Systemic lupus erythematosus, Pregnancy, Preeclampsia, Foetal growth restriction, Regulatory T cells, Th17 cells

Resumo

A gravidez é um estado transitório onde se verificam alterações fisiológicas profundas que têm como objetivo sustentar o crescimento fetal. Estas alterações são transversais a todos os sistemas fisiológicos maternos, pelo que, o sistema imunitário não é exceção. Desde a nidação, o crescimento trofoblástico e embrio-fetal obrigam a uma resposta imunitária materna dinâmica e complexa que permita à mãe tolerar o desenvolvimento de um “transplante” repleto de antígenos paternos. Este paradoxo imunológico, descrito pela primeira vez em 1953 por Medawar, ainda hoje se encontra envolto em mistério, mas o estabelecimento de mecanismos de tolerância parece ser fulcral ao desenvolvimento de uma gravidez saudável. A falência do desenvolvimento de um ambiente uterino promotor de tolerância imunológica tem-se associado à ocorrência de patologia obstétrica. Dos múltiplos mecanismos envolvidos e já descritos, um dos mais importantes parece relacionar-se com a atividade das células reguladoras T (Treg) e Th17 maternas. Efetivamente, uma preponderância da resposta pró-inflamatória mediada pelas células Th17 tem-se associado ao estabelecimento de complicações obstétricas como abortamento, parto prematuro ou desenvolvimento de patologia hipertensiva da gravidez. Estes achados são em parte também encontrados em doentes com diversas patologias autoimunes. O Lúpus Eritematoso Sistémico (LES) é uma destas patologias. Esta síndrome que se caracteriza por apresentar um envolvimento multissistémico que afeta predominantemente mulheres em idade fértil parece ter na sua etiologia a presença de uma resposta pró-inflamatória exagerada mediada por células Th17. Assim, uma vez que o LES se cruza frequentemente com a gravidez, esta patologia foi utilizada como modelo de estudo nesta tese.

O principal objetivo desta tese foi estudar as populações celulares T reguladoras e Th17 em grávidas saudáveis e em grávidas com patologia imunológica prévia, neste caso usando como modelo o LES. Foi ainda estudada a frequência destas populações linfocitárias em grávidas com patologia obstétrica. Para cumprir estes objetivos, foi inicialmente realizada a caracterização das grávidas do coorte de doentes com Lúpus do nosso centro. Este estudo permitiu conhecer a nossa realidade bem como descrever o risco real de complicações obstétricas associado ao LES em doentes portuguesas.

Verificou-se assim que, mesmo na presença de doença controlada ou em remissão, o risco de desenvolver complicações obstétricas é significativamente maior nestas doentes.

De forma a permitir a caracterização das células T circulantes em grávidas com patologia autoimune ou obstétrica foi inicialmente efetuada a análise destas populações celulares

em grávidas saudáveis ao longo de toda a gestação. Foi assim publicado o primeiro estudo prospetivo com a quantificação de populações linfocitárias Th17 e Treg durante toda a gestação e pós-parto em grávidas saudáveis. Este estudo permitiu verificar o estabelecimento de um provável estado pro-inflamatório por um aumento relativo do número de células Th17 na fase final da gravidez e no pós-parto. Estes achados podem ajudar a explicar o maior risco de desenvolvimento de patologia inflamatória ou imunológica no final da gravidez e no puerpério, bem como pode ser importante no estabelecimento dos mecanismos de parto.

Foi ainda avaliada a evolução do número circulantes destas populações celulares em grávidas com LES, durante toda a gravidez até ao pós-parto. Este estudo permitiu constatar que o comportamento observado em grávidas saudáveis não foi verificado nas grávidas com Lúpus, existindo desde o início da gestação um estado pró-inflamatório nestas doentes. Tal facto pode ser eventualmente justificado por uma desregulação imunológica intrínseca presente nas doentes com Lúpus, ou eventualmente a influencia de terapêutica imunomoduladora utilizada por estas doentes.

Nas grávidas sem antecedentes patológicos que desenvolveram durante a gravidez pré-eclampsia e restrição de crescimento fetal verificamos que o diagnóstico destas patologias obstétricas se associou a um aumento relativo do número de células Th17, especialmente na presença de envolvimento sistémico materno como na pré-eclampsia.

Com os nossos estudos, verificamos que a gravidez com LES se associa a um risco elevado de maus desfechos obstétricos, mesmo na presença de doença controlada. O aumento relativo de células Th17 associa-se ao desenvolvimento de patologia obstétrica como a pré-eclampsia e a restrição de crescimento fetal. Esta tese agrega em si um conjunto de publicações originais, destacando-se o primeiro estudo caso-controlo português publicado com grávidas com Lúpus, o primeiro estudo prospetivo publicado com avaliação da variação do número de células Th17 e T^{reg} em circulação em grávidas saudáveis ao longo de toda a gestação até ao pós-parto e o primeiro estudo publicado com avaliação do número destas células em grávidas com restrição de crescimento fetal.

Obstetrícia encerra em si ainda um dos maiores segredos da medicina moderna, que se prende com o desconhecimento etiológico da pré-eclâmpsia e da insuficiência placentária. As diferenças encontradas nas populações linfocitárias Treg e Th17 descritas nesta tese apontam para mais um eventual caminho a explorar, eventualmente com caris terapêutico com o objetivo de melhorar os desfechos obstétricos de grávidas com patologia autoimune prévia ou que desenvolvem patologia obstétrica.

Palavras-Chave:

Lúpus eritematoso sistêmico, Gravidez, Pré-eclâmpsia, Restrição do crescimento fetal, Células T reguladoras, Células Th17

Acronyms list

ACOG – The American College of Obstetricians and Gynecologists
ACR – American College of Rheumatology
AIDS – Acquired Immunodeficiency Syndrome
ANA – Anti-nuclear antibodies
Anti- β 2GPI – Anti- β 2-glycoprotein I
Anti-dsDNA – Anti-double-stranded DNA
Anti-La/SSB – Anti-Sjögren's-syndrome-related antigen B
Anti-Ro/SSA – Anti-Sjögren's-syndrome-related antigen A
APC – Antigen-presenting cell
aPL – Antiphospholipid antibodies
APO – Adverse pregnancy outcomes
APS – Antiphospholipid syndrome
ASA – Acetylsalicylic acid
CCL – CC-chemokine ligand
CCL2 - CC-chemokine ligand 2
CDC – Centers for Disease Control and Prevention
CHB – Congenital heart block
CXCL – Chemokine ligand
CXCL8 – CXC-chemokine ligand 8
CXCL10 – CXC-chemokine ligand 10
DCs – Dendritic cells
DNA – Deoxyribonucleic acid
dNK – decidual NK cells
ECD – Phycoerythrin-Texas Red
EULAR – The European Alliance of Associations for Rheumatology
FGR – Foetal growth restriction
FITC – Fluorescein isothiocyanate
FOXP3 – X-chromosome-encoded transcription factor Forkhead box P3
FSC – Forward scatter
hCG – human chorionic gonadotropin
HCQ – Hydroxychloroquine
HDP – Hypertensive disorders of pregnancy
HELLP – Hemolysis, Elevated Liver enzymes, and Low Platelet count
HIV – Human immunodeficiency virus
HLA – Human leukocyte antigen

IADPSG – The International Association of the Diabetes and Pregnancy Study Groups
ICU – Intensive care unit
IFN- γ – Interferon gamma
IL – Interleukin
IL1 β – Interleukin 1 β
IL-1RA – Interleukin-1 receptor antagonist
IL2 – Interleukin 2
IL4 – Interleukin 4
IL6 – Interleukin 6
IL10 – Interleukin 10
IL12 – Interleukin 12
IL15 – Interleukin 15
IL17a – Interleukin 17^a
IL18 – Interleukin 18
IL21 – Interleukin 21
IL22 – Interleukin 22
iTreg – Induced regulatory T cells
LMWH – Low molecular weight heparin
mAbs – monoclonal Antibodies
NA – Not available
NIH – National Institutes of Health
NK cells – Natural Killer cells
NS – Not significant
nTreg – natural regulatory T cells
PBS – Phosphate-buffered saline
PE – Pre-eclampsia
PE-anti-CD4 – Phycoerythrin-anti-CD4
PE-anti-CD56 – Phycoerythrin-anti-CD56
PE-anti-FOXP3 – Phycoerythrin-anti-FOXP3
PE-anti-IL-17A – Phycoerythrin anti-IL17a
PE-Cy5 – Phycoerythrin-cyanin 5.1
PGE2 - Prostaglandin E2
PIGF – Placental growth factor
PMA – Phorbol 12-myristate13-acetate
PTD – Preterm delivery
RBC – Red blood cells
ROR – Receptor-related orphan receptor

RT – Room temperature
sFlt-1 – soluble Fms-like tyrosine kinase 1
SLE – Systemic lupus erythematosus
SSC – Side scatter
TCR – T cell receptor
TGF – Transforming growth factor
TGF β – Transforming growth factor beta
Th – T helper lymphocytes
Th1 – T helper lymphocytes 1
Th2 – T helper lymphocytes 2
Th17 – T helper lymphocytes 17
TNF – Tumor necrosis factor
TNF- α – Tumor necrosis factor alpha
Treg - Regulatory T cells
ULSSA – Unidade Local de Saúde de Santo António
uNK – uterine NK cells
vs – *Versus*

Chapter I

Introduction

Introduction

1 – Background

1.1 – Immune tolerance and pregnancy

Pregnancy is a challenging state for the immune system. Since the beginning of gestation, the maternal immune system must tolerate the development of a semi-allogeneic trophoblast; otherwise, the success of the current gestation will be compromised. The foetus inherits half of its genetic material from the father, exposing foreign antigens to the mother. The natural pregnancy progression requires the maternal immune system to tolerate the exposed foetal antigens and then accept a healthy trophoblast growth. Medawar first recognised this immunological paradox in 1953 (Medawar, 1953) and despite the extensive research conducted to date, this phenomenon is not yet fully understood. In this paradigm, the placenta is a natural "transplant". The maternal immune system experienced a highly dynamic and regulated transformation throughout the three trimesters of pregnancy. During this process, it must avoid the rejection of the semi-allogeneic foetus and placenta while remaining competent to fight infections. The breakage of maternal immune tolerance seems to be associated with the development of adverse pregnancy outcomes (APO) like miscarriage, pre-eclampsia (PE), or preterm delivery (PTD) (Cao et al., 2015; Chen et al., 2022; Cornelius et al., 2013; Figueiredo and Schumacher, 2016; Roomandeh et al., 2018; Saifi et al., 2014; Saito et al., 2023; Zare et al., 2018).

An abnormal trophoblast growth and differentiation leads to an inadequate placentation. This is a significant fact in the pathogenesis of miscarriage, PE, and foetal growth restriction (FGR) (Darmochwal-Kolarz et al., 2012b; Figueiredo and Schumacher, 2016; Saito, 2010; Saito et al., 2023; Toldi et al., 2012; Wang et al., 2020). The trophoblast invasion necessary to develop a fully functional placenta demands an intricate network of immune tolerance that allows the growth of cells expressing paternal antigens through the maternal decidua. Several mechanisms with diverse protagonists are involved in this complex tolerance game. Nevertheless, regulatory T cells (Treg cells) and effector T cells that produce interleukin (IL) 17a (Th17 cells) appear to have a critical role in these tolerance mechanisms throughout pregnancy (Cornelius et al., 2013; Figueiredo and Schumacher, 2016; Saifi et al., 2014; Zare et al., 2018).

Nidation is the critical first step in placentation. Evidence has shown that inflammation is necessary for successful embryo implantation. After fecundation, the uterus is receptive to embracing the implanting blastocyst during the implantation window. During this window,

immune cells are recruited to the implantation site and produce pro-inflammatory signals in conjugation with endometrial stromal cells. These are prostaglandin E2 (PGE2), pro-inflammatory cytokines, such as IL-6, IL-15, tumour necrosis factor (TNF), and chemokines, including CXC-chemokine ligand 8 (CXCL8), CXCL10 and CC-chemokine ligand 2 (CCL2). This pro-inflammatory response seems essential to ensure a successful nidation (Erlebacher, 2013; K et al., 1995; Mor et al., 2011; Piccinni et al., 2021; Racicot et al., 2014; Vilella et al., 2013; Woon et al., 2023). Also, it has been demonstrated that IL-15 and IL-18 can stimulate the activation of uterine natural killer cells (uNK), which also seem to have an essential role in regulating the inflammatory process during implantation (Hanna et al., 2006; Woon et al., 2023). In addition, TNF- α , IL-1 β , IL-12, and Interleukin-1 receptor antagonist (IL-1RA) are involved in the trophoblast invasion and extracellular remodeling that characterised the implantation and placenta development (Dimitriadis et al., 2005; Karmakar et al., 2004; Lédée et al., 2011; Piccinni et al., 2021; Woon et al., 2023). In *in vitro* models of implantation, TNF α is a critical inflammatory cytokine that induces the expression of inflammatory factors by endometrial stroma cells, which intervene directly in the trophoblast differentiation, migration and invasion into the uterine compartment (You et al., 2021). It was also demonstrated that TNF α induces the uterine production of IL-17. IL-17 pathway is enriched in trophoblasts during implantation and IL-17 interacts with these cells and induces the expression of genes responsible for promoting trophoblast migration and invasion (Gonçalves et al., 2018; You et al., 2021). This data has shown that a pro-inflammatory environment is essential for effective embryo implantation.

Nonetheless, this inflammatory process needs to be tightly coordinated. The failure of the equilibrium between pro and anti-inflammatory mediators seems to be the genesis of implantation failure and poor placentation that could justify several obstetric syndromes (Clark, 2016; Hosseini et al., 2018; Lédée et al., 2016; Lee et al., 2011; Piccinni et al., 2021; Rafiee et al., 2015a; Saito, 2010; Saito et al., 2023; Wang et al., 2013, 2020). In a normal pregnancy, this early proinflammatory profile is followed by a shift toward an anti-inflammatory profile, characterised by an increase in IL-10. Recent findings showed that the IL-10 to TNF α ratio was significantly higher during early gestation in patients with normal pregnancies than in patients with pregnancy loss (Kaislasuo et al., 2021). A central unanswered question in reproductive immunology is which factors regulate or promote the shift from pro-inflammatory to anti-inflammatory profile and how the absence or incomplete shifting may affect normal placental and foetal development.

At the onset of pregnancy, semen seems to be very important in modulating the maternal immune response and promoting a foetus friendly environment (Schjenken and Robertson, 2015; Schumacher and Zenclussen, 2015). In the same way, since the beginning of gestation, the production of human chorionic gonadotropin (hCG) also seems to be

fundamental for the function and differentiation of T cells, B cells, and dendritic cells to an anti-inflammatory and tolerogenic profile (Dauven et al., 2016; Saito et al., 2023; Sauss et al., 2018; Schumacher et al., 2013, 2009). The trophoblast invasion and development demand an intricate network of immune tolerance that allows the growth of cells expressing paternal antigens through the maternal decidua. One mechanism that helps trophoblasts to evade recognition by the maternal immune system is the lack of the polymorphic human leukocyte antigen (HLA)-A, HLA-B, and HLA class II molecules on their cell surface (Krop et al., 2020). The classical HLA class I molecules HLA-A, -B, and -C are present on virtually all nucleated cells and present intracellular antigens to surveilling T-cells. Non-classical HLA molecules are selectively present and have been described on trophoblasts (Ellis et al., 1990) and other tissues (Cirulli et al., 2006; Le Discorde et al., 2003; Mallet et al., 1999). HLA class II is mainly expressed by antigen-presenting cells (APCs), including dendritic cells (DCs), macrophages and B cells. Since the foetus inherits half of its genes from the father, it also inherits half of the paternal HLA alleles, which can potentially be recognised as foreign by the maternal immune system (Krop et al., 2020). In order to evade recognition by the maternal immune system, trophoblast cells lack the expression of HLA-A and HLA-B and express polymorphic HLA-C, which possibly also leads to allorecognition of the inherited paternal HLA-C by maternal T cells (Krop et al., 2020; Papúchová et al., 2019). Also, they express non-classical HLA-E, HLA-G, and possibly HLA-F (Apps et al., 2009; Hackmon et al., 2017). Expression of these HLA molecules allows NK cell recognition, and the presentation of non-classical HLA has important tolerogenic properties to evade the immune destruction of these cells (Hunt et al., 2005; Rouas-Freiss et al., 1997). The mechanisms responsible for the presence or absence of the specific HLA I types on trophoblasts have not been fully elucidated (Krop et al., 2020). Despite these mechanisms, foetus-specific immune recognition has been observed. Therefore, to inhibit the effect of maternal immune rejection, it is thought that local immune regulation is also required to prevent anti-foetal immunity (Moldenhauer et al., 2010).

Foetal cells and the composition of maternal immune cells are essential for foetal tolerance. Even before conception, since seminal plasma exposure, activation and proliferation of foetus-specific maternal T cells have been observed in uterine-draining lymph nodes (Guerin et al., 2011; Moldenhauer et al., 2009; Robertson et al., 2013). Maternal CD8⁺ T cells are also recruited in the ectocervix upon coitus, but their specificity remains unknown (Sharkey et al., 2012). In the first trimester of gestation, 30-40% of all cells in the decidua are leukocytes (Bulmer et al., 1991). During this period, the most noticeable immune cells are decidual NK (dNK) cells (~60%), macrophages (~20%), and T cells (~10%) (Bulmer et al., 1991; Heikkinen et al., 2004; Williams et al., 2009). With the development of placenta, T cell lymphocytes count increase, dNK cell number decreases and macrophage quantity

remain relatively stable (Williams et al., 2009). Foetus-specific CD8⁺ T cells and inherited paternal antigen-specific antibodies are found in maternal peripheral blood during pregnancy (Nakajima et al., 1982; Regan et al., 1991; Regan and Braude, 1987). Maternal CD8⁺ T cells may recognise the paternally inherited HLA-C from the foetus and, if not suppressed, are likely to attack foetal trophoblasts (Lissauer et al., 2012; Tilburgs and Strominger, 2013). Besides this, ~30% of pregnancies result in the production of paternal HLA-specific IgG antibodies (Regan et al., 1991; van Kampen et al., 2001). Alloantibodies against foetal HLA-C have been associated with spontaneous PTD and recurrent pregnancy loss (Lee et al., 2013; Meuleman et al., 2016). Consequently, to inhibit trophoblast aggression by the maternal immune system, it is stated that local immune regulation is essential to prevent anti-foetal immunity and poor placental development. The establishment of an acquired immune response tailored to the trophoblast cell in development is vital, and failure to develop this response is associated with recurrent miscarriages and placental pathology (Fu et al., 2013; Hosseini et al., 2018; Rafiee et al., 2015b; Wang et al., 2010). Treg cells are crucial for blastocyst implantation and trophoblast development in a normal pregnancy. Therefore, a shift towards T helper cells and an elevation of Treg in maternal decidua and circulation are usually observed (Redman and Sargent, 2010, 2001; Sargent et al., 2007). Nevertheless, a lower Treg or a higher Th17 profile is associated with adverse obstetric outcomes like PTD (Koucký et al., 2014), PE (Gleicher, 2007; Sibai et al., 2005; Vargas-Rojas et al., 2016) or recurrent pregnancy loss (Bansal et al., 2012, 2011a; Qian et al., 2018; Wang et al., 2010, 2020).

1.2 – Treg and Th17 cells in pregnancy

T helper lymphocytes (Th cells) are essential for the control of the adaptive immune response. These cells may be categorised by the cytokines that they produce (Crotty, 2011; Harrington et al., 2005; Korn et al., 2009). Th1 cells classically produce interferon gamma (IFN- γ), IL-2 and TNF α , activating cytotoxic T lymphocytes, macrophages, and natural killer cells. Th2 cells are classically linked to the activation of B cells by producing IL-4. Th17 cells have a pro-inflammatory phenotype associated with the production of IL-17, IL-21, and IL-22. Treg is a subset of CD4⁺ T cells with the ability to suppress immune reactions and maintain peripheral tolerance (Bennett et al., 2001; Sakaguchi et al., 1995). Treg cells have an anti-inflammatory and immunomodulatory effect and characteristically express high levels of the IL-2 receptor (CD25^{high}) on cell surface, the transcription factor Foxhead box P3 (FOXP3), which is a "master switch" for Treg development (Adeegbe and Nishikawa,

2013; Fontenot et al., 2005; Littman and Rudensky, 2010) and produce transforming growth factor- β (TGF- β) and IL-10 (Crotty, 2011; Harrington et al., 2005; Korn et al., 2009).

Treg cells are usually classified into two subpopulations: induced regulatory T cells (iTreg), which develop in the periphery from *naïve* T cells upon exposure to TGF- β and IL-2, and natural Treg (nTreg), which grow from thymus T cell receptor (TCR) high-affinity T cells and represent a necessary mechanism of peripheral tolerance to self-antigens (Adeegbe and Nishikawa, 2013; Chien and Chiang, 2017). These cells have been proposed as one of the most critical immunosuppressive agents involved in the maternal immune tolerance to foetal antigens, and recent studies suggest that maladaptation in these immune responses is crucial for the development of adverse obstetrical outcomes like miscarriage (Bansal et al., 2011a; Wang et al., 2013, 2010) or PE (Darmochwal-Kolarz et al., 2012a; Gleicher, 2007; Sibai et al., 2005).

Th17 cells represent a lineage of CD4⁺ T lymphocytes characterised by expressing the transcription factor retinoic acid receptor-related orphan receptor (ROR) γ t and producing IL-17a. These cells have a pro-inflammatory profile and play a vital role in host defence against extracellular bacteria, *Mycobacterium tuberculosis*, and fungus (Saito et al., 2011). An increment in the number and activity of Th17 cells has been implicated in the pathophysiology of several autoimmune disorders, like multiple sclerosis (Fletcher et al., 2009), systemic lupus erythematosus (SLE) (Marinho et al., 2017; Xing et al., 2012), or rheumatoid arthritis (Wang et al., 2012). Th17 and Treg cells present a certain level of plasticity, and in the presence of certain stimuli, Treg cells can differentiate into Th17 cells and *vice versa* (Bellemore et al., 2015; Komatsu et al., 2014). However, lowering Treg and raising Th17 dynamic profiles seems to be associated with several adverse obstetric outcomes like PTD (Koucký et al., 2014; Muyayalo et al., 2020), PE (Chen et al., 2022; Eghbal-Fard et al., 2019; Poordast et al., 2017; Vargas-Rojas et al., 2016), or recurrent pregnancy loss (Mjösberg et al., 2010; Wang et al., 2020).

1.2.1 – Th17/Treg ratio in pregnancy

In a normal pregnancy, Treg cells and pro-inflammatory T cells seem essential to the tolerance mechanism throughout pregnancy (Cornelius and Lamarca, 2014; Figueiredo and Schumacher, 2016; Muyayalo et al., 2020; Roomandeh et al., 2018; Saifi et al., 2014; Saito et al., 2023). As previously stated, the maternal immune system is challenged to tolerate a semi-allogeneic foetus during pregnancy. The normal progression of gestation demands that the maternal immune system must be able to accept the exposed foetal antigens and then allow trophoblast growth. Pregnancy is an anti-inflammatory state, and Treg cells seem

essential for the implantation and trophoblast development, so a shift toward a Th2/Treg phenotype is observed as an elevation of Treg cells in maternal decidua and circulation is present (Boij et al., 2015; Redman and Sargent, 2010; Sargent et al., 2007; Wegmann et al., 1993).

The breakage of a tolerogenic immune response is associated with the development of APO (Cornelius and Lamarca, 2014; Figueiredo and Schumacher, 2016; Roomandeh et al., 2018; Saifi et al., 2014; Saito et al., 2023). A lower Treg or a higher Th17 profile is associated with PTD (Koucký et al., 2014), PE (Gleicher, 2007; Saito et al., 2023; Sibai et al., 2005; Vargas-Rojas et al., 2016) or recurrent pregnancy loss (Bansal et al., 2011b; Wang et al., 2013, 2010, 2020).

It is also known that the balance between the anti-inflammatory FOXP3⁺ Treg cells and the pro-inflammatory Th17 cells is crucially involved in the pathogenesis of several autoimmune disorders like SLE (M. Chen et al., 2018; Koga et al., 2019; Shin et al., 2011; Tsokos et al., 2016). Despite the improvement in obstetrical outcomes with better medical care, pregnancy in SLE patients is still associated with high maternal and foetal morbidity (Arkema et al., 2016a; Braga et al., 2021a, 2021b; Bundhun et al., 2017a; Ostensen and Clowse, 2013). The incidence of miscarriage, PTD, or placental insufficiency is increased in these patients (Aggarwal et al., 2011; Arkema et al., 2016a; Braga et al., 2021a, 2021b; Bundhun et al., 2017b, 2017a; Buyon et al., 2015a; Canti et al., 2012; Clowse et al., 2008; Feld et al., 2015; Khan et al., 2018a; Sylvia J. Kroese et al., 2017; Naseri et al., 2018a; Ostensen and Clowse, 2013; Pastore et al., 2019; Phansenee et al., 2018; Rezk et al., 2017a; Wu et al., 2018a; Zhan et al., 2017). This fact may be explained by an intrinsic unbalance between pro-inflammatory and anti-inflammatory profiles. SLE is classically characterised by a defect in cellular apoptotic debris clearance and failure in self-tolerance mechanisms resulting in the generation of autoantibodies. Innate and adaptive immune responses are involved in the production of autoantibodies by B cells. The chronic inflammation caused by this abnormal immune response leads to tissue damage (Accapezzato et al., 2023; Tsokos, 2011; Tsokos et al., 2016). T lymphocytes play an essential role in SLE pathogenesis by secreting pro-inflammatory cytokines, activating B cells to generate autoantibodies, and maintaining autoimmune response by memory T cells (Koga et al., 2019; Mak, 2022; Petrić and Radić, 2023). Even though the pathogenesis of SLE is incompletely understood, it has been described that a reduction in immunosuppressive Treg cells and TGF- β and a concomitant increase in the pro-inflammatory T cells correlates with disease activity (Accapezzato et al., 2023; Mak, 2022; Tower et al., 2011; Varghese et al., 2011). There is scarce data about Treg cell evolution in pregnant women with SLE. However, it has been shown that circulating CD4⁺CD25⁺FOXP3 Treg cells are markedly reduced in nonpregnant women with SLE compared with healthy

counterparts. These levels remain low in SLE patients during pregnancy. This immunologic imbalance characteristic of SLE may be implicated as a potential trigger for pregnancy complications (Fesel et al., 2012; Tower et al., 2013, 2011; Varghese et al., 2011) and could help explain the worse gestational outcomes in these patients, even with controlled disease.

1.3 – High-risk and complex pregnant patients

A high-risk pregnancy is usually defined as a gestation where the mother, the foetus, or both are at increased risk of complications during the gestation, delivery, or postpartum (Bayrampour et al., 2013; Holness, 2018; Lee et al., 2016; Rodrigues et al., 2016). It is estimated that worldwide 20 million women experience a high-risk gestation, and approximately 800 women die daily from perinatal complications (World Health Organization, 2019). The incidence of high-risk pregnancies ranges extensively in the literature (from 6% to 33%). What may be explained by the lack of a consensus definition and due to a wide variety of conditions that constitute a high-risk pregnancy (Holness, 2018; Soh and Nelson-Piercy, 2014; Zadeh et al., 2012).

There are several classification systems to classify pregnancy as high-risk (Holness, 2018). The National Institutes of Health (NIH) defined four broad categories associated with risk during gestation (Eunice Kennedy Shriver National Institute of Child Health and Human Development, 2018). These risks are outlined in Table1.

Table 1 – NIH High-risk pregnancy categories

Categories	Factors
Existing health conditions	High blood pressure, polycystic ovary syndrome, diabetes, kidney disease, autoimmune disease, thyroid disease, infertility, obesity, HIV/AIDS, Zika infection
Age	Young age, first pregnancy after 35 years
Lifestyle factors	Alcohol use, tobacco use, drug use
Conditions of pregnancy	Multiple gestation, gestational diabetes, pre-eclampsia and eclampsia, birth defects and genetic conditions in the foetus

Centers for Disease Control and Prevention (CDC) also specifies several indicators of severe maternal-foetal morbidity (CDC, 2021) – illustrated in table 2. Other important risk

factors that should be assessed when determining pregnancy risk are outlined in table 3, and they may be classified as biophysical, psychosocial, sociodemographic, or environmental factors. These indicators are even more important for women from vulnerable populations (Holness, 2018).

Table 2 – CDC Severe maternal morbidity indicators

System	Maternal morbidity indicators
Cardiovascular	Acute myocardial infarction; cardiac arrest/ventricular fibrillation; disseminated intravascular coagulation; thrombotic embolism; conversion of cardiac rhythm.
Pulmonary	Adult respiratory distress syndrome; pulmonary oedema; temporary tracheostomy; mechanical ventilation.
Injuries	Intracranial injuries; internal injuries of thorax, abdomen, or pelvis.
Pregnancy specific	Amniotic fluid embolism; pre-eclampsia/eclampsia; puerperal cerebrovascular disorders.
Operative	Severe anaesthesia complications; hysterectomy; heart and pericardium surgeries; cardiac arrest during procedure or surgery.
Other	Acute renal failure; sepsis; shock; sickle cell anaemia with crisis; aneurysm

Screening in pregnancy is the gold standard to identify any condition that may harmfully affect gestation development (Jordan and Murphy, 2009). A detailed history, physical examination, and laboratory findings may reveal potential risks for the mother or foetus, possibly classifying a gestation as a high-risk pregnancy (CDC, 2021). Previous medical conditions that may be associated with end-organ damage are also significant risk factors for adverse materno-foetal outcomes (Eunice Kennedy Shriver National Institute of Child Health and Human Development, 2018).

Table 3 – General categories of high-risk pregnancy

General categories	Examples
Biological	General health status, genetic, medical or obstetric disorders
Psychological	Maternal behaviours, lifestyle, psychological and mental disorders
Sociodemographic	Lack of prenatal care, low income, lower social level
Environmental	Hazards in the workplace and general environmental exposures

Optimal care for a high-risk pregnancy strongly depends on the associated condition or conditions that define a particular gestation as high-risk. Pregnant women with high-risk pregnancies face many prenatal and postpartum (physiological, psychosocial, and health-related) challenges. Medical and nursing care of women with high-risk diagnoses must be specific and individualised, following the most recent surveillance protocols for each condition (Ahluwalia et al., 2015). Challenges to effective care include adherence to medical therapies, reduction of high-risk behaviours, and attending prenatal, early, and frequent care (Holness, 2018). In many conditions, these risks may be reduced with adequate care, and pregnancy success may be achieved. There are, however, a few conditions where maternal mortality risk is too high. In these cases, pregnancy is usually contradicted (ACOG, 2019; Ramlakhan et al., 2020; Soh and Nelson-Piercy, 2014) – see Table 4.

SLE is an example of a condition associated with adverse pregnancy outcomes (Bundhun et al., 2017b). SLE patients have a high rate of maternal and foetal morbidity (Arkema et al., 2016a). In these high-risk pregnancies, the incidence of miscarriage, stillbirth, PTD, FGR, or hypertensive disorders increases (Bundhun et al., 2017a; Buyon et al., 2015b). Furthermore, lupus flares increase during pregnancy and postpartum (Clowse et al., 2019). Due to these facts, and considering that SLE is an autoimmune pathology where immune regulation is affected, SLE was considered the model to use in this thesis.

Table 4 – Examples of absolute contraindications for pregnancy

Examples of absolute contraindications for pregnancy
Chronic kidney disease stage 4 or 5 Active autoimmune diseases Women with antiphospholipid syndrome with recurrent placenta-mediated adverse pregnancy outcomes Pulmonary arterial hypertension Severe systemic ventricular dysfunction (ejection fraction <30%) Previous peripartum cardiomyopathy with any residual left ventricular dysfunction Severe mitral stenosis Severe symptomatic aortic stenosis Systemic right ventricle with moderate to severely decreased ventricular function Severe aortic dilation (>45 mm in Marfan syndrome or other hereditary thoracic aortic disease; >50 mm in bicuspid aortic valve; Turner syndrome with aortic size index >25 mm/m²; Tetralogy of Fallot > 50 mm) Vascular Ehlers-Danlos Severe (re)coarctation Fontan circulation with any complication

1.4 – Pregnancy in Systemic Lupus Erythematosus

1.4.1 – Context and epidemiology

SLE is a systemic autoimmune connective tissue disorder characterised by a broad clinical phenotype including dermatological, musculoskeletal, haematological, renal, cardiovascular, pulmonary, and neuropsychiatric manifestations (Tsokos, 2011; Tsokos et al., 2016). SLE is not rare, with an overall prevalence in the general population ranging from 100 to 500 cases per 100,000 individuals (Danchenko et al., 2006; Knight and Nelson-Piercy, 2017a; Vasconcelos, 2007). This prevalence has increased over the last 40 years due to a better diagnosis of milder cases, improved survival rates, and enlarged disease burden. SLE prevalence varies significantly with ethnicity, age, and gender. Traditionally, SLE affects women more frequently than men, with a seven to ten times higher prevalence rate and an overall ten-year survival rate of 92% (Danchenko et al., 2006; Rahman and Isenberg, 2008; Vasconcelos, 2007). In these patients, lupus flares and infections are associated with increased mortality at a younger age. In contrast, the increased incidence

of myocardial infarction and stroke are associated with premature death in later life (Knight and Nelson-Piercy, 2017a; Tan et al., 1982).

SLE is the most typical autoimmune rheumatic disease encountered in pregnancy. With actual state of care, many women with SLE of childbearing age are able to conceive. Therefore, SLE association with pregnancy is not rare, with an estimated prevalence of 1-2 cases per 1000 pregnancies (Cortes-Hernandez, 2002; Knight and Nelson-Piercy, 2017a; Varghese et al., 2011).

During pregnancy, SLE is associated with significant maternal and foetal morbidity (Arkema et al., 2016a; Bundhun et al., 2017b, 2017a; Knight and Nelson-Piercy, 2017a). The incidence of early miscarriage, stillbirth, PTD, FGR, or hypertensive disorders of pregnancy (HDP) is increased (Arkema et al., 2016b; Bundhun et al., 2017b; A. M. Eudy et al., 2018; Lateef and Petri, 2017a; Nahal et al., 2018).

Since the 1970s, many authors have provided specific care for pregnant women with SLE. These experiences showed that a multidisciplinary team has better disease control of the disease, improving obstetric outcomes (Madazli et al., 2010). Nonetheless, pregnancy outcomes in patients with SLE still showed a 20-40% increased risk of maternal and foetal complications in the form of lupus flares, hypertension, nephritis, venous thromboembolism, FGR, miscarriage, stillbirth, preterm labour or PE (Bundhun et al., 2017a; Clowse et al., 2008; Smyth et al., 2010). The presence of comorbidities such as preexisting hypertension, renal disease, pulmonary arterial hypertension, increased titers of antiphospholipid antibodies or active disease at conception are associated with worse pregnancy outcomes (Al-Riyami et al., 2021; Arkema et al., 2016a; Attia et al., 2019; Barnabe et al., 2011; Barnado et al., 2014; Chen et al., 2015; Danza et al., 2016; Jakobsen et al., 2015; Kleinman et al., n.d.; Sylvia J Kroese et al., 2017; Kwok et al., 2011; Pastore et al., 2019; Rezk et al., 2017b; Ruiz-Irastorza et al., 2000; Sugawara et al., 2019; Tincani et al., n.d.; Wallenius et al., 2014; Wu et al., 2018a; Yuen et al., 2009; Zhang et al., 2023). Therefore, pregnancy still constitutes a major physiological challenge for these women.

1.4.2 – SLE diagnostic criteria and pregnancy

SLE is a multisystemic autoimmune pathology. The disease phenotype is variable, with a wide variation of organ involvement, making SLE diagnosis particularly difficult. In his PhD thesis, Carlos Vasconcelos found 125 different organ cumulative combinations (Vasconcelos, 2007). Alongside the clinical picture that characterises the lupus patient, SLE is associated with high titers of autoantibodies to an array of ubiquitously nuclear antigens and a limited number of tissue-specific antigens. These antibodies are critical diagnostic

criteria and anti-dsDNA could be useful as a clinical marker of disease activity (Aringer et al., 2019a; Fanouriakis et al., 2019). The importance of these serologic markers is expressed in the revised classification criteria published in 2019 by the European Alliance of Associations for Rheumatology (EULAR) and American College of Rheumatology (ACR), where the presence of anti-nuclear antibodies (ANA) are now considered a must-have SLE diagnostic criteria (Aringer et al., 2019a; Fanouriakis et al., 2019).

Diagnosis of SLE during pregnancy can be very challenging. The physiological changes of pregnancy affect several diagnostic descriptors making the diagnosis elusive. The same reason may also challenge the evaluation of SLE activity during pregnancy. Several scales, like the Systemic Lupus Erythematosus Pregnancy Disease Activity Index (SLEPDAI), have been proposed and validated in clinical practice (Buyon et al., 1999). However, the correct diagnosis of a lupus flare during pregnancy still relies mainly on clinical grounds, which may be recognised by experienced physicians (Ruiz-Irastorza and Khamashta, 2004). The improvement of standard care of these patients with early diagnosis and reduced end-organ damage at an early age helps to justify the improved obstetric outcomes described in recent years. Furthermore, the diagnosis of mild lupus cases at younger age reduces the number of SLE cases diagnosed during pregnancy, which is associated with improved maternal and foetal outcomes (Laura Andreoli et al., 2017; Petri, 2007; Petri et al., 1987; Ruiz-Irastorza et al., 2000).

1.4.3 – Physiological changes induced by pregnancy and SLE

A healthy pregnancy induces dramatic physiological changes that affect almost every system. The most significant changes are related to the increased intra and extravascular volume, the presence of a prothrombotic state, and the immunologic adaptations necessary for foetal and placental tolerance. These physiological adaptations may contribute to decompensating an otherwise asymptomatic patient (Aringer et al., 2019a; Bundhun et al., 2017b). The intravascular volume could increase by as much as 50% during gestation so it may induce a clinical worsening in patients with lung-heart or kidney impairment. In the same way, weight increase will contribute to joint pain, primarily the axial, hip, or knee articulations (Ruiz-Irastorza and Khamashta, 2011, 2009). Being a prothrombotic state, pregnancy is also associated with a higher risk of thrombotic complications. This concern is particularly relevant in patients with serum antiphospholipid antibodies (aPL) or those with clinical manifestations of antiphospholipid syndrome (APS) (Tektonidou et al., 2019). Differentiating SLE manifestations from gestational physiological changes may be complex as usual complaints, clinical and/or laboratory changes could simulate SLE flares.

Symptoms like fatigue, arthralgia, hair loss, dyspnea, headache or peripheral oedema, and analytical abnormalities like anaemia or thrombocytopenia are frequently seen in healthy pregnant women. These may be easily mistaken for manifestations of a lupus flare (Knight and Nelson-Piercy, 2017a).

Table 5 – 2019 EULAR/ACR classification criteria for SLE

Classification criteria for SLE	
Entry criteria	ANA at a titer of $\geq 1:80$ on HEp-2 cells or an equivalent positive test (if absent, do not classify as SLE; if present, apply additional criteria. Additional criteria within the same domain will not be counted)
Clinical domain	Criteria (weight)
Constitutional	Fever (2)
Haemathologic	Leukopenia (3) Thrombocytopenia (4) Autoimmune hemolysis (4)
Neuropsychiatric	Delirium (2) Psychosis (3) Seizure (5)
Mucocutaneous	Non-scarring alopecia (2) Oral ulcers (2) Subacute cutaneous OR discoid lupus (4) Acute cutaneous lupus (6)
Serosal	Pleural or pericardial effusion (5) Acute pericarditis (6)
Musculoskeletal	Joint involvement (6)
Renal	Proteinuria $>0.5\text{g}/24\text{h}$ (4) Renal biopsy: Class II or V lupus nephritis (8) Renal biopsy: Class III or IV lupus nephritis (10)
Immunology domains	Criteria (weight)
Antiphospholipid antibodies	Anti-cardiolipin antibodies OR Anti- $\beta 2\text{GPI}$ antibodies OR Lupus anticoagulant (2)
Complement proteins	Low C3 OR low C4 (3) Low C3 AND low C4 (4)
SLE-specific antibodies	Anti-dsDNA antibody OR Anti-Smith antibody (6)
Classify as SLE with a score of 10 or more if entry criteria fulfilled	

Gestational haematologic changes may also mimic SLE flares. During pregnancy, haematocrit reduces, and anaemia can emerge, especially during the second half of gestation. Similarly, a reduction in platelet count is observed in approximately 10% of uncomplicated pregnancies, secondary to hemodilution (gestational thrombocytopenia). Inflammatory markers are also elevated, with a physiological increase of the circulatory leucocyte number, an increment of the liver fibrinogen production, and an elevation of the erythrocyte sedimentation rate. These expected changes are common in an uneventful pregnancy and may easily mimic a SLE flare, so these physiological changes need to be considered when a pregnant lupus patient is evaluated (Borella et al., 2014; Danza et al., 2016; Knight and Nelson-Piercy, 2017a).

Serum complement system C3 and C4 levels also change during pregnancy. The seric levels of these markers rise due to increased hepatic production. Therefore, comparing baseline levels to assess lupus activity is essential because it may remain within the normal range during a gestational SLE flare (Buyon et al., 1999; Kim et al., 2018).

Evaluation of kidney function and lupus nephritis activity may also be challenging. Proteinuria increases during pregnancy due to increased renal blood flow and glomerular filtration rate. This finding is also a marker of renal involvement in specific pregnancy complications like PE or HELLP (Hemolysis, Elevated Liver enzymes, and Low Platelet count) syndrome, which may mimic SLE flares, particularly lupus nephritis (Arkema et al., 2016a). Both PE and SLE are associated with arterial hypertension, proteinuria, thrombocytopenia, oedema, and elevated creatinine concentration (Knight and Nelson-Piercy, 2017a; Ruiz-Irastorza and Khamashta, 2009). Understanding when SLE is responsible for these manifestations is crucial for the prevention of inadequate preterm deliveries and the institution of an adequate immunosuppressive treatment (Aringer et al., 2019a). Table 6 summarizes the primary physiological and SLE-related changes observed during pregnancy.

1.4.4 – SLE effect on maternal and foetal outcomes

Pregnancy in SLE patients is associated with a significant risk of adverse maternal and foetal outcomes (Bundhun et al., 2017a) and a twofold greater cumulative pregnancy complication rate than a non-SLE control population (Bundhun et al., 2017a; Moroni et al., 2016c; Moroni and Ponticelli, 2016). SLE does not affect fertility, but it is associated with an increased risk of APO and a lower live birth rate when compared with a healthy control group (Bundhun et al., 2017a).

Table 6 – Primary physiological and SLE-related changes observed during pregnancy

	Pregnancy physiological changes	SLE-related changes
Clinical symptoms	Fatigue Facial flush Palmar erythema Mild arthralgia or myalgia Mild oedema Mild resting dyspnea Hair loss (postpartum)	Fever without evidence of infection Lymphadenopathy Photosensitive skin rash Oral or nasal ulcerations Inflammatory arthritis Serositis
Laboratory findings	Mild anaemia (usually microcytic hypochromic anaemia) Mild thrombocytopenia (>100 000/uL) Proteinuria <300 mg/day Increase in complement factors	Haemolytic anaemia Severe thrombocytopenia Leukopenia and/or lymphopenia Active urinary sediment Proteinuria >300 mg/day Decline in complement factors Increase in dsDNA antibodies

1.4.4.1 – Maternal outcomes

Pregnancy in lupus patients is associated with an increased risk of adverse maternal outcomes. The most ominous result is maternal death, and maternal mortality risk is 17- to 20-fold higher in lupus patients during pregnancy (Clowse et al., 2008). However, although the mortality risk increases in these patients compared with uncomplicated pregnant women, this is much lower than the annual mortality rate of nonpregnant SLE patients (Clowse et al., 2008; Knight and Nelson-Piercy, 2017a). Medical complications such as stroke, deep vein thrombosis, pulmonary embolism, severe infections, and haemorrhage are two to eight times more frequent during pregnancy and puerperium in SLE patients (Clowse et al., 2008). The presence of periconceptional comorbidities like arterial hypertension, lupus nephritis, chronic kidney failure, recent flare, or APS increase the risk of these complications (Andreoli et al., 2012).

Hypertensive disorders of pregnancy, especially PE, are important causes of maternal and foetal morbidity in lupus patients. The incidence of PE is two to fourfold higher than in the

general population (Askie et al., 2007; Bundhun et al., 2017a; Clowse et al., 2008; Nili et al., 2013; Wallenius et al., 2014; Wu et al., 2018a; Yuen et al., 2009). This risk may be even higher if SLE patients have additional risk factors like previous history of PE or APS (Milne et al., 2005). Other risk factors are lupus nephritis, chronic hypertension, chronic kidney disease, treatment with a high dose of oral steroids, or active disease (Andrade et al., 2008; Moroni et al., 2016a). Eclampsia risk is also increased in SLE patients, with a fourfold increased risk than the general population (0.5% vs. 0.09%)(Clowse et al., 2008).

Nevertheless, it is essential to note that although maternal complications are increased in women with SLE compared to a low-risk pregnant population, pregnancy by itself does not increase the risk of mortality or severe morbidity than in any other year of that woman's life (Clowse et al., 2008). Therefore, SLE in remission without significant organ involvement is likely associated with a normal gestation with pregnancy outcomes comparable to those of a healthy population (Clowse, 2007; Eudy et al., 2018).

1.4.4.2 – Maternal Lupus Flare

In SLE pregnant patients, the incidence of lupus flare seems to be increased (Eudy et al., 2018). Significant risk factors for the development of a lupus flare are the presence of active disease at conception, previous history of lupus nephritis and the necessity to use chronic medication to maintain lupus remission (Buyon et al., 2015c; Cortes-Hernandez, 2002; Eudy et al., 2018; Imbasciati et al., 2009; Khan et al., 2018b; Naseri et al., 2018b; Pastore et al., 2019; Ruiz-Irastorza et al., 1996; Ruiz-Irastorza and Khamashta, 2011). Women with quiescent and well-controlled SLE have less risk of flares during pregnancy and postpartum (Ruiz-Irastorza et al., 2004). These flares are usually non-severe and involve articular, dermatological, or mild haematological changes frequently controlled with oral steroids (Ruiz-Irastorza et al., 1996; Ruiz-Irastorza and Khamashta, 2011, 2009). Nonetheless, severe flares with significant organ involvement may threaten the mother's life or cause cumulative organ damage (Ruiz-Irastorza et al., 1996). In recent years, the percentage of patients who became pregnant with better disease control increased, which justifies the better results of the most recent published data (Chen et al., 2015; Danza et al., 2016; Kwok et al., 2011; Pastore et al., 2019; Rezk et al., 2017b; Ruiz-Irastorza et al., 2000; Sugawara et al., 2019). The use of hydroxychloroquine (HCQ) seems to be associated with these better results. HCQ use during pregnancy seems beneficial in reducing lupus flares, even in the presence of controlled disease at conception (L. Andreoli et al., 2017a; Aringer et al., 2019a; Clowse, 2007; Clowse et al., 2006; Østensen et al., 2015). The diagnosis of a lupus flare during pregnancy may be difficult. As previously stated, distinguishing pregnancy-

associated signs and symptoms from a SLE clinical or analytical deterioration is not always straight, and an assessment by an experienced team is essential – table 6 (Ruiz-Irastorza et al., 2004; Ruiz-Irastorza and Khamashta, 2011).

Preconceptional or an early pregnancy evaluation with a baseline haematological, renal, and serological analysis with proteinuria quantification should be obtained because they are essential to monitor disease status and identify flares (Knight and Nelson-Piercy, 2017a). Gestational C3, C4, and anti-dsDNA quantification may help to identify SLE flares. Serum C3 and C4 levels also increase during pregnancy, so their relative variation should be assessed rather than absolute levels. A reduction superior to 25% in the serum baseline complement levels may suggest lupus flare (L. Andreoli et al., 2017a; Buyon et al., 1999; Knight and Nelson-Piercy, 2017a).

1.4.4.3 – Pregnancy with lupus nephritis and renal flare during pregnancy

Lupus nephritis is an independent risk factor for adverse obstetric outcomes and a lower live birth rate even in women in remission (Bramham et al., 2011; Wu et al., 2018c). In fact, in patients with lupus nephritis, foetal loss, FGR, gestational hypertension, PE, or PTD significantly increase (Bramham et al., 2011; Wu et al., 2018c). Lupus nephritis was also associated with an increased risk of SLE flare, renal flare, treatment with steroids or immunosuppressants, proteinuria, and hypocomplementemia during pregnancy (Bramham et al., 2011; Wu et al., 2018c). Pregnancy is not an independent risk factor for long-term renal deterioration in these patients, especially if kidney function is normal at conception (Chiu et al., 2016; Gianfreda et al., 2017; Moroni et al., 2016b). At conception, kidney function, hypertension, or the presence of disease activity are the most critical determinants of kidney deterioration and pregnancy outcomes (Gladman et al., 2010; Moroni et al., 2016d). Proteinuria may also be associated with thrombosis and adverse maternal outcomes. In the presence of severe proteinuria (>1–2g/day), thromboprophylaxis with low molecular weight heparin (LMWH) may be considered (Brunskill, 2019; Lightstone and Hladunewich, 2017).

One particular challenge in pregnant patients with SLE is the differential diagnosis between PE and lupus nephritis flare (Lateef and Petri, 2017b; Saavedra Salinas et al., 2015; Stojan and Baer, 2012). As previously stated, these two conditions share clinical and analytical findings and may coexist, so the differential diagnosis is complex and, sometimes, impossible. A renal biopsy might be indicated if the result would affect management. However, this procedure is associated with a high risk of bleeding during pregnancy. New biomarkers, including angiogenic factors associated with PE screening are helpful in the

differential diagnosis (Lateef and Petri, 2017b; Saavedra Salinas et al., 2015; Stojan and Baer, 2012). Table 7 addresses the elements used for the differential diagnosis.

Table 7 – Differentiating PE from lupus flare

Features suggestive of PE	Features suggestive of SLE flare	Features shared by both conditions
Onset >20 weeks (more suggestive of PE) Abnormal liver function tests Rising uric acid level Elevated sFlt-1/PIGF ratio Visual symptoms (including flashing lights) Epigastric or right upper quadrant tenderness Severe headache Clonus	Onset <20 weeks (more suggestive of SLE) Active urinary sediment/cellular casts Haematuria Low or reduction of complement levels High or increasing anti-dsDNA antibodies titers Evidence of flare involving other organs	Hypertension Proteinuria oedema Haemolysis Renal function impairment Thrombocytopenia Presence of FGR

PIGF – placental growth factor; sFlt-1 – Soluble fms-like tyrosine kinase 1

1.4.4.4 – Pregnancy complicated by lupus with antiphospholipid antibodies

The presence of aPL is an independent risk factor for thromboembolism and adverse obstetric outcomes in SLE pregnant patients, particularly PTD, development of HDP, miscarriage, stillbirth, and FGR (Buyon et al., 2015a; D. Chen et al., 2018; Deguchi et al., 2018; Gonzalez-Echavarri et al., 2015; Saccone et al., 2017). APS definition includes laboratory and clinical criteria. Diagnosis requires the presence of aPL with additional clinical features of thrombotic or obstetric complications (Miyakis et al., 2006). In the presence of aPL and absence of complete APS, the addition of acetylsalicylic acid (ASA) during pregnancy may be enough to ensure similar outcomes to the general population. APS criteria in lupus patients represent an increased risk factor for adverse obstetric outcomes, which may be mitigated by adding LMWH to ASA during pregnancy (Ambrósio et al., 2010; Cordeiro et al., 2009; Palma Dos Reis et al., 2020; Serrano, 2013; Serrano et al., 2009; Soh et al., 2013).

1.4.4.5 – Foetal and neonatal outcomes

Lupus patients have a greater risk of miscarriage, stillbirth, FGR, PTD, lower birth weight, neonatal intensive care unit admission, neonatal and perinatal death (Tincani et al., 2016; Zhang et al., 2023). SLE patients' live birth rate is significantly reduced compared with healthy control groups (Bundhun et al., 2017a; Georgiou et al., 2000). Spontaneous abortion and stillbirth are increased in SLE pregnancies and affect approximately 20% of the cases (Aggarwal et al., 2011; Barbhaiya and Bermas, 2013; Barnado et al., 2014; Buyon et al., 2015a; D. Chen et al., 2018; Chen et al., 2015; Deguchi et al., 2018; McGrory et al., 2003; Molokhia et al., 2007; Ruiz-Irastorza et al., 2000; Ruiz-Irastorza and Khamashta, 2004; Yuen et al., 2009). Miscarriage and stillbirth risk is increased in the presence of aPL, even without APS criteria (Buyon et al., 2015a; Yelnik et al., 2016). A lower birth rate is also associated with a poorly controlled disease at conception (Buyon et al., 2015a; D. Chen et al., 2018) and a longer disease evolution (Bundhun et al., 2017a; Deguchi et al., 2018). PTD risk is almost 12 times higher in lupus patients (Bundhun et al., 2017a). It is associated with active disease at conception, the development of lupus flare, and other obstetric complications like PE or FGR (Buyon et al., 2015a; D. Chen et al., 2018; Deguchi et al., 2018). Due to these adverse obstetric outcomes, the relative risk of neonatal admission at the intensive care unit is also increased (Bundhun et al., 2017a).

1.4.4.6 – Anti-Ro/SSA and La/SSB antibodies

Adverse neonatal outcomes are associated with Anti-Ro/SSA and La/SSB antibodies. These antibodies are present in about 40% of SLE patients (Vasconcelos, 2007). They are involved in neonatal lupus etiology, which may be found in 1–2% of children born to mothers with these antibodies (Brucato et al., 2002). These antibodies may cross the placenta between 16 to 30 weeks of gestation (Lateef and Petri, 2017a). The neonatal lupus spectrum is vast, ranging from benign cutaneous manifestations to severe cardiac involvement with congenital heart block (CHB). Skin manifestations are present in 30-40% of children, are self-limited, and resemble lupus typical rash (Ribero et al., 2017; Vanoni et al., 2017). The most severe form of neonatal lupus is the development of CHB (Brito-Zerón et al., 2015). The main clinical presentation in these cases is foetal bradycardia associated with congestive heart failure (Brito-Zerón et al., 2015). The risk of neonatal cardiac lupus increases in women with a previously affected child, with an estimated recurrence rate of

12 to 17% (Brito-Zerón et al., 2015). The use of HCQ seems to be protective in these cases (Barsalou et al., 2017).

1.4.5 – Pregnancy management plan in SLE patients

Pregnancy management of lupus patients is a complex and challenging problem. The best maternal, foetal and neonatal outcomes are obtained with a cohesive multidisciplinary approach (Knight and Nelson-Piercy, 2017a). These multidisciplinary teams usually include internal medicine specialists or rheumatologists dedicated to lupus, maternal-foetal specialists, nephrologists, foetal medicine specialists, and neonatologists. Pregnancy planning must start before conception, with an individualised risk assessment, considering the activity of disease, organ damage, the medications used, previous comorbidities, and previous obstetric history (L. Andreoli et al., 2017a). This preconception assessment is crucial to ensure the best obstetric outcomes and is essential for accurate and tailored counselling.

There are extensive recommendations for the planning and surveillance of pregnancy in SLE patients (L. Andreoli et al., 2017a; Skorpen et al., 2016). The most crucial pregnancy preparation topic for a SLE patient is to ensure that gestation occurs with well-controlled disease, under of safe drugs for the foetus, in the lowest possible dosage. Comorbidities like hypertension and impaired kidney function must be considered and controlled before conception. HCQ should be initiated before pregnancy; in fact, all lupus patients without contraindication should be on HCQ, as it is associated with the best maternal and foetal outcomes. ASA should be started as a preventive drug for PE and placental insufficiency. The presence of APS, as well as previous thromboembolism or severe placental insufficiency, are indications for the use of LMWH through pregnancy to puerperium (L. Andreoli et al., 2017a; Cruz-Lemini et al., 2022; Skorpen et al., 2016).

1.4.5.1 – Preconception evaluation

Preconception evaluation has the main objective of gathering actual and precise information to allow a tailored assessment of the individual gestational risk (L. Andreoli et al., 2017a; Bertsias et al., 2008; Fanouriakis et al., 2019; Knight and Nelson-Piercy, 2017a). This evaluation should include lupus activity, especially information about previous and recent flares, prior organ damage, pharmacological history, and serologic profile. Obstetric history is also vital to stratify a future pregnancy risk. The presence of any additional medical

disorders, like hypertension, diabetes, renal disease, or previous thromboembolism, should be addressed and stratified as well (L. Andreoli et al., 2017a; Bertsias et al., 2008; Fanouriakis et al., 2019; Knight and Nelson-Piercy, 2017a).

Physical examination and complementary exams should be performed to complete actualised clinical information. Baseline blood pressure, urinalysis, total blood count, creatinine, urea and electrolytes, liver function tests, and serological profile should be assessed. Additional organ-specific tests like cardiac sonography, electrocardiography, or pulmonary function tests may be required on an individual basis (L. Andreoli et al., 2017a; Bertsias et al., 2008; Fanouriakis et al., 2019; Knight and Nelson-Piercy, 2017a). Patients with SLE in remission or stable, well-controlled disease can be advised that conceiving is safe. Patients with a recent diagnosis or with active disease should be encouraged to postpone pregnancy. A control plan with HCQ and immunosuppressive treatment, if necessary, must be undertaken before conception. Further evaluation is required in these patients, and pregnancy may be possible and safe when SLE is well-controlled for at least six months before conception (L. Andreoli et al., 2017a; Aringer et al., 2019b).

Severe impairment of organ function or preexisting severe organ damage represents a relative or absolute contraindication to pregnancy. Those women should be informed about health and pregnancy-related risks. Clinical scenarios associated with a high risk of adverse maternal or foetal outcomes include moderate-to-severe lupus flare or stroke within the past six months, moderate-to-severe heart failure (left ventricular ejection fraction <40%), severe heart valve disease, pulmonary hypertension (estimated systolic pulmonary artery pressure >50 mmHg, or symptomatic), chronic kidney disease with creatinine >2.5-2.8 mg/dL, estimated glomerular filtration rate <30 mL/min, severe restrictive lung disease (forced vital capacity <50% predicted or <1 L), or uncontrolled hypertension (L. Andreoli et al., 2017a; Bertsias et al., 2008; Fanouriakis et al., 2019; Knight and Nelson-Piercy, 2017a; Regitz-Zagrosek et al., 2018). A previous history of severe early-onset PE or HELLP syndrome must also be considered an extremely high-risk factor (Knight and Nelson-Piercy, 2017a; Rodríguez-Almaraz et al., 2018; Lisa R. Sammaritano, 2017).

These patients should be informed that although a pregnancy complicated by SLE is considered a high-risk pregnancy, a tailored management approach with close multidisciplinary surveillance will result in higher rates of successful pregnancies (Knight and Nelson-Piercy, 2017a).

1.4.5.2 – Medications used in pregnancy and breastfeeding

In a SLE patient planning a pregnancy, therapeutic adjustment is a mandatory aspect of preconception evaluation. The main goal is to use safe drugs for the foetus that allow disease control in the mother. Medications that are considered safe throughout pregnancy and during lactation are HCQ, azathioprine, cyclosporine, and tacrolimus (Skorpen et al., 2016). Mycophenolate mofetil, cyclophosphamide, and methotrexate must be discontinued before conception due to proven teratogenicity (Götestam Skorpen et al., 2017). The aim should be to avoid the use of steroids and to use steroid-sparing immunosuppressants (such as azathioprine) or steroids in the lowest effective maintenance dose (ideally <7.5 mg or even less than 6 mg prednisolone per day). The use of the lowest possible dosage of steroids is associated with improved obstetric outcomes (D. Chen et al., 2018; Deguchi et al., 2018; Skorpen et al., 2016). They are also safe to control active disease, particularly at low dosages (Götestam Skorpen et al., 2017). Mild flares during pregnancy may be controlled with HCQ or low-dose of oral steroids. Moderate to severe flares may require the usage of pulsed intravenous methylprednisolone, intravenous immunoglobulin, or high-dose oral steroids in combination with immunosuppressants (L. Andreoli et al., 2017b; Knight and Nelson-Piercy, 2017a). Severe and refractory cases may require a risk/benefit assessment, and prioritisation of maternal welfare over foetal concerns and pregnancy termination may be taken into consideration (L. Andreoli et al., 2017b; Aringer et al., 2019a; Bundhun et al., 2017a; Knight and Nelson-Piercy, 2017a).

Antihypertensive medications are also frequently used by lupus patients, especially in the presence of renal involvement. Labetalol, methyldopa, or nifedipine are safe first-line antihypertensive treatments for preexisting or pregnancy-induced hypertension at any stage, from conception to lactation. Angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers should be stopped before conception because they are associated with congenital abnormalities. Angiotensin-converting enzyme inhibitors can be used safely postpartum, including during lactation (Knight and Nelson-Piercy, 2017a; Skorpen et al., 2016).

1.4.5.3 – HCQ use during pregnancy in SLE patients

The use of HCQ has been associated with a protective effect in SLE patients, reducing the risk of organ damage, SLE flares, venous thrombosis, and increased long-term survival (Ruiz-Irastorza et al., 2010). The beneficial effect of HCQ in pregnant lupus patients was

also described (Andreoli et al., 2019; L. Andreoli et al., 2017b; Aringer et al., 2019a; Borella et al., 2014; Lazzaroni et al., 2016; Luo et al., 2015). HCQ use during pregnancy is considered safe and not linked with foetal malformations when compared to the general population (Diav-Citrin et al., 2013). The use of HCQ is associated with lower disease activity scores and lower prednisolone requirement by the end of gestation, lower incidence of PE (Levy et al., 2001; Seo et al., 2019), prematurity or FGR (Diav-Citrin et al., 2013; Leroux et al., 2015; Seo et al., 2019).

HCQ has been associated with a protective effect against neonatal lupus. In utero, exposure to HCQ is associated with a reduced risk of CHB in patients with anti-SSA/SSB antibodies (Barsalou et al., 2018, 2017; Izmirly et al., 2012).

1.4.5.4 – LMWH

The risk of thromboembolism should also be assessed before and throughout pregnancy. Those at high risk should receive thromboprophylaxis with LMWH (RCOG, 2015).

LMWH should be initiated at conception in patients with thrombotic APS, obstetric APS with recurrent early pregnancy loss, or a miscarriage after 10 weeks of gestation or with a premature birth of a morphologically normal foetus before the 34th week due to placental insufficiency or PE (Arslan and Branch, 2019; Bundhun et al., 2018; Cruz-Lemini et al., 2022; Knight and Nelson-Piercy, 2017b, 2017a). Thromboprophylaxis with LMWH may also be considered in SLE patients with significant proteinuria (Arslan and Branch, 2019; Bundhun et al., 2018; Knight and Nelson-Piercy, 2017b, 2017a). Calcium therapy is recommended in patients with long-term use of LMWH (Arslan and Branch, 2019; Bundhun et al., 2018; Knight and Nelson-Piercy, 2017b, 2017a).

1.4.5.5 – ASA

ASA use is associated with a significant risk reduction in the development of PE, PTD, perinatal death, or FGR, particularly if started before the 16th week of gestation throughout pregnancy (ACOG, 2020; Lene Seidler et al., 2018; Poon et al., 2019; Rolnik et al., 2017). As a high-risk group for the development of HDP, ASA is recommended for all SLE women during pregnancy (ACOG, 2019; L. Andreoli et al., 2017a; Aringer et al., 2019b; Knight and Nelson-Piercy, 2017a; Mackillop et al., 2007). ASA is also associated with better obstetric

outcomes in patients with aPL and APS, so it is also recommended in these patients (Arslan and Branch, 2019; Bundhun et al., 2018; Knight and Nelson-Piercy, 2017b, 2017a).

1.4.5.6 – Foetal surveillance

In lupus patients, the standard foetal evaluation must be individually tailored, considering the comorbidities present at conception and the maternal and foetal complications that could develop throughout gestation. Therefore, in addition to the first-trimester ultrasound and the morphology mid-trimester scan, regular growth scans should be performed in the second half of pregnancy (L. Andreoli et al., 2017b; Aringer et al., 2019b; Lisa R Sammaritano, 2017). Special care should be taken in women with hypertension, renal disease, or APS. In women with anti-Ro/SSA or anti-La/SSB antibodies, foetal echocardiography should be offered to identify any cardiac conduction abnormality, and regular rhythm evaluation should be obtained (Lees et al., 2020; Mackillop et al., 2007; Nohuz et al., 2020).

1.4.5.7 – Intrapartum management

Intrapartum management should be tailored accordingly. Every labour plan should consider the pregnancy progression and the comorbidities present at the end of gestation. SLE is not a contraindication for vaginal delivery, and spontaneous labour is preferred in the absence of other obstetric complications (L. Andreoli et al., 2017b). However, as the risk of PE and FGR is more significant in these patients, a programmed pregnancy termination between 39 or 40 weeks of pregnancy may be considered (Aringer et al., 2019b; Tektonidou et al., 2019).

PTD is frequent in these patients, either spontaneous or iatrogenic, due to maternal or foetal conditions. A woman in preterm labour or with a scheduled PTD should receive a cycle of steroid injections (betamethasone or dexamethasone) to induce foetal maturity and reduce morbidity (Areia et al., 2018; El-Sayed et al., 2017). This course of intramuscular glucocorticosteroids should be administered independently of any other maternal oral steroids that only reach the foetus in a minimal dose due to placental metabolism (El-Sayed et al., 2017; Knight and Nelson-Piercy, 2017a).

Pregnancy in lupus patients is also associated with an increased risk of caesarean delivery (Bundhun et al., 2017a; Wu et al., 2018b). This may be explained by the higher incidence

of PTD, FGR, or the necessity to terminate gestations due to maternal complications. Caesarean delivery represents an additional risk factor because it is associated with an increased risk of venous thromboembolism, blood loss and infection, so preventive measures should be applied (RCOG, 2015). Considering this, in SLE patients, caesarean delivery should only be performed for obstetric indications (Knight and Nelson-Piercy, 2017a; Mackillop et al., 2007).

During labour, special care must be taken with patients under long-term oral steroids or LMWH. Labour and delivery are physiological stress conditions, so patients under a long course of a high dosage of oral corticosteroids may require intravenous hydrocortisone to prevent adrenal insufficiency. Treatment with LMWH also mandates special care to reduce the risk of bleeding and allow regional anaesthesia. A prophylactic dose of LMWH must be stopped 12 hours before regional anaesthesia and labour, while therapeutic doses must be stopped 24 hours before a procedure (RCOG, 2015). LMWH can be reinitiated 4 to 6 hours after the removal of an epidural catheter or after a spinal injection. The epidural catheter should be removed >12 hours after the last LMWH application (Knight and Nelson-Piercy, 2017a).

1.4.5.8 – Postpartum

The puerperium comprehends the 6-8 weeks after delivery and may also add specific risks for lupus patients. Besides the increased risk of venous thromboembolism characteristic of the physiological state of postpartum, in lupus patients, puerperium may also increase the risk of a lupus flare. In some studies, lupus activity may increase in the first weeks after delivery, so special care must be taken for these patients (L. Andreoli et al., 2017a; Knight and Nelson-Piercy, 2017a; Mackillop et al., 2007).

Breastfeeding is allowed and recommended, and patients medicated with HCQ, azathioprine, or steroids should be reassured that these medications are safe for the baby (Skorpen et al., 2016). At discharge, a review of the progression of pregnancy and labour should be performed; a follow-up outpatient appointment by four to six weeks postpartum should be scheduled. This postpartum assessment is important to detect and manage lupus complications and ensure a smooth return to autoimmune/rheumatology outpatient services for ongoing care (Knight and Nelson-Piercy, 2017a).

Lupus patients with adverse obstetric outcomes linked to placental insufficiency (HDP, FGR, placental abruption, or stillbirth) have an increased risk of cardiovascular events later in life (coronary artery disease, stroke, peripheral vascular disease, or death from cardiovascular causes) when compared with women who had uncomplicated pregnancies

(Soh et al., 2016). All women should undergo a postpartum venous thromboembolism risk assessment, and all patients who received antenatal LMWH should continue it for six weeks. Patients with previous long-term anticoagulation may return to oral anticoagulation when the risk of bleeding is reduced, usually at 5-7 days postpartum (Knight and Nelson-Piercy, 2017a).

1.4.5.9 – Unidade Local de Saúde de Santo António (ULSSA) SLE pregnancy surveillance protocol

As previously stated, SLE increases the risk of adverse obstetric outcomes, particularly in patients with associated lupus nephritis or APS. Pregnancy preparation starts before conception to achieve optimal control of all comorbidities, and a trained multidisciplinary team with specific surveillance protocols must monitor pregnancy. A well-controlled disease and the prompt treatment of any flare are vital to the success of the gestation. The risks associated with pregnancy do not end with delivery, so the postpartum period also demands careful surveillance. Our institutional protocol respects this multidisciplinary approach and is in line with the most recent evidence available (Appendix 1).

Chapter II

Aims

Aims

The major aims of this thesis are:

- ➔ Assess the clinical impact of pregnancy in a cohort of Portuguese SLE patients;
- ➔ Assess the maternal and foetal outcomes of pregnancies in a cohort of Portuguese SLE patients and compare it with a group of healthy pregnant women;
- ➔ To evaluate prospectively the number of circulating Treg, Th17 cells, and Th17/Treg ratio during pregnancy and postpartum in a group of healthy pregnant women;
- ➔ To evaluate prospectively the number of circulating Treg, Th17 cells, and Th17/Treg ratio during pregnancy and postpartum in a group of SLE patients;
- ➔ To evaluate prospectively the number of circulating Treg, Th17 cells, and Th17/Treg ratio during pregnancy and postpartum in a group of pregnant patients diagnosed with pre-eclampsia;
- ➔ To evaluate prospectively the number of circulating Treg, Th17 cells, and Th17/Treg ratio during pregnancy and postpartum in a group of pregnant patients diagnosed with foetal growth restriction.

Chapter III

Methods

Methods

3.1 – Patient selection

The studies incorporated in this thesis included pregnant women with SLE managed at the Obstetric Department and the Immunology Clinical Unit of Centro Hospitalar Universitário de Santo António (nowadays named ULSSA), Oporto, Portugal. All patients met the most recent European Alliance of Associations for Rheumatology (EULAR) and American College of Rheumatism (ACR) classification criteria for SLE (Aringer et al., 2019b, 2019a; Fanouriakis et al., 2019). The control groups were randomly selected from healthy women with singleton pregnancies who attended the same hospital and during the same period.

In the lupus patient group, SLE-related data was collected: SLE evolution time, current and cumulative manifestations, previous clinical and laboratory criteria for APS, and arterial hypertension. The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score was used at preconception to assess SLE activity (Gladman et al., 2013). During pregnancy, SLE activity was evaluated by the Systemic Lupus Erythematosus Pregnancy Disease Activity Index (SLEPDAI) (Buyon et al., 1999). Baseline laboratory data included haematological parameters, serum levels of creatinine, urinalysis – notably microscopy and proteinuria, immunological evaluation including anti-dsDNA, anti-Ro/SS-A, anti-La/SS-B, anti-cardiolipin, anti- β 2 glycoprotein antibodies, lupus anticoagulant test, and C3 and C4 complement components. In SLE patients and healthy pregnant women, baseline maternal data was collected: age, past obstetric history, and anterior medical conditions.

3.2 – Obstetrics and Internal Medicine management protocol

According to the ULSSA institutional protocol designed for SLE pregnant women (see appendix 1), all lupus patients were evaluated based on EULAR/ACR recommendations for the management of SLE patients (L. Andreoli et al., 2017b; Aringer et al., 2019b, 2019a; Fanouriakis et al., 2019; Sammaritano et al., 2020). This protocol specifies the frequency of medical visits, the recommended primary and subsidiary complementary exams, the endorsed evaluation of foetal growth, and recommendations on delivery and puerperium care.

Table 8 – SLEDAI descriptors and scores

SLEDAI-2K score	Descriptor	SLEDAI-2K score	Descriptor
8	Seizure	4	Proteinuria
8	Psychosis	4	Pyuria
8	Organic brain syndrome	2	Rash
8	Visual disturbance	2	Alopecia
8	Cranial nerve disorder	2	Mucosal ulcers
8	Lupus headache	2	Pleurisy
8	Cerebrovascular accident	2	Pericarditis
8	Vasculitis	2	Low complement
4	Arthritis	2	Increased DNA binding
4	Myositis	1	Fever
4	Urinary casts	1	Thrombocytopenia
4	Haematuria	1	Leukopenia

Summarized from Gladman (Gladman et al., 2002)

Preconception consultation is mandatory, and pregnancy should be planned after at least six months of disease remission. Pregnancy follow-up consists of regular obstetric and internal medicine appointments. The main objective is an early diagnosis of SLE flares, hypertensive disorders of pregnancy, FGR, CHB, gestational diabetes, and spontaneous PTD. Serial monitoring of haematological, chemical, and immunological parameters was performed (with intervals no longer than 3 months). Foetal regular sonographic evaluation with Doppler and foetal echocardiography was also implemented. Before conception, HCQ was initiated as soon as possible, if not already prescribed, and low-dose ASA was started as soon as pregnancy was documented. LMWH was prescribed in all patients with APS or if other risk factors for thromboembolic disease were present.

In the SLE group, our institution supports a low threshold for patient hospitalisation in the presence of maternal or foetal complications and programmed delivery for termination of pregnancy up to 39 weeks of gestation. All patients are reevaluated in the first weeks of postpartum.

The control group was managed according to the Portuguese recommendations for low-risk pregnancies. Serial laboratory monitoring was performed, and foetal evaluation consisted of one ultrasound scan in each trimester (Direção-Geral da Saúde, 2015).

3.3 – Assessment of SLE flare

SLE activity was assessed according to the SLEDAI score (Gladman et al., 2002) up to six months before conception. Categorization of disease as remission / low-level active disease (up to 4 clinical criteria points), mild to moderate (5–12 points), and severe (more than 12 points) was made in all patients. SLE activity during pregnancy was assessed according to the SLEPDAI score (Buyon et al., 1999) calculated at the end of each trimester. The necessity to add new immunosuppressors during pregnancy or the necessity of hospitalisation for SLE-related complications were considered as severe flare criteria.

3.4 – Foetal and Obstetric outcomes

Maternal and foetal outcomes were assessed. Foetal outcomes included birth weight, gestational age at delivery, Apgar scores at 1 and 5 minutes after birth, admission requirement to the neonatal intensive care unit, development of neonatal lupus or CHB, presence of congenital malformation, or respiratory distress syndrome. APO as a composite outcome was defined as the occurrence of miscarriage, stillbirth, FGR, PTD, or the development of hypertensive disorders of pregnancy (gestational hypertension/PE/HELLP syndrome). Spontaneous abortion was defined as the involuntary loss of a foetus before the 20th week of gestation, stillbirth as the foetal death after 20 weeks of pregnancy. Preterm birth was a live birth before the 37th week of gestation. FGR was defined as birth weight below the 10th percentile, using the curves defined for the Portuguese population (Sousa-Santos et al., 2016). PE and HELLP syndrome were defined according to the American College of Obstetricians and Gynecologists Committee (ACOG) criteria (ACOG, 2020). PE was defined as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg on two occasions at least 4 hours apart after 20 weeks of gestation in a woman with previously normal blood pressure and proteinuria (defined as urinary excretion of 300 mg or more in a 24-hour urine collection or a protein/creatinine ratio of 0.3 mg/dL or more or a dipstick reading of 2 +). Otherwise, in the absence of proteinuria, PE was defined as new-onset hypertension with new-onset of target organ damage (platelet count less than $100 \times 10^3/L$; serum creatinine concentration greater than 1.1 mg/dL or doubling of the serum

creatinine concentration in the absence of other renal disease; elevated blood concentrations of liver transaminases to twice the upper limit of normal concentration; severe persistent right upper quadrant or epigastric pain not accounted for by alternative diagnoses; pulmonary edema; new-onset headache unresponsive to acetaminophen and not accounted for by alternative diagnoses; or visual disturbances) (ACOG, 2020). ACOG defines gestational hypertension as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg, or both, on two occasions at least 4 hours apart after 20 weeks of gestation, in a woman with previously normal blood pressure in the absence of proteinuria or organ dysfunction, and that returns to normal in the postpartum period (ACOG, 2020). Gestational diabetes was defined according to the Portuguese Endocrinology Society guidelines for the diagnosis of gestational diabetes (Catarina et al., 2017) and adapted from the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria (Shang and Lin, 2014).

3.5 – Lymphocyte immunophenotyping

3.5.1 – Blood samples collection

Blood samples were collected in each trimester during routine investigations (at 11-14 weeks, 24-28 weeks, and 32-34 weeks of gestation) and at 3-4 weeks postpartum.

Three millilitres of venous peripheral blood was collected into tubes coated with K2EDTA (Vacuette® tube K3E K3EDTA) for lymphocyte immunophenotyping and Treg cells detection and 4 mL into tubes coated with sodium heparin (Vacuette® Tube NH Sodium Heparin) for Th17 cells assay. Samples were analysed within 4 hours of venipuncture.

3.5.2 – Flow cytometry

3.5.2.1 – Fluorochrome labelled monoclonal antibodies

Cells were assessed according to the expression of surface and intracellular markers by flow cytometry, using different combinations of fluorochrome labelled monoclonal antibodies (mAbs).

For blood T, B and NK lymphocytes phenotyping, fluorescein isothiocyanate (FITC)-anti-CD45 (clone B3821F4A), phycoerythrin-cyanin 5.1 (PE-Cy5)-anti-CD3 (clone UCHT1), phycoerythrin-anti-CD4 (PE-anti-CD4) (clone SFC112T4D11), phycoerythrin-Texas Red (ECD)-anti-CD8 (clone SFC121Thy2D3), ECD-anti-CD19 (clone J4.119) and phycoerythrin-anti-CD56 (PE-anti-CD56) (clone N901/NKH-1) mAbs were used, all from Beckman Coulter (Fullerton, California, United States of America - USA).

Th17 analysis was carried out with PE-Cy5-anti-CD3 (clone UCHT1; Immunotech, Marseille, France), FITC-anti-CD4 (clone 13B8.2; Immunotech, Marseille, France), and phycoerythrin anti-IL17a (PE-anti-IL-17A) (clone eBio64-DEC17; eBioscience inc San Diego, CA, USA) mAbs. For Treg evaluation, PE-Cy5-anti-CD4 (clone 13B8.2; Immunotech, Marseille, France), FITC-anti-CD25 (B1.49.9; Immunotech, Marseille, France), and phycoerythrin-anti-FOXP3 (PE-anti-FOXP3) (clone PCH10; eBioscience San Diego, CA, USA) mAbs were used.

3.5.2.2 – T, B, and NK cells subsets immunophenotyping

Whole blood (100 µL) was incubated for 10 minutes at room temperature (RT) in the dark with two different combinations of mAbs (FITC-anti-CD45/PE-Cy5-anti-CD3/PE-anti-CD4/ECD-anti-CD8) and (FITC-anti-CD45/PE-Cy5-anti-CD3/ECD-anti-CD19/PE-anti-CD56), followed by cell fixation and red blood cells (RBC) lysis for 10 minutes, using the IMMUNOPREP Reagent System, from Beckman Coulter (Fullerton, California, USA).

T (CD3+/CD4+ and CD3+/CD8+), B (CD19+), NK (CD56+) and NKT-like (CD3+/CD56+) cell counts (cells/µL), and proportions (%) were established in a 4-colour standard protocol analysis, using a gating strategy of lymphocytes in CD45/side scatter (SSC) on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, California, USA).

3.5.2.3 – Detection of Treg cells

Treg cells were quantified by intracellular staining for FOXP3. Whole blood (100 μ L) was incubated with 10 μ L of anti-CD4 and 10 μ L of anti-CD25 mAbs for 15 minutes at RT in the dark, followed by RBC lysis with 3 mL of RBC Lysis Buffer (eBioscience, San Diego, California, USA). After centrifugation at 1600 rpm for 5 minutes, the supernatant was removed, and cells were washed twice with 3 mL of Flow Cytometry Staining Buffer (eBioscience, San Diego, California, USA) and incubated with 5 μ L of anti-FOXP3.

According to the manufacturer's instructions, cells were fixed and permeabilized using the FOXP3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent system from eBioscience (San Diego, California, USA).

Before flow cytometry analysis, cells were washed once with 3 mL of Flow Cytometry Staining Buffer. After centrifugation at 1600 rpm for 5 minutes, the supernatant was discarded, and the sediment was suspended in 500 μ L of Flow Cytometry Staining Buffer. At least 50 000 events were acquired on an Epics XL-MCL flow cytometer (Beckman Coulter, California, USA), and a first gate was set up on the forward scatter (FSC/SSC) plot to remove debris. Gates were then set only to include lymphocytes with CD4-specific fluorescence and CD4-gated cells were further analysed based on CD25 and FOXP3 expression. CD4+CD25high/FOXP3+ cells were identified as Treg cells (Fig. 1).

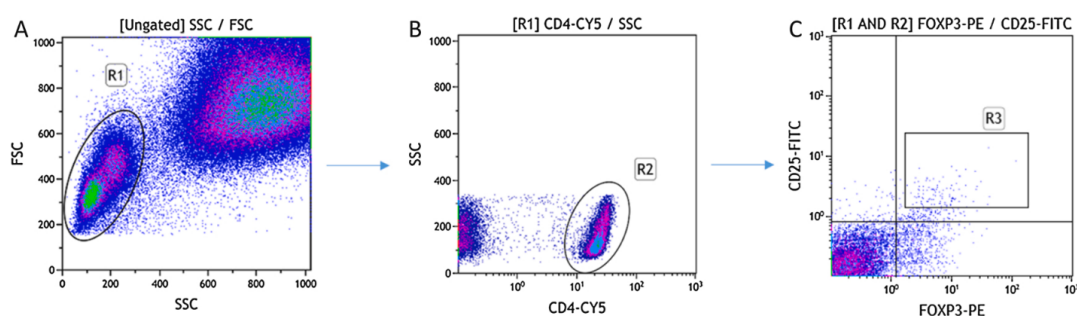


Figure 1 – Cytometric analysis of Treg cells. A first gate [R1] was set on a forward vs. side scatter (FSC/SSC) plot (A) and a second gate [R2] on CD4+ cells (B), followed by the analysis of CD25 and Foxp3 expression on CD4+ lymphocytes. CD4+/CD25high/Foxp3+ cells [R3] were considered as Treg cells (C).

3.5.2.4 – Th17 cells assay

Th17 cell analysis relied on intracellular staining for IL-17A after cell stimulation with phorbol 12-myristate13-acetate (PMA) and ionomycin in the presence of the protein transport inhibitor – brefeldin A.

To activate cells and induce cytokine production, 500 µL heparinised whole blood was added to 500 µL of RPMI 1640 medium supplemented with 2mM L-glutamine (Gibco™, United Kingdom) and incubated with 10 µg/mL brefeldin A, 25ng/mL PMA and 1 µg/mL ionomycin (Sigma Chemical Corporation, St. Louis, Missouri, USA) for 4 hours at 37 °C in an atmosphere of 5% CO₂.

A second whole blood sample (500 µL) was also cultured in the same conditions, without adding PMA and ionomycin stimulants, and used as a control.

For cell surface phenotyping, 100 µL of stimulated and control samples were incubated with 5 µL of anti-CD3 and 10 µL of anti-CD4 mAbs for 15 minutes at RT in the dark. Cells were washed once with 5 mL of phosphate-buffered saline (PBS) buffer (Inova Diagnostics, San Diego, California, USA), followed by centrifugation at 1600 rpm for 5 minutes.

For intracellular staining of IL-17A, cells were suspended, fixed, and permeabilized using the IntraPrep Permeabilization Reagent (Beckman Coulter, California, USA), according to the manufacturer's instructions. After centrifugation at 1600 rpm for 5 minutes, the supernatant was removed, and cells were suspended in 100 µL of IntraPrep Fixation and Permeabilization Buffer and incubated with 5 µL of anti-IL-17A mAb for 30 minutes at RT in the dark.

Finally, cells were washed once, in the same conditions, suspended in a PBS buffer, and examined by flow cytometry on an Epics XL-MCL flow cytometer (Beckman Coulter, California, USA). A minimum of 50 000 events were acquired.

A first gate was set up on the FSC/SSC plot to remove debris, then T/CD4+ cells were gated, followed by analysis of IL17a expression on CD4+ T cells (Fig. 2).

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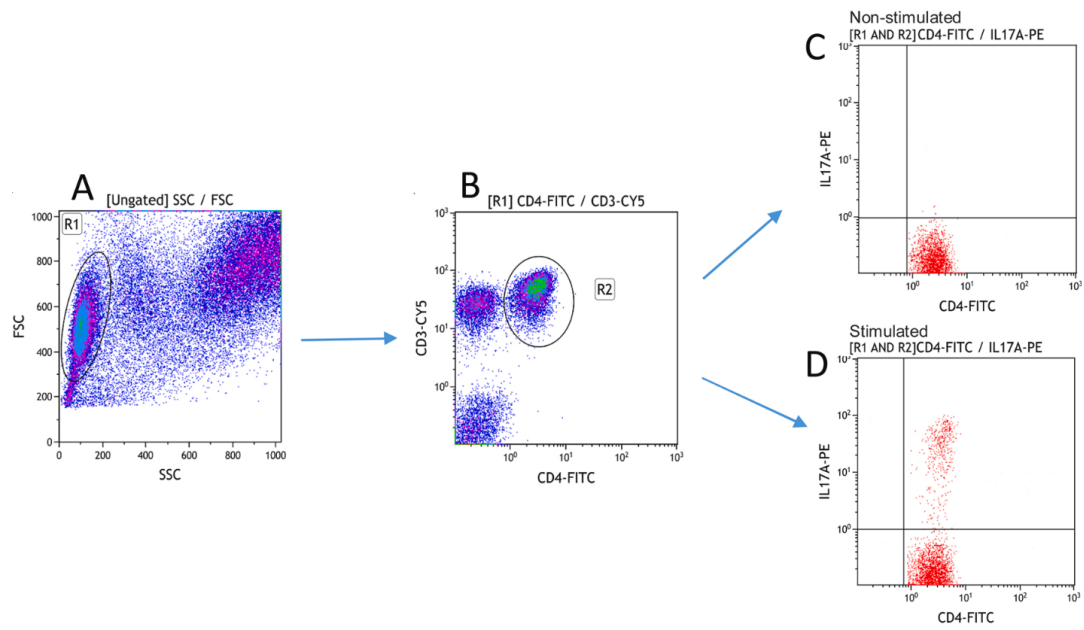


Figure 2 – Cytometric analysis of Th17 cells. A first gate [R1] was set on a forward vs. side scatter (FSC/SSC) plot (A) for lymphocytes and a second gate [R2] on CD3+ / CD4+ cells (B), followed by the analysis of IL17A expression on CD4+ T lymphocytes, in non-stimulated (C) and stimulated samples (D).

Chapter IV

Results

Article 1

Braga A, Barros T, Faria R, Marinho A, Carvalheira G, Rocha G, Farinha F, Neves E, Vasconcelos C, Braga J. **Systemic lupus erythematosus and pregnancy: A retrospective single-center study of 215 pregnancies from Portugal.** *Lupus*. 2021 Nov;30(13):2165-2175. doi: 10.1177/09612033211050340

Systemic lupus erythematosus and pregnancy: A retrospective single-center study of 215 pregnancies from Portugal

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Abstract

Objective: Systemic lupus erythematosus (SLE) is a life-threatening disorder that affects women at reproductive age. We evaluate the clinical impact of pregnancy in a cohort of Portuguese SLE patients and the risk factors associated with maternal and fetal adverse outcomes.

Methods: A retrospective observational study that included all pregnant women with SLE managed at a Portuguese tertiary hospital, between January 1993 and December 2019. Baseline maternal information was collected, and maternal–fetal and neonatal outcomes were evaluated. Disease activity before and during pregnancy was assessed.

Results: We included 215 pregnancies from 143 patients. Lupus nephritis was present in 20.0% and antiphospholipid syndrome (APS) in 21.9% of the cases. Preconception consultation was performed in 86.9% of the pregnancies, and 92.5% of the patients had no or low disease activity at conception. During gestation, 79.6% of the patients were under treatment, and hydroxychloroquine (HCQ) was the most commonly used drug (63.7%). Low-dose acetylsalicylic acid (ASA) was prescribed at conception in 87.9% of the patients. The live birth rate was 84.2%. An adverse pregnancy outcome (APO) occurred in 41.4% of the pregnancies. A miscarriage rate of 15.3% and a preterm delivery rate of 15.4% were found. Preeclampsia and fetal growth restriction complicated 13.1% and 14.0% of the gestations, respectively. Neonatal lupus occurred in 7.1% of the newborns, and there were 2 cases of congenital heart block. Significant risk factors for the development of APO were disease activity at conception, lupus flare, hypocomplementemia, positivity for lupus anti-coagulant, and APS. The use of ASA was significantly associated with a reduced incidence of miscarriage. An SLE flare was diagnosed in 16.3% of the cases. We identified as risk factors for lupus flares the presence of active disease at conception, a previous history of lupus nephritis, and the use of chronic medication. HCQ use during pregnancy was associated with a significant reduction of flare incidence during pregnancy and postpartum.

Conclusions: Pregnancy in an SLE patient is associated with an increased incidence of adverse obstetric outcomes. Good disease control before pregnancy and adequate treatment, especially with HCQ, is crucial to achieving the best obstetric results.

Keywords

Pregnancy, systemic, lupus erythematosus, antiphospholipid syndrome, renal lupus

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Introduction

Systemic lupus erythematosus (SLE) is a multisystemic disorder with strong female preponderance that typically affects women of childbearing age.^{1–3} The real impact of SLE on women's fertility is not well defined, with some studies showing that it may be reduced.^{2,4} Nevertheless, pregnancy is frequent in lupus patients, and SLE is present in 1:660 to 1:2952 of all pregnancies.⁵

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Despite the improvement in maternal and fetal outcomes, pregnancy in SLE patients continues to be associated with a high rate of maternal and fetal morbidity.^{6–8} In these high-risk pregnancies, the incidence of miscarriage, stillbirth, preterm delivery (PTD), fetal growth restriction (FGR), or hypertensive disorders is increased.^{7–9} Furthermore, the incidence of lupus flares is increased during pregnancy and postpartum.¹⁰ SLE clinical and analytical deterioration during pregnancy seems to be closely related to lupus activity before pregnancy, with remission or low disease activity associated with better gestational outcomes. With this in mind, to minimize the risk of adverse obstetric outcomes, the management of pregnancy in a lupus patient must start before conception to control lupus activity and improve the comorbidities that might be present. In fact, pregnancy is not contraindicated in SLE patients unless severe organ damage such as severe pulmonary hypertension or end-stage kidney disease is already installed.¹¹

We conducted a retrospective review of all pregnant patients with SLE followed in our center from 1993 through 2019. The purpose of this study was to assess the clinical impact of pregnancy in a cohort of Portuguese SLE patients and the maternal and fetal outcomes of these pregnancies. The available data were further analyzed, and the study group was divided into two subgroups with the objective to compare the maternal and fetal outcomes between the first and the last half of the follow-up period (pregnant patients before and after 2006).

Materials and methods

Patients

This study included pregnant women with SLE who were managed at the Obstetrics Department and at the Immunology Clinical Unit of our institution, a tertiary university hospital (Centro Hospitalar Universitário do Porto), between January 1993 and December 2019. All patients met the American College of Rheumatism criteria for SLE diagnosis.¹² Each pregnancy was considered individually because the preconception clinical status was included as a predictor of outcome.

Baseline maternal data was collected: age, past obstetric history, SLE disease evolution time, current and cumulative manifestations of SLE, previous clinical and laboratory criteria for antiphospholipid syndrome (APS), and arterial hypertension. Periconceptional SLE activity was assessed by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score.¹³ SLEDAI categorization of disease activity in the 6 months prior to conception as absent (up to four points), mild to moderate (5–12 points), and severe (up to 12 points) was performed in all patients. SLE activity during pregnancy was assessed using the Systemic Lupus

Erythematosus Pregnancy Disease Activity Index (SLEPDAI) scale.¹⁴

Baseline laboratory data were also assessed, in particular: hematological parameters, serum levels of creatinine, urinalysis notably microscopy and proteinuria, immunological evaluation including antinuclear (ANA), anti-double-stranded DNA (ads-DNA), anti-Ro/SS-A and anti-La/SS-B, anti-cardiolipin (aCL), and anti- β 2 glycoprotein (aB2GPI) antibodies, lupus anticoagulant test (LAC), and C3 and C4 complement components.

The study group was further divided into two subgroups (pregnant patients that delivered until 2006 inclusive versus pregnant patients that delivered after 2006), and the maternal and fetal outcomes were compared.

Internal medicine and obstetrics management protocol

All patients were evaluated according to our institutional protocol designed for pregnant patients with SLE, based on EULAR recommendations for the follow-up of pregnant patients with SLE. This protocol specifies the frequency of medical visits, the recommended basic and subsidiary complementary exams, the endorsed evaluation of fetal growth and vitality, as well as recommendations on delivery and puerperium care. A preconceptional consultation is mandatory, and pregnancy should be planned after 6 months of disease remission.

Pregnancy follow-up consists of regular obstetric and internal medicine appointments with the objective of early diagnosis of SLE flares, hypertensive disorders of pregnancy (HDP), gestational diabetes (GD), FGR, congenital heart block (CHB), and risk assessment for spontaneous PTD. Serial monitoring of hematological, chemical, and immunological parameters and regular fetal sonographic evaluation with Doppler and fetal echocardiography was performed. Before conception, hydroxychloroquine was introduced when possible, and low-dose acetylsalicylic acid (ASA) was started as soon as pregnancy was documented, if not already prescribed. Low-molecular-weight heparin (LMWH) was prescribed in all patients with APS or if other risk factors for thromboembolic disease were present. We support a low threshold for patient hospitalization in the presence of maternal or fetal complications and programmed delivery for termination of pregnancy at 39 weeks of pregnancy. All patients are reevaluated during the first weeks of postpartum.

Assessment of SLE flares

Periconceptional SLE activity was evaluated according to the SLEDAI score¹³ in the 6 months before conception, and SLE activity during pregnancy was assessed according to

the SLEPDAI score¹⁴ calculated at the end of each trimester. In addition to the SLEPDAI classification of severe flares, the necessity to add new immunosuppressors during pregnancy or the necessity of hospitalization by an SLE-related comorbidity was considered criteria for a severe flare.

Obstetric and fetal outcomes

Maternal and fetal outcomes were assessed. Fetal outcomes included birth weight, gestational age at delivery, Apgar scores at 1 and 5 min after birth, requirement of admission to the neonatal intensive care unit, development of neonatal lupus or CHB, presence of congenital malformation, or respiratory distress syndrome. adverse pregnancy outcome (APO) was defined as the occurrence of miscarriage, stillbirth, FGR, PTD, or the development of hypertensive disorders of pregnancy (HDP – gestational hypertension/preeclampsia/hemolysis, elevated liver enzymes, and a low platelet count syndrome (HELLP)).

Spontaneous abortion was defined as the unexpected loss of a fetus before 20 weeks of gestation, stillbirth as the fetus's death after 20 weeks of gestation, and elective abortion as the voluntary termination of pregnancy.

Preterm birth referred to a live birth before the 37th week of gestation. FGR was defined as a birth weight below the 10th percentile, using the Portuguese population's curves.¹⁵

Preeclampsia was defined using the American College of Obstetricians and Gynecologists Committee (ACOG) criteria (2020) as a systolic blood pressure ≥ 140 mmHg and/or a diastolic blood pressure ≥ 90 mmHg on two occasions at least 4 h apart after 20 weeks of gestation in a woman with previously normal blood pressure and proteinuria (defined as urinary excretion of 300 mg or more in a 24-h urine collection or protein/creatinine ratio ≥ 0.3 mg/dL or dipstick reading of 2+). Alternatively, in the absence of proteinuria, preeclampsia is defined as new-onset hypertension with the new onset of target organ damage (platelet count less than $100,000 \times 10^9/L$, serum creatinine concentration greater than 1.1 mg/dL or doubling of the serum creatinine concentration in the absence of other renal diseases, elevated blood concentrations of liver transaminases to twice the upper limit of normal concentration, severe persistent right upper quadrant or epigastric pain and not accounted for by alternative diagnoses, and pulmonary edema or new-onset headache unresponsive to acetaminophen and not accounted for by alternative diagnoses or visual disturbances).¹⁶ Gestational hypertension is defined by ACOG criteria (2020) as systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg, on two occasions at least 4 h apart after 20 weeks of gestation, in a woman with previously normal blood pressure in the absence of proteinuria and that returns to normal in the postpartum period.¹⁶

In our cohort, before 2011, GD was defined according to the Carpenter and Coustan criteria¹⁷ and, after 2011, by the Portuguese Endocrinology Society guidelines for the diagnosis of GD,¹⁸ which we adapted from the International Association of Diabetes and Pregnancy Study Groups criteria.¹⁹

Statistical analysis

Descriptive data analysis was performed. Categorical data between groups were compared by the chi-square test, and Fisher's exact tests and quantitative data were compared by analysis of variance, *t*-test, and Scheffe's test. The comparison of the continuous data was performed using nonparametric and parametric tests when indicated. Multivariate analysis was performed using one-way MANOVA. IBM® SPSS® Statistics version 25.0 was used to perform the analysis. The level of significance was set at $p < .05$.

Results

Demographic data and clinical characteristics of patients

We included in this study 215 pregnancies from 143 patients followed at our center between 1993 and 2019. The average age at conception was 31.7 ± 5.1 years, ranging from a minimum of 20 to a maximum of 48 years. The prevalence of primiparity was 49.3%. The mean age at SLE diagnosis was 23.9 ± 6.1 years, and the mean disease evolution time before pregnancy was 7.4 ± 4.9 years. Table 1 summarizes the main pregestational demographic and clinical characteristics.

Cumulative clinical manifestations of SLE included previous cutaneous manifestations in 84.7% of the patients, articular manifestations in 67.4%, hematologic manifestations in 41.9%, and lupus nephritis in 20.0%. The diagnosis of lupus nephritis occurred less than a year before pregnancy in 9.5% of the patients with SLE and kidney involvement. APS was present in 21.9% of the patients, 42.6% fulfilling obstetric criteria (recurrent miscarriage in 25.5%) and 57.4% with thrombotic criteria. Other autoimmune disorders were observed in 18.6% of patients, of particular relevance, Hashimoto's thyroiditis in 7.0%, and Sjogren syndrome in 6.5% of the cases. Arterial hypertension was present at conception in 43.1% of the patients.

At the time of conception, autoantibodies were present in all patients. Antinuclear antibodies were positive in 94.9% of the cases, anti-dsDNA in 38.6%, anti-SS-A in 28.4%, anti-SS-B in 4.7%. aCL in 34.0%, aB2GPI in 22.8%, and LAC in 13.5% of the patients. Complement at the time of conception was low in 30.4% of them. 92.5% of the SLE patients had no or low disease activity at the time of conception (SLEDAI ≤ 4). Preconception consultation was performed in 86.9% of the pregnancies.

Table 1. Baseline clinical characteristics of pregnant SLE patients.

Variable	SLE cases (215 pregnancies in 143 patients)
Age at conception (years, mean $\pm$ SD)	31.7 $\pm$ 5.1
Obstetric history (<i>n</i> (%))	
Nulliparity	106 (49.3)
History of previous miscarriage	59 (27.4)
Pregnancy after assisted reproductive technology	8 (3.7)
Previous comorbidities, <i>n</i> (%)	
Chronic arterial hypertension	93 (43.3)
APS	47 (21.9)
Concomitant autoimmune disorders	40 (18.6)
Periconceptional SLE status (<i>n</i> (%))	
SLEDAI $\leq$ 4 at conception	199 (92.5)
Remission on medication	118 (54.8)
Previous cutaneous manifestations	182 (84.7)
Previous articular manifestations	145 (67.4)
Previous hematologic manifestations	90 (41.9)
Previous lupus nephritis	43 (20.0)
Autoantibodies (<i>n</i> (%))	
ANA	204 (94.9)
Anti-DsDNA	83 (38.6)
Anti-SSA	61 (28.4)
Anti-SSB	10 (4.7)
Anti-cardiolipin	78 (34.0)
Anti-beta2-glycoprotein	49 (22.8)
Lupus anticoagulant	29 (13.5)
Medication (<i>n</i> (%))	
ASA	189 (87.9)
HCQ	137 (63.7)
Prednisolone	87 (40.5)
Azathioprine	19 (8.8)
Cyclosporine	4 (1.9)
Tacrolimus	1 (0.5)
LMWH	68 (31.6)
Vitamin D	29 (13.5)

ANA: antinuclear antibodies; APS: antiphospholipid syndrome; ASA: low-dose acetylsalicylic acid; HCQ: hydroxychloroquine; LMWH: low-molecular-weight heparin; SLE: systemic lupus erythematosus; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

SLE therapy consisted of antimalarial treatment (hydroxychloroquine (HCQ)), immunosuppressors (azathioprine, cyclosporine, and tacrolimus), and corticosteroid therapy.

At conception, 79.6% of the patients were under treatment, 63.7% with HCQ, and 40.5% with prednisone (dose range between 5 and 10 mg/day). Azathioprine was used in 8.8% of the cases, cyclosporine in 1.9%, and tacrolimus in 0.5% of them. ASA was prescribed at conception in 87.9% of the patients.

Pregnancy outcome

Pregnancy outcomes are expressed in Table 2. The live birth rate was 84.2%, and the mean gestational age at delivery

was 37.3 $\pm$ 2.1 weeks. The mean birth weight was 2843.0 $\pm$ 551.1 g (minimum 480 g, maximum 4238 g), and the cesarean rate was 46.7%. APO occurred in 41.4% of the pregnancies (risk factors for APO are summarized in Table 3).

The miscarriage rate was 15.3%, and there were two fetal deaths and five cases of elective termination of pregnancy before fetal viability: two cases secondary to severe fetal malformations (0.9%), two cases due to fetal aneuploidy (0.9%), and one case at 20 weeks of pregnancy due to severe lupus flare refractory to medical treatment. The occurrence of a lupus flare during the first trimester was significantly associated with embryo-fetal loss at the beginning of pregnancy.

In our study, the use of periconceptional ASA was significantly associated with a reduced incidence of

Table 2. Pregnancy outcomes of pregnant women with SLE.

Variable	Outcome
Pregnancy outcome	
Live birth rate (%)	84.2
Mean gestational age at delivery (weeks)	37.3±2.1
APO (%)	41.4
Miscarriage (%)	15.3
PTD (%)	15.4
Spontaneous PTD (%)	34.6
Gestational diabetes (%)	6.0
Preeclampsia (%)	13.1
HELLP syndrome (%)	2.2
FGR (%)	14.0
Cesarean delivery (%)	46.7
Neonatal outcomes	
Neonatal deaths (n)	1
Stillbirths (n)	2
Neonatal ICU admission rate (%)	10.4
Neonatal lupus (%)	7.1
Maternal outcomes	
Maternal deaths (n)	0
SLE flare during pregnancy (%)	16.3
SLE flare postpartum (%)	17.0
SLE improvement (%)	9.7

APO: adverse pregnancy outcome; FGR: fetal growth restriction; HELLP: hemolysis, elevated liver enzymes, and a low platelet count syndrome; ICU: intensive care unit; PTD: preterm delivery; SLE: systemic lupus erythematosus.

miscarriage. The same significant association was also observed in patients with a previous history of APS and the use of ASA or LMWH.

Preterm delivery (PTD) occurred in 15.4% of the cases. PTD was spontaneous in 34.6% (premature rupture of membranes in 7.7% of the cases) and therapeutic in 65.4% of the cases (34.6% for maternal indication and 22% for fetal indication). PTD was more frequent in patients with a history of lupus nephritis (24.3% vs 12.1%) and was significantly associated ($p < .05$) with the development of hypertensive disorders of pregnancy (gestational hypertension, preeclampsia, and HELLP syndrome), a SLEDAI score >4 at conception and the diagnosis of an SLE flare during pregnancy. In our cohort, the presence of LAC and anti-SSA antibodies were also significantly associated with PTD.

The prevalence of preeclampsia and HELLP syndrome in this cohort was 13.1% and 2.2%, respectively. During gestation, the development of preeclampsia was significantly associated with a personal history of arterial hypertension and a previous diagnosis of lupus nephritis. Periconceptional hypocomplementemia, treatment with prednisolone, SLEDAI >4 at conception, and the occurrence of an SLE flare during pregnancy were also

significantly associated with preeclampsia development. On the other hand, the use of HCQ and the reduction of SLEPDAI classification more than 2 points between the first and the third trimester were significantly associated with a decrease of preeclampsia incidence in this cohort.

FGR complicated 14.0% of the gestations. A personal history of APS was significantly associated with the development of FGR. FGR was also more frequent in the subgroup of patients who developed preeclampsia and HELLP syndrome and in patients with a preconception diagnosis of chronic arterial hypertension.

GD was diagnosed in 6% of the patients. In our study, the use of prednisolone was not significantly associated with the development of diabetes.

Cesarean delivery occurred in 46.7% of the cases. Obstetric contraindication for vaginal delivery was responsible for 29.4% of the cesareans (previous history of uterine surgery, fetal malpresentation, and placenta previa); 22.5% were secondary to non-reassuring fetal state and 11.7% for rapid and severe deterioration of maternal state. 70% of the vaginal deliveries occurred after induction of labor, and the rate of vaginal delivery after induction of labor was 63%.

Neonatal outcomes

Of the 183 newborns (one twin delivery), there was one neonatal death secondary to severe prematurity (24 weeks of gestation), and 10.4% needed admission to the neonatal intensive care unit. The Apgar score at the fifth minute of life was ≤ 7 in 4 cases; 2 of these cases had congenital sepsis. Six newborns had postnatal confirmation of interauricular communication, and three had pyelocaliceal dilation.

There was a total of 13 cases of neonatal lupus, including 2 cases of CHB, both diagnosed during pregnancy and whose mothers were positive for anti-SS-A/B antibodies. The remaining neonatal lupus cases had positive autoantibodies (anti-SSA) and photosensitive rash that reverted during the first year of life (11 cases). The use of HCQ was not significantly associated with a reduced frequency of neonatal lupus in our study.

Maternal outcomes

Maternal outcomes are expressed in Table 2. An SLE flare occurred in 16.3% of the patients during pregnancy (22.8% during the first trimester, 14.3% in the second trimester, and 31.1% in the third trimester) and in 17.0% during postpartum. At conception, 92.5% of the patients were in remission/low disease activity (SLEDAI ≤ 4). Disease activity was mild to moderate in 6.5% and severe in 1% of the patients. Among women who flared, one-third had renal involvement, 14.3% skin manifestations, 14.2% articular symptoms, and 40% hypocomplementemia. There were no maternal deaths.

Table 3. Risk factor for APO.

Variable	APO (n = 89)	Normal pregnancy (n = 126)	p-value
Previous comorbidities (n (%))			
Chronic arterial hypertension	26 (29.2)	9 (7.1)	<.05
APS	22 (24.7)	25 (19.8)	NS
Lupus nephritis	26 (29.2)	17 (13.5)	<.05
SLE status (n (%))			
SLEDAI $\leq$ 4 at conception	75 (84.3)	124 (98.4)	<.05
Flare during gestation	20 (22.5)	15 (11.9)	<.05
SLE improvement	6 (6.7)	15 (11.9)	NS
Post-gestation flare	12 (21.4)	19 (15.1)	NS
Low complement levels at conception	31 (34.8)	34 (27.0)	NS
Low complement levels during gestation	27 (30.3)	21 (16.7)	<.05
Medication (n (%))			
ASA	73 (82.0)	112 (88.9)	NS
HCQ	54 (60.7)	83 (65.9)	NS
Prednisone	39 (43.8)	48 (38.1)	NS
LMHW	26 (29.1)	42 (33.3)	NS
Vitamin D	12 (13.5)	17 (13.5)	NS
Autoantibodies (n (%))			
Anti-DsDNA	39 (43.8)	44 (34.9)	NS
Anti-SSA	33 (37.1)	28 (22.2)	<.05
Anti-SSB	3 (3.4)	7 (5.6)	NS
Anti-cardiolipin	29 (32.5)	49 (38.9)	NS
Anti-beta2-glicoprotein	17 (19.1)	23 (18.3)	NS
Lupus anticoagulant	16 (18.0)	13 (10.3)	NS

ANA: antinuclear antibodies; APS: antiphospholipid syndrome; ASA: low-dose acetylsalicylic acid; HCQ: hydroxychloroquine; LMWH: low-molecular-weight heparin; SLE: systemic lupus erythematosus; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

One patient had a stroke (patient positive for aCL and aB2GPI, without criteria for APS) at the 27th week of gestation. Three patients experienced severe, persistent headache nonresponsive to opioid analgesia (so-called lupus headache, in the absence of hypertension or other pre-eclampsia criteria). In these 4 cases of central nervous system involvement, there was 1 case of stillbirth (26th week of pregnancy, during the flare), 1 first trimester miscarriage, 1 PTD secondary to premature rupture of membranes at 31 weeks of gestation (in the patient with the stroke), and 1 vaginal delivery at the 37th week of pregnancy after effective flare control. All the cases of SLE flares were controlled with corticosteroids, and in 11.4% of the cases with the additional use of azathioprine. In two patients, termination of pregnancy was decided due to the difficulty in controlling the maternal condition. In one case, termination of gestation before viability was needed due to a severe renal flare unresponsive to immunosuppressive treatment.

SLE flare during pregnancy was significantly associated with periconceptional SLEDAI >4 , previous lupus nephritis, the use of chronic SLE treatment, and the use of prednisolone. The occurrence of an SLE flare during the first 6 months postpartum was associated with the development of hypocomplementemia during pregnancy and with the

diagnosis of FGR or hypertensive disorders during pregnancy. Postpartum flares were also associated with the development of an SLE flare during gestation.

During gestation, we observed maternal clinical and analytical improvement in 9.7% of the cases. SLE improvement was significantly associated with the maintenance of chronic medication and the use of azathioprine. The absence of anti-SSA and anti-SSB antibodies was significantly associated with a more benign course during pregnancy.

Comparison of pregnancy outcomes before and after 2006

In order to analyze the impact in maternal and fetal outcomes of the improvement of care in lupus patients, the studied group was divided into two subgroups: pregnant patients that delivered until 2006 inclusive versus pregnant patients that delivered after 2006, (mid-term between 1993 and 2019). Patients' characteristics as well as the maternal and fetal outcomes were compared. All data are summarized in [Tables 4](#) and [5](#).

Concerning the clinical characteristics of these patients, the group of lupus patients that conceived after 2006 had a

Table 4. Patient's characteristics before and after 2006.

Variable	SLE patients that become pregnant ≤2006 (n = 57)	SLE patients that become pregnant >2006 (n = 158)	p-value
Age (years, mean ± SD)	24.3±5.7	23.8±6.3	NS
Obstetric history (n (%))			
Nuliparity	33 (57.9)	73 (46.2)	NS
History of previous miscarriage	16 (28.1)	43 (27.2)	NS
ART pregnancy	0 (0)	8 (0.1)	NS
Pregestational SLE status (n (%))			
SLEDAI≤4 at conception	46 (85.2)	153 (96.8)	<.05
Lupus nephritis	19 (33.3)	24 (15.2)	<.05
Medication (n (%))			
ASA	48 (84.2)	141 (89.2)	NS
HCQ	24 (42.1)	113 (71.5)	<.05
prednisone	28 (49.1)	59 (37.3)	NS
azathioprine	4 (0.1)	15 (0.1)	NS
LMHW	14 (24.5)	54 (34.2)	NS
Vitamin D	0 (0)	29 (18.3)	<.05

ART: assisted reproductive technology; ASA: low-dose acetylsalicylic acid; HCQ: hydroxychloroquine; LMWH: low-molecular-weight heparin; NS: not significant; SLE: systemic lupus erythematosus; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

Table 5. Pregnancy outcomes before and after 2006.

Pregnancy outcome	SLE patients that conceived ≤2006	SLE patients that conceived >2006	p value
Mean gestational age at delivery (weeks)	36.5±2.9	37.6±1.8	<.05
Mean newborn birth weight (g)	2654.1±673.42	2914.58±476.54	<.05
APO (%)	54.4	36.7	<.05
Miscarriage (%)	21.1	13.3	NS
PTD (%)	20.9	12.6	NS
Gestational diabetes (%)	5.3	6.3	NS
Preeclampsia (%)	23.9	9.5	<.05
FGR (%)	18.6	12.6	NS

APO: adverse pregnancy outcome; FGR: fetal growth restriction; NS: not significant; PTD: preterm delivery.

significantly larger time interval between SLE diagnosis and gestation ($p < .05$), a lower SLEDAI score at conception ($p < .05$), a higher use of vitamin D at conception ($p < .05$), a lower incidence of lupus nephritis ($p < .05$), a reduced frequency of hypocomplementemia at conception ($p < .05$), and a higher use of HCQ ($p < .05$).

Maternal outcomes also showed several differences between both groups. In the group of lupus patients that became pregnant after 2006, we observed a lower frequency of SLE diagnosis during gestation ($p < .05$), a lower SLEDAI score during gestation and in postpartum ($p < .05$), a reduced frequency of hypocomplementemia during gestation, a higher use of HCQ during gestation ($p < .05$), and a higher rate of SLE improvement ($p = .05$).

Considering pregnancy and maternal–fetal outcomes, in the group of lupus patients that conceived after 2006, we observed a reduced incidence of APO ($p < .05$),

preeclampsia ($p < .05$), HELLP syndrome ($p < .05$), a higher mean gestational age ($p < .05$), and higher mean birth weight at delivery ($p < .05$).

Discussion

Our results emphasize that gestation in an SLE patient is a high-risk pregnancy, being associated with an increased incidence of prematurity, development of HDP, lower birth weight, cesarean delivery, and neonatal intensive care admission,^{7,20} even if SLE activity is in remission/low level at the time of conception. This fact is particularly highlighted in this study because even among women with well-controlled disease (in our cohort, the majority of the patients (92.5%) at the time of conception), perinatal outcomes are worse than those among the general population.^{21,22}

In our study, 92.5% of the SLE patients had a SLEDAI score ≤ 4 at conception, which is associated with no or low disease activity. In these patients, the most frequent SLE activity findings were serologic markers, particularly, low complement or high titers of anti-dsDNA antibodies. Of note, in our study, low complement levels at conception or high titers of dsDNA antibodies were not associated with APO.

In our cohort, live birth rate was 84.2%, similar to what is described in more recently published studies. At least one APO was found in 41.4% of the patients, with a miscarriage rate of 15.3%. PTD occurred in 15.4% of the patients. Preeclampsia and FGR complicated 13.1% and 14.0% of the gestations, respectively. This data is in accordance with the more recently published literature.^{7,23} We observed as a significant risk factor for the development of AOP, the presence of disease activity at the time of conception, the development of a lupus flare during pregnancy, and hypocomplementemia. The positivity for LAC and a previous diagnosis of APS was also significantly associated with the development of FGR and PTD during pregnancy. This is in accordance with the PROMISSE study published in 2015,⁹ where lupus patients with APS had an increased risk of FGR, PTD, early pregnancy loss, and HDP.

In our study, clinical deterioration of lupus activity, even if in the first trimester, was associated with the development of HDP. Other risk factors for the development of preeclampsia are also frequent in patients with lupus, such as arterial hypertension or renal impairment. These factors may help to explain the high incidence of obstetric complications in lupus pregnancies. In our cohort, preeclampsia incidence is in accordance with the more recent published data.^{24–26} The use of low-dose ASA in 87.9% of the patients since the beginning of gestation may explain our incidence of HDP, which is similar to the more recent studies and much lower than older studies.²⁷ One important note is that active SLE and preeclampsia share very identical signs and symptoms, which sometimes makes the differential diagnosis almost impossible. This may be a significant bias in the evaluation of these patients, especially in very severe cases, where termination of pregnancy has to be decided to control a severe lupus flare.

The prematurity rate was 15.6% in our study, which is lower than the rates described in the most recent literature data.^{24,25,27–31} PTD is associated with active disease at conception, development of lupus flare, and other obstetric complications such as preeclampsia or FGR. In our study, as previously stated, a disease in remission at the time of conception in association with regular and tight surveillance of these pregnant patients probably explains the lower PTD rate.

Our miscarriage rate was slightly higher when compared with the published data.^{26,28,31–33} This may be justified by the fact that a considerable percentage of our patients had a

periconceptional evaluation and underwent a very tight surveillance protocol. This, in association with a strict protocol for patient education, may allow us to track pregnancies from the very beginning and diagnose a large proportion of the spontaneous miscarriages that occur in the early first trimester. Our study also described a significant association with ASA use at the time of conception and a reduction in the miscarriage rate, especially in patients with concomitant antiphospholipid antibodies or APS.

Our cesarean rate was relatively high, but it is in accordance with some published data.^{28,33} However, it could be explained by the higher-than-average incidence of APO, especially HDP or FGR. During labor, fetal distress was also more frequent in lupus pregnancies. Nevertheless, a pregnant lupus patient with an uncomplicated pregnancy should be encouraged to have a vaginal delivery.

Clinical or analytical signs of neonatal lupus occurred in 7.1% of the newborns, which resolved during the first months of life. However, there were 2 cases of CHB, representing an incidence of 1%. The published rate of CHB among patients with anti-SSA/B antibodies is about 2% but increases up to 20% if the mother had a previous history of this complication.³⁴ The use of HCQ seems to be protective against the development of CHB,³⁵ which may help explain our low rate of this severe neonatal complication.

An SLE flare was diagnosed in 16.3% of the cases, and in our study, it was significantly more common in patients who were not in remission at the time of conception. The incidence of lupus flare seems to be increased during pregnancy.¹⁰ Eudy et al. showed that pregnant patients had an increased incidence of lupus flares during gestation and postpartum.¹⁰ In our cohort, we identified as significant risk factors for the development of lupus flares, the presence of active disease at the time of conception, a previous history of lupus nephritis, and the need for using chronic medication to maintain lupus remission. This data is similar to other published studies.^{5,9,10,28,33,36–39} The incidence of lupus flare in our population was slightly lower than that found in current published data.^{9,26,28,32,33,39–44} The use of HCQ during pregnancy was associated with a significant reduction of flares during pregnancy and postpartum. In our cohort, a large percentage of patients were on HCQ at the time of conception. In association with a good periconceptional control of the disease, it helps explain the reduced incidence of SLE flares compared with other studies.

In the last few years, the percentage of patients who become pregnant with better disease control is increasing, which justifies the better results of the most recent published data.^{24,25,27–31} In our institution, when we compare our current data with our previously published results,⁴⁵ we notice a marked reduction in the number of flares diagnosed during pregnancy and postpartum (31% vs 16.3%), in accordance with an increment in the use of HCQ during

pregnancy (39% vs 79.5%). The use of HCQ during pregnancy is safe, not related to fetal malformations, and it is essential to control the disease.^{4,46–48} Its use is crucial for the reduction of lupus flares, even in the presence of well-controlled disease at the time of conception.

Lupus nephritis was a significant risk factor for developing either an SLE flare during pregnancy or an AOP, notably HDP and PTD. According to the published literature, renal involvement was a frequent manifestation of lupus activity, occurring in 31% of our patient flares. A meta-analysis performed in 2018²³ reported that gestational hypertension, preeclampsia, SLE flare, proteinuria, and hypocomplementemia were significantly more frequent in pregnant women with lupus nephritis. Even in the presence of a well-controlled disease, lupus patients with a history of renal involvement seem to experience an increased risk of SLE flare and adverse obstetric outcomes.

In the comparative analysis of pregnancies delivered before and after 2006, we observed a better control of the disease, with a significantly lower SLEDAI score at conception, a lower incidence of lupus nephritis, and a higher use of HCQ in the latter subgroup. This reflects the continuous improvement in care of these patients with an optimal SLE control before conception. This fact justifies the better maternal and fetal outcomes observed in the more recent subgroup (conception after 2006).

It is essential to address some limitations of this study. The retrospective nature of this study may be biased by inaccurate or missed information. In addition, the total number of patients and pregnancies included is relatively small, which could be explained by the low prevalence of SLE in the general population. However, in our study, the sample size was able to provide statistical significance for several variables. On the other hand, the study's main strength relies on the fact that the patients had homogeneity in their prenatal follow-up provided by our institution's protocol of assistance, ensuring technical rigor in their follow-up.

Conclusions

This study supports the most recent data highlighting the improvement in pregnancy outcomes of lupus patients in the last decades. These better outcomes contrast with published data from the second half of the last century and point that a better disease control before pregnancy and an adequate treatment, especially with HCQ, is crucial to achieve the best obstetric outcomes. In spite of this, pregnancy in lupus patients remains a high-risk scenario, where SLE flares are still frequent and associated with APO development. Our study revealed a statistically significant association between active SLE and the occurrence of pregnancy loss, prematurity, and the development of HDP or FGR. The use of safe and effective immunosuppressive and immunomodulatory

therapies such as HCQ improved SLE activity during pregnancy and seemed to be associated with a reduction in the development of HDP. However, the use of these drugs was not sufficient to avoid significant adverse outcomes, and APO occurred even in patients with well-controlled disease. The improvement of these outcomes is only possible with the development of trained multidisciplinary teams, with specific surveillance protocols that start with a preconceptional evaluation, months before the actual pregnancy. This must be the basis for qualified prenatal care of SLE patients.

Declaration of conflicting interests

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Article 2

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Systemic Lupus Erythematosus and Pregnancy: a Portuguese Case–Control Study

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Abstract

Pregnancy in systemic lupus erythematosus (SLE) patients is associated with an increased risk of adverse outcomes. During pregnancy, SLE patients have a higher rate of miscarriage, stillbirth, preterm delivery, fetal growth restriction, or hypertensive disorders of pregnancy. To date, only a few case–control studies were published with the purpose to evaluate the magnitude of risk associated with pregnancy in lupus patients. The aim of our study was to evaluate the maternal and fetal outcomes in a cohort of Portuguese SLE patients and to compare it with a group of healthy pregnant women. We conducted a retrospective case–control study that included all pregnant women with SLE managed at a Portuguese tertiary center, between 2010 and 2019. Pregnancy outcomes were compared between SLE patients and a group of matched healthy pregnant women. Baseline maternal data was collected, and maternal–fetal and neonatal outcomes were evaluated. One hundred twenty-four SLE pregnancies were included. Of the patients, 95.2% were in remission at conception. In 13.7% of cases, a lupus flare was diagnosed during gestation and in 17.9% in the postpartum period. The live birth rate was 84.6%, and the incidence of adverse outcomes was 40.3% (OR 2.64, 95% CI 1.67–4.18). Considering only patients in remission at conception, the presence of adverse outcomes remained significantly higher (36.8% vs. 20.3%, $P < 0.01$). Miscarriage rate was 15.3% (OR 5.85, 95% CI 2.57–13.34) and preterm delivery occurred in 12.4% of the patients (OR 1.72, 95% CI 0.83–3.57). Preeclampsia prevalence was higher in SLE patients (OR 3.92, 95% CI 1.32–11.57). In the SLE group, the newborn admission to an intensive care unit rate was increased (OR 4.99, 95% CI 1.47–16.90). No neonatal or maternal deaths were reported. In our study, pregnancy with SLE was associated with an increased incidence of adverse outcomes, even in a population of SLE patients with well-controlled disease.

Keywords Systemic lupus erythematosus · Pregnancy · Miscarriage · Preeclampsia · Preterm delivery · Fetal growth restriction

Introduction

Systemic lupus erythematosus (SLE) is a life-threatening multisystemic autoimmune disorder that affects women of childbearing age [1–3]. The fertility of SLE patients is not

reduced, so the association between lupus and pregnancy is not rare, occurring in 1:660 to 1:3000 pregnancies [2, 4, 5].

Despite the improvement in maternal and fetal outcomes, pregnancy in SLE patients continues to be associated with a high rate of maternal and fetal morbidity [6–8]. The incidence of adverse outcomes like miscarriage, stillbirth, preterm delivery, fetal growth restriction, or hypertensive disorders of pregnancy seems to be increased in these patients [8–22]. During pregnancy, the incidence of lupus flares also appears to be increased [23] and it is closely related to the control of the disease before pregnancy. Optimal management at conception is associated with better gestational outcomes and reduced SLE clinical and analytical deterioration [24, 25].

The management of a pregnancy in a SLE patient must start before conception. A close follow-up of SLE activity

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before conception is crucial aspect for pregnancy success. This, in association with multidisciplinary surveillance throughout the pregnancy and prompt diagnosis and control of any lupus flare, is essential to reduce the incidence of adverse pregnancy outcomes (APO) [26].

Only a few case–control studies were published to date with the objective to evaluate the magnitude of risk associated with pregnancy in lupus patients. The aim of our study was to assess the maternal and fetal outcomes in a cohort of Portuguese SLE patients and compare it with a group of healthy pregnant women.

Materials and Methods Patients

Patients

This study included pregnant women with SLE who were managed at the Obstetric Department and the Immunology Clinical Unit of our institution, a tertiary Portuguese university hospital (Centro Hospitalar Universitário do Porto), between January 2010 and December 2019. All patients met the most recent European League Against Rheumatism (EULAR) and American College of Rheumatism (ACR) criteria for SLE diagnosis [27–29]. This study included 124 pregnancies from 53 SLE patients. Each pregnancy was considered individually because the preconception clinical status was incorporated as a predictor of outcome. Multiple gestations or elective terminations for personal reasons were excluded. The control group was randomly selected from healthy women with singleton pregnancies who attended the same hospital and during the same period. A group of 300 control participants with routine regular antenatal care in our institution was matched with the study group by the date of the first appointment.

In both groups, baseline maternal data was collected: age, past obstetric history, and anterior medical conditions. In the study group, SLE-related data was also collected: SLE evolution time, current and cumulative manifestations, previous clinical and laboratory criteria for antiphospholipid syndrome (APS), and arterial hypertension. Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score was used at preconception to assess SLE activity [30]. During pregnancy, SLE activity was evaluated by Systemic Lupus Erythematosus Pregnancy Disease Activity Index (SLEPDAI) scale [31]. Baseline laboratory data included the following: hematological parameters, serum levels of creatinine, urinalysis notably microscopy and proteinuria, immunological evaluation including antinuclear (ANA), anti-double-stranded DNA (anti-dsDNA), anti-Ro/SS-A, anti-La/SS-B, anti-cardiolipin, anti- β 2 glycoprotein antibodies, lupus anticoagulant test, and C3 and C4 complement components.

Obstetrics and Internal Medicine Management Protocol

According to our institutional protocol designed for SLE pregnant women, all lupus patients were evaluated based on EULAR/ACR recommendations for the management of SLE patients [24, 25, 27–29]. This protocol specifies the frequency of medical visits, the recommended primary and subsidiary complementary exams, the endorsed evaluation of fetal growth, as well as recommendations on delivery and puerperium care.

Preconception consultation is mandatory, and pregnancy should be planned after at least six months of disease remission. Pregnancy follow-up consists of regular obstetric and internal medicine appointments. The main objective is an early diagnosis of SLE flares, hypertensive disorders of pregnancy (HDP), fetal growth restriction (FGR), congenital heart block (CHB), gestational diabetes (GD), and spontaneous preterm delivery (PTD). Serial monitoring of hematological, chemical, and immunological parameters was performed. Fetal regular sonographic evaluation with Doppler and fetal echocardiography was also implemented. Before conception, hydroxychloroquine (HCQ) was introduced when possible, and low-dose acetylsalicylic acid (ASA) was started, if not already prescribed, as soon as pregnancy was documented. Low molecular weight heparin (LMWH) was prescribed in all patients with APS or if other risk factors for thromboembolic disease were present.

In the SLE group, we support a low threshold for patient hospitalization in the presence of maternal or fetal complications and programmed delivery for termination of pregnancy by 39 weeks of pregnancy. All patients are reevaluated in the first weeks of postpartum.

The control group was managed according to the Portuguese recommendations for low-risk pregnancies. Serial laboratory monitoring was performed and fetal evaluation consisted of one ultrasound scan in each trimester [32].

Assessment of SLE Flare

Performed according to the SLEDAI score [30] up to 6 months before conception. Categorization of disease as absent (up to 4 points), mild to moderate (5–12 points), and severe (up to 12 points) was made in all patients. SLE activity was assessed according to the SLEPDAI score [31] calculated at the end of each trimester. The necessity to add new immunosuppressors during pregnancy or the necessity of hospitalization for SLE-related complications were considered as severe flare criteria.

Fetal and Obstetric Outcomes

Maternal and fetal outcomes were assessed. Fetal outcomes included birth weight, gestational age at delivery, Apgar

scores at 1 and 5 min after birth, requirement of admission to the neonatal intensive care unit, development of neonatal lupus or CHB, presence of congenital malformation, or respiratory distress syndrome. APO as a composite outcome was defined as the occurrence of miscarriage, stillbirth, FGR, PTD, or the development of hypertensive disorders of pregnancy (HDP—gestational hypertension/preeclampsia/HELLP syndrome). Spontaneous abortion was defined as the involuntary loss of a fetus before the 20th week of gestation, stillbirth as the fetal death after 20 weeks of pregnancy. Preterm birth was a live birth before the 37th week of gestation. FGR was defined as birth weight below the 10th percentile, using the curves defined for the Portuguese population [33]. Preeclampsia and HELLP syndrome were defined according to the American College of Obstetricians and Gynecologists Committee (ACOG) criteria [34]. Preeclampsia was defined as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg on two occasions at least 4 h apart after 20 weeks of gestation in a woman with previously normal blood pressure and proteinuria (defined as a urinary excretion of 300 mg or more in a 24-h urine collection or a protein/creatinine ratio of 0.3 mg/dL or more or a dipstick reading of 2+). Otherwise, in the absence of proteinuria, preeclampsia was defined as new-onset hypertension with new-onset of target organ damage (platelet count less than $100 \times 10^3/L$; serum creatinine concentration greater than 1.1 mg/dL or doubling of the serum creatinine concentration in the absence of other renal disease; elevated blood concentrations of liver transaminases to twice the upper limit of normal concentration; severe persistent right upper quadrant or epigastric pain not accounted for by alternative diagnoses; pulmonary edema; new-onset headache unresponsive to acetaminophen and not accounted for by alternative diagnoses; or visual disturbances) [34]. ACOG defines gestational hypertension as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg, or both, on two occasions at least 4 h apart after 20 weeks of gestation, in a woman with previously normal blood pressure in the absence of proteinuria or organ dysfunction, and that returns to normal in the postpartum period [34].

GD was defined according to the Portuguese Endocrinology Society guidelines for the diagnosis of gestational diabetes [35] adapted from the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria [36].

Statistical Analysis

Descriptive data analysis was performed. Categorical data between groups were compared by chi-square, and Fisher's exact tests and quantitative data were compared by analysis of variance (ANOVA) and Scheffé's test. The comparison of the continuous data was performed using nonparametric and parametric tests when indicated. The denominator used in the

analyses of all maternal outcomes, stillbirths, and live births was all reported pregnancies. For all other maternal–fetal outcomes, the denominator was all live births. The IBM SPSS Statistics® version 25.0 was used to perform the analysis. The level of significance was set at $P < 0.05$.

This study used a retrospective, observational design, and it was approved by the Medical Ethical Committee of our center. All patient information remained confidential.

Results

Demographic and Clinical Characteristics of Patients

The study group included 124 pregnancies from 53 SLE patients. The average age at conception was 32.4 ± 5.0 years, ranging from a minimum of 20 to a maximum of 48 years. The mean age at SLE diagnosis was 23.3 ± 6.2 years, and the mean disease evolution time before pregnancy was 9.0 ± 4.9 years. The prevalence of primiparity was 42.7% ($n = 53$). Cumulative clinical manifestations of SLE included previous cutaneous manifestations in 85.5% of the patients, articular manifestations in 69.4%, hematologic manifestations in 45.2%, and lupus nephritis in 13.7%. Regarding concomitant morbidities, 10.5% of the patients had arterial hypertension and 16.9% secondary APS. Autoantibodies were positive in all patients. At conception, ANA were positive in 100% of the cases, anti-dsDNA in 41.1%, anti-cardiolipin in 24.2%, anti- β_2 glycoprotein in 12.9%, anti-Ro/SS-A in 28.3%, anti-La/SS-B in 6.5%, and lupus anticoagulant in 17.7%. Complement was low in 20.2% of the patients.

SLE therapy consisted of HCQ, immunosuppressors (azathioprine, cyclosporine, and tacrolimus), and corticosteroids. At conception, 83.1% of the patients were under treatment, 73.4% with HCQ and 37.1% with prednisone. Azathioprine was used in 10.5% of the cases, cyclosporine, and tacrolimus in 0.8%. ASA was prescribed at conception in 87.9% of the patients and LMWH in 36.3% of the patients.

Of the SLE patients, 95.2% had no or low disease activity at the time of conception ($SLEDAI \leq 4$). Preconception consultation was performed in more than 85% of the pregnancies.

The control group included 300 pregnancies with an average age at conception of 31.3 ± 5.8 years, ranging from a minimum of 18 to a maximum of 44 years old. The prevalence of primiparity in this group was 47.7% ($n = 143$). Table 1 summarizes the principal pregestational demographic and clinical characteristics of both groups.

Pregnancy Outcome

Pregnancy outcomes are expressed in Table 2. In the study group, the live birth rate was 84.6%, and the mean gestational

Table 1 Baseline clinical characteristics of pregnant women with and without SLE

Variable	SLE cases (n=124)	Control cases (n=300)	P-value
Age (years, mean $\pm$ SD)	32.4 $\pm$ 5.0	31.3 $\pm$ 5.8	NS
Obstetric history (n, %)			
Nuliparity	53 (42.7%)	143 (47.7%)	NS
History of previous miscarriage	35 (28.2%)	64 (21.3%)	NS
ART pregnancy	8 (6.5%)	5 (1.7%)	< 0.01
Previous comorbidities (n, %)			
Arterial hypertension	13 (10.5%)	0	< 0.01
APS	21 (16.9%)	0	< 0.01
Pregestational SLE status (n, %)			
SLEDAI= $\leq$ 4 at conception	118 (95.2%)	NA	
Cutaneous manifestations	106 (85.5%)	NA	
Articular manifestations	86 (69.4%)	NA	
Hematologic manifestations	56 (45.2%)	NA	
Lupus Nephritis	17 (13.7%)	NA	
Autoantibodies state (n, %)			
ANA	124 (100%)	NA	
Anti-dsDNA	51 (41.1%)	NA	
Anti-Ro/SS-A	35 (28.2%)	NA	
Anti-La/SS-B	8 (6.5%)	NA	
Anti-cardiolipin	30 (24.2%)	NA	
Anti- β 2 glycoprotein	16 (12.9%)	NA	
Lupus anticoagulant	22 (17.7%)	NA	
Medication (n, %)			
ASA	109 (87.9%)	11 (3.7%)	< 0.01
LMWH	45 (36.3%)	2 (0.7%)	
HCQ	91 (73.4%)	NA	
Prednisone	46 (37.1%)	NA	
Azathioprine	13 (10.5%)	NA	
Tacrolimus	1 (0.8%)	NA	
Cyclosporine	1 (0.8%)	NA	

ANA antinuclear antibodies, *Anti-dsDNA* anti-double-stranded DNA, *APS* antiphospholipid syndrome, *ART* assisted reproductive technology, *ASA* low-dose acetylsalicylic acid, *HCQ* hydroxychloroquine, *LMWH* low molecular weight heparin, *NA* not available, *NS* not significant, *SLE* systemic lupus erythematosus, *SLE-DAI* Systemic Lupus Erythematosus Disease Activity Index

age at delivery was 37.7 ± 1.8 weeks. In the control group, the mean gestational age at delivery was 38.5 ± 1.8 weeks ($P < 0.01$). The incidence of APO was 40.3% and 20.3% in the study and control groups, respectively ($P < 0.01$). Even analyzing the subgroup of pregnancies in remission at conception, and excluding patients with known independent risk factors for APO like previous hypertension and lupus nephritis, the presence of APO remained significantly higher in the lupus patients' group (36.8% vs. 20.3%, $P < 0.01$).

In the SLE group, the miscarriage rate was 15.3%. In the control group, the incidence of this adverse outcome was 3.0%, $P < 0.01$. This data corresponds to an OR of 5.9 (CI 2.6–13.3, $P < 0.01$).

PTD occurred in 12.4% of the patients in the SLE cohort and in 7.6% of the cases in the control group ($P=0.136$). In the study group, PTD was spontaneous in 38.6% and

therapeutic in 61.4% of the patients (37.5% for maternal indication and 62.5% for fetal causes). In the control group, PTD was spontaneous in 81.8% of the cases.

The prevalence of HDP was significantly higher in the SLE group (7.6% vs. 2.1%, $P < 0.01$). There were no cases of HELLP syndrome to report in any group. If we analyze the group of SLE patients without chronic arterial hypertension, the incidence of HDP remained two times higher than in the healthy cohort (4.2% vs. 2.1%).

FGR incidence was similar in both groups, complicating 8.6% of the gestations in lupus patients and 8.9% in the control group ($P=0.910$). Cesarean delivery occurred in 42.8% of the cases in the study group and 32.3% of the pregnancies in the control group ($P=0.05$).

Assisted reproductive technologies (ARTs) were performed in 6.5% of lupus patients. In this subgroup of

Table 2 Maternal–fetal outcomes of pregnant women with and without SLE

Variable	SLE cases (<i>n</i> = 124)	Control cases (<i>n</i> = 300)	OR (95% CI)	<i>P</i> value
Pregnancy outcome				
Live birth rate (%)	105 (84.6%)	290 (96.7%)	5.25 (2.36–11.65)	< 0.01
Mean gestational age at delivery (weeks)	37.7 ± 1.8	38.5 ± 1.8		< 0.01
Mean Newborn birthweight (gr)	2938.9 ± 462.9	3138.2 ± 517.1		< 0.01
APO (%)	124 (40.3%)	20 (20.3%)	2.64 (1.67–4.18)	< 0.01
Miscarriage (%)	19 (15.3%)	9 (3.0%)	5.85 (2.57–13.34)	< 0.01
PTD (%)	13 (12.4%)	22 (7.6%)	1.72 (0.83–3.57)	NS
Gestational diabetes (%)	6 (5.7%)	26 (8.9%)	0.54 (0.21–1.34)	NS
HDP (%)	8 (7.6%)	6 (2.1%)	3.92 (1.32–11.57)	< 0.01
FGR (%)	9 (8.6%)	26 (8.9%)	0.96 (0.43–2.11)	NS
Cesarean delivery (%)	45 (42.8%)	94 (32.3%)	1.57 (0.99–2.48)	0.05
Neonatal Outcomes				
Neonatal deaths (<i>n</i>)	0	0		-
Stillbirth (<i>n</i>)	0	1	0.92 (0.03–22.78)	NS
Admission to neonatal ICU (%)	7 (6.7%)	4 (1.3%)	4.99 (1.47–16.90)	< 0.01
Neonatal Lupus (<i>n</i>)	4	0		-
Maternal Outcomes				
Maternal deaths (<i>n</i>)	0	0		-
SLE flare during pregnancy	17 (13.7%)	NA		-
SLE flare postpartum	22 (17.9%)	NA		-
SLE improvement	15 (12.0%)	NA		-

APO adverse pregnancy outcome, FGR fetal growth restriction, HDP hypertensive disorders of pregnancy, ICU intensive care unit, PTD preterm delivery, NA not available, NS not significant, SLE systemic lupus erythematosus

patients, there were no differences between the maternal or fetal outcomes (HDP, prematurity, miscarriage, stillbirth, FGR, intensive care neonatal admission, cesarean delivery, global APO). Therefore, the use of ART to conceive was not associated with APO in our group of SLE patients.

The development of APO was more frequent in SLE patients with APS (26.0% vs. 14.9%, $P > 0.05$), and in patients with antiphospholipid antibodies (40.0% vs. 29.5%, $P > 0.05$), lupus anticoagulant (20.0% vs. 4.1%, $P < 0.01$), or antiphospholipid antibodies triple positivity (10.0% vs. 1.4%, $P < 0.05$). The periconceptional SLE-DAI, the use of corticosteroids, HCQ, or LMWH and the anti-Ro/anti-La status were similar in both the subgroups of SLE patients with vs without APO.

Neonatal Outcomes

In the study group, there were no stillbirths or neonatal deaths to report. There was one stillbirth of a fetus with severe growth restriction at the 24th week of gestation in the control group. There were no neonatal deaths in the control group. Apgar scores at the 1st and 5th minutes were similar in both groups. The newborn admission rate to the neonatal intensive care unit was significantly higher in the SLE group (6.5% vs. 1.3%, $P < 0.01$).

In the study group, there were four cases of neonatal lupus and one case of CHB. All of them were diagnosed during pregnancy and the mothers were all positive for anti-Ro/SS-A and La/B antibodies. With the exception of the CHB case that needed a neonatal intervention with an implementation of a pacemaker, the remaining cases reverted spontaneously in the first year of life. No severe malformations were diagnosed in any group.

Maternal Outcomes

Maternal outcomes are expressed in Table 2. At conception, 95.2% of the patients were in remission, and disease activity was mild to moderate in 4.8% of the cases. There were no cases with a SLEDAI score above eight at the time of conception. There were no maternal deaths to report. A SLE flare was diagnosed in 13.7% of the patients during pregnancy and in 17.9% during postpartum. Among the flares diagnosed during gestation, 52.9% of the patients experienced a clinical deterioration during the first trimester, 11.7% in the second trimester, and 35.3% in third trimester.

Patients that experience a SLE flare during pregnancy had a significant higher incidence of periconceptional SLEDAI > 4 ($P < 0.05$), higher frequency of lupus nephritis ($P < 0.05$), higher necessity to use chronic SLE medication

($P < 0.05$), higher incidence of prednisone prescription ($P < 0.05$), and higher frequency of HDP diagnosis during gestation ($P < 0.05$). The occurrence of a SLE flare during the first 6 months postpartum was more frequent in SLE patients with hypocomplementemia during pregnancy and with the diagnosis of FGR or HDP ($P < 0.05$). Postpartum flares were also related to the development of a SLE flare during gestation. During gestation, we observed maternal clinical and analytical improvement in 12.0% of the patients.

Discussion

Our results highlight that gestation in a SLE patient is a high-risk pregnancy, associated with an increased incidence of adverse obstetric outcomes, particularly prematurity, preeclampsia, lower birth weight, higher cesarean delivery rate, and admission to a neonatal intensive care unit (Table 2). This fact is particularly emphasized in this study because even among SLE patients with well-controlled disease (95% of the patients at conception), perinatal outcomes were worse than those of a healthy control group.

Our SLE cohort's live birth rate was 84.6%, similar to the more recently published data [12, 16, 17, 19]. The presence of at least one APO was significantly higher in SLE patients. Even when we evaluate a subgroup of patients with controlled disease at conception and excluding patients with known risk factors as chronic arterial hypertension and previous lupus nephritis, the development of APO remained significantly higher in lupus patients. These results are in line with published studies, particularly the meta-analyses published by Bundhun in 2017 [20].

In our study, the miscarriage rate was significantly higher in SLE patients, which is in accordance to previously published studies [22, 37–40]. In the studied SLE group, the miscarriage rate was slightly higher when compared to the published data [15, 21, 41–43]. In fact, it is important to consider that this finding may be a bias created by the implementation of a very tight surveillance protocol from early gestation where all miscarriages in the SLE population are diagnosed. On the other hand, in the control group, the number of miscarriages may be underreported. In our setting, early miscarriages occurring in general healthy population are usually not managed in tertiary centers.

PTD is also an adverse outcome associated with lupus. In the SLE cohort, preterm delivery occurred in 12.4% of the cases and was non-spontaneous in most patients (37.5% for maternal indications and 62.5% for fetal indications). This fact is also in line with the published studies [8, 22, 37, 40, 44–46]. In our research, the prematurity rate was lower when compared with the most recent data [15, 43, 47–51]. PTD is generally associated with active disease at conception, development of lupus flare, and other obstetric complications like

preeclampsia or FGR. In our study, as previously stated, a high rate of well-controlled disease at the time of conception in association with regular and tight surveillance may explain the lower PTD rate in our SLE cohort.

HDP are important causes of maternal and fetal morbidity. As noted in the published literature, the incidence of HDP was significantly higher in SLE patients [22, 40, 46, 52]. Besides the higher incidence of other known risk factors, like arterial hypertension or renal impairment, lupus being an autoimmune disorder, may represent an independent risk factor for the development of HDP. This fact is primarily expressed in our study, as the incidence of HDP in SLE patients without chronic arterial hypertension or lupus nephritis and in remission at conception was two-times the incidence of the HDP in control group. In our cohort, the incidence of preeclampsia was in consonance with the most recent published data and was lower when compared with older studies [21, 50, 51]. These findings may be explained by an almost universal prescription of ASA since the beginning of gestation and by a good lupus control at conception and during pregnancy [47].

The incidence of FGR in the study group was similar to the observed in the control group. This result was discordant from that obtained in previously published studies where the incidence of FGR was higher in SLE patients [22, 40, 46, 52]. A well-controlled disease at conception and a higher use rate of ASA may help to explain this finding. The protective effect of HCQ against placental disorders may also be a potential explanation for our lower FGR incidence [53].

As expected, and in accordance with the published literature, the cesarean delivery rate was higher in the study group [8, 44, 46, 52]. This could be explained by the higher-than-average incidence of APO. Nevertheless, a pregnant lupus patient with an uncomplicated pregnancy should be encouraged to have a vaginal delivery.

In our study, the neonatal intensive care unit admission was significantly higher in lupus patients. This fact is in line with published literature [22, 46, 52] and is justified by the higher incidence of PTD. In our study, there were four cases of neonatal lupus and 1 case of CHB, representing an incidence of 1%. The published rate of CHB among patients with anti-SSA/B antibodies is about 2%, but increases up to 20% if the mother has a previous history of this complication [54]. The use of HCQ seems to be protective against the development of CHB [55], which may help explain our lower rate of this severe neonatal complication.

In our cohort, there were no maternal deaths to report. A SLE flare was diagnosed in 13.7% of the patients during pregnancy and in 17.9% during postpartum. The incidence of lupus flares seems to be increased during pregnancy [23]. Eudy et al. showed that when compared with nonpregnant women, pregnant patients had an increased

incidence of lupus flares during gestation and postpartum [23]. Our cohort identified as significant risk factors for the development of lupus flares, the presence of active disease at conception, a previous history of lupus nephritis, and the necessity to use chronic medication to maintain lupus in remission. This data is comparable to other published studies [5, 15, 23, 42, 56–60]. In our cohort, the incidence of lupus flare was slightly lower than that found in current published data [15, 21, 22, 41, 42, 59–64]. This may be explained by a high rate of HCQ use during pregnancy—which is associated with a reduction of SLE flares during pregnancy—and by a good periconceptional control of the disease. In the last few years, the percentage of SLE patients who become pregnant with good disease control increased, which justifies the better results of the most recent published literature [15, 43, 47–51]. During pregnancy, the use of HCQ is safe, not related to fetal malformations, and helps to achieve disease control [4, 65–67]. The use of HCQ during gestation is crucial for the reduction of lupus flares, even in the presence of well-controlled disease at conception [25].

The present study is noteworthy for several reasons. Pregnancy has been traditionally reported to be risky for women with SLE due to the high rates of preeclampsia, fetal growth restriction, cesarean deliveries, lupus flares, and other APO. This study is the first case–control study in a cohort of SLE Portuguese patients. The present study included a single-center large sample of SLE patients, which adds homogeneity to the patient's follow-up.

Nevertheless, our research has some limitations associated with its retrospective nature. This justifies inherent biases, including selection bias and information bias. In addition, the clinical records lacked some relevant demographic details, including the level of education and patient income, which may be confounding factors. Finally, some laboratory data, such as complement and autoantibody state, were not available for the control group as we do not perform these laboratory tests on healthy pregnant women. The generalizability of this study should be interpreted with caution as it was conducted in a single tertiary university hospital in Portugal.

Conclusions

SLE is associated with an increased risk of APO, miscarriage, preeclampsia, prematurity, neonatal intensive care admission, and cesarean delivery, even in a population of SLE patients with well-controlled disease at conception.

Therefore, the planning of a pregnancy in SLE patients should start before conception to achieve optimal control of all comorbidities. In addition, pregnancy follow-up must be made by a well-trained multidisciplinary team, with specific

and tight surveillance protocols. This must always be the basis for a qualified prenatal care of SLE patients.

Future larger cohort studies should focus on antenatal management and medication use in pregnant women with SLE.

Recommendations

- SLE is associated with an increased risk of APO; therefore, the planning of a pregnancy in SLE patients should start before conception to achieve optimal control of all comorbidities
- Pregnancy follow-up must be made by a well-trained multidisciplinary team, with specific and tight surveillance protocols
- The development of APO was more frequent in SLE patients with APS or antiphospholipid antibodies, so the use of LMWH is particularly important in the subgroup of patients

Author Contribution AB, CV, and JB conceived and designed the study. AB and TB collected the patient data. AB, RF, CV, and JB contributed to the analysis and interpretation of data. All authors drafted the manuscript, revised it critically, and approved the final version for submission.

Declarations

Conflict of Interest The authors declare no competing interests.

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Article 3

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Short communication

Th17/Regulatory T cells ratio evolution: A prospective study in a group of healthy pregnant women

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ABSTRACT

During pregnancy, the maternal immune system is challenged to tolerate a semi-allogenic fetus. A shift toward a tolerogenic profile is essential to ensure a healthy fetal and placental development. One of the most important mechanisms involved in the maternal immune tolerance towards the fetal antigens is expressed in the activity of the regulatory T (Treg) and Th17 cells. The behavior and equilibrium of these two T lymphocyte populations were rarely studied in normal healthy pregnancies through the beginning of gestation to the postpartum period. We conducted a prospective longitudinal observational study where peripheral blood lymphocyte subsets were analyzed in each trimester of pregnancy and postpartum period in a group of healthy pregnant women. Our study observed a consistent reduction in peripheral Treg cell count through all pregnancy while the Th17 cell count remained stable. The Th17/Treg ratio increases significantly throughout pregnancy to the postpartum period. These changes could be justified by the migration of the immunotolerant Treg cells to the maternal decidua and lead to the establishment of a systemic pro-inflammatory profile by the end of pregnancy. This data could explain why systemic syndromes like preeclampsia develop in susceptible women during the second half of pregnancy or why many autoimmune disorders flourish in the first weeks postpartum.

1. Introduction

During pregnancy, the maternal immune system is challenged to tolerate a semi-allogenic fetus. The fetus inherits half of its genetic material from the father, exposing foreign antigens to the mother's immune system. The normal progression of gestation requires that the maternal immune system must be able to tolerate the exposed fetal antigens and then allow the normal trophoblast growth. Medawar first recognized this immunological paradox in 1953 (Medawar, 1953) and, despite the extensive research conducted, it is not yet fully understood. The breakage of maternal immune tolerance seems to be associated with the development of adverse obstetrical outcomes like miscarriage, preeclampsia, or preterm delivery (Cornelius and Lamarca, 2014; Figueiredo and Schumacher, 2016; Saifi et al., 2014; Wang et al., 2013; Zare et al., 2018). In this paradigm, the placenta is the real "transplant". An abnormal trophoblast growth and differentiation leads to an incompetent placenta, which is linked to the pathogenesis of miscarriage, preeclampsia, and fetal growth restriction (Figueiredo and Schumacher,

2016). The trophoblast invasion necessary to develop a fully functional placenta demands an intricate network of immune tolerance that allows the growth of cells expressing paternal antigens through the maternal decidua. Several mechanisms with diverse protagonists are involved in this complex tolerance game. Still, regulatory T cells (Treg cells) and effector T cells that produce interleukin (IL) 17a (Th17 cells) appear to have an essential role in the tolerance mechanism throughout pregnancy (Cornelius and Lamarca, 2014; Figueiredo and Schumacher, 2016; Saifi et al., 2014; Zare et al., 2018).

In a normal pregnancy, Treg cells are crucial for the blastocyst implantation and trophoblast development, therefore, a shift toward Th2 cells and an elevation of Treg in maternal decidua and circulation are usually observed (Redman and Sargent, 2010). Treg is a subset of CD4 + T cells with the ability to suppress immune reactions and maintain peripheral tolerance (Bennett et al., 2001; Sakaguchi et al., 1995). These cells are characterized by high cell membrane expression of the receptor for interleukin-2 (CD25^{high}) and of the transcription factor X-linked Foxhead box P3 (Foxp3), which is a "master switch" for Treg

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development and the most important marker of these cells (Adeegbe and Nishikawa, 2013; Fontenot et al., 2005; Littman and Rudensky, 2010).

Treg cells are usually classified into two subpopulations: induced regulatory T cells (iTreg), which develop in the periphery from naïve T cells upon exposure to transforming growth factor- β (TGF- β) and IL-2, and natural Treg (nTreg), which grow from thymus TCR high-affinity T cells and represent a key mechanism of peripheral tolerance to self-antigens (Adeegbe and Nishikawa, 2013; Chien and Chiang, 2017). These cells have been proposed as one of the most critical immunosuppressive agents involved in the maternal immune tolerance to the fetal antigens, and recent studies suggest that maladaptation in these immune responses is crucial for the development of adverse obstetrical outcomes like miscarriage (Bansal et al., 2011; Wang et al., 2013, 2010) or preeclampsia (Darmochwal-Kolarz et al., 2012; Gleicher, 2007; Kieffer et al., 2019; Sibai et al., 2005).

Th17 cells represent a lineage of CD4 + T lymphocytes characterized by expressing the transcription factor retinoic acid receptor-related orphan receptors (ROR) γ t and by the production of IL-17a. These cells have a pro-inflammatory profile and play a vital role in host defense against extracellular bacteria, *Mycobacterium tuberculosis*, and fungus (Saito et al., 2011). An increment in the number and activity of Th17 cells has been implicated in the pathophysiology of several autoimmune disorders, like multiple sclerosis (Fletcher et al., 2009), systemic lupus erythematosus (Marinho et al., 2017; Xing et al., 2012), or rheumatoid arthritis (Wang et al., 2012).

Th17 and Treg cells present a certain level of plasticity, and, in the presence of certain stimuli, Treg cells can differentiate into Th17 cells and *vice versa* (Bellemore et al., 2015; Komatsu et al., 2014). Notwithstanding, lowering Treg and raising Th17 dynamic profiles seems to be associated with several adverse obstetric outcomes like preterm birth (Koucký et al., 2014), preeclampsia (Vargas-Rojas et al., 2016), or recurrent pregnancy loss (Mjösberg et al., 2010).

The aim of this study was to prospectively evaluate the number of circulating Treg and Th17 cells in a group of healthy pregnant women during pregnancy and postpartum to compare these cells count and their ratio throughout gestation to puerperium. As far as we know, this is the first study to prospectively evaluate the frequency and variation of Treg and Th17 cells in a group of healthy uncomplicated pregnant women during all pregnancy and postpartum.

2. Material and methods

2.1. Subjects and study design

The study population consisted of 25 healthy pregnant women, whose pregnancy surveillance and delivery occurred at the Obstetric Department of Centro Hospitalar Universitário do Porto (CHUP - North of Portugal). By protocol, all the patients included should be healthy before pregnancy and without previous obstetric complications (excepting for personal history of a single early previous miscarriage). We analyzed the subgroup of patients with personal history of a single previous miscarriage and compared it with the remaining pregnant women to find differences in the distribution of the study cells.

This study had a prospective design. All pregnant patients were recruited when attending their first scheduled visit at the Obstetric Department of CHUP. Blood samples were obtained in each trimester during routine investigations (at 11–14 weeks, 24–28 weeks and 32–34 weeks of gestation), and at 3–4 weeks postpartum.

Ethical approval was obtained from the research ethics committee of Centro Hospitalar Universitário do Porto, and written informed consent for all the analysis was obtained from all subjects. The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki.

2.2. Blood samples collection

Three milliliters (mL) of venous peripheral blood was collected into tubes coated with K2EDTA (VACUETTE® TUBE K3E K3EDTA) for lymphocyte immunophenotyping and Treg cells detection and 4 mL into tubes coated with sodium heparin (VACUETTE® TUBE NH Sodium Heparin) for Th17 cells assay. Samples were analyzed within 4 h of venipuncture.

2.3. Flow cytometry

2.3.1. Fluorochrome labeled monoclonal antibodies

Cells were assessed according to the expression of surface and intracellular markers by flow cytometry, using different combinations of fluorochrome labeled monoclonal antibodies (mAbs).

For blood T, B and NK lymphocytes phenotyping, fluorescein isothiocyanate (FITC)-anti-CD45 (clone B3821F4A), phycoerythrin-cyanin 5.1 (PE-Cy5)-anti-CD3 (clone UCHT1), phycoerythrin (PE)-anti-CD4 (clone SFC112T4D11), phycoerythrin-Texas Red (ECD)-anti-CD8 (clone SFC121Thy2D3), ECD-anti-CD19 (clone J4.119) and PE-anti-CD56 (clone N901/NKH-1) mAbs were used, all from Beckman Coulter (Fullerton, California, United States of America - USA).

Th17 analysis was carried out with PE-Cy5-anti-CD3 (clone UCHT1; Immunotech, Marseille, France), FITC-anti-CD4 (clone 13B8.2; Immunotech, Marseille, France) and PE-anti-IL-17A (clone eBio64-DEC17; eBioscience inc San Diego, CA, USA) mAbs. For Treg evaluation, PE-Cy5-anti-CD4 (clone 13B8.2; Immunotech, Marseille, France), FITC-anti-CD25 (B1.49.9; Immunotech, Marseille, France) and PE-anti-FoxP3 (clone PCH10; eBioscience San Diego, CA, USA) mAbs were used.

2.3.2. T, B and NK cells subsets immunophenotyping

Whole blood (100 μ L) was incubated for 10 min at room temperature (RT) in the dark with two different combinations of mAbs (FITC-anti-CD45/PE-Cy5-anti-CD3/PE-anti-CD4/ECD-anti-CD8) and (FITC-anti-CD45/PE-Cy5-anti-CD3/ECD-anti-CD19/PE-anti-CD56), followed by cell fixation and red blood cells (RBC) lysis for 10 min, using the IMMUNOPREP Reagent System, from Beckman Coulter (Fullerton, California, USA).

T (CD3+/CD4+ and CD3+/CD8+), B (CD19+), NK (CD56+) and NKT-like (CD3+/CD56+) cells counts (cells/ μ L) and proportions (%) were established in a 4-colour standard protocol analysis, using a gate strategy of lymphocytes in CD45/side scatter (SSC) on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, California, USA).

2.3.3. Detection of Treg cells

Treg cells were quantified by intracellular staining for Foxp3. Whole blood (100 μ L) was incubated with 10 μ L of PE-Cy5-anti-CD4 and 10 μ L of FITC-anti-CD25 mAbs for 15 min at RT in the dark, followed by RBC lysis with 3 mL of RBC Lysis Buffer (eBioscience, San Diego, California, USA). After centrifugation at 1600 rpm for 5 min, the supernatant was removed and cells were washed twice with 3 mL of Flow Cytometry Staining Buffer (eBioscience, San Diego, California, USA).

Cells were fixed and permeabilized using Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent system from eBioscience (San Diego, California, USA), according to the manufacturer's instructions.

Before flow cytometry analysis, cells were washed once with 3 mL of Flow Cytometry Staining Buffer. After centrifugation at 1600 rpm for 5 min, the supernatant was discarded and the sediment was suspended in 500 μ L of Flow Cytometry Staining Buffer. A minimum of 50 000 events were acquired on an Epics XL-MCL flow cytometer (Beckman Coulter, California, USA) and a first gate was set up on the forward scatter (FSC)/SSC plot to remove debris. Gates were then set to only include lymphocytes with CD4-specific fluorescence and CD4-gated cells were further analyzed based on CD25 and Foxp3 expression. CD4+CD25high/Foxp3+ cells were identified as Treg cells (Fig. 1).

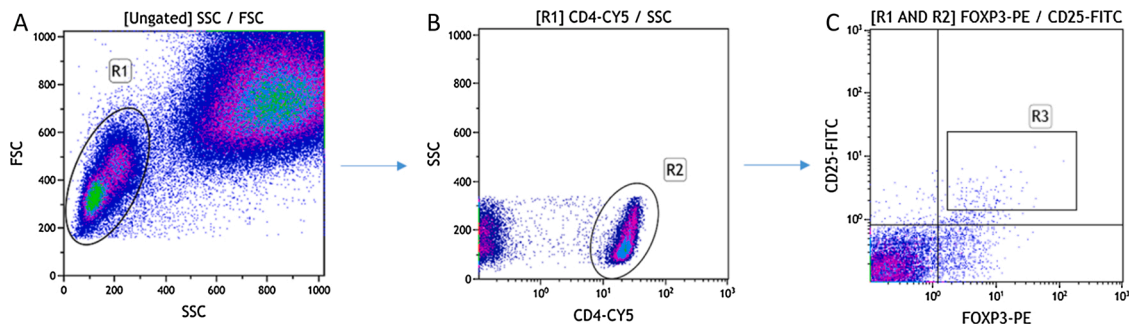


Fig. 1. Cytometric analysis of Treg cells. A first gate [R1] was set on a forward vs. side scatter (FSC/SSC) plot (A) and a second gate [R2] on CD4⁺ cells (B), followed by the analysis of CD25 and Foxp3 expression on CD4⁺ lymphocytes. CD4⁺/CD25^{high}/Foxp3⁺ cells [R3] were considered as Treg cells (C).

2.3.4. Th17 cells assay

Th17 cells analysis relied on intracellular staining for IL-17A after cell stimulation with phorbol 12-myristate13-acetate (PMA) and ionomycin in the presence of the protein transport inhibitor – brefeldin A.

To activate cells and induce cytokine production, 500 μ L heparinized whole blood was added to 500 μ L of RPMI 1640 medium supplemented with 2 mM l-glutamine (GibcoTM, United Kingdom) and incubated with 10 μ g/mL brefeldin A, 25 ng/mL PMA and 1 μ g/mL ionomycin (Sigma Chemical Corporation, St. Louis, Missouri, USA) for 4 h at 37 °C in an atmosphere of 5% CO₂.

A second whole blood sample (500 μ L) was also cultured in the same conditions, without the addition of PMA and ionomycin stimulants, and used as control.

For cell surface phenotyping, 100 μ L of stimulated and control samples were incubated with 5 μ L of anti-CD3 and 10 μ L of anti-CD4 mAbs, for 15 min at RT in the dark. Cells were washed once with 5 mL of PBS buffer (Inova Diagnostics, San Diego, California, USA), followed by centrifugation at 1600 rpm for 5 min.

For intracellular staining of IL-17A, cells were suspended, fixed and permeabilized using the IntraPrep Permeabilization Reagent (Beckman Coulter, California, USA), according to the manufacturer's instructions. After centrifugation at 1600 rpm for 5 min, the supernatant was removed, cells were suspended in 100 μ L of IntraPrep Permeabilization

Buffer and incubated with 5 μ L of anti-IL-17A mAb for 30 min at RT in the dark.

Finally, cells were washed once, in the same conditions, suspended in PBS buffer and examined by flow cytometry on an Epics XL-MCL flow cytometer (Beckman Coulter, California, USA). A minimum of 50 000 events were acquired.

A first gate was set up on the FSC/SSC plot to remove debris, then T/CD4⁺ cells were gated, followed by analysis of IL17A expression on CD4⁺ T cells (Fig. 2).

2.4. Statistical analysis

Nonparametric Mann-Whitney *U* test and *t*-Student test were used to compare means between groups when appropriate. Nonparametric tests were used to analyze the Th17/Treg ratio and parametric tests were used to analyze the lymphocyte distribution. The significance level was set at $p = 0,05$. Statistical evaluation and plotting were performed using the SPSS program (version 26.0).

2.5. Maternal and neonatal outcomes

In the study group, the median age at conception was $29,85 \pm 6,51$ years. 60 % were primiparas, and the median gestational age at delivery

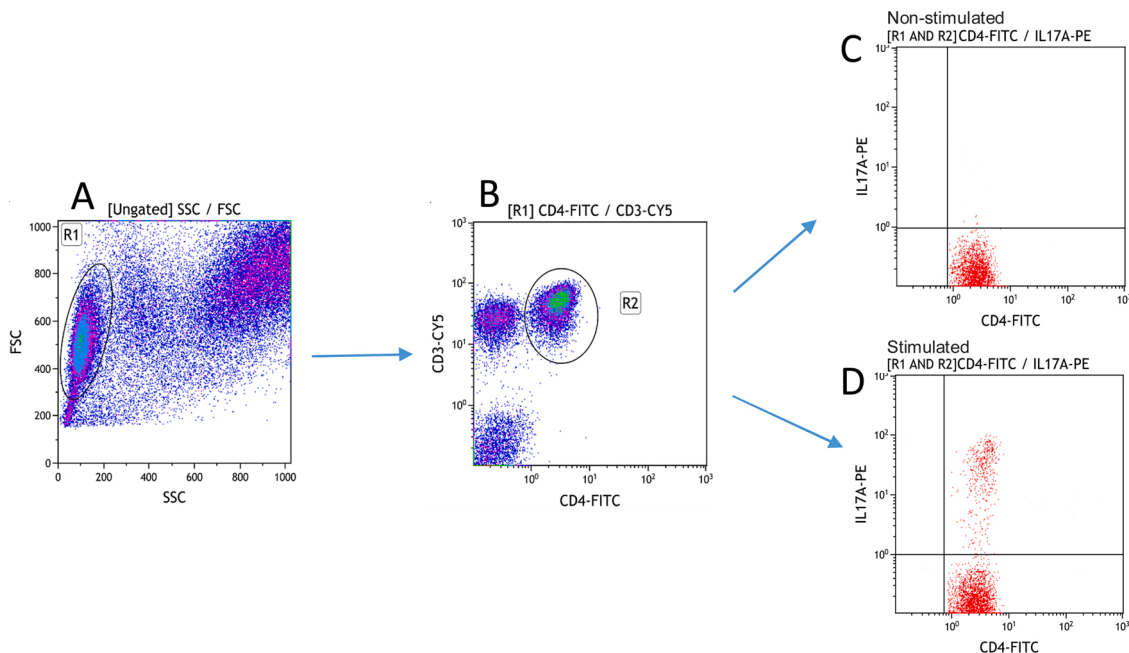


Fig. 2. Cytometric analysis of Th17 cells. A first gate [R1] was set on a forward vs. side scatter (FSC/SSC) plot (A) for lymphocytes and a second gate [R2] on CD3⁺/CD4⁺ cells (B), followed by the analysis of IL17A expression on CD4⁺ T lymphocytes, in non-stimulated (C) and stimulated samples (D).

was $39,25 \pm 1,02$ weeks. None of the pregnant women had a previous history of recurrent miscarriage, preeclampsia, or preterm delivery. In all cases, pregnancy was uneventful without the development of hypertensive disorders of pregnancy, fetal growth restriction, gestational diabetes, or preterm delivery. Spontaneous labor occurred in 70 % of the cases and the remaining cases were induced to prevent postdatism according to Portuguese pregnancy surveillance guidelines. All newborns were healthy, no malformations were diagnosed, and the Apgar Index at 5 min of life was >7 in all cases. Demographic and pregnancy outcomes are highlighted in Table 1.

3. Results

3.1. Evaluation of CD3+/CD4+ and CD3+/CD8 + T cells, CD3-/CD56 + NK cells and CD3+/CD56 + NKT-like cells during pregnancy

The evaluation of CD3+/CD4+ and CD3+/CD8 + T cells, CD3-/CD56 + NK cells and CD3+/CD56 + NKT-like cells during pregnancy and postpartum is expressed in Fig. 3. There were no significant differences in the distribution of these cells during pregnancy and postpartum.

3.2. Treg cells during pregnancy

Regarding the evolution of CD4⁺CD25^{high}Foxp3⁺ Treg cells count during pregnancy, we observed a decrease in number during pregnancy to postpartum. The highest value was observed in the first trimester and steadily decreased throughout pregnancy, with a significant reduction postpartum (Fig. 4). This data is similar to data published by Mjösberg (Mjösberg et al., 2010, 2009). This author also used Foxp3 as a marker for regulatory T cells during pregnancy and described a decline in these cells during the first and second trimesters.

3.3. Th17 cells and Th17/Treg ratio during pregnancy

Th17 cells number showed a different behavior when compared with CD4⁺CD25^{high}Foxp3⁺ Treg cells (Fig. 4). An increase in Th17 cells count was observed during the 2nd trimester, followed by a reduction at the end of pregnancy, and a second increase at postpartum, achieving its highest value after birth (Fig. 4). Considering the plasticity of Th17 and Treg cells, we compared the mean cells count of both cell types in each trimester and postpartum. In our study, the mean value of Th17 cells was always significantly higher ($p < 0,05$) than the CD4⁺CD25^{high}Foxp3⁺ Treg cells count.

When analyzing the Th17/Treg ratio, there was a trend to its increase throughout pregnancy to postpartum (Fig. 5). This increase was consistent in all cases and achieved statistical significance postpartum.

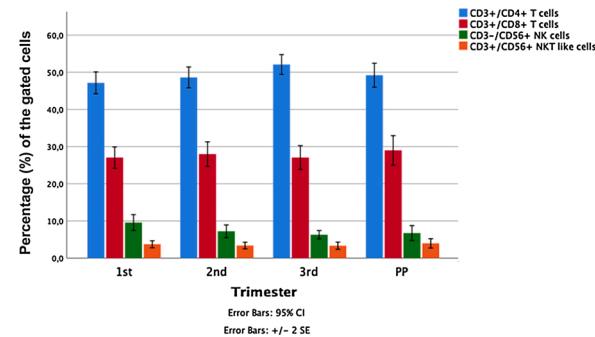


Fig. 3. Evaluation of CD3+/CD4+ and CD3+/CD8 + T cells, CD3-/CD56 + NK cells and CD3+/CD56 + NKT-like cells during pregnancy and postpartum.

3.4. Effect of history of previous miscarriage

When we analyze the subgroup of patients with personal history of previous miscarriage and compare it with remaining pregnant women, the rate of CD3+/CD4+ and CD3+/CD8 + T cells, CD3-/CD56 + NK cells and CD3+/CD56 + NKT-like cells during pregnancy and postpartum were similar in both groups.

Regarding the distribution of CD4⁺CD25^{high}Foxp3⁺ Treg and in Th17 cells count during pregnancy and postpartum, there were no differences between groups (data not shown).

4. Discussion

As far as we know, this is the first study to prospectively evaluate in a group of healthy uncomplicated pregnant women the behavior of Treg and Th17 cells counts and variation during pregnancy and postpartum.

The observed kinetics of Th17 and Treg cell numbers in this study suggest a dynamic balance between immune activation and suppression throughout pregnancy. This may reflect a physiological mechanism for maternal immune tolerance towards the fetus (Ji et al., 2019; Krechetova et al., 2020). Foxp3 transcription factor impairs the development of Th17 cells and assigns an anti-inflammatory effect to T cells, which justifies an essential tolerogenic function of regulatory T cells during pregnancy (Figueiredo and Schumacher, 2016; Liu et al., 2017). The consistent reduction on Treg cells counts during pregnancy here described highlights a possible migration of these cells from the peripheral blood to the maternal-fetal interface in the decidua, where intimate contact between maternal and fetus cells occurs (Hosseini et al., 2016; Liu et al., 2011).

The higher value of the Th17/Treg ratio by the end of pregnancy and postpartum may reflect a physiological pro-inflammatory state, where Th17 cells may be implicated, reinforcing an inflammatory scenario to facilitate parturition (Gomez-Lopez et al., 2020). These findings may also help to explain why systemic pro-inflammatory syndromes, like preeclampsia, develop in susceptible women during the second half of pregnancy (Anvari et al., 2015; Eghbal-Fard et al., 2019; Ribeiro et al., 2017; Zare et al., 2018) and the flourishing of many autoimmune disorders in the first months postpartum (Liu et al., 2017).

In our study there were no significant differences in the distribution of CD3+/CD4+, CD3+/CD8 + T cells, CD3-/CD56 + NK cells and CD3+/CD56 + NKT-like cells during pregnancy and postpartum. The importance of CD3-/CD56 + NK cells and CD3+/CD56 + NKT-like cells during pregnancy is well established (Bansal et al., 2012; Fu et al., 2013; Fukui et al., 2012; Hanna et al., 2006; Loewendorf et al., 2015; Yuan et al., 2015), and the presence of a pro-inflammatory profile has been associated with a poorer placentation, recurrent miscarriage and preeclampsia (Fu et al., 2014; Fukui et al., 2012; Sargent et al., 2007). We did not observe any significant variation throughout pregnancy in these cells' populations. There are few studies that evaluated the evolution of NK and NK-T like cells and their different phenotypes during pregnancy,

Table 1
Demographic and pregnancy outcome data.

Demographic data	
Age at conception (years $\pm$ SD)	29,85 $\pm$ 6,5
Primiparity (%)	15 (60,0)
Previous miscarriage rate (%)	5 (20,0)
Pregnancy outcomes	
Mean gestational age at delivery (gestational weeks $\pm$ SD)	39,3 $\pm$ 1,0
Cesarean rate (%)	5 (20,0)
Number of cases with Apgar Index <7 at 5 min (n)	0
Female newborns (%)	11 (44)
Newborns with weight $< 10^{\text{th}}$ percentile (%)	3 (8)
% Hypertensive disorders of pregnancy	0
% Preterm deliveries	0

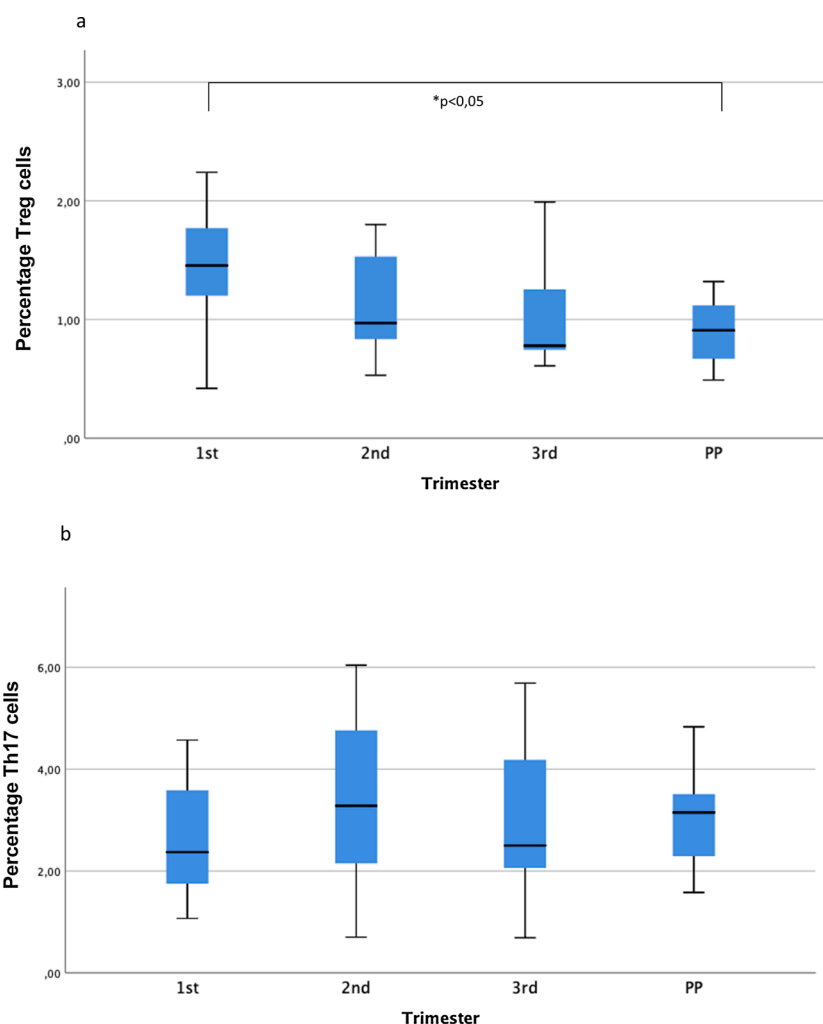


Fig. 4. (a) Boxplot representation of the distribution of $CD4^+CD25^{high}Foxp3^+$ Treg cells count during pregnancy and in postpartum. (b) Boxplot representation of the distribution of Th17 cells count during pregnancy and in postpartum.

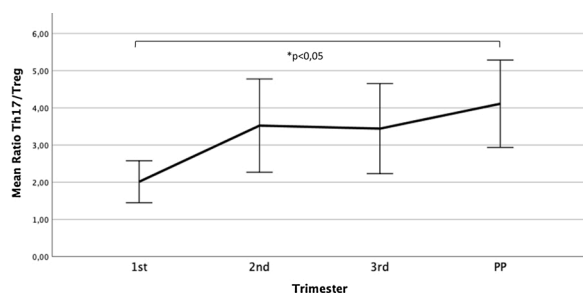


Fig. 5. Th17/CD4+CD25highFoxp3+ Treg ratio throughout pregnancy to postpartum.

especially in healthy pregnant women, so these findings may illustrate a physiologic tolerogenic progression of uncomplicated pregnancies.

We also evaluated the dynamics of $CD3^-/CD56^+$ NK cells, $CD3^+/CD56^+$ NKT-like, $CD4^+CD25^{high}Foxp3^+$ Treg and Th17 cells in patients with a previous miscarriage. In our study, the distribution of these cells during pregnancy and postpartum was similar in the subgroup of patients with a previous miscarriage. This finding may be justified by the design of the study, where all the enrolled pregnant women did not have any other previous obstetric adverse outcome. The occurrence of a single previous early miscarriage is usually explained by chromosomal defects (American Society for Reproductive Medicine, 2012; Gonçalves et al.,

2018), which are not immune-mediated, so, the absence of differences between groups may be justified by this fact.

The reduced number of enrolled women is one of the limitations of our study. However, a solid selection of participants highlighted by clinical sample homogeneity and the rigorous protocols for pregnancy surveillance strengthens our results. Furthermore, the major advantage of this study is the longitudinal evaluation of each pregnant women in each trimester and postpartum.

Our study showed a clear opposite behavior of $CD4^+CD25^{high}Foxp3^+$ Treg and Th17 cells throughout pregnancy to postpartum.

Declaration of Competing Interest

The authors have no financial or other conflicts of interest in the outcomes of this study.

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Article 4

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The dynamics of Th17 / Treg ratio in SLE patients during pregnancy

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ABSTRACT

Systemic lupus erythematosus (SLE) is a multisystemic autoimmune disorder that affects women at childbearing age. During pregnancy, maternal immune system is challenged to tolerate a semi-allogenic fetus and a shift toward a tolerogenic profile is essential. Failure to develop this tolerogenic profile seems to be associated with the development of adverse obstetric outcomes. We conducted a prospective longitudinal observational study where peripheral blood lymphocyte subsets were analyzed during pregnancy in a group of SLE patients and compared with healthy gestations. We observed a reduction in peripheral Treg cell count throughout all pregnancy in control patients, which was not observed in SLE patients. In contrast, the Th17 cell count remained stable in both groups. In the control group, the Treg/Th17 ratio decreased throughout pregnancy to the postpartum, which was not observed in the study group. These changes may be justified by the migration of the immunotolerant Treg cells to the maternal decidua and may lead to the establishment of a pro-inflammatory profile by the end of pregnancy in healthy pregnancies, which was not observed in the SLE pregnant patients. This pro-inflammatory state at the end of a healthy pregnancy may be necessary for the spontaneous beginning of labor and help to explain why systemic syndromes like preeclampsia develop during the second half of pregnancy. The lack of these findings in SLE patients may express a pro-inflammatory state from the beginning of pregnancy, the influence of immunomodulatory medication or an intrinsic deregulation of immune function, which is a characteristic of these patients.

1. Introduction

Systemic lupus erythematosus (SLE) is a multisystemic autoimmune disorder of unknown etiology where a complex combination of genetic, epigenetic, and environmental factors contribute to the breakdown of self-tolerance (Jiang et al., 2019; Suárez-Fueyo et al., 2016; Tsokos, 2011). SLE is classically characterized by a failure in self-tolerance mechanisms associated with a defect in apoptotic debris clearance and the generation of autoantibodies. The chronic inflammation caused by this abnormal immune response leads to tissue damage (Tsokos, 2011; Tsokos et al., 2016). T lymphocytes play an essential role in SLE pathogenesis through the secretion of pro-inflammatory cytokines, inducing the production of autoantibodies by B cells, and maintaining autoimmune response by accumulating memory cells (Koga et al., 2019).

T helper lymphocytes (Th cells) are crucial to the control of the adaptive immune system. These cells may be categorized by the

cytokines produced (Crotty, 2011; Harrington et al., 2005; Korn et al., 2009). Th1 cells classically produce IFN- γ and IL-2, resulting in the activation of cytotoxic T lymphocytes, macrophages, and natural killer cells. Th2 cells are classically linked to the activation of B cells by producing IL-4. Th17 cells have a pro-inflammatory phenotype associated with the production of IL-17, IL-21, and IL-22. Tregs cells have an anti-inflammatory and immunomodulatory effect and characteristically express high concentrations of IL 2 receptor (CD25^{high}), X-linked Fox-head box P3 (FOXP3) which is a "master switch" for Treg development (Adeegbe and Nishikawa, 2013; Fontenot et al., 2005; Littman and Rudensky, 2010) and produce TGF- β and IL-10 (Crotty, 2011; Harrington et al., 2005; Korn et al., 2009). In addition, Th17 and Treg cells present a certain level of plasticity, so, in specific situations, Treg cells can differentiate into Th17 cells (Komatsu et al., 2014) and vice versa (Bellemore et al., 2015). It is also known that the balance between the anti-inflammatory FOXP3 + Treg cells and the pro-inflammatory Th17

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cells is crucially involved in SLE pathogenesis (Chen et al., 2018; Koga et al., 2019; Shin et al., 2011; Tsokos et al., 2016).

The balance between these T lymphocytes also seems to be involved in the tolerogenic mechanisms required for the development of a semi-allogenic fetus during pregnancy. Furthermore, the breakage of immune tolerance is linked to the development of adverse outcomes such as miscarriage, preeclampsia, or preterm delivery (Cornelius and Lamarca, 2014; Figueiredo and Schumacher, 2016a; Saifi et al., 2014; Zare et al., 2018). Thus, the trophoblast invasion that is necessary to develop a healthy pregnancy demands an intricate network of immune tolerance. Despite the description of several mechanisms, Treg and Th17 balance appears to have an essential role in this tolerance game (Cornelius and Lamarca, 2014; Figueiredo and Schumacher, 2016a; Saifi et al., 2014; Zare et al., 2018).

Treg cells seem to be essential for the implantation and normal trophoblast development in a normal pregnancy, so a shift toward Th2 cells and an elevation of Tregs in maternal decidua and circulation are usually observed (Redman and Sargent, 2010). Notwithstanding, a lower Treg or a higher Th17 profile is associated with adverse obstetric outcomes like preterm birth (Koucký et al., 2014), preeclampsia (Gleicher, 2007; Sibai et al., 2005; Vargas-Rojas et al., 2016) or recurrent pregnancy loss (Bansal et al., 2011; Wang et al., 2010a, 2010b, 2013).

Despite improvement of maternal and fetal outcomes with better medical care, pregnancy in SLE patients is associated with a high maternal and fetal morbidity (Arkema et al., 2016; Braga et al., 2021a, 2021b; Bundhun et al., 2017a; Ostensen and Clowse, 2013). In addition, the incidence of adverse outcomes like miscarriage, preterm delivery (PTD) or placental insufficiency is increased in these patients morbidity (Aggarwal et al., 2011; Arkema et al., 2016; Braga et al., 2021a, 2021b; Bundhun et al., 2017b, 2017a; Buyon et al., 2015; Canti et al., 2012; Clowse et al., 2008; Feld et al., 2015; Khan et al., 2018; Kroese et al., 2017; Naseri et al., 2018; Ostensen and Clowse, 2013; Pastore et al., 2019; Phansensee et al., 2018; Rezk et al., 2017; Wu et al., 2018; Zhan et al., 2017). This fact may be associated with a basal unbalance between pro-inflammatory and anti-inflammatory profiles even during pregnancy.

In the present study, the authors prospectively evaluated the number of circulating Treg and Th17 cells in a group of SLE patients during pregnancy and postpartum. The pattern of evolution of these lymphocytes was compared with a group of healthy pregnant women. Several markers of regulatory T cells were used to compare the progression of these cells throughout gestation.

2. Material and methods

2.1. Subjects

The study population consisted of SLE pregnant patients who delivered in the Obstetric Department of Centro Hospitalar Universitário do Porto (CHUPorto), Portugal. A control group of healthy pregnant women was used. By protocol, all control pregnant women should be healthy before pregnancy and without previous obstetric complications.

Ethical approval was obtained from the research ethics committee of CHUPorto, and written informed consent was obtained from all subjects. The study adhered to the tenets of the most recent revision of the Declaration of Helsinki.

The diagnosis of SLE was made according to the 2002 American College of Rheumatism criteria.

Our comparison cohort was randomly selected from healthy women with singleton pregnancies who attended the same hospital during the same period.

2.2. Study design

This study had a prospective design. All pregnant patients were recruited when attending their first scheduled visit at the Obstetric

department of CHUPorto between 2016 and 2019.

SLE activity was evaluated according to the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scale descriptors. The SLEDAI index was calculated based on data obtained up to six months before gestation, categorizing disease activity as absent (up to 4 points), mild to moderate (5–12 points), or severe (up to 12 points). In addition, SLE activity during pregnancy was assessed according to the descriptors of the Systemic Lupus Erythematosus Pregnancy Disease Activity Index (SLEPDAI) scale (Buyon et al., 1999).

Our institutional protocol was in accordance with the international recommendations for the follow-up of pregnant patients with SLE during the study period and nowadays summarizes the main EULAR recommendations for follow-up pregnant patients with SLE (Andreoli et al., 2017). Serial assessment of hematological, renal, hepatic, and immunological parameters and regular fetal sonographic evaluation with Doppler and fetal echocardiography was performed. The pregnancy follow-up consisted of frequent visits to timely diagnose SLE flares, adverse pregnancy outcomes (APO), congenital heart block, and for the assessment of risk of spontaneous PTD. Low-dose acetylsalicylic acid was introduced as soon as pregnancy was documented, if not previously prescribed.

APO, the primary composite outcome, was defined as the occurrence of miscarriage, stillbirth, fetal growth restriction, PTD or the development of hypertensive disorders of pregnancy (gestational hypertension/preeclampsia). Spontaneous abortion was defined as the involuntary loss of a fetus before the 20th week of gestation, stillbirth as the fetal death after 20 weeks of pregnancy. Preterm birth was a live birth before the 37th week of gestation. FGR was defined as a birth weight below the 10th percentile, using the curves defined for the Portuguese population (Sousa-Santos et al., 2016). Preeclampsia and HELLP syndrome were defined according to the American College of Obstetricians and Gynecologists Committee (ACOG) criteria (ACOG, 2020). Preeclampsia was defined as a systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg on two occasions at least 4 h apart after 20 weeks of gestation in a woman with previously normal blood pressure and occurrence of proteinuria (defined as a urinary excretion of 300 mg or more in a 24-hour urine collection or a protein/creatinine ratio of 0.3 mg/dL or more or a dipstick reading of 2+). Otherwise, in the absence of proteinuria, preeclampsia was defined as new-onset hypertension with new-onset target organ damage (platelet count less than $100 \times 10^9/L$; serum creatinine concentration greater than 1.1 mg/dL or doubling of the serum creatinine concentration in the absence of other renal diseases; elevated blood concentrations of liver transaminases to twice the upper limit of normal concentration; severe persistent right upper quadrant or epigastric pain not accounted for by alternative diagnoses; pulmonary edema; new-onset headache unresponsive to acetaminophen and not accounted for by alternative diagnoses; or visual disturbances) (ACOG, 2020). ACOG defines gestational hypertension as a systolic blood pressure ≥ 140 mmHg and/or a diastolic blood pressure ≥ 90 mmHg, on two occasions at least 4 h apart after 20 weeks of gestation, in a woman with previously normal blood pressure in the absence of proteinuria or organ dysfunction, and that returns to normal in the postpartum period (ACOG, 2020).

Gestational diabetes was defined according to the Portuguese Endocrinology Society guidelines for the diagnosis of gestational diabetes (Catarina et al., 2017) which are adapted from the International Association of Diabetes and Pregnancy Study Groups criteria (Shang and Lin, 2014).

The surveillance of pregnancies from the control group was made according to the Portuguese recommendations for low-risk pregnancies (Direção-Geral da Saúde, 2015). Serial laboratorial screening and fetal evaluation was made in each trimester.

Patients with any APO in the previous gestations were excluded. Blood samples for lymphocytic analysis were obtained in each trimester, during routine investigations, and during the first month postpartum.

2.3. Blood samples collection

Blood samples were obtained in each trimester during routine investigations (at 11–14 weeks, 24–28 weeks and 32–34 weeks of gestation), and at 3–4 weeks postpartum.

Three milliliters (mL) of venous peripheral blood was collected into tubes coated with K2EDTA (VACUETTE® TUBE K3E K3EDTA) for lymphocyte immunophenotyping and Treg cells detection and 4 mL into tubes coated with sodium heparin (VACUETTE® TUBE NH Sodium Heparin) for Th17 cells assay. Samples were analyzed within 4 h of venipuncture.

2.4. Flow cytometry

2.4.1. Fluorochrome labeled monoclonal antibodies

Cells were assessed according to the expression of surface and intracellular markers by flow cytometry, using different combinations of fluorochrome labeled monoclonal antibodies (mAbs).

For blood T, B and NK lymphocytes phenotyping, fluorescein isothiocyanate (FITC)-anti-CD45 (clone B3821F4A), phycoerythrin-cyanin 5.1 (PE-Cy5)-anti-CD3 (clone UCHT1), phycoerythrin (PE)-anti-CD4 (clone SFC112T4D11), phycoerythrin-Texas Red (ECD)-anti-CD8 (clone SFC121Thy2D3), ECD-anti-CD19 (clone J4.119) and PE-anti-CD56 (clone N901/NKH-1) mAbs were used, all from Beckman Coulter (Fullerton, California, United States of America - USA).

Th17 analysis was carried out with PE-Cy5-anti-CD3 (clone UCHT1; Immunotech, Marseille, France), FITC-anti-CD4 (clone 13B8.2; Immunotech, Marseille, France) and PE-anti-IL-17A (clone eBio64-DEC17; eBioscience inc San Diego, CA, USA) mAbs. For Treg evaluation, PE-Cy5-anti-CD4 (clone 13B8.2; Immunotech, Marseille, France), FITC-anti-CD25 (B1.49.9; Immunotech, Marseille, France) and PE-anti-FOXP3 (clone PCH10; eBioscience San Diego, CA, USA) mAbs were used.

2.4.2. T, B and NK cells subsets immunophenotyping

Whole blood (100 µL) was incubated for 10 min at room temperature (RT) in the dark with two different combinations of mAbs (FITC-anti-CD45/PE-Cy5-anti-CD3/PE-anti-CD4/ECD-anti-CD8) and (FITC-anti-CD45/PE-Cy5-anti-CD3/ECD-anti-CD19/PE-anti-CD56), followed by cell fixation and red blood cells (RBC) lysis for 10 min, using the IMMUNOPREP Reagent System, from Beckman Coulter (Fullerton, California, USA).

T (CD3 +/CD4 + and CD3 +/CD8 +), B (CD19 +), NK (CD56 +) and NKT-like (CD3 +/CD56 +) cells counts (cells/µL) and proportions (%) were established in a 4-colour standard protocol analysis, using a gate strategy of lymphocytes in CD45/side scatter (SSC) on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, California, USA).

2.4.3. Detection of Treg cells

Treg cells were quantified by intracellular staining for FOXP3. Whole blood (100 µL) was incubated with 10 µL of PE-Cy5-anti-CD4 and 10 µL of FITC-anti-CD25 mAbs for 15 min at RT in the dark, followed by RBC lysis with 3 mL of RBC Lysis Buffer (eBioscience, San Diego, California, USA). After centrifugation at 1600 rpm for 5 min, the supernatant was removed and cells were washed twice with 3 mL of Flow Cytometry Staining Buffer (eBioscience, San Diego, California, USA).

Cells were fixed and permeabilized using FOXP3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent system from eBioscience (San Diego, California, USA), according to the manufacturer's instructions.

Before flow cytometry analysis, cells were washed once with 3 mL of Flow Cytometry Staining Buffer. After centrifugation at 1600 rpm for 5 min, the supernatant was discarded and the sediment was suspended in 500 µL of Flow Cytometry Staining Buffer. A minimum of 50 000 events were acquired on an Epics XL-MCL flow cytometer (Beckman Coulter, California, USA) and a first gate was set up on the forward scatter (FSC)/SSC plot to remove debris. Gates were then set to only include

lymphocytes with CD4-specific fluorescence and CD4-gated cells were further analyzed based on CD25 and FOXP3 expression. CD4 + CD25high/FOXP3 + cells were identified as Treg cells (Fig. 1).

2.4.4. Th17 cells assay

Th17 cells analysis relied on intracellular staining for IL-17A after cell stimulation with phorbol 12-myristate13-acetate (PMA) and ionomycin in the presence of the protein transport inhibitor – brefeldin A.

To activate cells and induce cytokine production, 500 µL heparinized whole blood was added to 500 µL of RPMI 1640 medium supplemented with 2 mM L-glutamine (Gibco™, United Kingdom) and incubated with 10 µg/mL brefeldin A, 25 ng/mL PMA and 1 µg/mL ionomycin (Sigma Chemical Corporation, St. Louis, Missouri, USA) for 4 h at 37 °C in an atmosphere of 5% CO₂.

A second whole blood sample (500 µL) was also cultured in the same conditions, without the addition of PMA and ionomycin stimulants, and used as control.

For cell surface phenotyping, 100 µL of stimulated and control samples were incubated with 5 µL of anti-CD3 and 10 µL of anti-CD4 mAbs, for 15 min at RT in the dark. Cells were washed once with 5 mL of PBS buffer (Inova Diagnostics, San Diego, California, USA), followed by centrifugation at 1600 rpm for 5 min.

For intracellular staining of IL-17A, cells were suspended, fixed and permeabilized using the IntraPrep Permeabilization Reagent (Beckman Coulter, California, USA), according to the manufacturer's instructions. After centrifugation at 1600 rpm for 5 min, the supernatant was removed, cells were suspended in 100 µL of IntraPrep Permeabilization Buffer and incubated with 5 µL of anti-IL-17A mAb for 30 min at RT in the dark.

Finally, cells were washed once, in the same conditions, suspended in PBS buffer and examined by flow cytometry on an Epics XL-MCL flow cytometer (Beckman Coulter, California, USA). A minimum of 50 000 events were acquired.

A first gate was set up on the FSC/SSC plot to remove debris, then T/CD4 + cells were gated, followed by analysis of IL17A expression on CD4 + T cells (Fig. 2).

2.5. Statistical analysis

Descriptive data analysis was performed. Nonparametric Mann-Whitney U test and t-student test were used to compare means between groups when appropriate. Categorical data were compared by chi-square and Fisher's exact tests. The denominator used in the analysis of all the maternal outcomes, and in the rates of stillbirths and live births was the total of reported pregnancies. For all the other maternal-fetal outcomes, the denominator was the total of live births. The IBM SPSS Statistics® version 26.0 was used to perform statistical analysis. The level of significance was set at $P < 0.05$.

3. Results

3.1. Maternal and neonatal outcomes

A total of 20 SLE patients were included. The control group consisted of 20 healthy pregnant women matched by age and date of conception. In the study group, the median maternal age at delivery was $32,8 \pm 2,5$ years, and in the control group was $29,9 \pm 6,5$ years ($p > 0,05$). As defined in the study design, none of the SLE or control pregnant women had previous history of APO. All SLE patients were under acetylsalicylic acid treatment during pregnancy and 75% were under hydroxy-chloroquine. The median gestational age at delivery was $37,6 \pm 1,9$ weeks in the study group and $39,3 \pm 1,0$ weeks in the control group ($p = 0,01$). In both groups, pregnancy was uneventful and no cases of hypertensive disorders of pregnancy, fetal growth restriction, gestational diabetes, or preterm delivery were reported. All newborns were healthy. No malformations were diagnosed at birth, and the Apgar Index

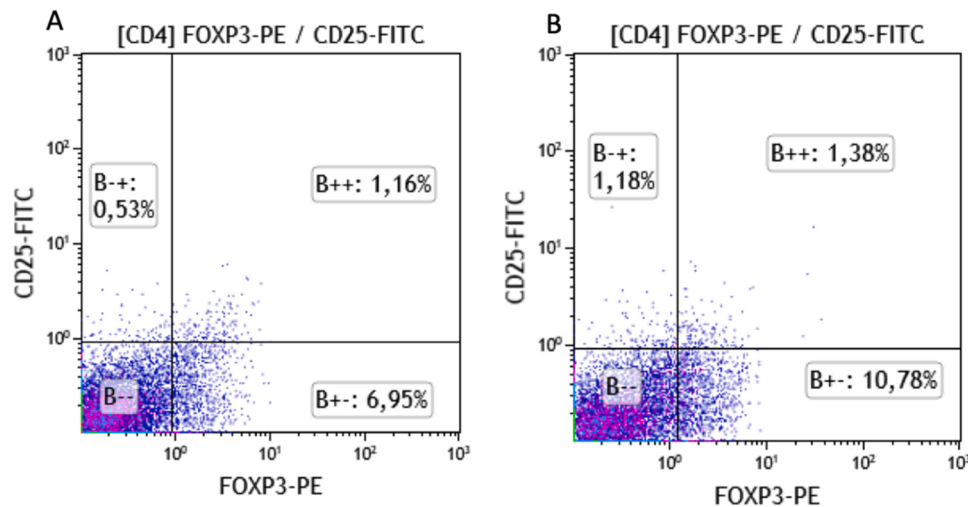


Fig. 1. Cytometric analysis of Treg cells. Flow cytometry cytograms of CD4⁺CD25^{high}FOXP3⁺ Treg in the 3rd trimester (A) and postpartum (B). Analysis of CD25 and FOXP3 expression on CD4⁺ lymphocytes where CD4⁺/CD25^{high}/FOXP3⁺ cells [B+ +] were considered as Treg cells.

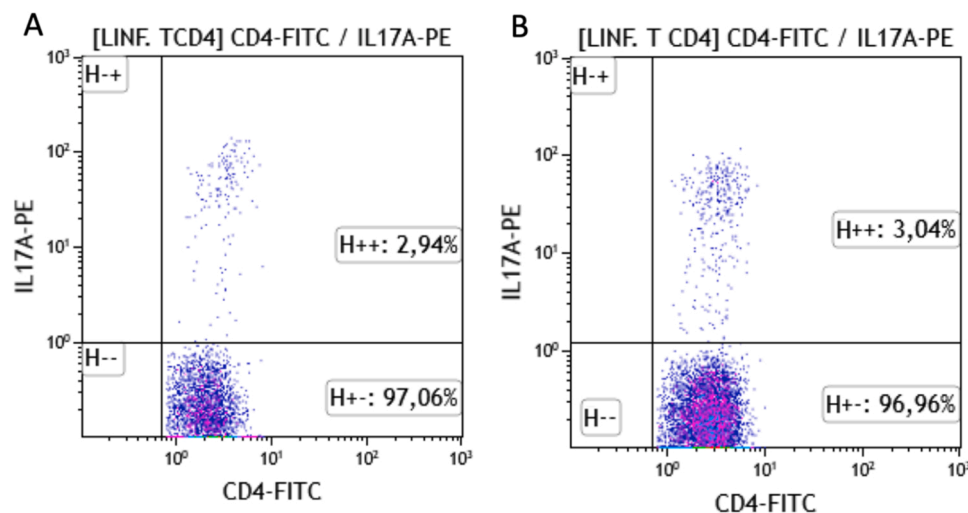


Fig. 2. Cytometric analysis of Th17 cells. Flow cytometry cytograms of Th17 cells in the 3rd trimester (A) and postpartum (B). Analysis IL17A expression on CD4⁺ T lymphocytes, in stimulated samples [H+ +] were considered as Th17 cells.

was > 7 at 5th minute of life in all cases. Demographic and pregnancy outcomes for both groups are expressed in Table 1.

Table 1
Demographic and pregnancy outcomes.

Demographic data	SLE	Controls	P
Age at conception (years±SD)	32,8 ± 2,5	29,9 ± 6,5	NS
Primiparity (%)	40,0	60,0	NS
Previous miscarriage rate	20,0	20,00	NS
Pregnancy outcomes			
Mean gestational age of delivery (gestational age±SD)	37,6 ± 1,9	39,3 ± 1,0	< 0,05
Cesarean rate (%)	50,0	20,0	< 0,05
Number of cases with Apgar Index < 7 at 5th minute (n)	0	0	–
% Female newborn	60%	45%	–
% Hypertensive disorders of pregnancy	0	0	–
% Pre-term deliveries	0	0	–

NS – non significative.

3.2. CD4⁺CD25^{high}FOXP3⁺ Treg and TH17 cells behavior during pregnancy

When analyzing the behavior of CD4⁺CD25^{high}FOXP3⁺ Treg cells during pregnancy in SLE patients, we observed that the number of these cells remained stable throughout all pregnancy and postpartum. In the control group, the number of CD4⁺CD25^{high}FOXP3⁺ Treg cells decreased from pregnancy to postpartum (Fig. 3), with this reduction achieving statistical significance at postpartum. Comparing both groups, in SLE patients, the mean CD4⁺CD25^{high}FOXP3⁺ Treg cells count was significantly higher in these patients in the postpartum period. No significant differences were found during pregnancy between groups.

Concerning Th17 cells count, it increased slightly during all pregnancy in lupus patients, decreasing in postpartum. Nevertheless, these results did not achieve statistical significance. In the control group, Th17 cells showed a similar behavior (Fig. 4). There were no significant differences in the Th17 cell count during pregnancy in any group. There were also no differences in the Th17 cell count between both groups at any trimester.

Having in consideration the plasticity of these cells and its antagonistic effect, the relationship between CD4⁺CD25^{high}FOXP3⁺ Treg

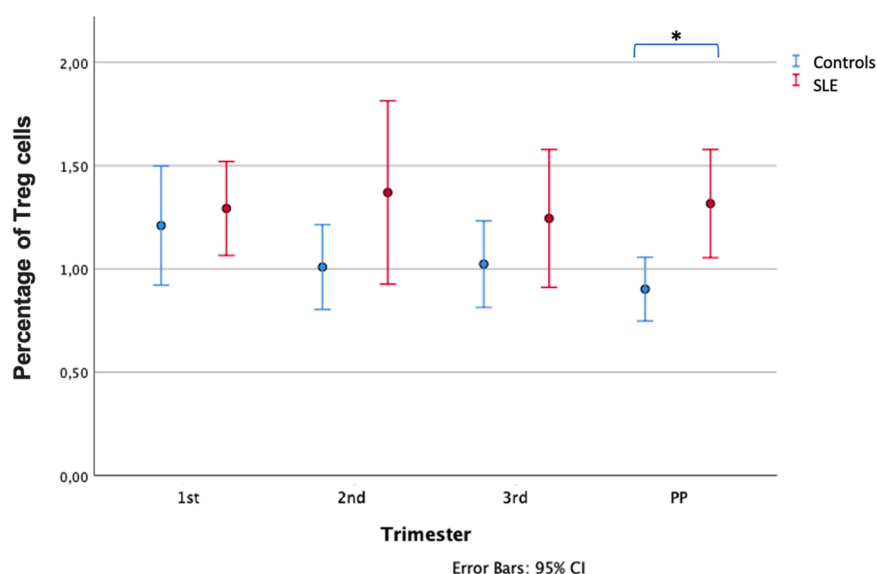


Fig. 3. Dynamics of CD4 +CD25highFOXP3 + Treg cells during pregnancy. * $p < 0,05$.

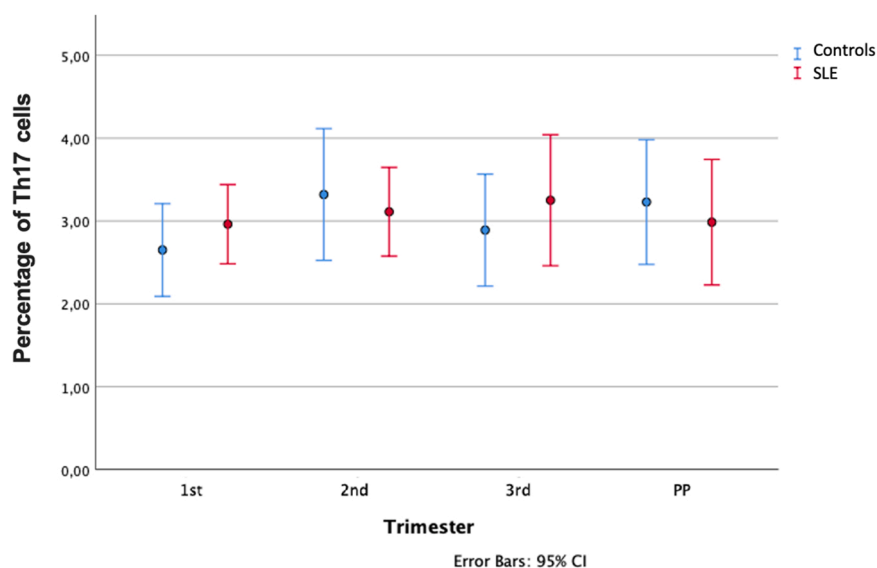


Fig. 4. Dynamics of Th17 cells during pregnancy.

and Th17 cells was analyzed. In our study, the CD4 +CD25highFOXP3 + Treg/Th17 ratio remained stable in the study group during pregnancy and increased postpartum. However, in the control group, the observed CD4 +CD25highFOXP3 + Treg cell count reduction during pregnancy and postpartum led to an inverse progression of this ratio. In the control group, CD4 +CD25highFOXP3 + Treg/Th17 ratio decreased during gestation to the postpartum period. In our study, the CD4 +CD25highFOXP3 + Treg/Th17 ratio expressed a statistically different behavior between groups ($p < 0,05$ – Fig. 5).

4. Discussion

As far as we know, this is the first study to prospectively evaluate the variation of Treg and Th17 cells counts during pregnancy and postpartum in a group of SLE patients and to compare it to a group of healthy uncomplicated pregnant women.

The observed kinetics of Th17 and Treg cell numbers suggested a dynamic balance between immune activation and suppression throughout pregnancy. This was particularly evident in the control

group, where the changes in the Treg cells count were more marked, with a constant reduction during pregnancy. These findings were consistent with previous results from our group (Braga et al., 2021c). This findings may reflect a physiological mechanism for maternal immune tolerance towards the fetus (Braga et al., 2021c; Figueiredo and Schumacher, 2016b; Ji et al., 2019; Krechetova et al., 2020; Liu et al., 2017). The consistent decrease in CD4 +CD25highFOXP3 + Treg cells counts during pregnancy in the control group may be explained by the migration of these cells from the peripheral blood to the maternal-fetal interface in the decidua, promoting a normal growth of the placenta (Hosseini et al., 2016; Liu et al., 2011). These results were similar to the data published by Mjosberg (Mjösberg et al., 2010). The importance of these cells for the invasion of the trophoblast and normal development of the placenta has been previously postulated and seems to be implicated in the development of several adverse obstetric outcomes (Gluhovschi et al., 2015; Tower et al., 2013, 2011; Varghese et al., 2011).

In the SLE patients' group, the dynamics of CD4 +CD25highFOXP3 + Treg cells during pregnancy was quite different. We observed that the number of these cells remained stable throughout pregnancy

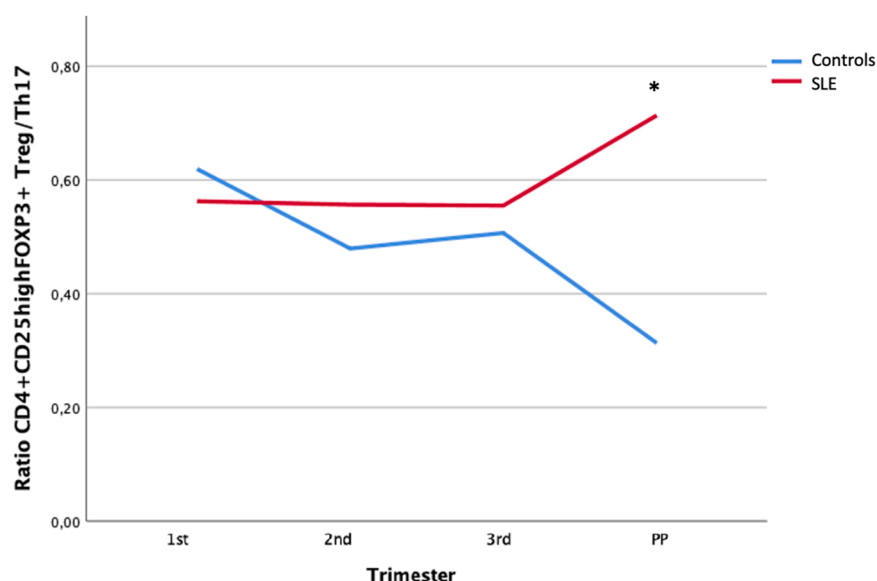


Fig. 5. Dynamics of CD4 + CD25highFOXP3 + Treg/Th17 ratio during pregnancy. *p < 0.05.

and postpartum. Therefore, the progressive reduction in the number of CD4 + CD25highFOXP3 + Treg cells observed in healthy women from pregnancy to postpartum was not observed in lupus patients with controlled disease. In our study, this was emphasized by a significantly higher number of CD4 + CD25highFOXP3 + Treg cells in the puerperium when compared to healthy pregnant women. Several mechanisms may explain these findings. Considering that the reduction of the peripheral CD4 + CD25highFOXP3 + Treg cells count in a healthy pregnancy is probably a result of the migration of these cells to the decidua, this migration process may be impaired in SLE patients. This may justify a worse tolerance of a semiallogenic fetoplacental unit, and may explain why placental insufficiency and related clinical manifestations are increased in these patients (Braga et al., 2021b; Bundhun et al., 2017c). This fact is also highlighted in our study because, even without fulfilling criteria for fetal growth restriction, the mean neonatal weight at delivery was significantly reduced in the SLE group (Table 1). These data may also express an inherent deregulation of immune function, which is a well-known characteristic of lupus patients (Álvarez-Rodríguez et al., 2019; Chen et al., 2018; Li et al., 2015; Talaat et al., 2015; Yang et al., 2018). In SLE patients, the balance between Treg/Th17 cells favors a pro-inflammatory profile (Álvarez-Rodríguez et al., 2019; Chen et al., 2018; Li et al., 2015; Talaat et al., 2015; Yang et al., 2018). In our study, patients had well-controlled disease, therefore, the absence of episodes of lupus flares during pregnancy reduced the probability that this pro-inflammatory profile could be explained by a worsening of the clinical status. In addition, the use of immunomodulatory therapy, particularly hydroxychloroquine, also modulates the Treg and Th17 differentiation (Yang et al., 2018), which may also interfere with the dynamics of these cells during pregnancy. Nevertheless, further studies are required to clarify these findings.

Concerning the Th17 cells count, we did not find any significant difference inside any group and between groups during gestation. The lack of APO or lupus flares may help to explain these findings. A Th17 profile has been associated with APO or lupus flares in previous studies (Bansal et al., 2011; Gleicher, 2007; Koucký et al., 2014; Sibai et al., 2005; Vargas-Rojas et al., 2016; Wang et al., 2010a, 2013), so the absence of differences is probably explained by the uneventful nature of the gestations included in the study.

The differences found in the CD4 + CD25highFOXP3 + Treg/Th17 ratio are justified by the dynamics of the CD4 + CD25highFOXP3 + Treg count. In the control group, the reduced CD4 + CD25highFOXP3 + Treg/Th17 ratio at the end of pregnancy and in postpartum reflects a

physiological pro-inflammatory state at this stage, where Th17 cells may be implicated, reinforcing the importance of an inflammatory scenario to facilitate parturition (Gomez-Lopez et al., 2020). These findings may also help to explain, on one side, the development of systemic pro-inflammatory syndromes, like preeclampsia, in susceptible women during the second half of pregnancy (Anvari et al., 2015; Eghbal-Fard et al., 2019; Ribeiro et al., 2017; Zare et al., 2018) and, on the other site, the flourishing of many autoimmune disorders in the first months postpartum (Liu et al., 2017). However, the lack of these dynamics in SLE patients may be justified by several mechanisms: an intrinsic deregulation of immune function, which is a well-known characteristic of these patients (Álvarez-Rodríguez et al., 2019; Chen et al., 2018; Li et al., 2015; Talaat et al., 2015; Yang et al., 2018); the presence of a pro-inflammatory state since the beginning of gestation, which may justify the higher incidence of APO (Braga et al., 2021b) in these pregnancies; or the influence of immunomodulatory medication (Yang et al., 2018).

Nevertheless, our research work presents some limitations. One possible limitation is the characterization of Treg cells population. Treg cells are a heterogeneous group where several subsets with different markers have been described (Giganti, 2021). These subsets may have different functions in the control of the immune response and in the induction of peripheral tolerance (Giganti, 2021). The use of other Treg markers, such as CD127 (IL-7 receptor) or CD45R, could enhance the identification of these cells and even identify active subpopulations of Treg group. These markers should be used in future studies to refine the identification of Treg cells population. The reduced number of enrolled patients is another limitation of our study. However, a solid selection of participants highlighted by the homogeneity of the clinical sample and the implementation of rigorous protocols for pregnancy surveillance strengthens our results. The homogeneity of the sample allowed us to demonstrate differences in the behavior of CD4 + CD25highFOXP3 + Treg and Th17 cells during pregnancy and postpartum between healthy and SLE pregnant women, highlighting a different immune response in SLE patients, even in the absence of adverse obstetric outcomes. Future investigations addressing these limitations will be important to confirm these findings.

5. Conclusion

Very few published studies have evaluated the behavior of CD4 + CD25highFOXP3 + Treg cells and Th17 cells throughout

gestation and postpartum in healthy women (Figueiredo and Schumacher, 2016a; Saito, 2010; Wang et al., 2010a). To our knowledge, this is the first study to analyse these cells in SLE patients during pregnancy.

In our results, circulating CD4 + CD25 + FOXP3 + Treg cells decreased steadily during pregnancy in healthy women, probably representing the migration of these cells to the decidua. These findings were not observed in SLE patients. Concerning Th17 cells, their count remained relatively stable during pregnancy in both groups, which explains the differences in the CD4 + CD25highFOXP3 + Treg/Th17 ratio present throughout pregnancy to postpartum. The pro-inflammatory status associated with this relative excess of Th17 cells at the end of healthy pregnancies and especially at postpartum was not observed in the group of SLE patients. The constitutional pro-inflammatory profile of SLE patients, the absence of pregnancies complicated by adverse obstetric outcomes or lupus flares in our cohort, and the regular use of immunomodulatory therapy may explain the results observed in SLE patients and may be considered in future investigations.

Declarations of Interest

None

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Article 5

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Th17 / Treg ratio: A prospective study in a group of pregnant women with preeclampsia and fetal growth restriction

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ABSTRACT

Introduction: During pregnancy, the maternal immune system is challenged to tolerate a semi-allogenic fetus. A proinflammatory profile has been linked to adverse pregnancy outcomes and poor placental development. In this study, the authors evaluated the number of circulating Tregs and Th17 cells in a group of patients diagnosed with preeclampsia (PE) and fetal growth restriction (FGR).

Methods: Prospective longitudinal observational study where peripheral blood lymphocyte subsets were analyzed in a cohort of pregnant patients with PE, FGR, and a control group of healthy pregnant women.

Results: The diagnosis of PE was associated with a significative higher number of circulating Th17 cells and a significative relative reduction in the Treg cell count. This proinflammatory profile was also expressed in the evolution of the Th17/CD4⁺CD25^{high}FOXP3⁺ Treg ratio. In the FGR group, the Th17 cell count was significantly higher during the third trimester of pregnancy. This proinflammatory profile was also expressed in the evolution of the Th17/CD4⁺CD25^{high}FOXP3⁺ Treg ratio. When we compare the immunological profiles of patients with PE and FGR we observed a higher number of proinflammatory Th17 cells and a significative lower number of Treg cells in PE patients. This is particularly expressed in the differences found between the Th17/CD4⁺CD25^{high}FOXP3⁺ Treg ratios of these two groups.

Discussion/Conclusion

Our data showed a that a proinflammatory profile and a relative excess of Th17 cells was associated with the diagnosis of PE and FGR. A more exuberant systemic proinflammatory profile present in the PE patients is absent in patients with FGR without preeclampsia

1. Introduction

Pregnancy is a dynamic state characterized by multiple adaptations which are necessary to the development of a healthy newborn. The involvement of the maternal immune system in this process is no exception. Medawar was the first to highlight the paradoxical non-rejection of an intrauterine fetus that express paternal histocompatibility antigens (Medawar, 2018). The immunological mechanisms involved in the protection of the semi allogeneic fetus and placenta are not fully understood, however, the breakage of maternal immune tolerance seems to be associated with the development of adverse obstetrical

outcomes like miscarriage, preeclampsia, or preterm delivery (Figueiredo and Schumacher, 2018a; Cornelius and Lamarca, 2018; Zare et al., 2018; Saifi et al., 2017; Wang et al., 2018a).

Preeclampsia (PE) is the most common hypertensive disorder of pregnancy, affecting approximately 3–10% of pregnant women and it is a leading cause of both maternal and perinatal morbidity and mortality (ACOG, 2020; Dekker and Robillard, 2018). This syndrome is characterized by hypertension associated with proteinuria or other signs of maternal end-organ dysfunction or uteroplacental dysfunction after 20 weeks of gestation (ACOG, 2020; Sibai et al., 2018; Poon et al., 2019; Brown et al., 2018). After decades of investigation, the ethology of PE

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remains largely unknown. Several factors have been proposed to contribute to the pathogenesis of this syndrome, including poor placentation, poor angiogenesis, chronic inflammation or endotheliosis (Burton et al., 2019; Agrawal et al., 2018; Gómez-Arriaga et al., 2014; Redman and Sargent, 2001, 2018; Staff et al., 2014). Epidemiological findings suggests that immune maladaptation is the main cause of PE (Dekker and Robillard, 2018; Saito et al., 2018). In fact, PE occurs more commonly in the first pregnancy, when there is a new partner, and when there is a long interval since the last pregnancy (Poon et al., 2019). The breakage of maternal immune tolerance seems to be associated with the development preeclampsia (Figueiredo and Schumacher, 2018a; Cornelius and Lamarca, 2018; Zare et al., 2018; Saifi et al., 2017; Wang et al., 2018a). In fact, the normal progression of gestation requires maternal immune tolerate towards fetal antigens, which is essential to a normal trophoblast growth. An abnormal trophoblast growth and differentiation may lead to an incompetent placenta, which is linked to the pathogenesis of miscarriage, preeclampsia, and fetal growth restriction (Figueiredo and Schumacher, 2018a).

Fetal growth restriction (FGR) defines fetuses who have failed to achieve normal growth during intra-uterine life (ACOG, 2019). In the absence of a better tool that measures the growth potential of each fetus, FGR is usually considered when the estimated fetal weight falls below a predefined threshold for its gestational age (ACOG, 2019; Salomon, Jun 1 et al., 2019; Lees et al., 2020). The American College of Obstetrics and Gynecology (ACOG) defines FGR as an estimated fetal weight below the 10th percentile for gestational age (ACOG, 2019). FGR is a major concern in maternal-fetal medicine. It is associated with an increased risk of perinatal morbidity and death and long-term adverse outcomes (Francis et al., 2014; Miller et al., 2016; Barker et al., 2005; Flenady et al., 2016), notably to prematurity (Francis et al., 2014) and newborns experience worse neurological development (Barker et al., 2005). In adulthood, FGR has been linked to an increased risk of cardiovascular morbidity, in particular to hypertension, type-2 diabetes mellitus, coronary heart disease, and stroke (Barker et al., 2005). FGR is also a significant risk factor for fetal death, and up to 30% of the diagnosed stillbirths in the third trimester are related to FGR (Nohuz et al., 2020). In fact, the prenatal diagnosis of FGR is an essential feature in prenatal surveillance aimed to reduce fetal death (Salomon, Jun 1 et al., 2019; Nohuz et al., 2020). Unfortunately, up to 50% of these newborns will not be diagnosed before delivery (ACOG, 2019). FGR has been associated with several conditions that could be broadly categorized into maternal, fetal, or placental factors. Of the possible causes, abnormal placenta development is the most common pathologic finding in FGR (Salafia et al., 1995). This results in placental insufficiency and poor placental perfusion (Salafia et al., 1995) and is associated with abruptio placentae, placental hematoma, and preeclampsia (Maulik, 2006; Pollack and Divon, 1992; Ananth and Wilcox, 2001). The trophoblast invasion necessary to develop a fully functional placenta requires an intricate network of immune tolerance that allows the growth of cells expressing paternal antigens through the maternal decidua. T helper lymphocytes (Th cells) are essential cells in the control of the adaptative immune system and may be categorized mainly by the cytokines produced (Crotty, 2011; Korn et al., 2009; Harrington et al., 2005). Th1 cells classically produce IFN- γ and IL-2, resulting in the activation of cytotoxic T lymphocytes, macrophages, and natural killer cells; Th2 cells are linked to the activation of B cells by producing IL-4; Th17 cells have a proinflammatory phenotype associated with the production of IL-17, IL-21, and IL-22; Tregs cells have an anti-inflammatory and immunomodulatory effect and characteristically express high concentrations of IL-2 receptor (CD25high), X-linked Foxhead box P3 (Foxp3) which is a "master switch" for Treg development (Fontenot et al., 2018; Adeegbe and Nishikawa, 2018; Littman and Rudensky, 2018) and produce TGF- β and IL-10 (Crotty, 2011; Korn et al., 2009; Harrington et al., 2005). Treg cells and Th17 cells appear to have an essential role in the fetal tolerance mechanism throughout pregnancy (Figueiredo and Schumacher, 2018a; Cornelius and Lamarca, 2018; Zare et al., 2018; Saifi et al., 2017). In a

normal pregnancy, Treg cells are crucial for blastocyst implantation and trophoblast development. Therefore, a shift toward Th2 cells and Treg elevation in maternal decidua and circulation is usually observed (Redman and Sargent, 2018). Abnormal placentation and abnormal placental perfusion have been associated with an imbalance between Treg and Th17 cells (Cornelius and Lamarca, 2018; Zare et al., 2018; Toldi et al., 2018; Darmochwal-Kolarz et al., 2018a; Santner-Nanan et al., 2018; Figueiredo and Schumacher, 2018b; Saifi et al., 2018). Th17 and Treg cells present a certain level of plasticity, and in specific situations, Treg cells are able to differentiate into Th17 cells (Komatsu et al., 2018) and vice-versa (Bellemore et al., 2018). Notwithstanding, diminishing the Treg and/or augmenting the Th17 is linked to an abnormal trophoblast development and with adverse obstetric outcomes like preterm birth (Koucký et al., 2018), PE (Sibai et al., 2018; Vargas-Rojas et al., 2018; Gleicher, 2018) or recurrent pregnancy loss (Wang et al., 2018b, 2018c; Bansal et al., 2018). Some authors consider that PE and FGR share the same pathophysiology, corresponding of different phenotypes of the same disorder (Ness and Sibai, 2006). The published data regarding the balance between Th17 and Treg cells in pregnancies with the diagnosis of PE and FGR secondary to placental insufficiency is still sparse. Previous published data confirmed the presence of a proinflammatory status in patients with PE which is expressed by an higher concentration of IL-17 and a higher rate of Th17 cell expression (Poordast et al., 2018; Cornelius et al., 2016; Saito, 2018; Zhang et al., 2018; Darmochwal-Kolarz et al., 2018b; Molvarec et al., 2015; Liu et al., 2017). However there were no publish data to compare the immunological profile of these patients, which could be one possible answer for the different phenotype expressed in PE versus FGR.

In the present study, the authors evaluated the number of circulating Tregs and Th17 cells in a group of patients diagnosed with PE and FGR during the third trimester of pregnancy and compared the immunologic profile of these pregnancies with a control group of healthy pregnant patients. At the physiological changes experienced during pregnancy usually restitute to the natural basal state during puerperium. Physiological puerperium classically extends to the 6th to the 8th week after birth. To access this evolution, immunological profile was also evaluated after birth in all group of patients.

2. Material and methods

2.1. Subjects

The study population consisted of pregnant women diagnosed with PE and FGR and pregnancy surveillance and delivery at the Obstetric Department of Centro Hospitalar Universitário do Porto (CHUPorto), Portugal. A control group of healthy pregnant women matched to the cases by age and date of conception was used. By protocol, all control

Table 1
Maternal characteristics.

Demographic data	PE	FGR	Control	p value
Age at conception (years $\pm$ SD)	31.9 $\pm$ 5.1	31.7 $\pm$ 4.2	29.8 $\pm$ 6.5	NS
Primiparity (%)	17 (85.0)	16 (80.0)	15 (60.0)	NS
Smoking	0	0	0	-
Body Mass Index above 35 kg/m ²	3 (15.0)	4 (20.0)	3 (15.0%)	NS
Prior FGR	0	0	0	-
Prior essential hypertension	0	0	0	-
Prior pre-eclampsia	0	0	0	-
Prior autoimmune disease	0	0	0	-
Prior stillbirth	0	0	0	-
Pre-existing diabetes	0	0	0	-
Pre-existing vascular disease	0	0	0	-
Pre-existing renal impairment	0	0	0	-
Gestation after ART	0	2 (10.0)	0	-

pregnant women must be healthy before pregnancy and without previously diagnosed medical or obstetric complications (Table 1).

Ethical approval was obtained from the research ethics committee of CHUPorto and written informed consent was obtained from all subjects.

Preeclampsia were defined according to the American College of Obstetricians and Gynecologists Committee (ACOG) criteria (ACOG, 2020, 2021). FGR was defined as birthweight below the 10th percentile, using the curves defined for the Portuguese population (Sousa-Santos et al., 2016). Fetal weight was estimated using Hadlock formula (Hadlock et al., 1985). Our comparison cohort was randomly selected from healthy women with singleton pregnancies who attended the same hospital during the same period.

3. Study design

This study had a prospective design. All pregnant patients in the study group were recruited when attending their scheduled visit at the Obstetric Department of CHUP after the diagnosis of PE or FGR. Blood samples were obtained in the third trimester during routine investigations (after PE or FGR diagnosis in the study group and at 30–32 weeks of gestation in the control group), and at 3–4 weeks postpartum.

Ethical approval was obtained from Centro Hospitalar Universitário do Porto (404–12 (280-DEFI/ 342-CES) and written informed consent for all the analyses was obtained from all subjects. The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki.

Maternal characteristics were collected in both groups in accordance with previous published recommendations (Khalil et al., 2019) (Table 1). FGR was diagnosed when the estimated fetal weight according to the Portuguese fetal growth curves was below the 10th percentile (Sousa-Santos et al., 2016) and associated with fetal Doppler abnormalities (Lees et al., 2020). FGR was diagnosed at the third-trimester ultrasound (between 28 and 32 weeks of gestation), which is performed universally according to the Portuguese guidelines (Direção-Geral da Saúde, 2015). Confirmation of FGR was established in all cases after birth with the newborn weight evaluation (Sousa-Santos et al., 2016). After the diagnosis of FGR, serial monitoring of hematologic, renal, hepatic, and immunologic parameters and regular fetal sonographic evaluation with Doppler was performed. FGR pregnancy follow-up was according to the standard of care and the ISUOG guidelines (ACOG, 2019; Lees et al., 2020). The development of adverse pregnancy outcomes (APO) was recorded. APO was defined as the occurrence of stillbirth, preterm delivery (PTD) or the development of hypertensive disorders of pregnancy (gestational hypertension/preeclampsia). Stillbirth was defined as the involuntary loss of a fetus after 20 weeks of pregnancy. Preterm birth was a live birth before the 37th week of gestation.

Preeclampsia and HELLP syndrome were defined according to the ACOG criteria (ACOG, 2020). Gestational diabetes was defined according to the Portuguese Endocrinology Society guidelines for the diagnosis of gestational diabetes (Catarina et al., 2017) adapted from the International Association of Diabetes and Pregnancy Study Groups criteria (Shang and Lin, 2014).

Pregnancies from the control group were surveilled according to the Portuguese recommendation for low-risk pregnancies (Direção-Geral da Saúde, 2015). In this group, serial laboratory evaluation was performed, and fetal evaluation and ultrasound scan executed in each trimester. Patients with previous APO or a history of PE or FGR in a previous gestation were excluded.

4. Blood samples collection

Blood samples for lymphocyte analysis were obtained after the diagnosis of PE or FGR and during the first month postpartum in the study group and in the third trimester and during the first month postpartum in the control group.

Three milliliters (mL) of venous peripheral blood was collected into tubes coated with K2EDTA (VACUETTE® TUBE K3E K3EDTA) for lymphocyte immunophenotyping and Treg cells detection and 4 mL into tubes coated with sodium heparin (VACUETTE® TUBE NH Sodium Heparin) for Th17 cells assay. Samples were analyzed within 4 h of venipuncture.

5. Flow cytometry

5.1. Fluorochrome labeled monoclonal antibodies

Cells were assessed according to the expression of surface and intracellular markers by flow cytometry, using different combinations of fluorochrome labeled monoclonal antibodies (mAbs).

For blood T, B and NK lymphocytes phenotyping, fluorescein isothiocyanate (FITC)-anti-CD45 (clone B3821F4A), phycoerythrin-cyanin 5.1 (PE-Cy5)-anti-CD3 (clone UCHT1), phycoerythrin (PE)-anti-CD4 (clone SFC12T4D11), phycoerythrin-Texas Red (ECD)-anti-CD8 (clone SFC121Thy2D3), ECD-anti-CD19 (clone J4.119) and PE-anti-CD56 (clone N901/NKH-1) mAbs were used, all from Beckman Coulter (Fullerton, California, United States of America - USA) – Fig. 1.

Th17 analysis was carried out with PE-Cy5-anti-CD3 (clone UCHT1; Immunotech, Marseille, France), FITC-anti-CD4 (clone 13B8.2; Immunotech, Marseille, France) and PE-anti-IL-17A (clone eBio64-DEC17; eBioscience inc San Diego, CA, USA) mAbs. For Treg evaluation, PE-Cy5-anti-CD4 (clone 13B8.2; Immunotech, Marseille, France), FITC-anti-CD25 (B1.49.9; Immunotech, Marseille, France) and PE-anti-FoxP3 (clone PCH10; eBioscience San Diego, CA, USA) mAbs were used.

5.2. T, B and NK cells subsets immunophenotyping

Whole blood (100 µL) was incubated for 10 min at room temperature (RT) in the dark with two different combinations of mAbs (FITC-anti-CD45/PE-Cy5-anti-CD3/PE-anti-CD4/ECD-anti-CD8) and (FITC-anti-CD45/PE-Cy5-anti-CD3/ECD-anti-CD19/PE-anti-CD56), followed by cell fixation and red blood cells (RBC) lysis for 10 min, using the IMMUNOPREP Reagent System, from Beckman Coulter (Fullerton, California, USA).

T (CD3⁺/CD4⁺ and CD3⁺/CD8⁺), B (CD19⁺), NK (CD56⁺) and NKT-like (CD3⁺/CD56⁺) cells counts (cells/µL) and proportions (%) were established in a 4-colour standard protocol analysis, using a gate strategy of lymphocytes in CD45/side scatter (SSC) on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, California, USA).

5.3. Detection of Treg cells

Treg cells were quantified by intracellular staining for Foxp3. Whole blood (100 µL) was incubated with 10 µL of PE-Cy5-anti-CD4 and 10 µL of FITC-anti-CD25 mAbs for 15 min at RT in the dark, followed by RBC lysis with 3 mL of RBC Lysis Buffer (eBioscience, San Diego, California, USA). After centrifugation at 1600 rpm for 5 min, the supernatant was removed and cells were washed twice with 3 mL of Flow Cytometry Staining Buffer (eBioscience, San Diego, California, USA).

Cells were fixed and permeabilized using Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent system from eBioscience (San Diego, California, USA), according to the manufacturer's instructions.

Before flow cytometry analysis, cells were washed once with 3 mL of Flow Cytometry Staining Buffer. After centrifugation at 1600 rpm for 5 min, the supernatant was discarded and the sediment was suspended in 500 µL of Flow Cytometry Staining Buffer. A minimum of 50 000 events were acquired on an Epics XL-MCL flow cytometer (Beckman Coulter, California, USA) and a first gate was set up on the forward scatter (FSC)/SSC plot to remove debris. Gates were then set to only include lymphocytes with CD4-specific fluorescence and CD4-gated cells were further analyzed based on CD25 and Foxp3 expression.

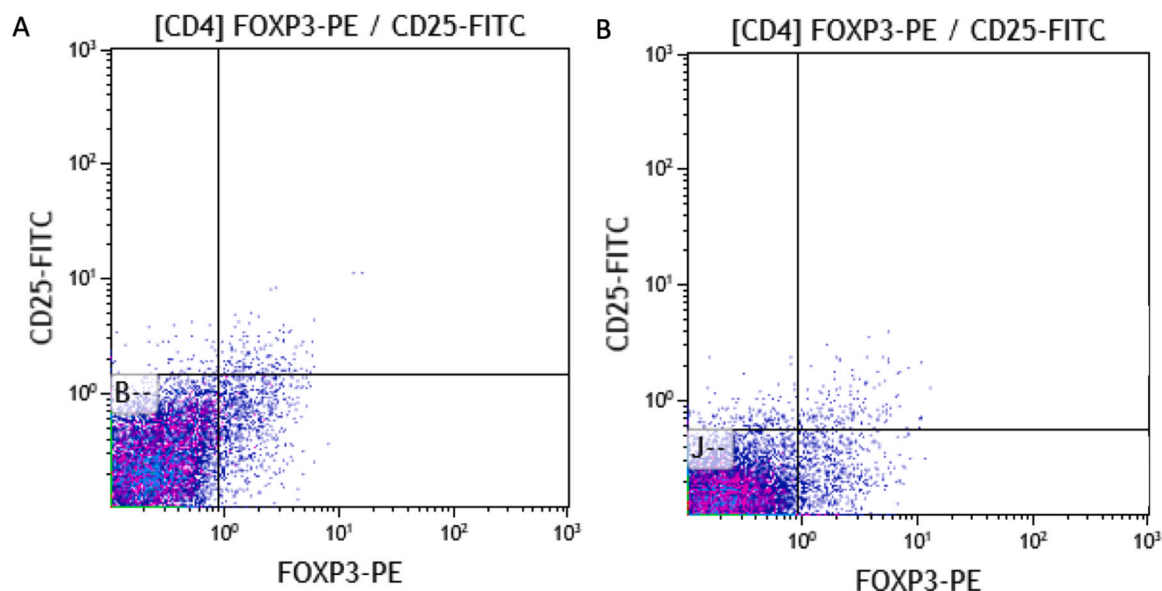


Fig. 1. Cytometric analysis of Treg cells. Flow cytometry cytograms of CD4⁺CD25^{high}FOXP3⁺ Treg in the 3rd trimester (A) and postpartum (B).

CD4⁺CD25^{high}FOXP3⁺ cells were identified as Treg cells (Fig. 2).

5.4. Th17 cells assay

Th17 cells analysis relied on intracellular staining for IL-17A after cell stimulation with phorbol 12-myristate13-acetate (PMA) and ionomycin in the presence of the protein transport inhibitor – brefeldin A.

To activate cells and induce cytokine production, 500 μ L heparinized whole blood was added to 500 μ L of RPMI 1640 medium supplemented with 2 mM L-glutamine (GibcoTM, United Kingdom) and incubated with 10 μ g/mL brefeldin A, 25 ng/mL PMA and 1 μ g/mL ionomycin (Sigma Chemical Corporation, St. Louis, Missouri, USA) for 4 h at 37 °C in an atmosphere of 5% CO₂.

A second whole blood sample (500 μ L) was also cultured in the same conditions, without the addition of PMA and ionomycin stimulants, and used as control.

For cell surface phenotyping, 100 μ L of stimulated and control

samples were incubated with 5 μ L of anti-CD3 and 10 μ L of anti-CD4 mAbs, for 15 min at RT in the dark. Cells were washed once with 5 mL of PBS buffer (Inova Diagnostics, San Diego, California, USA), followed by centrifugation at 1600 rpm for 5 min.

For intracellular staining of IL-17A, cells were suspended, fixed and permeabilized using the IntraPrep Permeabilization Reagent (Beckman Coulter, California, USA), according to the manufacturer's instructions. After centrifugation at 1600 rpm for 5 min, the supernatant was removed, cells were suspended in 100 μ L of IntraPrep Permeabilization Buffer and incubated with 5 μ L of anti-IL-17A mAb for 30 min at RT in the dark.

Finally, cells were washed once, in the same conditions, suspended in PBS buffer and examined by flow cytometry on an Epics XL-MCL flow cytometer (Beckman Coulter, California, USA). A minimum of 50 000 events were acquired.

A first gate was set up on the FSC/SSC plot to remove debris, then T/CD4⁺ cells were gated, followed by analysis of IL17A expression on

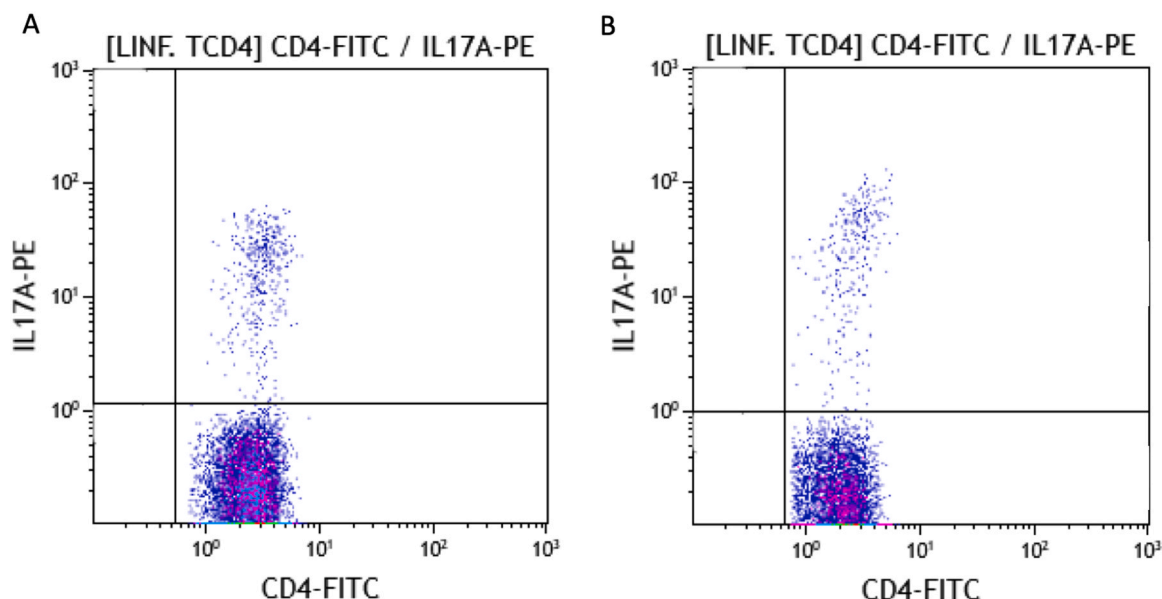


Fig. 2. Cytometric analysis of Th17 cells. Flow cytometry cytograms of Th17 cells in the 3rd trimester (A) and postpartum (B).

CD4 + T cells (Fig. 3).

6. Statistical analysis

Descriptive data analysis was performed. Nonparametric Mann-Whitney U test and t-student test were used to compare means between groups when appropriate. Categorical data were compared between groups by chi-square and Fisher's exact tests. Nonparametric tests were used to analyze the Th17/Treg ratio and parametric tests were used to analyze the lymphocyte distribution. The significance level was set at $p = 0,05$. Statistical evaluation and plotting were performed using the SPSS program (version 26.0).

7. Results

7.1. Maternal characteristics

A total of 20 cases of PE and 20 cases of FGR were recruited. The control group consisted of 20 healthy pregnant women matched to the cases by age and date of conception. In the PE group, the maternal median age at delivery was 31.9 ± 5.1 years and in the FGR group was 31.7 ± 4.2 years. In the control group it was 29.8 ± 6.5 years ($p > 0.05$). There were no smokers in the study group nor previous medical conditions. As defined in the study design, none of the study or control pregnant women had previous history of APO. Maternal characteristics of both groups are summarized in Table 1.

7.2. PE assessment

In all cases, serial monitoring of hematologic, renal, hepatic, and immunologic parameters and regular fetal sonographic evaluation with Doppler was performed. Clinical e analytical findings are summarized in Table 2.

7.3. FGR surveillance and sonographic assessment

In all cases, serial monitoring of hematologic, renal, hepatic, and immunologic parameters and regular fetal sonographic evaluation with Doppler was performed. Patients with the diagnosis of hypertensive disorder of pregnancy were excluded from this group. In the study group there were umbilical Doppler abnormalities in all cases. In the control group there were no Doppler abnormalities detected in the routine ultrasound. Sonographic findings are summarized in Table 3.

Table 2

Clinical evolution.

Clinical and analytical characteristics	PE	FGR	Control
Severe Hypertension	15 (75.0)	0	0
Mean systolic arterial pressure (mmHg $\pm$ SD)	152.3 $\pm$ 10.3	110.5 $\pm$ 10.7	108.5 $\pm$ 11.8
Mean diastolic arterial pressure (mmHg $\pm$ SD)	95.5 $\pm$ 4.8	66.7 $\pm$ 5.1	64.8 $\pm$ 9.1
Thrombocytopenia	7 (35.0)	0	0
Elevated Creatinine	8(40.0)	0	0
Eclampsia	0	0	0
HELLP syndrome	4 (20.0)	0	0
Fetal Growth Restriction	10(50.0)	20(100%)	0
Gestational Diabetes	0	0	0
Stillbirth	0	0	0

Table 3

Sonographic assessment.

Sonographic data	FGR	Control	p value
Amniotic fluid - single deepest pocket < 2 cm (%)	4 (20.0)	0	$p < 0.05$
Uterine artery Doppler – pulsatility index > 95th centile	20 (100.0)	0	$p < 0.05$
Fetal Cerebro placental Index < 5th centile	19 (90.0)	0	$p < 0.05$
Ductus Venosus Doppler – pulsatility index > 95th centile	3 (15.0)	0	$p < 0.05$

7.4. Perinatal outcomes

In the PE group, the mean gestational age at delivery was 34.7 ± 3.4 weeks ($p < 0.05$), and the mean newborn weight was 2261 ± 862 g ($p < 0.05$). In the FGR the mean gestational age at delivery was 34.9 ± 2.5 weeks ($p < 0.05$), and the mean newborn weight was 1768.0 ± 446.4 g($p < 0.05$). In the control group, mean gestational age at delivery was 39.3 ± 1.0 weeks and the mean newborn weight was 3290.8 ± 378.5 g.

In both study groups, no cases of stillbirth or neonatal deaths were diagnosed. In the control group, pregnancy evolution was normal and no APO were diagnosed including hypertensive disorders of pregnancy, fetal growth restriction, gestational diabetes, or preterm delivery. All newborns in both groups were healthy. No malformations were diagnosed at birth, and the Apgar Index at the 5th minute of life was > 7 in both groups. Pregnancy outcomes for both groups are expressed in Table 4.

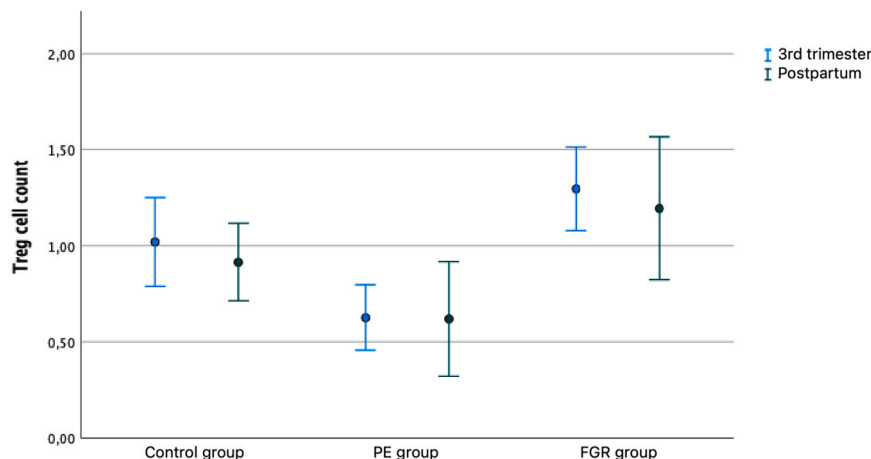


Fig. 3. CD4 +CD25highFOXP3 + Treg cell number during 3rd trimester and postpartumFGR – Fetal Growth Restriction.

Table 4

Pregnancy outcome.

Pregnancy outcome	PE	FGR	Control	p value
Mean newborn weight	2261 ± 862 g	1768.0 ± 446.4 g	3290 ± 378.5 g	p < 0.05
Mean Gestation age at delivery	34.7 ± 3.4	34.9 ± 2.5	39.2 ± 1.0	p < 0.05
Mean Apgar Index at 5 min (Apgar Index ±SD)	9.1 ± 1.0	9.5 ± 0.5	9.9 ± 0.4	p < 0.05
Number of cases with Apgar Index < 7 at 5 min (n)	2	0	0	NS
Preterm deliveries (%)	17 (85%)	13 (65%)	0	p < 0.05
Perinatal newborn deaths	0	0	0	-
Perinatal maternal deaths	0	0	0	-
Maternal Intensive/Intermediate Care Unit admission	20 (100%)	0	0	p < 0.05
Neonatal Intensive Care Unite admission	8 (40%)	7 (35%)	0	p < 0.05

7.5. Evaluation of CD3⁺/CD4⁺ and CD3⁺/CD8⁺ T cells, CD3⁺/CD56⁺ NK cells and CD3⁺/CD56⁺ NKT-like cells during pregnancy

The evaluation of CD3⁺/CD4⁺ and CD3⁺/CD8⁺ T cells, CD3⁺/CD56⁺ NK cells and CD3⁺/CD56⁺ NKT-like cells during pregnancy and postpartum were analyzed and compared between groups. In the PE group we observed that the circulating number of CD3⁺/CD56⁺ NK cells were significantly higher in the group of patients with preeclampsia during the third trimester ($6.5 \pm 2.9\%$ Vs $8.5 \pm 3.7\%$; $p < 0.05$). In these group of patients, the circulating number of these cells reduced significantly postpartum. This difference was not observed in the control group. Concerning the FGR group there were no significant differences in the distribution of these cells during the 3rd trimester of pregnancy and postpartum between groups.

7.6. CD4⁺CD25^{high}FOXP3⁺ Treg and Th17 cells evolution during pregnancy

When analyzing the evolution of CD4⁺CD25^{high}FOXP3⁺ Treg (Fig. 1e 2), we observed that the number of these cells were significantly lower at diagnosis in the PE group when compared with the FG and control group (Fig. 3). At the postpartum period, we observed the same findings, with a significantly lower CD4⁺CD25^{high}FOXP3⁺ Treg cell count in the PE group (Fig. 3).

In the FGR group, we observed that the number of these cells were similar at FGR diagnosis and during postpartum. Comparing FGR group

and control group, no significant differences in CD4⁺CD25^{high}FOXP3⁺ Treg cells were found during pregnancy or postpartum (Fig. 3).

Concerning, Th17 cells count, in the PE group, we observed a significantly higher cell count in the PE group during the third trimester (Fig. 4). At the postpartum period we did not find significant differences in the Th17 cell count between groups.

In the FGR group, we also observed a significantly higher cell count during the third trimester. In this group a reduction of the Th17 cell count was observed, which was not observed at any other studied group. This finding did not achieve statistical significance (Fig. 4).

7.7. Th17 / CD4⁺CD25^{high}FOXP3⁺ Treg ratio evolution during pregnancy

Considering the plasticity characteristic of these cells and its antagonistic effect, the relationship between these two groups of cells was analyzed. The Th17/CD4⁺CD25^{high}FOXP3⁺ Treg ratio was significantly higher in the PE group during pregnancy and in the postpartum (Fig. 5). The Th17/Treg ratio increased in the control group towards puerperium, showing a relative increase in the proinflammatory cell count. This finding was not observed in the PE or FGR group. This difference achieved statistical significance during postpartum (Fig. 5). These findings may express a relative decrease of the proinflammatory cells in the study groups after pregnancy, which was more noticeable in the PE group.

8. Discussion

To our knowledge, this is the first study to prospectively evaluate the variation of Treg and Th17 cells counts during pregnancy and postpartum in a group of FGR patients and to compare it to a group of paired PE and healthy uncomplicated pregnant women. The study design excluded maternal and environmental risk factors for PE and FGR. This fact helped to ensure homogeneity in the study group and allowed a better evaluation of the immune dynamics and its correlation with PE and FGR. The observed kinetics of Th17 and Treg cell numbers suggested a dynamic balance between immune activation and suppression throughout pregnancy. This may reflect a physiological mechanism for maternal immune tolerance of the fetus (Figueiredo and Schumacher, 2018a; Liu et al., 2017; Ji et al., 2019; Krechetova et al., 2020).

Development of PE may express a proinflammatory state associated with a poor placental development (Saito, 2018; Liu et al., 2017; Robillard et al., 2007; Fu et al., 2018a; Hosseini et al., 2018; Collier et al., 2021). Several studies have demonstrated the association between a proinflammatory profile with a relative excess of Th17 cell counts and the failure of a normal trophoblast development in recurrent pregnancy

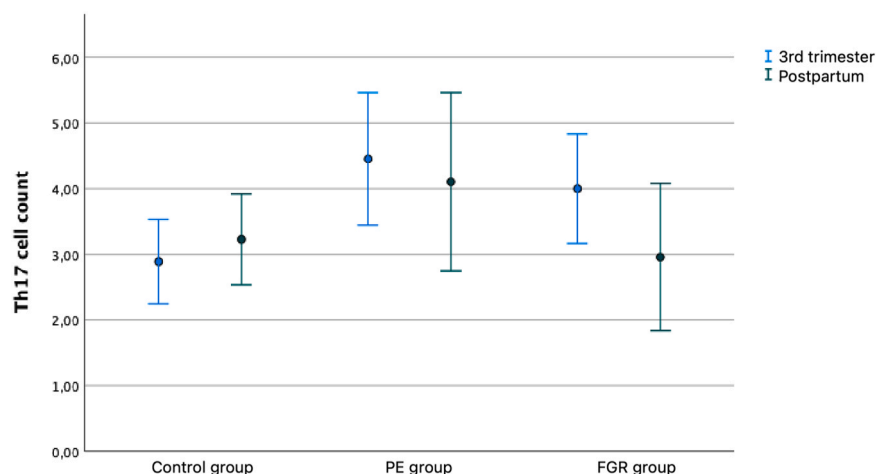


Fig. 4. Th17 cell number during 3rd trimester and postpartum. FGR – Fetal Growth Restriction.

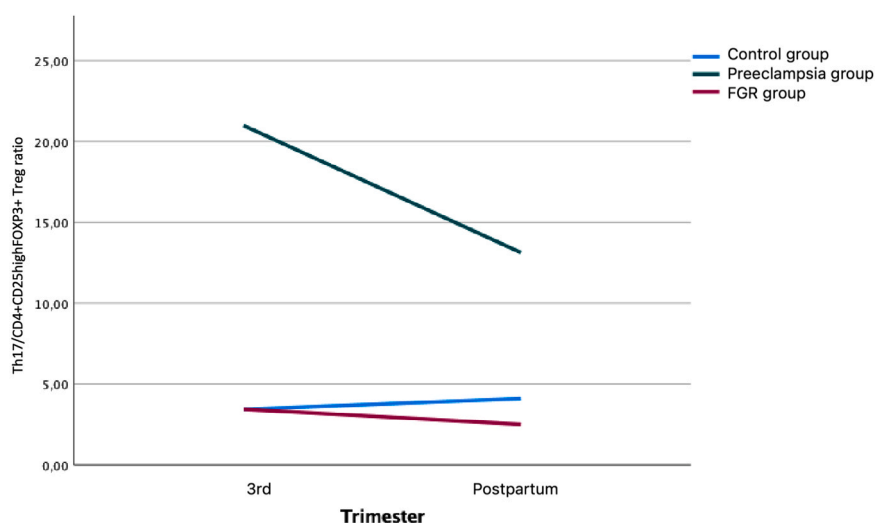


Fig. 5. Evolution of Th17/CD4 +CD25^{high}FOXP3 + Treg ratio. *p < 0.05 FGR – Fetal Growth Restriction.

loss cases (Saifi et al., 2018; Fu et al., 2018b; Qian et al., 2018; Wang et al., 2020). Similarly, previous studies described a proinflammatory profile associated with a relative increase of Th17 cell count associated with a reduced Treg cell in patients with PE (Toldi et al., 2018; Darmochwal-Kolarz et al., 2018a; Poordast et al., 2018; Eghbal-Fard et al., 2019; Muyayalo et al., 2019). In fact, a tolerogenic profile seems to be essential to promote a healthy trophoblast growth, preventing the development of placental disorders like PE.

In our study, the diagnosis of PE was associated with a significant higher number of circulating Th17 cells during pregnancy, and a significant relative reduction in the Treg cell count during pregnancy and postpartum. This proinflammatory profile was also expressed in the evolution of the Th17/CD4⁺CD25^{high}FOXP3⁺ Treg ratio. In the control group we observed a significant lower expression of proinflammatory cells. This may reflect a physiological maternal immune tolerance towards the fetus (Ji et al., 2019; Krechetova et al., 2020). In fact, Foxp3 transcription factor impairs the development of Th17 cells and assigns an anti-inflammatory effect to T cells, which justifies an essential tolerogenic function of regulatory T cells during pregnancy (Figueiredo and Schumacher, 2018a; Liu et al., 2017). These findings align with the published previous studies addressing other pathologies linked with poor placental growth, like recurrent pregnancy loss or preeclampsia (Figueiredo and Schumacher, 2018a; Sibai et al., 2018; Fu et al., 2018b; Qian et al., 2018; Wang et al., 2020, 2010; Figueiredo and Schumacher, 2018b; Saifi et al., 2018; Koucký et al., 2018; Vargas-Rojas et al., 2018; Gleicher, 2018; Wang et al., 2018b, 2018c; Bansal et al., 2018).

In our study, we also observed a reduction in the Th17/CD4⁺CD25^{high}FOXP3⁺ Treg ratio at postpartum in the PE group. This variation was not observed in the control group and may highlight the presence of a pathologic proinflammatory state in PE patients that will disappear after birth. The observed kinetics of Th17 and Treg cell numbers suggested a dynamic balance between immune activation and suppression throughout pregnancy that may reflect a physiological mechanism for maternal immune tolerance towards the fetus (Figueiredo and Schumacher, 2018a; Liu et al., 2017; Ji et al., 2019; Krechetova et al., 2020), which seems to fail in PE patients (Collier et al., 2021).

This proinflammatory state in PE patients is also expressed in our study by a relative increase in the CD3⁺/CD56⁺ + NK cells in PE group during pregnancy which disappear at postpartum. Higher levels of NK cells was also described in PE patients (Laganà et al., 2017; Fukui et al., 2012). This findings are associated with poor trophoblast growth and placental disorders like preeclampsia (Robillard et al., 2018; Loewendorf et al., 2018).

Traditionally, the development of FGR in the absence of a clear fetal

pathology expresses a problem with the placenta development. The normal growth of trophoblast usually needs a tolerogenic profile. A relative excess of proinflammatory cells, like Th17, has previously been implicated in the development of APO, like preeclampsia or recurrent pregnancy loss (Sibai et al., 2018; Koucký et al., 2018; Vargas-Rojas et al., 2018; Gleicher, 2018; Wang et al., 2018b, 2018c; Bansal et al., 2018).

Several studies have demonstrated the association between a proinflammatory profile with a relative excess of Th17 cell counts and the failure of a normal trophoblast development in recurrent pregnancy loss cases (Saifi et al., 2018; Fu et al., 2018b; Qian et al., 2018; Wang et al., 2020). FGR as a consequence of placental insufficiency is a different spectrum of the same disease (Liu et al., 2017). In our study, the diagnosis of FGR was associated with a significant higher number of circulating Th17 cells. This proinflammatory profile was also expressed in the evolution of the Th17/CD4 +CD25^{high}FOXP3 + Treg ratio. In the FGR group, a reduction of the relative proportion of proinflammatory cells in the postpartum may express that during pregnancy, in these patients, the normal tolerogenic profile was absent. This was not observed in the healthy control group.

There are no previously published studies to date addressing this relationship. The relative excess of Th17 cells in the presence of FGR, also expressed in the evolution of the Th17/CD4⁺CD25^{high}FOXP3⁺ Treg ratio towards postpartum may represent a worse tolerance of a semi-allogenic fetus in the FGR group. These findings align with the published previous studies addressing other pathologies linked with poor placental growth, like recurrent pregnancy loss or PE (Figueiredo and Schumacher, 2018a; Sibai et al., 2018; Fu et al., 2018b; Qian et al., 2018; Wang et al., 2020, 2010; Figueiredo and Schumacher, 2018b; Saifi et al., 2018; Koucký et al., 2018; Vargas-Rojas et al., 2018; Gleicher, 2018; Wang et al., 2018b, 2018c; Bansal et al., 2018).

When we compare the immunological profiles of patients with PE and FGR we observed a higher number of proinflammatory Th17 cells and a significant lower number of Treg cells in PE patients. This is particularly expressed in the differences found between the Th17/CD4⁺CD25^{high}FOXP3⁺ Treg ratios of these two groups. This data may reflect a systemic proinflammatory profile present in the preeclampsia patients that is absent, or at least not so exuberant in patients with FGR without preeclampsia. These findings may help to explain why in the presence of a higher number of proinflammatory Th17 cells, the relative reduction of Treg cells that we observe in the PE group (but not in the FGR group), could be the base of the systemic syndrome that characterize PE.

Nevertheless, some limitations may be pointed to this study, notably,

the reduced number of enrolled patients. Another limitation is the use of corticosteroids for fetal lung maturation in the presence of preterm PE or severe FGR. The use of corticosteroids in these settings is mandatory to improve fetal outcomes and are expressed in the respective surveillance guidelines. The impact of this treatment was not considered in this study and is one possible source of bias. In spite of this limitations, the homogeneity of the sample allowed us to clearly demonstrate an opposite evolution of CD4⁺CD25^{high}FOXP3⁺ and Th17 cell counts during pregnancy and postpartum between groups. The results that are now published show us a clearly different immune response in PE and FGR patients when compared to healthy controls.

9. Conclusion

As far as we know, this is the first study to evaluate the variation of Treg and Th17 cells counts prospectively during pregnancy to the postpartum in a group of PE patients, and the first study to evaluate prospectively the variation of Treg and Th17 cells counts during pregnancy and postpartum in a group of FGR patients. As far as we know, this is also the first study to compare Treg and Th17 cells counts between a group of FGR patients and a group of healthy uncomplicated pregnant women.

We showed that the presence of a proinflammatory profile and a relative excess of Th17 cells was associated with the diagnosis of PE and FGR. This fact may be the underlying cause of poor placental development, a reduced fetal growth and systemic inflammatory response present in preeclampsia.

Ethics approval

This study was approved by the ethic committees of Centro Hospitalar Universitário do Porto and Instituto Ciência Biomédicas Abel Salazar, Oporto University (404-12 (280-DEFI/ 342-CES).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication

All participants signed informed consent regarding publishing their anonymized data.

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CRedit authorship contribution statement

AB, JB, EN and CV conceived and designed the study. AB and JG collected the patient data. AB, JB, JG, EM and CV contributed to the analysis and interpretation of data. All authors drafted the manuscript, revised it critically, and approved the final version for submission.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Data Availability

Not applied.

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Chapter V

Discussion

Discussion

A detailed discussion of each study is presented within the body of the corresponding article. An integrated discussion of the main findings of the studies and their clinical relevance is given below and directed by the aims of this thesis.

Main findings and their clinical relevance

- ➔ Assess the clinical impact of pregnancy in a cohort of Portuguese SLE patients;
- ➔ Assess the maternal and foetal outcomes of pregnancies in a cohort of Portuguese SLE patients and compare it with a group of healthy pregnant women.

SLE is a systemic autoimmune connective tissue disorder affecting mainly women and frequently associated with pregnancy. Pregnancy in SLE patients is a high-risk event because adverse maternal and foetal outcomes increase (Bundhun et al., 2017a; Zhang et al., 2023). Gestation in a SLE patient is associated with an increased incidence of prematurity, development of HDP, lower birth weight, caesarean delivery, and neonatal intensive care admission even if SLE is in remission or with low level activity at the time of conception (Bundhun et al., 2017a; Moroni et al., 2016; Moroni and Ponticelli, 2016). In our population, our studies show similar data. Even among SLE patients with well-controlled disease, APO are significantly more frequent than the general population, as particularly highlighted in our publications (Braga et al., 2021a, 2021b).

This study was the first publication where SLE pregnancy outcomes were compared with a group of healthy pregnant women in Portugal. In our study, the global incidence of adverse outcomes in SLE pregnant patients was 40.3% (OR 2.64, 95% CI 1.67-4.18). Miscarriage rate was 15.3% (OR 5.85, 95% CI 2.57-13.34). Preterm delivery occurred in 12.4% of the patients (OR 1.72, 95% CI 0.83-3.57), and PE incidence was almost four times higher in SLE patients (OR 3.92, 95% CI 1.32-11.57). In the SLE group, the rate of newborn admission to an intensive care unit was also increased (OR 4.99, 95% CI 1.47-16.90) – table 9. This data clearly expresses that, despite an elevated live birth rate, SLE pregnancies remain a high-risk scenario. The development of APO was more frequent in SLE patients with lupus nephritis, APS or antiphospholipid antibodies (Braga et al., 2021a, 2021b).

Table 9 – Maternal-foetal outcomes of pregnant women with and without SLE

Variable	SLE cases (n=124)	Control cases (n=300)	OR (95% CI)	P-value
Pregnancy outcomes				
Live birth rate (%)	105 (84.6%)	290 (96.7%)	5.25 (2.36-11.65)	<0.01
Mean gestational age at delivery (weeks)	37.7±1.8	38.5±1.8		<0.01
Mean Newborn birthweight (g)	2938.9±462.9	3138.2±517.1		<0.01
APO (%)	124 (40.3%)	20 (20.3%)	2.64 (1.67-4.18)	<0.01
Miscarriage (%)	19 (15.3%)	9 (3.0%)	5.85 (2.57-13.34)	<0.01
PTD (%)	13 (12.4%)	22 (7.6%)	1.72 (0.83-3.57)	NS
Gestational Diabetes (%)	6 (5.7%)	26 (8.9%)	0.54 (0.21-1.34)	NS
HDP (%)	8 (7.6%)	6 (2.1%)	3.92 (1.32-11.57)	<0.01
FGR (%)	9 (8.6%)	26 (8.9%)	0.96 (0.43-2.11)	NS
Caesarean delivery (%)	45 (42.8%)	94 (32.3%)	1.57 (0.99-2.48)	0.05
Neonatal Outcomes				
Neonatal deaths (n)	0	0		-
Stillbirth (n)	0	1	0.92 (0.03-22.78)	NS
Admission to neonatal ICU (%)	7 (6.7%)	4 (1.3%)	4.99 (1.47-16.90)	<0.01
Neonatal Lupus (n)	4	0		-
Maternal Outcomes				
Maternal deaths (n)	0	0		-
SLE flare during pregnancy	17 (13.7%)	NA		-
SLE flare postpartum	22 (17.9%)	NA		-
SLE improvement	15 (12.0%)	NA		-

The use of safe and effective immunosuppressive and immunomodulatory therapies like HCQ improved SLE activity during pregnancy and was associated with a reduction in the development of PE. However, using these drugs was insufficient to avoid completely significant adverse outcomes. Our study (Braga et al., 2021a) also supports the most recent data highlighting the improvement in pregnancy outcomes of lupus patients in the last decades. These better outcomes contrast with published data from the second half of the previous century and point out that better disease control before pregnancy and adequate treatment, especially with HCQ, are crucial to achieve the best obstetric outcomes. Despite this, even when we evaluated a subgroup of patients with controlled disease at conception and excluded patients with known risk factors such as chronic arterial hypertension and previous lupus nephritis, the development of APO remained significantly higher in lupus patients (Braga et al., 2021b).

This data allows us to conclude that the basis for improving prenatal care of SLE patients is only possible by trained multidisciplinary teams with specific surveillance protocols that start with a preconception evaluation months before pregnancy and must continue tightly throughout gestation.

- ➔ To evaluate prospectively the number of circulating Treg, Th17 cells, and Th17/Treg ratio during pregnancy and postpartum in a group of healthy pregnant women.

During pregnancy, the maternal immune system is challenged to tolerate a semi-allogeneic foetus. A shift towards a tolerogenic profile is essential to ensure healthy foetal and placental development. The trophoblast invasion necessary to develop a fully functional placenta demands an intricate network of immune tolerance that allows the growth of cells expressing paternal antigens through the maternal decidua. One of the most important mechanisms involved in the maternal immune tolerance to foetal antigens is defined by the activity of Treg and Th17 cells. Treg cells are crucial for blastocyst implantation and trophoblast development in a normal pregnancy. Therefore, a shift towards Th2 cells and an elevation of Treg in maternal decidua and circulation are usually observed (Redman and Sargent, 2010).

Despite this, the behaviour and balance of these two T lymphocyte populations were rarely studied in normal healthy human pregnancies, particularly from the beginning of gestation to the postpartum period. We conducted the first study to prospectively evaluate in a group of healthy, uncomplicated pregnant women the variation of Treg and Th17 cell counts during pregnancy and postpartum (Braga et al., 2021c).

We observed a consistent reduction in peripheral Treg cells count throughout all pregnancies while the Th17 cells number remained relatively stable. Our study showed an explicit different behaviour between CD4⁺CD25^{high}Foxp3⁺ Treg and Th17 cells throughout pregnancy to postpartum. This is expressed in the peripheral blood Th17/Treg ratio that increased significantly throughout pregnancy to postpartum. The observed kinetics of Th17 and Treg cells may suggest a dynamic balance between immune activation and suppression throughout pregnancy. These findings may reflect a physiological mechanism for maternal immune tolerance towards the foetus (Ji et al., 2019; Krechetova et al., 2020). The consistent reduction in Treg cell counts during pregnancy here described highlights a possible migration of these cells from the peripheral blood to the maternal-foetal interface in the decidua, where intimate contact between maternal and foetus cells occurs (Hosseini et al., 2016; F. Liu et al., 2011; Y.-S. Liu et al., 2011; Mjösberg et al., 2010). In addition, Foxp3 transcription factor impairs the development of Th17 cells and assigns an anti-inflammatory effect to T cells, which promote an essential tolerogenic function of regulatory T cells during pregnancy (Figueiredo and Schumacher, 2016a; Liu et al., 2017). The higher value of the Th17/Treg ratio by the end of pregnancy and postpartum may reflect a physiological pro-inflammatory state, where Th17 cells may be implicated, reinforcing an inflammatory scenario to facilitate parturition (Gomez-Lopez et al., 2020). These findings may also help to explain why systemic pro-inflammatory syndromes, like pre-eclampsia, develop in susceptible women during the second half of pregnancy (Anvari et al., 2015; Eghbal-Fard et al., 2019; Ribeiro et al., 2017; Zare et al., 2018) and the flourishing of many autoimmune disorders in the first months postpartum (Braga et al., 2022; Liu et al., 2017).

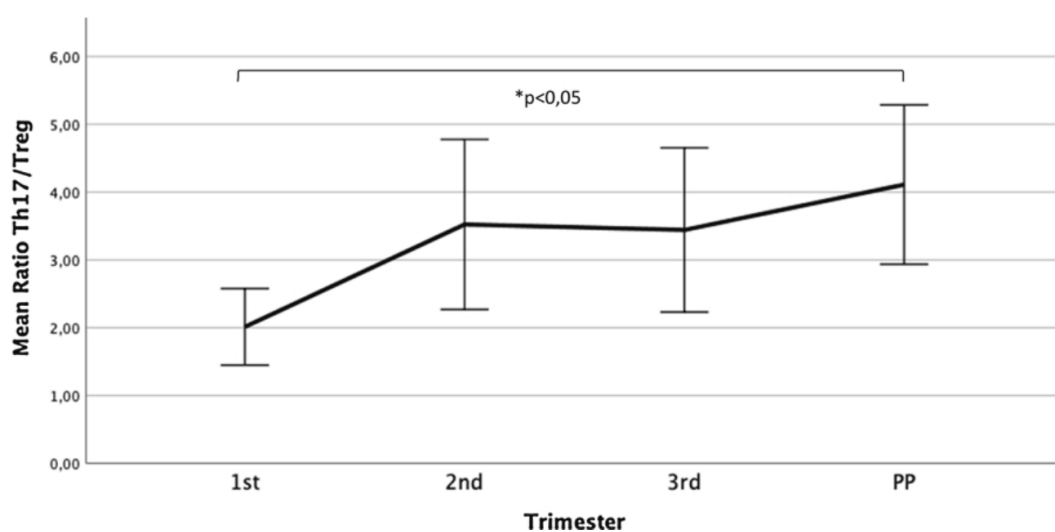


Figure 3 – Th17/CD4⁺CD25^{high}Foxp3⁺ Treg ratio throughout pregnancy to postpartum.

- ➔ To evaluate prospectively the number of circulating Treg, Th17 cells, and Th17/Treg ratio during pregnancy and postpartum in a group of SLE patients.

SLE is classically characterised by a failure in self-tolerance mechanisms associated with a defect in apoptotic debris clearance and the generation of autoantibodies. The chronic inflammation caused by this abnormal immune response leads to tissue damage (Tsokos, 2011; Tsokos et al., 2016). T lymphocytes play an essential role in SLE pathogenesis through the secretion of pro-inflammatory cytokines, inducing the production of autoantibodies by B cells, and maintaining autoimmune response by accumulating memory cells (Koga et al., 2019). During pregnancy, the maternal immune system is challenged to tolerate a semi-allogeneic foetus, and a shift towards a tolerogenic profile is essential. Failure to develop this tolerogenic profile seems to be associated with the development of APO, which is far more common in SLE pregnant women (Braga et al., 2021b, 2021a). As far as we know, we conducted the first published, prospective, longitudinal, observational study where peripheral blood lymphocyte subsets were analysed during pregnancy in a group of SLE patients and compared with healthy gestations. The observed kinetics of Th17 and Treg cell numbers suggests a dynamic balance between immune activation and suppression throughout pregnancy. This was particularly evident in the control group, where the Treg cell count changes were more marked, with a constant reduction during pregnancy (Braga et al., 2021c). As previously stated, these findings may reflect a physiological mechanism for maternal immune tolerance towards the foetus (Braga et al., 2021c; Figueiredo and Schumacher, 2016b; Ji et al., 2019; Krechetova et al., 2020; Liu et al., 2017). The consistent decrease in CD4+CD25^{high}FOXP3⁺ Treg cell counts during pregnancy in the control group may be explained by the migration of these cells from the peripheral blood to the maternal-foetal interface in the decidua, promoting normal trophoblast growth (Hosseini et al., 2016; F. Liu et al., 2011; Mjösberg et al., 2010). However, these dynamics were not observed in SLE patients. These cells remained stable throughout pregnancy in the lupus patients' group. Therefore, the progressive reduction in CD4+CD25^{high}FOXP3⁺ Treg cells observed in healthy women from pregnancy to postpartum was not observed in lupus patients. The dynamics of the CD4+CD25^{high}FOXP3⁺ Treg count justify the differences found in the Th17/Treg ratio. In the control group, the peripheral increase of the Th17/Treg ratio at the end of pregnancy and in postpartum may reflect a physiological pro-inflammatory state by the end of gestation, reinforcing the importance of an inflammatory scenario to facilitate parturition (Gomez-Lopez et al., 2020). As earlier noticed, these findings may also help to explain the development of systemic pro-inflammatory syndromes, like PE, in susceptible women

during the second half of pregnancy (Anvari et al., 2015; Eghbal-Fard et al., 2019; Ribeiro et al., 2017; Zare et al., 2018). However, the lack of these dynamics in SLE patients may be justified by several mechanisms. Immunological findings in SLE patients reveal that the balance between Th17/Treg cells favours a pro-inflammatory profile (Álvarez-Rodríguez et al., 2019; Chen et al., 2018; Li et al., 2015; Talaat et al., 2015; Yang et al., 2018). In our study, patients had well-controlled disease. Therefore, the absence of episodes of lupus flares during pregnancy reduced the probability that these differences could be explained by a worsening of the clinical status and may be the basal Th17/Treg profile in SLE pregnant patients. Considering that the reduction of the peripheral CD4⁺CD25^{high}FOXP3⁺ Treg cell counts in a healthy pregnancy is probably the result of the migration of these cells to the decidua, this migration process may be impaired in SLE patients. These immunological differences could also help to clarify the higher incidence of adverse pregnancy outcomes found in the SLE gestations (Braga et al., 2022, 2021c, 2021b). A worse tolerance of a semi-allogeneic foetoplacental unit may explain why placental insufficiency and related clinical manifestations are increased in these patients (Braga et al., 2021b; Bundhun et al., 2017b). This may be the expression, or the consequence, of the intrinsic deregulation of immune tolerance, which is a well-known feature of SLE pathophysiology (Álvarez-Rodríguez et al., 2019; Chen et al., 2018; Li et al., 2015; Mak, 2022; Talaat et al., 2015; Yang et al., 2018). The lack of these dynamics, as we observed in healthy gestations, may justify the higher incidence of APO (Braga et al., 2021b) in these pregnancies.

In addition, immunomodulatory therapy, particularly HCQ, modulates Treg and Th17 differentiation (Yang et al., 2018), which may also interfere with these cells during pregnancy. It is well known that HCQ reduces the number and function of Th17 cells (Yang et al., 2018). Nowadays, it is *gold standard* to prescribe hydroxychloroquine during pregnancy to SLE patients because it is associated with a significant improvement in SLE and pregnancy outcomes. Our findings reaffirm the relevance of this recommendation because, in our cohort of SLE patients, HCQ was linked to a better pregnancy outcome (Braga et al., 2021b). The different behaviour of the Th17/Treg ratio in SLE patients already under HCQ (Braga et al., 2022) may help us to postulate that in the absence of this influence, the pro-inflammatory profile may be much more evident and linked to worse clinical outcomes.

In our studies we did not evaluate the Treg or Th17 cells' function. A future field of investigation would be to correlate this data with functional studies to understand if the relative higher number of pro-inflammatory cells observed is associated with a higher pro-inflammatory activity in these patients.

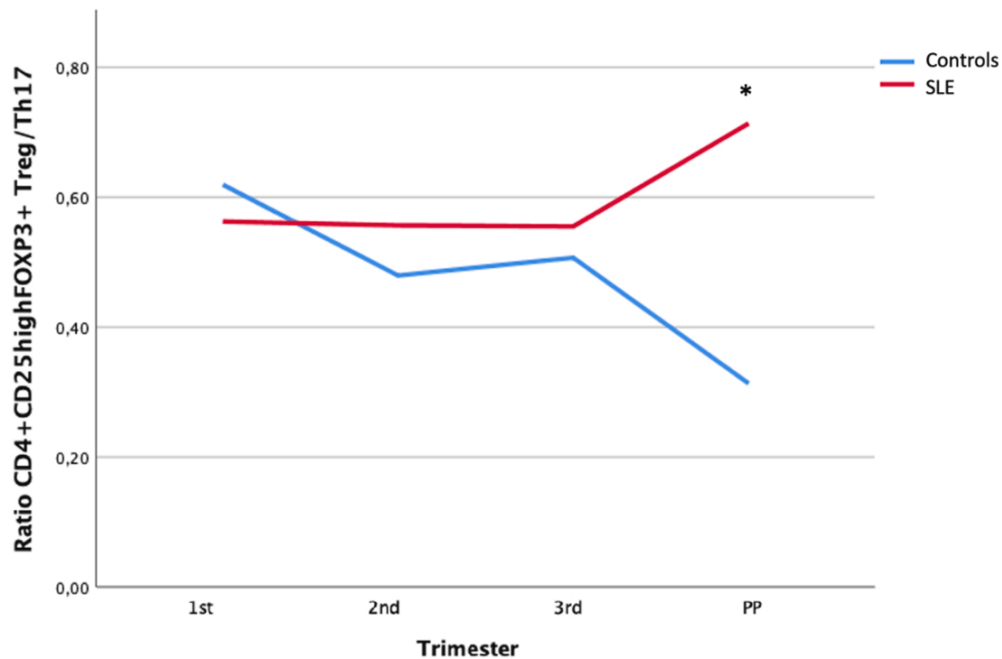


Figure 4 – Dynamics of CD4 +CD25highFOXP3 + Treg/Th17 ratio during pregnancy.

*p < 0,05 (in the published article we used the inverted CD4 +CD25highFOXP3 + Treg/Th17 ratio and not the Th17/Treg ratio, as recommended by the Editor).

- ➔ To evaluate prospectively the number of circulating Treg, Th17 cells, and Th17/Treg ratio during pregnancy and postpartum in a group of pregnant patients diagnosed with pre-eclampsia;
- ➔ To evaluate prospectively the number of circulating Treg, Th17 cells, and Th17/Treg ratio during pregnancy and postpartum in a group of pregnant patients diagnosed with foetal growth restriction.

In our study the kinetics of Th17 and Treg cells count suggested a dynamic balance between immune activation and tolerance throughout pregnancy. This may reflect a physiological mechanism for maternal immune tolerance of the foetus (Figueiredo and Schumacher, 2016b; Ji et al., 2019; Krechetova et al., 2020; Liu et al., 2017). Our findings highlight the presence of a proinflammatory profile in PE or FGF pregnant patients.

Development of PE is associated with a proinflammatory state where there is with poor placental development (Collier et al., 2021; Fu et al., n.d.; Hosseini et al., 2018; Liu et al., 2017; Robillard et al., 2007; Saito, 2010; Saito et al., 2023). Several authors have demonstrated the association between a proinflammatory profile with a relative excess of Th17 cells count and the failure to develop a normal trophoblast in recurrent pregnancy loss

cases (Chen et al., 2022; Fu et al., 2014; Li et al., 2021; Qian et al., 2018; Saifi et al., 2014; Wang et al., 2020; Zhang et al., 2023). Similarly, previous studies described a proinflammatory state associated with a relative increase of Th17 cells count associated with reduced Treg cells in patients with PE (Darmochwal-Kolarz et al., 2012; Eghbal-Fard et al., 2019; Muyayalo et al., 2019; Poordast et al., 2017; Saito et al., 2023; Toldi et al., 2011). In fact, a clear tolerogenic profile during pregnancy seems to be essential to promote a healthy trophoblast growth, preventing the development of placental disorders like PE. In our study, the diagnosis of PE was associated with a significantly higher number of circulating Th17 cells during pregnancy, and a significant reduction in the Treg cell count during pregnancy and postpartum, in comparison with healthy pregnant patients. This proinflammatory profile was also expressed in the evolution of the Th17/Treg ratio.

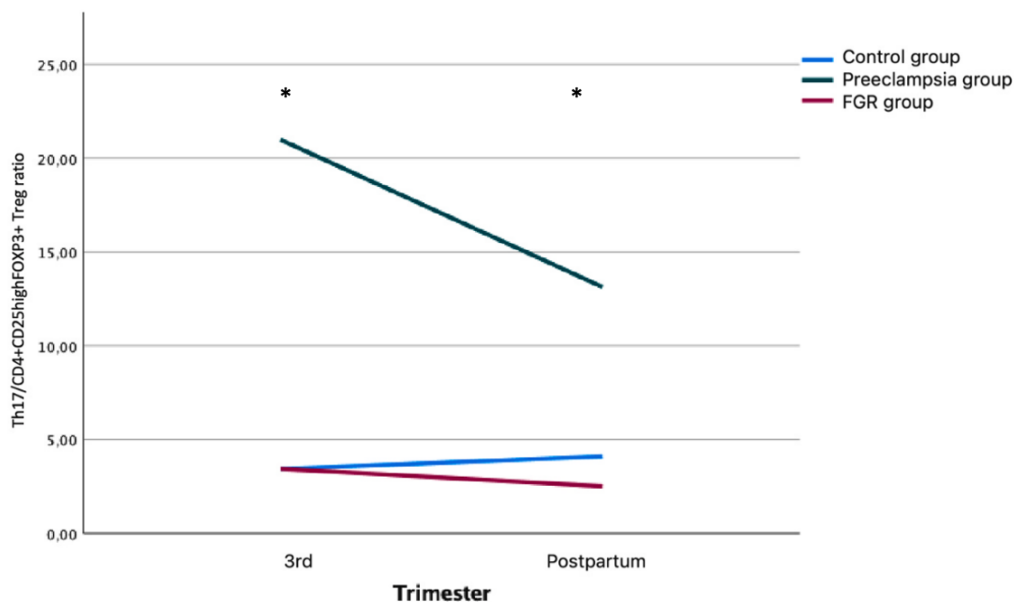


Figure 5 – Dynamics of CD4 +CD25highFOXP3 + Treg/Th17 ratio during pregnancy.

*p < 0,05.

Foxp3 transcription factor impairs the development of Th17 cells and assigns an anti-inflammatory effect to T cells, which explains the essential tolerogenic function of regulatory T cells during pregnancy (Figueiredo and Schumacher, 2016b; Liu et al., 2017). In our findings, the relative excess of Th17 cells is linked to poor placental growth and the development of PE, which is aligned with the published previous studies (Bansal et al., 2011; Figueiredo and Schumacher, 2016b, 2016a; Fu et al., 2014; Gleicher, 2007; Koucký et al., 2014; Piccinni et al., 2021; Qian et al., 2018; Saifi et al., 2014; Saito et al., 2023; Sibai

et al., 2005; Vargas-Rojas et al., 2016; Wang et al., 2013, 2020; W. J. Wang et al., 2010). We also observed a reduction in the Th17/Treg ratio at postpartum in the PE group. This variation highlights the presence of a pathological proinflammatory state in PE patients that disappears after birth, which is the opposite of what is normally observed in normal pregnancies (Collier et al., 2021; Figueiredo and Schumacher, 2016b; Ji et al., 2019; Krechetova et al., 2020; Liu et al., 2017).

Traditionally, the development of FGR in the absence of a clear foetal pathology expresses abnormal placenta development. As previously stated, the normal growth of trophoblast needs a systemic and/or local immunological tolerogenic profile (Liu et al., 2017). Our study showed that the diagnosis of FGR was associated with a significantly higher number of circulating Th17 cells. This proinflammatory profile was also expressed in the Th17/Treg ratio. In the FGR group, a reduction of the relative proportion of proinflammatory cells in the postpartum may express that during pregnancy the normal tolerogenic profile was also impaired and there is a relative excess of Th17 cells. In the presence of FGR, the evolution of the Th17/Treg ratio towards postpartum was the inverse of the ratio progression in normal pregnancy and may represent a worse tolerance of a semi-allogeneic foetus in the FGR group.

When we compared the immunological profiles of patients with PE and FGR, we observed a higher number of proinflammatory Th17 cells and a significantly lower number of Treg cells in PE patients. This is particularly highlighted in the differences found between the Th17/Treg ratios of these two groups. This data may reflect a systemic proinflammatory profile that is less exuberant in patients with FGR without PE (Fig. 5). These findings may help to explain why in the presence of a higher number of proinflammatory Th17 cells, the relative reduction of Treg cells that we observe in the PE group (but not in the FGR group), could be the basis of the systemic syndrome that characterises PE.

Table 10 – Summary of Th17/Treg ratios of the present studies

Study group	Th17/Treg ratios
Healthy pregnancies	Th17/Treg ratio increased significantly throughout pregnancy to the postpartum period
SLE patients	The lack of these findings in SLE patients could express an immune dysfunction from the beginning of pregnancy
PE patients	The presence of a proinflammatory profile and a relative excess of Th17 cells was associated to the diagnosis of PE We observed a higher number of proinflammatory Th17 cells and a significative lower number of Treg cells in PE patients
FGR patients	The presence of a proinflammatory profile and a relative excess of Th17 cells was associated to the diagnosis of FGR We observed a higher number of proinflammatory Th17 cells and a non-significative different number of Treg cells in FGR patients

Chapter VI

Conclusions

Conclusions

- ➔ This study supports the most recent data highlighting the improvement in pregnancy outcomes of lupus patients in the last decades. These better outcomes contrast with published data from the second half of the last century and show that having a better disease control before pregnancy and adequate therapeutic measures, especially HCQ, are crucial to achieving the best obstetric outcomes.
- ➔ Pregnancy in lupus patients remains a high-risk scenario, where SLE flares are still frequent and associated with the development of APO. Our study revealed a statistically significant association between SLE and the occurrence of pregnancy loss, prematurity, and the development of HDP or FGR.
- ➔ The development of APO was more frequent in SLE patients with lupus nephritis, APS or antiphospholipid antibodies positivity.
- ➔ The use of safe and effective immunosuppressive and immunomodulatory therapies such as HCQ improved SLE activity during pregnancy and seemed to be associated with a reduction in the development of HDP.
- ➔ However, the use of these drugs was not sufficient to avoid significant adverse outcomes, and APO occurred even in patients with well-controlled disease.
- ➔ The improvement of these outcomes is only possible with the development of trained multidisciplinary teams, with specific surveillance protocols that start with a preconceptional evaluation, months before the planned pregnancy. This must be the basis for qualified prenatal care of SLE patients.
- ➔ A consistent reduction in peripheral Treg cells count throughout all pregnancy is observed in healthy pregnant women while the Th17 cells count remained stable.
- ➔ The Th17/Treg ratio increases significantly throughout pregnancy to the postpartum period. These changes could be justified by the migration of the immunotolerant Treg cells to the maternal decidua and lead to the establishment of a systemic proinflammatory profile by the end of the pregnancy.

- ➔ This proinflammatory state at the end of a healthy pregnancy may be necessary for the spontaneous beginning of labour and helps to explain why systemic syndromes like PE develop during the second half of pregnancy.
- ➔ The lack of these findings in SLE patients may express an intrinsic deregulation of the immune function, a pro-inflammatory state from the beginning of the pregnancy, or the influence of immunomodulatory medication.
- ➔ The presence of a proinflammatory state since the beginning of gestation may justify the higher incidence of APO in SLE pregnancies.
- ➔ The presence of a proinflammatory profile and a relative excess of Th17 cells was associated with the diagnosis of PE and FGR.
- ➔ When we compared the immunological profiles of patients with PE and FGR we observed a higher number of proinflammatory Th17 cells and a significant lower number of Treg cells in PE patients.
- ➔ This fact may be the underlying cause of the systemic inflammatory response that characterises PE.

Chapter VII

Future Perspectives

Future

The continuous accumulation of knowledge regarding the pathophysiology of autoimmune disorders like SLE brings to the clinical practice new therapeutic strategies useful to control this disease that still causes great morbidity and mortality. Pregnancy is characterised by profound physiological changes, particularly in the immune system. The concept that there is a necessity to establish a tolerogenic medium to allow the development of a new foetus and placenta is already well-established in the clinical and scientific community. However, the mechanisms involved in the establishment of this tolerance, even in healthy pregnant women, are still largely unknown. The clarification of these mechanisms, and the perception that the failure of tolerance is the origin of several APO, is mandatory to establish new therapeutic targets for frequent obstetric syndromes, like PE or FGR. This thesis contributes to highlighting that common disruptive proinflammatory pathways are shared between autoimmune disorders and PE or FGR patients. Therefore, the development of prospective studies that elucidate these mechanisms, namely about the functional state of T cells subsets, may help to open the window to justify the use of several immunological therapeutic weapons in obstetric patients.

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Appendix I

CMIN surveillance protocol for SLE pregnant patients

1. OBJETIVO / ENQUADRAMENTO

O Lúpus Eritematoso Sistémico (LES) é uma patologia autoimune multissistémica que afeta maioritariamente mulheres. Diagnosticada habitualmente em idade jovem, é a doença autoimune que mais frequentemente se associa à gravidez. Apresenta uma prevalência estimada entre 20 a 150 casos por 100.000 e uma incidência de 1 a 25 novos casos por 100.000 pessoas/ano. Por si só, o LES não diminui a fertilidade feminina, no entanto, algumas das complicações inerentes a evolução desta patologia ou o uso de fármacos gonadotóxicos podem comprometer o futuro reprodutivo destas doentes.

Doentes com LES têm um risco global de mortalidade 20 vezes superior ao da população geral. O risco de desenvolverem trombozes, infeções graves, trombocitopenia ou necessitarem de transfusão de sangue está aumentado 3 a 7 vezes. Têm também um maior risco de desenvolverem hipertensão arterial, diabetes ou insuficiência renal. Neste sentido, a incidência de complicações obstétricas está significativamente aumentado nestas gestações.

A incidência de flare lupico durante a gravidez varia entre 15-63%, e está aumentado nas gestações em doentes com patologia ativa (50 vs. 20%) ou que suspendem abruptamente a medicação no início da gestação.

Neste sentido, uma correta avaliação e orientação preconcepcional e uma adequada vigilância obstétrica efetuada por uma equipa multidisciplinar treinada é essencial na redução de incidência de complicações durante a gravidez.

2. ÂMBITO

O presente documento destina-se a todos os profissionais envolvidos na actividade assistencial desenvolvida no CHP e especialmente aos médicos do CMIN-UMMF e UIC

3. SIGLAS / ABREVIATURAS / DEFINIÇÕES / EVIDÊNCIAS

SIGLAS

AAS – ácido acetilsalicílico

AVC – acidente vascular cerebral

C3 – fator 3 do complemento

C4 – fator 4 do complemento

CH50 – complemento total

CPK - creatinofosfocinase

CVF – capacidade vital forçada

FDA – “Food and Drug Administration”

HBPM – heparina de baixo peso molecular

LES – lúpus eritematoso sistémico

PAP – pressão artéria pulmonar

PCR – proteína C reativa

RCF – restrição de crescimento fetal

TFG – taxa filtração glomerular

TSH- hormona estimulante da tiroide

VS – velocidade de sedimentação

VDRL – teste “Venereal Disease Research Laboratory”

4. RESPONSABILIDADES

É da responsabilidade do Serviço de Obstetrícia e da Unidade de Imunologia Clínica coordenar a elaboração e atualização do presente documento e promover a sua divulgação e utilização.

5. METODOLOGIA

FATORES DE RISCO

- **Fatores associadas a aumento do risco obstétrico:**

- Dano de órgão irreversível
 - Insuficiência renal
 - Insuficiência cardíaca
 - Hipertensão pulmonar
 - Patologia pulmonar intersticial
- HTA
- Nefrite lúpica
- Presença de atividade lúpica
- Corticosteroides em alta dose
- Presença de anticorpos anti fosfolípidos ou presença de síndrome anti fosfolípidos
- Presença de anticorpos SSA ou SSB
- Antecedentes de complicações obstétricas prévias
- Idade superior a 40 anos
- Eventos tromboembólicos passados

- **Contraindicação para gravidez em doentes com LES:**

- Hipertensão pulmonar grave (PAP sistólica estimada >50mmHg ou sintomática)
- Patologia pulmonar restritiva (CVF < 1L)
- Insuficiência cardíaca
- Insuficiência renal crónica com diminuição grave da TFG (creatinina sérica superior a 2,8mg/dL)
- Icterícia nos 6 meses anteriores

- Flare grave nos 6 meses anteriores
- AVC nos 6 meses anteriores

PRECONCEÇÃO

Objetivos da avaliação preconcecional

- Avaliação do dano orgânico
- Avaliação da presença de fatores preditores de maus desfechos obstétricos
- Clarificação dos riscos inerentes à gestação em função da situação clínica individual
- Ajuste terapêutico
- Garantir remissão da doença de base pelo menos 6 meses antes da gestação

AValiação de Dano – Estudo Inicial

Estudo analítico preconcecional obrigatórios

- Hemograma
 - Proteínas de fase aguda – VS
- Bioquímica
 - Glicose
 - Creatinina
 - Ácido úrico
 - Perfil lipídico
 - Perfil hepático com bilirrubinas
 - Ionograma
 - Proteínas totais e albumina sérica
- Estudo coagulação
- Análise Urina
 - Sedimento urinário
 - Avaliação proteinúria – especialmente se antecedentes de nefrite lúpica
- Perfil imunológico
 - Anti ds-DNA
 - Complemento (C3, C4)
 - Anticorpos anti fosfolípidos (anticardiolipina IgG e IgM; anti β 2-glicoproteína IgG e IgM; anticoagulante lúpico)

- SSA e SSB
- VDRL
- Coombs

Avaliação complementar a ponderar em função do atingimento de base

- Ecocardiograma
- Ecografia abdominal e reno-vesical
- Rx tórax e provas funcionais respiratórias

AVALIAÇÃO DO TRATAMENTO PRÉ-CONCECIONAL

Substituição de fármacos potencialmente teratogénicos por outros seguros durante a gravidez. Se doente sob tratamento com metotrexato ou micofenolato de mofetil, optar por inicial azatioprina em substituição e esperar pelo menos 6 meses antes da próxima gravidez, de forma a assegurar estabilidade clínica e analítica pré-concecional. Ver anexo 1

Orientações terapêuticas pré-concecional

- Corticoides – prednisolona em doses entre 10-20mg (a mais baixa possível)
- Não aumentar dose de corticoides com fim preventivo
- Não usar betametasona nem dexametasona
- Azatioprina em dose não superior a 1,5mg /Kg/dia
- Assegurar tratamento com Hidroxicloroquina
- Iniciar AAS mini dose desde a preconceção
- Avaliar introdução de HBPM (ver protocolo gravidez com SAF)

DURANTE A GESTAÇÃO

Gestação a ser vigiada na consulta de Obstetrícia e Doenças Autoimunes

Objetivos da vigilância materno-fetal multidisciplinar

- Avaliação do dano na 1ª consulta da gestação
 - Avaliar presença de nefrite, alterações hematológicas ou presença de anticorpos anti fosfolípidos
- Avaliação da presença de fatores preditores de maus desfechos obstétricos
- Clarificação dos riscos inerentes à gestação em função da situação clínica individual

- Ajuste terapêutico
- Rastreio e orientação de pré-eclampsia
- Rastreio e orientação da RCF
- Rastreio e tratamento precoce de flare

AVALIAÇÃO INICIAL

Avaliação Obstétrica

- Exame físico com avaliação tensional
- Confirmação de viabilidade embrio-fetal
- Substituição de fármacos não aconselhados na gravidez
 - Manutenção da restante medicação prévia à gravidez
- Prescrição de AAS (100mg/dia ao deitar)
- HBPM (protocolo gravidez com SAF, protocolo profilaxia TVP)
- Suplementos de ácido fólico, iodo, cálcio e Vit.D

Estudo analítico a efetuar na 1ª consulta de Obstetrícia

Avaliação obstétrica de rotina

Estudo adicional:

- Bioquímica
 - Creatinina
 - Avaliação da TFG
 - Ácido úrico
 - Perfil hepático com bilirrubinas
 - Ionograma
 - TSH, T4
- Estudo coagulação
- Análise Urina
 - Sedimento urinário
 - Avaliação proteinúria
- Perfil imunológico
 - Anti ds-DNA
 - Complemento (C3, C4)

- Anticorpos anti fosfolipídeos (anticardiolipina IgG e IgM; anti β 2-glicoproteína IgG e IgM; anticoagulante lúpico) – se não efetuados recentemente
- SSA e SSB

Avaliação complementar a ponderar em função do atingimento de base

- Ecocardiograma
- Ecografia abdominal e reno-vesical

ESQUEMA DE VIGILÂNCIA

Periodicidade das consultas

- Mensais até às 24 semanas
- Quinzenais das 24 às 36 semanas
- Semanais após as 37 semanas
- Vigilância mais intensiva caso necessário

Avaliação clínica

- Avaliação clínica cuidada
 - Avaliação tensional
 - Analise urina em todas as consultas
 - Procurar sinais ou sintomas sugestivos de flares
 - Adaptar questionário clínico a cada situação clínica específica
- Explicitar riscos inerentes à situação clínica de cada grávida

Avaliação analítica:

- Avaliação trimestral de:
 - Função renal
 - Perfil e função hepática
 - Índices hematológicos
 - Índices imunológicos
- Se necessário adequar frequência a cada situação clínica

Avaliação placentar e fetal:

- Ecos seriadas (biometria, avaliação do líquido amniótico e doppler umbilical)
 - A partir das 20 semanas com periodicidade adaptada a cada caso
- Doppler utero-placentar
 - 20-23 semanas
- Monitorização dos movimentos fetais
- Ecocardiografia fetal nas pacientes antiSSA/SSB +
- CTG semanal a partir das 36 semanas
 - PB de acordo com cada caso clínico

PARTO E PUERPERIO

- Via de parto definida por indicação obstétrica
- Indução de trabalho de parto às 40 semanas
 - A ajustar caso a caso
- Ter em atenção os intervalos de segurança entre última dose de HBPM e a intervenção de neuro-eixo/parto

Avaliação puerperal:

- 1ª consulta no 1º mês pós-parto

ATITUDE PERANTE FLARE LUPICO

- Avaliação clínica em cada consulta de sinais sugestivos de flare
 - Artralgias
 - Artrite
 - Mialgias
 - Fadiga
 - Lesões cutâneas
 - Febre
 - Aftas orais
 - Dor torácica
 - Edemas
 - Hematúria

- Correlacionar clínica com avaliação analítica/imunológica
- Orientação em conjunto com UIC

Flare ligeiro

- Fadiga, artralgias, artrite, lesões cutâneas, mialgias
- Medidas gerais:
 - Repouso relativo
 - Assegurar cumprimento da medicação pela grávida
 - Corticoterapia tópica para controlo sintomas cutâneos
 - Paracetamol SOS
 - Sem em melhoria em 2 semanas com medidas gerais
 - Iniciar prednisolona oral/aumento de dose
 - Intensificar vigilância fetal

Flare Moderado a Grave

- Serosite, trombocitopenia, anemia hemolítica, lúpus neuropsiquiátrico
- Medidas gerais referidas
- Vigilância em regime de internamento
- Corticoterapia em alta dose (0,5-1mg/kg/dia)
 - Ponderar uso de pulsos de metilprednisolona

Nefrite Lúpica

- Diagnóstico diferencial com pré-eclampsia difícil (por vezes impossível)
 - Ver tabela 1
- Medidas gerais referidas
- Vigilância em regime de internamento
- Corticoterapia em alta dose (0,5-1mg/kg/dia)
 - Ponderar uso de pulsos de metilprednisolona
- Iniciar imunossuppressores e anti hipertensor
 - Azatioprina 1ª linha
- Terminar gravidez se resposta insuficiente ao tratamento ou diagnóstico concomitante de pré-eclampsia.

Tabela 1 – Diferencias entre nefrite lúpica e pré-eclampsia

Alterações clínicas/analíticas	Nefrite lúpica	Pré-eclampsia
Pressão arterial	Normal – Elevada	Elevada
Início da elevação tensional	Início pode ser anterior às 20 semanas de gestação	Após as 20 semanas de gestação
Proteinúria	Presente	Presente
Sedimento urinário	Ativo	Inativo
Ácido úrico	Normal	Elevado
Anticorpos anti-dsDNA	Elevado (a aumentar)	Normal
Complemento	Diminuído	Normal
Atividade doença extra-renal	Pode estar presente	Ausente

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ANEXO 1

FARMACOS A UTILIZAR NA GRAVIDEZ E POS-PARTO

Fármaco	Seguro na gravidez	Categoria FDA	Amamentação
Hidroxicloroquina	Sim	C	Seguro
Prednisolona	Sim	B	Seguro
Azatioprina	Sim	D	Provavelmente seguro
Ciclosporina	Sim	C	Provavelmente seguro
Tacrolimos	Sim	C	Provavelmente seguro
Metotrexato	Não	X	Não amamentar
Micofenolato de Mofetil	Não	D	Não amamentar
Ciclofosfamida	Não	D	Não amamentar
Rituximab	Não	C	Não amamentar
Belimumab	Não	C	Não amamentar
Leflunamida	Não	X	Não amamentar
IVIG	Sim	C	Não amamentar
AAS mini dose	Sim	C	Seguro
Clopidogrel	Sim	B	Seguro
Heparina	Sim	C	Seguro
IECAs/ARAs	Não	D	Seguro
Nifedipina	Sim	C	Seguro
Alfametildopa	Sim	B	Seguro
Omeprazol	Sim	C	Seguro
Pantoprazol	Sim	B	Seguro
Esomeprazol	Sim	B	Seguro
Lansoprazol	Sim	B	Seguro