NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTMENT OF LIFE SCIENCES

TOWARDS UNDERSTANDING OF GENETIC SUSCEPTIBILITY LINKING PRETERM BIRTH AND PERIODONTITIS

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Master in Molecular Biology in Health

DOCTORATE IN BIOLOGY

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DOCTORATE IN BIOLOGY

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Aos meus pais

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ABSTRACT

Preterm Birth (PTB) is a major public health challenge involving a strong inflammatory component, as in chronic diseases such as Crohn's disease and periodontitis (PD). The traditional clinical approach treats these as distinct conditions based on their symptoms. However, evidence suggests a potential shared genetic predisposition underlying some chronic diseases. Interestingly, several of these – especially inflammatory ones – have also been considered risk factor for PTB. This thesis investigates the hypothesis that PTB and PD may stem from a common genetic susceptibility to inflammatory dysregulation rather than a direct causal relationship. A case-control study was conducted in a Portuguese cohort, focusing on inflammatory-related genetic variants. An initial panel of 73 variants in 36 genes was selected via literature review and bioinformatic tools. Two *KCNN3* variants, rs1218585 and rs1218584, were associated with increased spontaneous PTB (SPTB) odds. Haplotype analysis further supported the association, aligning with the role of *KCNN3* in regulating contractility through potassium channels. In the PD context, a novel association was found with *IL1RN* rs4251961, highlighting its potential role in modulating inflammatory responses in periodontal tissues. Furthermore, in line with this thesis hypothesis, a composite inflammatory phenotype including both SPTB and PD was analyzed, revealing a consistent association with the *TLR1* rs5743618. Although not as robust, *IL1RN* rs4251961 also showed a moderate association, potentially supporting a broader inflammatory susceptibility. Despite the limited sample size, this study supports a common genetic predisposition to complex inflammatory conditions, manifesting as SPTB and PD. These findings advance understanding of the genetic basis of inflammatory-driven diseases and may inform future preventive and therapeutic strategies.

Keywords: Spontaneous preterm birth, Periodontitis, Inflammation, Genetic susceptibility, *KCNN3*, *IL1RN*, *TLR1*

RESUMO

O parto pré-termo espontâneo (SPTB) constitui um importante problema de saúde pública, estando associado a uma resposta inflamatória exacerbada, tal como ocorre em doenças crónicas como a doença de Crohn e a periodontite (PD). A abordagem clínica atual encara estas condições como processos distintos, com base nos seus sintomas específicos. No entanto, evidências sugerem uma predisposição genética comum subjacente a algumas delas. Curiosamente, várias destas doenças – sobretudo inflamatórias – são apontadas como fatores de risco para o SPTB. Esta tese testa a hipótese de que o SPTB e a PD possam resultar de uma suscetibilidade genética comum à desregulação inflamatória, mais do que uma relação causal direta. Para isso foi realizado um estudo caso-controlo num coorte português, focado na análise de variantes genéticas associadas à inflamação. Um painel de 73 variantes em 36 genes foi inicialmente selecionado com base na literatura e ferramentas bioinformáticas. Duas variantes do gene *KCNN3* (rs1218585 e rs1218584) mostraram associação com o aumento das chances de SPTB, reforçada pela análise de haplótipos e em consonância com o papel deste gene na contratilidade uterina. No contexto da PD, foi identificada uma nova associação com a variante *IL1RN* rs4251961, salientando o seu envolvimento na resposta inflamatória dos tecidos periodontais. De acordo com a hipótese da tese, foi ainda analisado um fenótipo inflamatório composto (SPTBxPD), revelando associação consistente com o *TLR1* rs5743618. Embora menos robusta, o *IL1RN* rs4251961 também mostrou associação, reforçando a hipótese de uma suscetibilidade inflamatória comum. Apesar das limitações amostrais, este estudo apoia uma predisposição genética comum para condições inflamatórias complexas, que se manifestam como SPTB e PD. Estes resultados permitem compreender melhor a base genética de doenças inflamatórias e podem informar futuras estratégias preventivas e terapêuticas.

Palavras chave: Parto pré-termo espontâneo, Periodontite, Inflamação, Suscetibilidade genética, *KCNN3*, *IL1RN*, *TLR1*

CONTENTS

ACKNOWLEDGMENTS IX

FUNDING XI

ABSTRACT XIII

RESUMO XV

CONTENTS XVII

LIST OF FIGURES XXI

LIST OF TABLES XXIII

ABBREVIATIONS AND ACRONYMS XXV

THESIS OUTLINE XXXI

1 INTRODUCTION: INFLAMMATORY FACTORS CONCURRING TO PRETERM BIRTH 1

1.1 Inflammatory dynamics in pregnancy 2

1.1.1 Structure of the human fetal membranes 4

1.2 Birth as an inflammatory event 6

1.3 Preterm birth (PTB) 10

1.3.1 Epidemiology 10

1.3.2 Etiology 12

1.3.3 Autoimmune and inflammatory diseases related to PTB 33

1.3.4 Periodontitis (PD) 36

1.4 Relationship between PD and PTB 42

1.4.1 Epidemiology results 42
1.4.2 Biological hypothesis 44
1.4.3 A bidirectional hypothesis between PD and PTB 45
1.5 Final considerations 48
2 AIMS OF THE PROJECT 51
3 INFLAMMATORY-RELATED GENES ASSOCIATED TO PTB IN A PORTUGUESE POPULATION: A PILOT STUDY 53
3.1 Introduction 54
3.2 Materials and methods 55
3.2.1 Study design 55
3.2.2 DNA extraction and genotyping 57
3.2.3 Statistical analysis 59
3.3 Results 60
3.3.1 Screening self-report questionnaire 60
3.3.2 Demographic and lifestyle information of the PTB and control groups 66
3.3.3 Bioinformatic analysis for candidate gene variants 68
3.3.4 Genetic association analysis in SPTB patients and controls 70
3.4 Discussion 74
3.4.1 Development of the questionnaire 74
3.4.2 Selection of genetic variants 75
3.4.3 Pilot study and *KCNN3* selection 75
3.4.4 Limitations and future directions 76
4 *KCNN3* RS1218585 AND RS1218584 ASSOCIATE WITH PTB IN A PORTUGUESE POPULATION: A CASE-CONTROL STUDY 77
4.1 Introduction 78
4.2 Materials and methods 79
4.2.1 Study population 79

4.2.2 DNA extraction and genotyping 79
4.2.3 Statistical analysis 79
4.3 Results 80
4.3.1 Demographic and lifestyle information of the control group and PTB group 80
4.3.2 Genotypes and haplotypes distributions in SPTB patients and controls 83
4.4 Discussion 88
4.5 Final considerations 90
5 ASSOCIATION OF INTERLEUKIN-1 RECEPTOR ANTAGONIST (*IL1RN*) RS4251961 WITH RISK OF PERIODONTITIS IN A PORTUGUESE POPULATION 93
5.1 Introduction 94
5.2 Materials and methods 95
5.2.1 Study population 95
5.2.2 Selection of cases and control subjects 96
5.2.3 DNA extraction and genotyping 97
5.2.4 Statistical analysis 97
5.3 Results 98
5.3.1 Demographic and lifestyle information of the control group and PD group 98
5.3.2 Genotype frequencies in PD patients and healthy controls 99
5.4 Discussion 103
5.5 Conclusion 105
6 TOWARDS UNDERSTANDING OF GENETIC SUSCEPTIBILITY LINKING PRETERM BIRTH AND PERIODONTITIS 107
6.1 Introduction 108
6.2 Materials and methods 109
6.2.1 Study design 109
6.2.2 Genotype and variants selection 110
6.2.3 Statistical analysis 110

6.3 Results 111
6.3.1 Demographic and lifestyle information of the control group and the Inflammation group 111
6.3.2 Variants filtering and selection 114
6.3.3 Genotypes distribution in inflammation group and controls 114
6.4 Discussion 123
6.5 Conclusion 129
7 GENERAL DISCUSSION AND FINAL CONSIDERATIONS 131
BIBLIOGRAPHY 141
A SUPPLEMENTARY MATERIAL 181

LIST OF FIGURES

Figure 1.1. The three different stages of pregnancy. 4
Figure 1.2. The arrangement and structure of the human fetal membranes. 5
Figure 1.3. Inflammatory pathway leading to birth. 8
Figure 1.4. Zone of altered morphology (ZAM). 10
Figure 1.5. Estimated national preterm birth rates in 2020. 11
Figure 1.6. Causes and interplay of several elements contributing to PTB.. 14
Figure 1.7. Human populations according to genetic ancestry 33
Figure 1.8. Representation of periodontal disease, which includes gingivitis and PD. 38
Figure 1.9. The role of cytokines in the PD pathogenesis. 39
Figure 1.10. Biological hypothesis relating PD and PTB. 45
Figure 3.1. Overview of iPLEX® genotyping assay. 59
Figure 3.2. MMP9 network based on molecular actions. 69
Figure 3.3. A protein-protein interaction network of some inflammatory-related proteins that may be involved in SPTB and PD (based on evidence). 69
Figure 4.1. Linkage disequilibrium (LD) pattern among *KCNN3* variants. 87
Figure 5.1. The genotype distributions of *IL1RN* rs4251961 in healthy controls and patients with PD are summarized. 102

LIST OF TABLES

Table 1.1. Case-control studies with genetic variants in inflammatory-related genes associated with higher risk of PTB. **21**
Table 1.2. Genetic variants related to PTB and inflammatory or autoimmune diseases. **35**
Table 3.1. Socio-demographic and lifestyle characteristics of SPTB and control groups **66**
Table 3.2. Candidate genes and respective variants **69**
Table 3.3. Genotype distributions of significant polymorphisms in patients with SPTB and controls **71**
Table 3.4. Genetic association test results for significant polymorphisms in SPTB patients and controls **71**
Table 3.5. Crude and adjusted OR from logistic regression for the association of KCNN3 rs1218585, KCNN3 rs1218584, IFNGR1 rs1327474, and IFNG rs2430561 polymorphisms with SPTB risk **73**
Table 4.1. Demographic characteristics of mothers with PTB and controls (mothers with full term babies) **81**
Table 4.2. Genotype distributions of KCNN3 rs1218585 and rs1218584 in patients with SPTB and controls and power calculations **84**
Table 4.3. Association test results for KCNN3 rs1218585 and rs1218584 under genetic inheritance models (allelic and trend tests) **84**
Table 4.4. Crude and adjusted OR from logistic regression under the additive model for the association of KCNN3 rs1218585 and rs1218584 polymorphisms with SPTB risk **85**
Table 4.5. Haplotype frequencies of KCNN3 rs1218585-rs1218584 in patients with SPTB and controls **87**
Table 5.1. Demographic and lifestyle characteristics of patients with PD and healthy controls **98**

Table 5.2. Results of likelihood tests of global effects of genetic predictors: ordered p-values and their adjustments for the 10 logistic models **100**
Table 5.3. Genotype distributions of IL1RN rs4251961 and TNFA rs1800629 in patients with PD and controls, significances of the Wald test results and power calculations **101**
Table 6.1 Distribution of participants by group and conditions **110**
Table 6.2 Demographic and lifestyle characteristics of patients with PD and healthy controls **112**
Table 6.3 Genotype distributions and logistic regression results for IL1RN rs4251961, TLR1 rs5743618, IL6 rs2069827, and IL6R rs4845617 in women with inflammation and controls.. **115**
Table 6.4 Genotype distributions and logistic regression results for the association between IL1RN rs4251961, TLR1 rs5743618, IL6 rs2069827, and IL6R rs4845617 with inflammation, adjusted for confounding factors. **116**
Table 6.5 Exploratory analysis of genetic associations in women with inflammation and controls, under additive, dominant, and recessive models, adjusted for confounding factors. **117**
Table 6.6 Logistic regression results for the association between IL1RN rs4251961, TLR1 rs5743618, IL6 rs2069827, and IL6R rs4845617 with inflammation, adjusted for the SPTBxPD interaction term..... **121**
Table 6.7 Exploratory analysis of genetic associations in women with inflammation and controls, under additive and recessive models, with SPTBxPD as covariate. **122**
Table S. 1 Minor allele frequencies and association results for 56 SNPs tested for association with the inflammation phenotype, using PLINK genotypic association test (chi-square) **186**
Table S. 2 Chi-square test results for confounding variables significantly associated with the inflammation phenotype **188**

ABBREVIATIONS AND ACRONYMS

AGTR2	Angiotensin II receptor type 2
aOR	Adjusted odds ratio
APOs	Adverse pregnancy outcomes
AR	Allergic rhinitis
ASTN1	Astrotactin 1
ATM	Ataxia telangiectasia mutated serine/threonine kinase
B	Bonferroni correction
BH	Benjamini-Hochberg
BY	Benjamini-Yekutieli
CAL	Clinical attachment loss
CARD6	Caspase recruitment domain family member 6
CD	Crohn's disease
CD14	Cluster of differentiation 14
CI	Confidence intervals
CR1	Complement receptor 1
CTLA4	Cytotoxic T-lymphocyte associated protein 4
DALYs	Disability-adjusted life-years
DAMPs	Damage-associated molecular patterns

ddNTP	Dideoxynucleotide
DEFB1	Defensin beta 1
DF	Degrees of freedom
DM	Adult dermatomyositis
dNK	Decidual natural killer
dNTPs	Dideoxyribonucleotides
EBF1	EBF transcription factor 1
EEFSEC	Eukaryotic elongation factor, selenocysteine-tRNA specific
FCAR	Fc alfa receptor
FDR	False discovery rate
FUT2	Fucosyltransferase 2
FWER	Family-wise error rate
GWAS	Genome-wide association studies
GWL	Genome-wide linkage
HGO	Hospital Garcia de Orta
HLA	Human leukocyte antigen
HSPA1L	Heat shock protein family A (Hsp70) member 1 like
HWE	Hardy-Weinberg equilibrium
IBD	Inflammatory disease is inflammatory bowel disease
IFNG	Interferon gamma protein-coding gene
IFNGR1	Interferon gamma receptor 1
IFN-γ	Interferon gamma
IGF1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor 1 receptor
IL	Interleukin

IL1A	Interleukin 1 alpha protein-coding gene
IL1B	Interleukin 1 beta protein-coding gene
IL-1RA	Interleukin 1 receptor antagonist
IL1RN	Interleukin 1 receptor antagonist protein-coding gene
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL6	Interleukin 6 protein-coding gene
IL6R	Interleukin 6 receptor protein-coding gene
IQGAP2	IQ motif containing GTPase activating protein 2
JIA	Juvenile idiopathic arthritis
KCNN3	Potassium calcium-activated channel subfamily N member 3
LD	Linkage disequilibrium
LPS	Lipopolysaccharide
MAF	Minor allele frequency
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MBL	Mannose-binding lectin
MMP	Matrix metallopeptidase
MTHFR	Methylenetetrahydrofolate reductase
NF-κB	Nuclear factor kappa B
NGS	Next-generation sequencing
NK	Natural killer
NLRP	Nucleotide-binding oligomerization domain-like receptor pyrin domain containing
NOD2	Nucleotide-binding oligomerization domain containing 2
OR	Odds ratio
PAMPs	Pathogen-associated molecular patterns

PCR	Polymerase Chain Reaction
PD	Periodontitis
PGE2	Prostaglandin E2
PON	Paraoxonase
PPD	Periodontal probing depth
PPROM	Preterm premature rupture of membranes
PRKCA	Protein kinase C alpha
PTB	Preterm birth
PTGER3	Prostaglandin E receptor 3
PTGFR	Prostaglandin F2α receptor
RA	Rheumatoid arthritis
REC	Gingival recession
SAP	Shrimp alkaline phosphatase
SD	Standard deviation
SK	Small-conductance calcium-activated potassium channel
SK3	Small-conductance calcium-activated potassium channel protein 3
SLE	Systemic lupus erythematosus
SPTB	Spontaneous preterm birth
TIMP	TIMP metallopeptidase inhibitor
TLR	Toll-like receptors
TNFA	Tumor necrosis factor alpha protein-coding gene
TNFRSF1A	Tumor necrosis factor receptor superfamily member 1A
TNFRSF1B	Tumor necrosis factor receptor superfamily member 1B
TNF-α	Tumor necrosis factor alpha
TOF	Time of flight

T_{reg}	Regulatory T cells
UC	Ulcerative colitis
VEGF	Vascular endothelial growth factor
VNTR	Variable number of tandem repeats
WES	Whole-exome sequencing
WGS	Whole-genome sequencing
WHO	World Health Organization
YLDs	Years lived with a disability
YLLs	Years of life lost to due to premature mortality
ZAM	Zone of altered morphology
ZIM	Zone of intact morphology

THESIS OUTLINE

This thesis is structured into seven main chapters and a final section containing supplementary materials. Chapters 1 is an introductory section; Chapter 2 describes the thesis aim and Chapters 3 to 6 present the investigational work conducted. Chapter 7 presents the final discussion and conclusions.

In detail:

- **Chapter 1** provides a comprehensive review of the pregnancy, particularly parturition, and the inflammatory factors, genetic variants, and predisposition for preterm birth (PTB). This chapter contextualized the study by exploring key aspects of inflammatory dynamics in pregnancy and the role of inflammation in labor. It also delves into PTB epidemiology and etiology, focusing on genetic predisposition, and genetic associations between inflammatory-related genes and PTB. Additionally, this chapter discusses the influence of women's ancestry on inflammatory responses in this condition and the relationship between autoinflammatory and inflammatory diseases and PTB, especially periodontitis (PD).
- **Chapter 2** presents the aims of the thesis.
- **Chapter 3** describes the study design and the methodological framework of the study. It details the development of the screening questionnaire used for participant selection, the identification and selection of candidate genes for analysis, and the pilot study conducted on a small population sample to validate the genetic variant panel. This chapter establishes the foundation for the subsequent genetic association studies.

- **Chapter 4** presents the main findings of the genetic association study focusing on two variants of the *KCNN3* gene (rs1218585 and rs1218584) and their potential role in spontaneous PTB (SPTB).
- **Chapter 5** explores the genetic association study on PD. This chapter identifies specific polymorphisms linked to PD susceptibility in a Portuguese cohort and discusses their possible implication in inflammatory pathways and disease mechanisms.
- **Chapter 6** investigates the relationship between SPTB and PD by analyzing shared genetic variants and potential overlapping inflammatory pathways, this chapter integrates on the interplay between these two conditions.
- **Chapter 7** presents a general discussion of the thesis results, final considerations and future research directions.
- **Supplementary material** contains additional data and supplementary information to support the main chapters.

1

INTRODUCTION: INFLAMMATORY FACTORS CONCURRING TO PRETERM BIRTH

The content of this chapter contains part of the following publication:

Couceiro J, Matos I, Mendes JJ, Baptista PV, Fernandes AR, Quintas A. Inflammatory factors, genetic variants, and predisposition for preterm birth. Clin Genet. 2021 Oct;100(4):357-367. doi: 10.1111/cge.14001. Epub 2021 May 28. PMID: 34013526.

1.1 Inflammatory dynamics in pregnancy

Inflammatory processes play key physiological roles in pregnancy [1]. Implantation and placentation, fetal growth, and parturition are distinct processes, each requiring a specific immune environment and presenting different immunological challenges. The success of a pregnancy hinges on the maternal immune system's ability to adapt and respond appropriately to the unique needs of each developmental phase [2]. The three stages of pregnancy are summarized in Figure 1.1. During the first trimester of pregnancy, a pro-inflammatory state is required for the implantation of the blastocyst and placentation [2,3]. The process of implantation begins when the trophoblast cells penetrate the decidua's surface epithelial layer, attaching and invading to form the placenta. The success of a pregnancy is dependent on the coordinated balance between the invading trophoblast and a receptive maternal decidua [2]. During implantation and the subsequent development of the placenta, the endometrium is remodeled, involving leukocytes such as natural killer (NK) cells and macrophages. These maternal leukocytes are recruited by chemokine gradients produced by trophoblasts and decidual stromal cells, and their phenotype and function are generally different from those of their peripherally circulating counterparts [4]. Approximately 70% of decidual leukocytes are NK cells [2,4] and unlike their circulating counterparts, decidual NK (dNK) cells produce a vast array of growth factors (e.g. vascular endothelial growth factor (VEGF)), angiogenic factors, and cytokines (e.g. interferon gamma (IFN-γ) and interleukin (IL) 8, IL-8), helping the endometrium remodeling, promoting and controlling the trophoblast invasion, and increasing the availability of maternal blood at the implementation site [4–6]. In addition, these cells are highly cytotoxic when activated in other tissues and in circulation. Thus, there are factors produced at the implantation site that are essential for maintaining the pro-pregnancy phenotype of these cells. Although the underlying mechanisms are not well defined yet, one factor involved in promoting pregnancy by controlling excessive inflammation is IL-10 [7,8]. Decidual macrophages are the predominant subset of maternal decidual leukocytes (20-25%) and the primary antigen-presenting cells in early pregnancy. Decidual macrophages are believed to exist as

regulatory/homeostatic and anti-inflammatory cells of an M2-like* phenotype [4,9]. They express IL-10 and produce VEGF and matrix metallopeptidase (MMP) 9, MMP-9, which may promote angiogenesis and tissue remodeling [4]. Additionally, decidual macrophages are proposed to perform "cleanup" functions by phagocytosing apoptotic trophoblasts, which prevents the release of paternal antigens that could trigger a maternal immune response against the fetus [2,4]. Successful implantation necessitates a finely regulated inflammatory response since reduced levels of inflammation may result in implantation failure, while excessive levels may lead to miscarriage. A pro-inflammatory environment is also required for trophoblast invasion into the decidua [3]. Moreover, at the implementation site, inflammation is characterized by the presence of IL-6, IL-8, IL-15, granulocyte-macrophage colony-stimulating factor, CXC-chemokine ligand 1, CC-chemokine ligand 4, osteopontin and tumor necrosis factor alpha (TNF-α), which are derived from both endometrial stromal cells and infiltrating immune cells [10]. These findings demonstrate how crucial local inflammation is for regulating the maternal decidua's receptivity, as well as for implantation and early placentation success [2].

During the second trimester, the longest period of the pregnancy (from week 13 to week 27), fetal growth and development take place. At this stage, the maternal immune system switches to an anti-inflammatory state to allow the fetus to mature [2–4]. Several immune cell types, including dNK, decidual macrophages, and regulatory T (T_{reg}) cells, contribute to the establishment of an anti-inflammatory microenvironment, which is characterized by a T helper 2-type tissue. T_{reg} cells are key players in the tissue repair process due to their anti-inflammatory and anti-apoptotic capabilities [2]. Like decidual macrophages, T_{reg} cells play a central role in maintaining an anti-inflammatory environment, preventing effector-type immune responses against paternal antigens from the early pregnancy [2,11]. Therefore, T_{reg} cells play a critical role in both implantation and fetal development [2]. Any ongoing pro-inflammatory signals at this stage of pregnancy can lead to miscarriage, and any subsequent pro-inflammatory insult, such as infection, can lead to PTB [2,12].

The final stage of the third trimester, when the fetus has completed its development, corresponds to a second pro-inflammatory stage that is responsible for the initiation of parturition

* Macrophages can have either primarily pro-inflammatory (M1-like) or anti-inflammatory (M2-like) attributes. The M2-like phenotype is associated with tissue renewal and predominantly secretes anti-inflammatory cytokines.

[2,3]. The pro-inflammatory nuclear factor kappa B (NF-κB) signaling pathway initiates labor, it is crucial for the continued progress of labor and for delivery. The uterus contracts, the baby is born, and the placenta separates due to the immune cells that infiltrate the myometrium* [2]. The last stage of pregnancy, parturition/birth, will be further developed in the next section. Pregnancy is, therefore, a process that involves at least three phases, each corresponding to a different gestation stage and having its own inflammatory profile. Therefore, the immune system's capacity to preserve the status quo at each stage is crucial to the success of pregnancy. The intricate process of immune modulation during pregnancy encompasses several aspects, including genetic background, epigenetic changes [13], and microRNAs [14], among others.

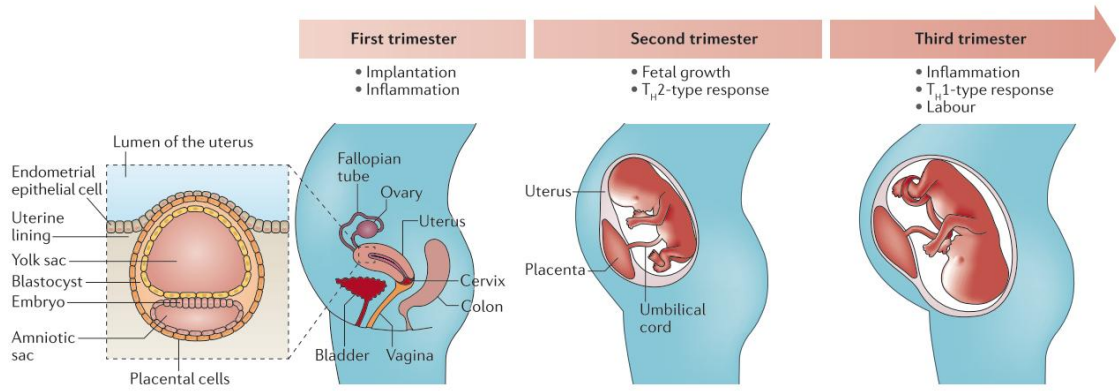

Figure 1.1. The three different stages of pregnancy. The first trimester of pregnancy is associated with inflammation, which is required for implementation and placentation. The second trimester is characterized by an anti-inflammatory environment that is necessary for fetal growth and development. In the third trimester, there is a second pro-inflammatory stage, responsible for the initiation of parturition (Mor et al., 2017 [2]).

1.1.1 Structure of the human fetal membranes

As described by Amber and colleagues (2021), the fetal membranes surround the fetus during pregnancy and play a crucial role in maintaining gestation until term (Figure 1.2). The innermost membrane is the amnion, which provides most of the structural integrity. This, in turn, is made up of the amniotic epithelium that lines the surface of the amnion facing the fetus and

* The myometrium is the middle layer of the uterine wall, consisting mainly of uterine smooth muscle cells, and its main function is to induce uterine contractions.

is held in place by the basal membrane. Beneath the basal membrane is a compact layer made up of type I and III collagen, which gives the amnion tensile strength. This compact layer is produced and maintained by fibroblasts in an adjacent layer of loose collagen. The amnion is almost avascular, obtaining nutrients and exchanging waste with amniotic fluid. The amnion is separated from the outer chorionic membrane by a space known as the intermediate layer, a space filled with fluid until 12-15 weeks of gestation, when the expanding amnion adheres to the chorion. The chorion surrounds the amnion and is formed by the fusion of the mesoderm (an embryonic layer formed in the third week of gestation) with the outer cells of the embryo, the trophoblasts. The chorionic mesoderm differentiates to become a layer of loose reticular collagen and fibroblasts that face the amnion. This collagen is supplied by a network of fetal blood vessels, and its outer surface is covered by trophoblasts. The outer surface of the chorion is covered by a layer of maternal cells known as decidua, which are rich in maternal blood vessels and immune cells. The decidua merges with the chorion during the early stages of pregnancy, forming the choriodecidua [15].

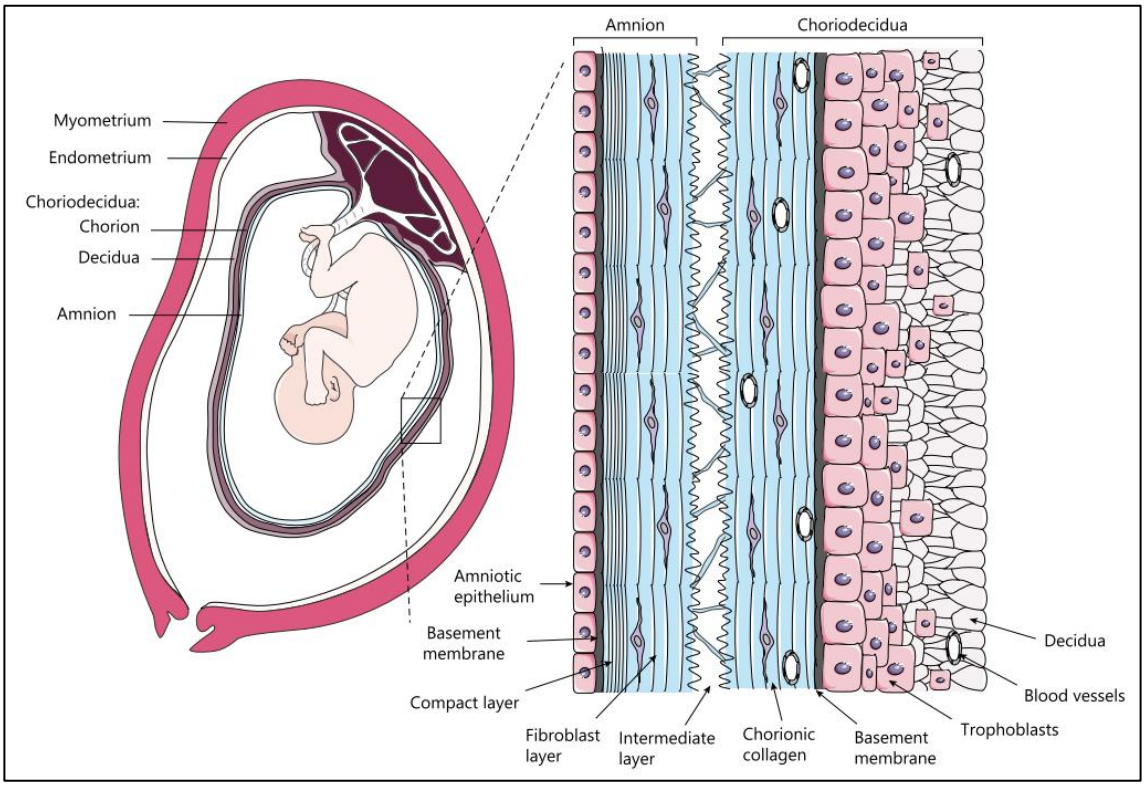

Figure 1.2. The arrangement and structure of the human fetal membranes. Amnion and choriodecidua are components of human fetal membranes. The amnion consists of a monolayer arrangement of amniotic epithelium, basement membrane, and collagen beneath it. Positioned against the amnion, the chorionic collagen lies above a layer

of trophoblasts originating from the placenta. The choriodecidua is formed when the decidua merges with the outer surface of the chorion (Amber et al., 2021[15]).

1.2 Birth as an inflammatory event

The rapid transition from pregnancy to parturition occurs through the amplification of pro-inflammatory signals that activate the uterus for labor [16]. This shift from a quiescent to a pro-inflammatory environment initiates labor and involves a three-step process characterized by uterine contractility, cervical ripening and rupture of membranes [17,18]. Increased synthesis of pro-inflammatory mediators, invasion of immune cells, and reduced sensitivity to the anti-inflammatory effects of progesterone contribute to this significant change [3,18], and this inflammatory event is not limited to a single gestational tissue, as demonstrated by several human microarray studies [19–24]. Sharp and colleagues (2016) identified 1761 genes differentially expressed in the myometrium [20], while Stephen and colleagues (2015) identified 796 choriodecidual genes, which were significantly altered in labor [24]. Haddad and colleagues (2006) had previously shown that labor chorioamniotic membranes genes involved in neutrophil and monocyte recruitment are upregulated, even when peripheral blood did not change inflammatory gene expression, suggesting that labor induces changes in gene expression consistent with a localized acute inflammatory response [23]. These significant changes of gene expression in the feto-maternal tissues during labor are predominantly associated with inflammatory pathways [16,20,22,24]. Overall, the increased expression of pro-inflammatory mediators in the intrauterine tissues include: (i) pro-inflammatory cytokines (TNF-α, IL-1 beta (IL-1β), IL-6, IL-8) and monocyte chemoattractant protein-1 in the cervix, decidua, fetal membranes, myometrium and placenta [25–28], (ii) prostaglandins (prostaglandin F2$_\alpha$ and prostaglandin E2 (PGE2)) in the myometrium, cervix and fetal membranes [29], and (iii) MMP-1 and MMP-9 in the cervix, fetal membranes and placenta [30–32]. Taken together, these findings confirm that uterine transition is a specific inflammatory reaction occurring within the uterine cavity.

As depicted in Figure 1.3, the exact inflammatory pathway leading to birth starts with the activation of the innate immune system. In infectious conditions, the immune system is activated by pathogen-associated molecular patterns (PAMPs), which are unique to microorganisms and are expressed on their surfaces [33,34]. In normal conditions, i.e. in a state of sterile

inflammation, damage-associated molecular patterns (DAMPs) are produced. When parturition approaches, DAMPs are released by a uterus that is becoming more physiologically stressed, a fetus that is maturing, and an ageing placenta [35,36]. Furthermore, fetal membrane senescence is suggested to be a signal that plays a significant role in the initiation of labor [37]. Senescent cells change into a secretory phenotype that is linked to pro-inflammatory senescence, without dying, allowing the continuous release of sterile inflammation markers. This, in turn, can stimulate positive feedback pathways linked to inflammation and senescence [37]. Regardless of whether the initial stimulus is infectious or sterile, both PAMPs and DAMPs are part of the triggers that set off the onset of labor through the activation of toll-like receptors (TLR) [2,16,34]. The TLR are expressed abundantly in the decidua, placenta, and membranes throughout pregnancy, in immune and non-immune cells, and are upregulated during parturition. TLR activation in macrophages induces inflammasome assembly through NF-κβ activation [38], which in turn controls the expression of genes encoding pro-inflammatory cytokines (such TNF-α, IL-1β, IL-6 and, IL-8) and chemokines [11]. The production of pro-inflammatory mediators leads to further recruitment of pro-inflammatory leukocytes such as macrophages, monocytes, neutrophils, and T cells to the decidua, placenta, and amniotic cavity. As more leukocytes invade the uterus, the inflammatory response is amplified through increased secretion of pro-inflammatory mediators [11], thereby recruiting and activating more leukocytes. This feed-forward loop activation of leukocytes promotes the release of more chemokines and cytokines, including other ones, like IFN-γ. These mediators also exert their effects on adjacent intrauterine tissues, which, as a reaction, release additional inflammatory mediators. The amplification of inflammatory signals promotes altered expression of uterine activation proteins, including cyclooxygenase-2, connexin 43, prostaglandin F2α receptor, and oxytocin receptor, among many others [16]. The uterine activation proteins, along with the rising amounts of cytokines and ligands for pro-contractile receptors produced by the myometrium and extraplacental membranes (amnion and choriodecidua), stimulate the transformation of the uterus from a quiescent organ into a contractile muscle, enabling the coordinated and regular contractions to occur [1,39,40]. Cytokines like IL-1β and TNF-α stimulates not only the production of prostaglandins, but also the expression of MMPs, promoting tissue remodulation, inducing cervical ripening and fetal membrane rupture [29,41,42]. Together, these changes comprise the transition of the uterus of pregnancy into the active and contractile uterus of delivery, stimulating the physiological events of delivery.

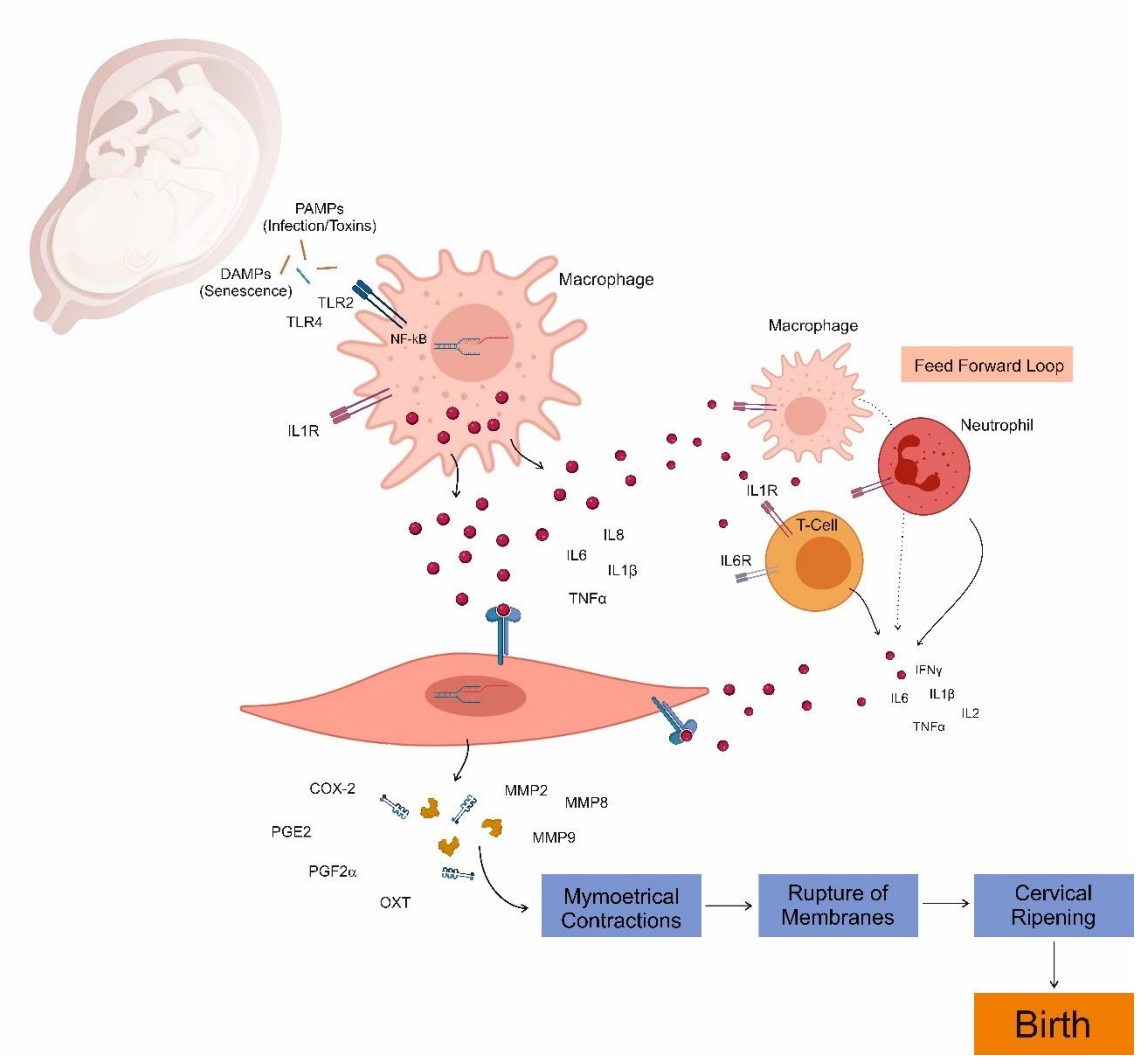

Figure 1.3. Inflammatory pathway leading to birth. PAMPs or DAMPs are the triggers of the inflammatory process, activating TLRs, which in turn induces inflammasome assembly and stimulation of pro-inflammatory mediators. There is then a positive feedback loop with the recruitment and activation of more leukocytes and the release of more pro-inflammatory mediators, not only in the uterus but also in the underlying tissues. The amplification of inflammatory signals also promotes the activation of uterine proteins. Together, these changes cause the uterus to transition to an active and contractile state, stimulating the physiological events of delivery (Adapted from Leimert et al, 2021 [16]).

Although this is the most widely accepted explanation in the scientific community, a recent study challenges whether human labor at term is in fact an inflammatory condition. Kyathanahalli and colleagues (2023) carried out a review with the aim of determining whether inflammation is a driver of labor, a consequence of labor, or an epiphenomenon (e.g., whether it occurs during labor in preparation for resolution of pregnancy) [43]. In sum, the conclusions of this study suggest that there is not a clear pattern of inflammatory immune cells infiltrating

the uterine tissues before labor begins, and there are no signs of higher levels of inflammatory cytokines and chemokines in healthy individuals before labor starts. During active labor, markers of inflammatory activation, including NF-κB, chemokines, cytokines, and influx of immune cells, were evident in the uterine myometrium, fetal membranes, and choriodecidua cells. However, it remains unclear whether these markers are essential for the onset of labor or if they emerge only after labor processes have started. Proinflammatory cytokines are elevated in the postpartum period compared to the antepartum, leading to the conclusion that inflammation plays a role in postpartum resolution and tissue remodeling. To make research results easier to compare, the authors suggest that future studies should use standard definitions of labor beginning, such as cervical dilation and/or effacement, the frequency of uterine contractions, and clinically relevant markers. They conclude that further research is necessary to understand the events leading to normal human parturition, as the question of whether inflammation is a necessary precursor to the onset of spontaneous term labor remains unclear [43].

Furthermore, a study conducted by Marcellin and colleagues (2017) proposes that in addition to inflammation, other processes, such as immunological changes, may be implicated in the process of parturition. The study demonstrated immune activation and graft rejection molecular signatures before labor, limited to the zone of altered morphology (ZAM). ZAM, composed mainly of trophoblasts, is the region of fetal membranes overlying the cervix (Figure 1.4), which presents physical weaknesses accompanied by the swelling and disruption of connective tissue. In ZAM, dNK cells become activated, and primarily M2-like macrophages abruptly decline, which is concomitant with a switch in human leukocyte antigen (HLA) presentation at the trophoblast membrane surface, where poorly polymorphic HLA-F molecules are replaced with classical, highly polymorphic HLA-A, -B, and -C molecules. This study showed that acute inflammation only occurs after these first steps of immune modifications, suggesting a local immune remodeling at the beginning of parturition [44].

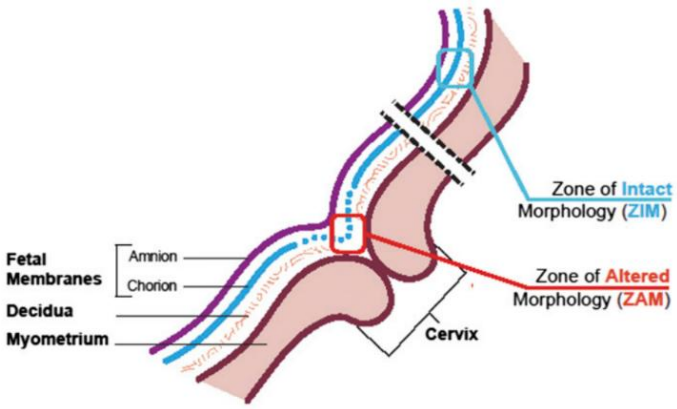

Figure 1.4. Zone of altered morphology (ZAM). The choriodecidia overlying the cervix is known as the ZAM, while the zone of intact morphology (ZIM) is the region located away from the cervix, which, as the name implies, remains unchanged at delivery. (Adapted from Marcellin et al, 2017 [44]).

1.3 Preterm birth (PTB)

According to the World Health Organization (WHO) [45], preterm birth (PTB) is defined as any birth before 37 completed weeks of gestation, or fewer than 259 days since the first day of woman's last menstrual period. PTB can be further subcategorized based on gestational age at birth: extremely preterm (<28 weeks of gestation), very preterm (28 weeks to <32 weeks), and late preterm (32 weeks to <37 weeks).

1.3.1 Epidemiology

Preterm Birth (PTB) is a major clinical and public health challenge [46], considered a leading health indicator [47].

The actual prevalence of PTB is unknown due to the lack of present data in many countries [48]. Chawanpaiboon and colleagues (2019) showed that the global PTB rate increased from 9.8% in 2000 to 10.6% in 2014 [49]. A recent study by Wu and colleagues (2024) confirmed, in a retrospective cohort study involving 1,385,979 births, that the PTB incidence has gradually increased in Taiwan, from 8.85% in 2004 to 10.73% in 2014 [50]. The estimated regional PTB prevalence for 2014 ranged from 8.7% in Europe to 13.4% in North Africa [49]. A study following the incidence of PTB in the US after 2014 showed a consistent upward trend, rising from 9.63% in 2015 to 10.1% in 2020 [51]. The latest systematic analysis on the worldwide prevalence

of PTB, by Ohuma and colleagues (2023) [46], estimated a prevalence of 9.9% in 2020, meaning that 1 in 10 babies born preterm. The highest PTB rate was seen in southern Asia (13.2%), almost double the lowest PTB rate reported in the Sustainable Development Goal region of eastern Asia, south-east Asia, and Oceania, excluding Australia and New Zealand (6.8%) (Figure 1.5). Nevertheless, significant national disparities remained in 2020, since over 50% of all PTB cases occurred in only eight countries. India represented the largest proportion, over 20% of all PTB cases globally, followed by Pakistan, Nigeria, China, Ethiopia, Bangladesh, the Democratic Republic of Congo, and the US. The authors point out that although the majority of elevated PTB rates are found in low- and middle-income countries, rates of 10% or higher were also recorded in high-income countries, including the US (10.0%) and Greece (11.6%) [46]. PTB is also the leading cause of death in children younger than five years old (18%) [52] and represents 70% of neonatal deaths [53]. Its prevention is paramount to achieve the United Nations Sustainable Development Goal 3.2 (committing to reduce neonatal mortality to 12 or fewer neonatal deaths per 1000 livebirths in every country) [54]. PTB is also responsible for up to 75% of neonatal morbidity [53], causing long-term neurocognitive deficits, pulmonary and cardiovascular disorders, and behavioral problems, among others [55–58]. Additionally, these infants are at increased risk of various adult-onset metabolic diseases such as obesity, diabetes, and hypertension [59–61]. Overall, preterm babies have substantially higher risks of adverse outcomes compared with babies born at term, and the risks of mortality and morbidity increase according to the degree of prematurity – babies born extremely preterm at the highest risk, followed by babies born very preterm, and finally babies born moderate or late preterm [46].

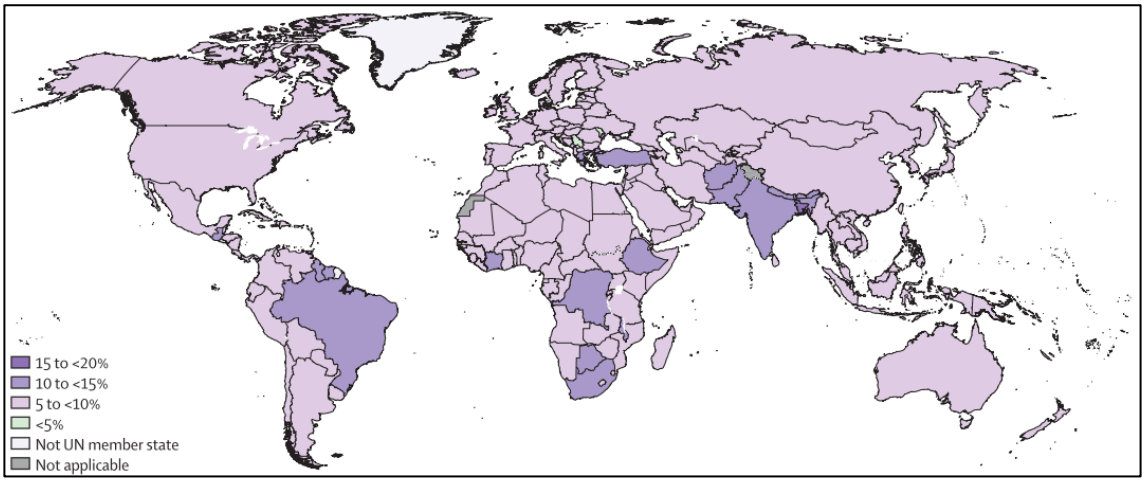

Figure 1.5. Estimated national preterm birth rates in 2020. (Ohuma et. al 2023 [46])

Moreover, PTB imposes tremendous short and long-term difficulties on families, resulting in enormous physical, psychological, and economic costs [62,63]. In 2005, the US Institute of Medicine estimated that the US annual cost of PTB was 26 billion dollars [64]. More recent data indicated that PTB imposes a substantial burden on the US healthcare system with an average lifetime additional cost of $65,000 per PTB [65]. In 2017, a German study set forth that the median price of extremely preterm infants during the first year after birth was 73,905 EUR, whereas full-term infants had an average of 1590 EUR [66]. Considering the impact of PTB on families and society [67,68], PTB represents a major burden worldwide and remains a unmet medical need that urgently requires effective therapeutic interventions [3,67–69].

1.3.2 Etiology

The etiology of PTB is still unclear, but several risk factors have been associated, including stress, inflammatory diseases, socioeconomic factors, maternal medical conditions, and genetic predispositions, among others. About two-thirds of PTB are spontaneous (SPTB), and the vast majority are idiopathic [11,56]. The remaining cases are attributable to medical intervention (i.e., iatrogenic), such as caesarean section or labor induction due to maternal or fetal conditions that compromise the health of the mother or infant [56]. The underlying molecular mechanisms are far from understood, although an imbalance between anti-inflammatory and pro-inflammatory pathways is involved in the labor trigger [55,70,71]. Notwithstanding PTB worldwide importance, there has been little advancement in the prevention of prematurity, most likely attributed to a lack of comprehension of the typical regulating mechanisms for pregnancy, the onset of labor, and the pathways by which these mechanisms are disturbed, resulting in PTB [65]. PTB is the result of preterm labor, a syndrome encompassing several etiologies. Among these, intra-amniotic inflammation due to microbial invasion (i.e., intrauterine infection) is the most well-established cause, accounting for 25 to 40% of PTB cases [56,72]. Additional risk factors include placental lesions, premature senescence of the decidua, placental and cervical insufficiencies, resistance to progesterone, genetic predispositions, epigenetic, and maternal risk factors, such as stress, high blood pressure, smoking, low socioeconomic status, among others (
Figure 1.**6**) [3,73–75]. SPTB thus results from a complex interaction of genetic, environmental, social, and behavioral factors, although the exact etiology remains unknown [76]. In the following two subsections, intra-amniotic inflammation will be explored, as it is the most

extensively studied etiology of PTB, and the genetic predisposition to PTB, which is the central focus of this thesis.

At the molecular level, the inflammatory pathway leading to birth, illustrated in Figure 1.3, is common to both term and preterm labor [3], although preterm labor is frequently characterized by the premature pathological activation of the same processes [1,77]. The scale of the inflammatory reaction can be unusually high or acute, the nature of the cellular inflammatory response may be modified, and coordination between tissues may be disrupted [1]. As previously mentioned in this chapter, while the molecular mechanisms underlying PTB remain unclear, it is known that an unbalance between anti-inflammatory and pro-inflammatory pathways plays a key role in triggering labor [55,70,71]. Actually, pro-inflammatory mediators (cytokines and chemokines) produced by leucocytes, and increased in PTB, may be enough to trigger the inflammatory pathway in delivery, despite uterine stretch and fetal maturation, leading to premature uterine activation and contractility, and eventual PTB [11,78]. Studies using mouse models have supported this evidence and highlighted the critical role of IL-1 cytokines in inflammation-induced PTB [79], probably through the activation of NF-κB signaling pathways [80]. IL-6 is another cytokine that also seems to be essential for PTB, since IL-6-deficient mice exhibit delayed parturition and demonstrate resistance to lipopolysaccharide (LPS)-induced PTB [81] (common mouse models of PTB involve the administration of LPS).

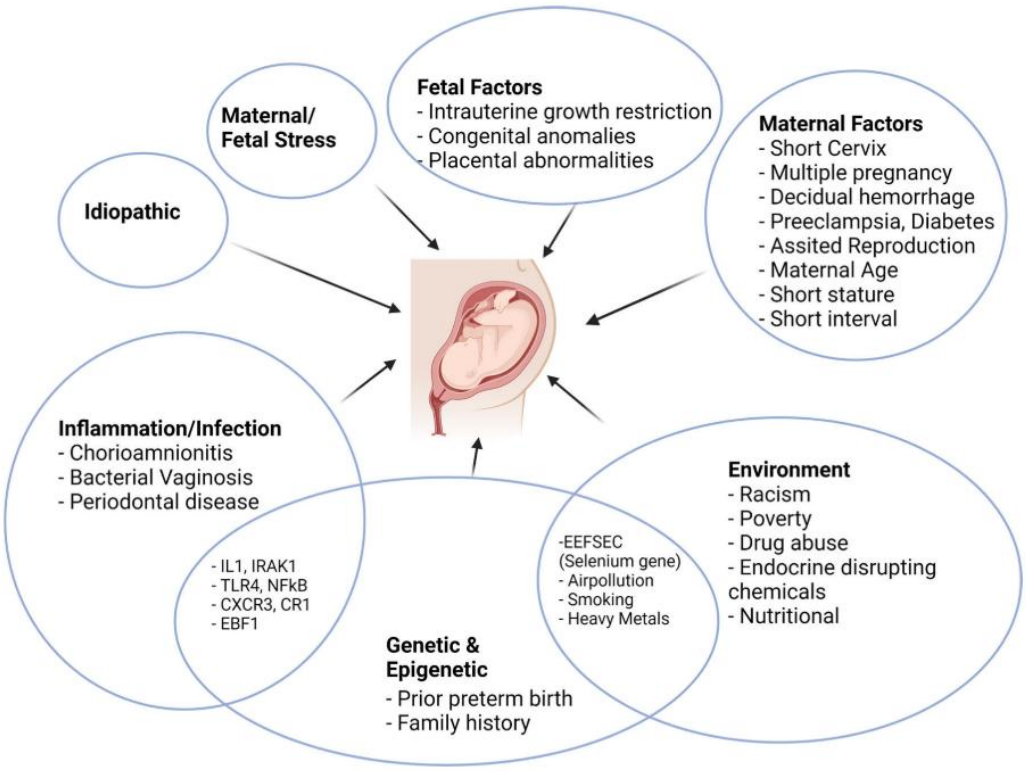

Figure 1.6. Causes and interplay of several elements contributing to PTB. PTB arises from a multitude of causes, many of which coincide. Several genes involved in inflammatory and immune systems have been associated with PTB; many environmental factors are believed to cause genetic and epigenetic alterations that lead to PTB (Jain et al, 2022 [65]).

1.3.2.1 Intra-amniotic inflammation in PTB

Intra-amniotic inflammation occurs in 30-50% of women with preterm labor [82], and its occurrence can be categorized into two distinct situations: intra-amniotic infection, which arises from the invasion of the amniotic cavity by microbes, and sterile intra-amniotic inflammation, which occurs in the absence of microbes and is characterized by a rise in alarmins or DAMPs [36,56,72,78].

In intra-amniotic infection, the predominant mode of infection is via the ascending route, as the microorganisms isolated from the amniotic fluid resemble those present in the lower genital tract [56,78,83,84]. In addition, other routes have been proposed through which microorganisms can reach the intra-amniotic space: (i) hematogenous dissemination with the transplacental transmission, demonstrated for the first time through the detection of periodontal pathogens and by-products in the amniotic fluid [85]; (ii) placental damage or disruption and/or fetal-maternal hemorrhage [86]; (iii) retrograde seeding from the peritoneal cavity

through the fallopian tubes; and (iv) accidental inoculation (i.e., iatrogenic) at the time of invasive procedures such as amniocentesis, cordocentesis, or chorionic villous sampling [78]. The amniotic cavity is a sterile compartment [87], and therefore, the detection of microorganisms in the amniotic fluid is considered to be abnormal. This can elicit a local inflammatory response, also known as microbial-associated intra-amniotic inflammation [78,88,89], which is strongly associated with preterm labor and PTB [56,72,78]. In general, microbial-induced PTB is mediated by the inflammatory process characteristic of birth, through the activation of the immune system by its PAMPs. The threat posed by the infiltration of bacterial, parasitic and viral pathogens during pregnancy can result in significant adverse prenatal and maternal outcomes [78,86]. In addition to the overall spectrum of PTB and its associated neonatal complications, the consequences include limited fetal growth, congenital anomalies and fetal and neonatal death. In fact, the spread of infection from the maternal host to the fetus, known as vertical transmission, is the leading cause of morbidity and mortality in pregnancy [86].

The sterile intra-amniotic inflammation is more common than intra-amniotic infection in women with preterm labor with intact membranes [36]. Moreover, women with sterile intra-amniotic inflammation have pregnancy and neonatal outcomes similar to women with intra-amniotic infection [36,90]. Alarmins can be released to the amniotic cavity during cellular senescence [78,91], a physiological mechanism of birth described in the previous section, or as a result of tissue injury or programmed cellular death [78,92]. Actually, decidual senescence has been suggested as a separate process implicated in SPTB that is not caused by infection [56,93]. As mentioned before, alarmins, like DAMPs, can also induce the activation of the immune system. Essentially, in sterile intra-amniotic inflammation, the inflammatory response is triggered by alarmins and involves the activation of the nucleotide-binding oligomerization domain-like receptor pyrin domain containing (NLRP) family, specifically the NLRP3 inflammasome, in the amniotic cavity [78].

1.3.2.2 Genetic predisposition to PTB

The genetic predisposition is considered a risk factor for PTB [65,73,94–96]. While some data had emerged in the 1980s [97], the body of evidence supporting the genetic impact on gestation duration and PTB began to consolidate by the late 1990s. A study developed by Porter and colleagues in 1997 showed that women who themselves born preterm, have a nearly 20% higher risk of PTB. The risk increases with the decrease in maternal gestational age at birth, with a more than two-fold increase for women born before 32 weeks [98]. In another large

population study, Winkvist and colleagues (1998) demonstrated that mothers who delivered preterm, are at a higher risk of developing a future SPTB. Furthermore, the authors also found that mothers with an older sister who had a PTB have an 80% higher risk of giving birth to a preterm infant [99]. Similarly, Mercer and colleagues (1999) showed that mothers with a prior SPTB carried a 2.5-fold increased risk of SPBT in the current gestation, and the recurrence is even higher for previous pregnancies of 23 to 27 weeks [100]. Although some studies failed to demonstrate a significant intergenerational transmission of PTB [101–104], the majority have, over the years, corroborated previous results showing that personal and family history is an important risk for PTB. Several of them validate that women who have been preterm babies themselves have an increased risk of PTB [75,76,98,105–108]. Additionally, various studies have also confirmed the increased risk of PTB in women who have a sister who was born preterm [75,76,108–110] or a sister who delivered a PTB [105,108,111]. In 2019, a massive Canadian retrospective intergenerational cohort study indicated that family history is the real risk factor for PTB. Their findings suggested that it is not the fact of having been born preterm that puts women at higher risk of delivering preterm, but the fact of having been born to a mother who ever delivered preterm [112]. The same concept could be deduced from the findings of a prior study [110]. Finally, a 2021 study found that PTB risk is significantly associated with a maternal family history of PTB among female relatives within three generations [113]. Family history as a risk factor for PTB seems to be mainly associated with the maternal lineage [76,94,107,108,111,114]. Along with intergenerational investigations, several studies have demonstrated variance in PTB rates according to genetic ancestry background [51,109,115,116], and studies involving twins have shown heritability for PTB in a range from 25% to 40% [114,117]. Finally, the most powerful evidence supporting a genetic predisposition to PTB is the discovery of genes that increase the risk of the condition. Different genome-wide approaches have been used over the years, to find the specific genes associated with PTB. The first one was a genome-wide linkage (GWL) study [96], involving seven large northern Finnish families with multiple SPTB [118]. A GWL study, like the common genome-wide association studies (GWAS), involve genotyping specific and pre-selected variants using microarrays [119]. The difference is that GWL studies involve either single large pedigrees or a large number of nuclear families to identify disease genes with large effects [65]. In the Finnish study, the authors reported a linkage between SPTB and the insulin-like growth factor 1 (IGF1) receptor (*IGF1R*) gene in the fetal genome [118]. These findings were replicated in a subsequent case-control study with several families' representative of the Finnish population. The IGF1 is

a major regulator of fetal growth and exerts its actions mainly through the receptor IGFR1 [120]. Additionally, these results suggest that IGFR1 potentially regulates signaling cascades involved in the onset of labor, influencing the risk of SPTB [118]. After that, different GWAS were performed, but low or no evidence was found, or the results could not be replicated [65]. The first large genome-wide study with robust PTB association was performed in women with European ancestry and identified three loci, EBF transcription factor 1 (*EBF1*), eukaryotic elongation factor, selenocysteine-tRNA specific (*EEFSEC*), and angiotensin II receptor type 2 (*AGTR2*). These findings were replicated in a separate northern European cohort with SPTB [121]. None of these genes directly influence immune or inflammatory processes associated with SPTB, but they may be involved in other disease mechanisms with implications on PTB and offer new avenues for research. More recently, Gupta and colleagues (2022) performed a GWAS also with European ancestry's women and identified a single nucleotide polymorphism (SNP) in the astrotactin 1 (*ASTN1*) gene, rs14675645, associated with SPTB with genome-wide significance [122]. The latest study, a large maternal genome-wide meta-analysis, identified 22 *loci* associated with gestational duration and 7 *loci* associated with SPTB, with a genome-wide significance [123].

GWAS studies have not been very effective in discovering genetic variants associated with SPTB. This limited effectiveness may be attributed to the hypothesis that the correlation between SPTB and neonatal morbidity and mortality indicates that SPTB is likely to be influenced by negative selection. Thus, the effect size for common alleles influencing phenotype attribution is expected to be weak [65]. Furthermore, GWAS on PTB have been underpowered so far [65,124,125].

The expanding approaches to genomic studies include whole-exome sequencing (WES) and whole-genome sequencing (WGS) analyses. These studies assess families with a high SPTB occurrence [65,96], using next-generation sequencing (NGS) methods to identify all genetic variations (i.e., also include rare variants) [126]. Thus far, WES or WGS studies conducted to examine the genetic factors in SPTB have been limited, perhaps due to the elevated expenses associated with genetic sequencing [125]. The first studies of WES and SPTB were carried out on Finnish mothers with a history of SPTB. McElroy and colleagues (2013) performed WES analysis in PTB mothers and found deleterious variants in the complement and coagulation pathway. In a larger case-control dataset, aiming to observe more common coding-region variants, they confirm a significant association of SPTB with three SNPs in the complement receptor 1 (*CR1*) gene [127]. A previous case-control study also suggested the relationship between

genes of the complement-coagulation pathway and PTB [128]. Then, in 2016, a WES study compared variants, identified by targeted sequencing, of Finnish women with 2-3 generations of SPTB with controls without a history of SPTB. The genes *IGF1*, ataxia telangiectasia mutated serine/threonine kinase (*ATM*), and IQ motif containing GTPase activating protein 2 (*IQGAP2*) were identified as the most promising to be connected with SPTB [129]. These genes are involved in growth, metabolic, and inflammation pathways [129]. Finally, Huusko and colleagues (2018) identified rare, possibly damaging, variants in glucocorticoid receptor signaling pathway genes. In a replication Danish cohort, variants in the heat shock protein family A (Hsp70) member 1 like (*HSPA1L*) gene, were identified in several families affected by SPTB. The association of one of *HSPA1L* variants with SPTB was further supported by large GWAS dataset, and *in silico* and *in vitro* analysis [130]. In the US, a group carried out WES studies with newborns of mothers of African ancestry with preterm premature rupture of membranes (PPROM). Damaging or potentially damaging variants contributing to PTB associated with PPROM were identified in fibrillar collagen genes [131] and in innate immunity and host defense genes (caspase recruitment domain family member 6 (*CARD6*), fucosyltransferase 2 (*FUT2*), *NLRP10*, nucleotide-binding oligomerization domain containing 2 (*NOD2*) [132], mannose-binding lectin (MBL) 2, *MBL2*, defensin beta 1 (*DEFB1*) [132,133]).

Overall, studies using NGS methods have shown that, although there is still a need for further validation, multiple genes involved in the inflammatory and innate systems are associated with SPTB.

Finally, over the years, several genetic association studies have also suggested that multiple variants, mostly SNPs in innate immune or inflammatory-related genes, are linked to the risk of PTB.

1.3.2.3 Genetic association studies between inflammatory genes and PTB

Perturbations in the labor inflammatory pathway can occur, leading to premature activation of essential pathway components, resulting in PTB. Therefore, variants in genes associated with the inflammatory pathway can increase the susceptibility to this condition [38,134], and several association studies have attempted to identify them. Despite the efforts, the identification of genes predisposing to PTB has been limited [96,135,136]. Focusing only on the birth inflammatory pathway, described in section 1.2 "Birth as an Inflammatory Event" and depicted in Figure 1.3, over the years several genomic loci related to each phase of this process, both maternal and fetal, have been positively linked with the gestational duration and a higher risk

of PTB. As mentioned previously in this chapter, both PAMPs and DAMPs are part of the triggers that set off the onset of labor. Some spectra of PAMP and DAMP ligands, including bacterial LPS, interact with TLR4, making it the most studied TLR regarding its role in PTB [11]. A polymorphism in the coding region of the *TLR4* gene (rs4986790) that causes an amino acid change at position 299 (Asp299Gly) in the respective protein may interfere with normal NF-κB activation, was associated with higher risk to PTB in the fetal genome [137]. Other case-control studies suggested a possible association between fetal or maternal polymorphisms in *TLR1* [138] and *TLR2* [139] genes with PTB or PPROM leading to PTB. As cytokines are the main players in initiating and regulating the inflammatory process related to birth [70], many studies have demonstrated a positive association of SNPs in their genes with PTB. TNF-α protein coding gene (*TNFA*) is one of the most extensively studied genes related to pro-inflammatory cytokines, and it contains a SNP in the promotor region (rs1800629) that enhances transcription. The minor allele (A) is associated with higher levels of *TNFA* gene expression (OMIM #191160), leading to an up-regulation of the inflammatory pathway, and it was related to PTB risk [140–144]. Other two SNPs in *TNFA* gene, rs1799724 [145] and rs361525 [146], were additionally suggested to be associated with PTB. Additionally, the findings of Menon and colleagues (2006) indicated that TNF-α receptors are also related to PTB, namely two SNPs in TNF receptor superfamily member 1A (*TNFRSF1A*), rs3764874, and in TNF receptor superfamily member 1B (*TNFRSF1B*), rs653667 [147]. Later, other studies associated new polymorphisms in *TNFRSF1B* gene, rs1061622 [148] and rs5746053 [149], with increased odds of SPTB. Another highly studied polymorphism in PTB is an intronic variable number of tandem repeats (VNTR) of 86 base-pair in the interleukin 1 receptor antagonist (IL-1RA) protein coding gene (*IL1RN*). Carriers of *IL1RN**2 allele have higher levels of IL-1β compared to noncarriers [150–152], and several studies linked this allele to the increased risk of PTB in both mother [148,153,154] and fetus [148,151,155]. Several authors focused their studies on genes encoding the pro-inflammatory cytokines IL-1 alpha (IL-1α) and IL-1β, the *IL1A* and *IL1B*, respectively – (i) two SNPs in the promotor region of the gene *IL1B*, rs1143634 [155] and rs16944 [145,156], and (ii) two SNPs in the *IL1A* gene, rs17561 [134,156,157] and rs1800587 [156,157]. Another widely studied pro-inflammatory cytokine gene associated with the risk of PTB is the IL-6 protein-coding gene (*IL6*) – two SNPs in the promotor region, rs1800795 [134,154,158,159] and rs1800796 [160], were identified. Other two SNPs suggested to be related to PTB increased risk were found in the IL-6 receptor protein coding gene (*IL6R*), in both fetal (rs8192282 [138]) and mother (rs4553185 [149]) genomes. Other polymorphisms were associated with the risk of PTB,

either in genes that encode pro-inflammatory cytokines or their receptors [149,159,161,162], or in genes encoding anti-inflammatory cytokines [144,163,164]. The MMPs-encoding genes have also been studied since inappropriate or premature MMP expression and activity are proposed to contribute to the pathological PPROM and PTB [42]. A VNTR in *MMP9* (rs2234681) increases the promoter activity, thus increasing *MMP9* gene transcription and promoting PTB [165]. Two other *MMP9* variants, rs3918242 [148,166] and rs3918260 [149], were associated with PTB increased risk. Finally, SNPs within genes involved in the synthesis and inhibition of other MMPs have also exhibited associations with PTB [138,149,161,167]. Information from candidate gene studies on variants in inflammatory-related genes associated with a significantly increased risk of PTB from 1999 to date is listed in Table 1.1.

Further studies also showed the involvement of other immune response-related genes, such as cytotoxic T-lymphocyte associated protein 4 (*CTLA4*) [149], cluster of differentiation 14 (*CD14*) [149], paraoxonase (PON) 1 (*PON1*) [168], *PON2* [149,168], and Fc alfa receptor (*FCAR*) [169]. Lastly, additional genes that are not part of the inflammatory pathway but can indirectly influence it, have also been associated with PTB risk, such as genes involved in cell growth [170,171], the genes methylenetetrahydrofolate reductase (*MTHFR*) [172–175], potassium calcium-activated channel subfamily N member 3 (*KCNN3*) [176–178], and protein kinase C alpha (*PRKCA*) [179], among others.

Overall, the VNTR polymorphism of the *IL1RN* gene, rs2234663, and the *TNFA* promoter polymorphism rs1800629, were the most often associated variants with PTB, followed by the *IL1A* rs17561 missense variant. Despite the advances, many studies have failed to identify genetic variants associated with PTB, and many others have not been replicated or confirmed [65]. Thus, further studies are needed to uncover the influence of specific variants on pregnancy duration and PTB.

Table 1.1. Case-control studies with genetic variants in inflammatory-related genes associated with higher risk of PTB.

Study area, population	Polymorphism	Genotypes	Cases Clinical condition	N	Control group (N)	Sample	Degree of risk with significance	References
USA, African ancestry	*TNFA* (-308 G/A) rs1800629		PTB + PPROM	26	110	Maternal	OR=3.18 (1.33-7.83)	Roberts, 1999 [140]
Kenya, African ancestry	*TNFA* (-308 G/A) rs1800629	AA	IPTB	33	926	Infant	RR=7.3 (2.85-18.9) ǀ RR=6.7 (2.0-23) ††	Aidoo, 2001 [141]
USA, multiple genetic ancestry	*IL1B* (+3953 C/T) rs1143634	allele 1	PTB	52	197	Infant	Not given (p=.033, only in infants with African ancestry)	Genç, 2002 [155]
	IL1RN rs2234663	*IL1RN*2*	PPROM + PTB				OR=6.5 (1.25-37.7) (only infants with American ancestry)	
USA, African ancestry	*MMP9* rs2234681	14 CA-repeat	PPROM	74	215	Infant	OR=3.06 (1.77-5.27) †	Ferran, 2002 [165]
USA, African ancestry	*MMP1 (*-1607) rs1799750	---	PPROM	75	235	Infant	OR=2.29 (1.09-4.82)	Fujimoto, 2002 [167]
Finland, European ancestry	*TLR4* (+896A/G ǀ Asp299Gly) rs4986790	299Gly allele	PTB	282	345	Infant	Not given (p=.024)	Lorenz, 2002 [137]

		Asp/Gly, Gly/Gly					Not given (p=.028)	
Switzerland, European ancestry	*IL1RN* rs2234663	*IL1RN**2 homozygosity	PTB	18	245	Infant	Not given (p<.0001)	Witkin, 2003 [151]
Greece, European ancestry	*VEGF* (+936 C/T) rs3025039	CC, CT	PTB	54	79	Maternal	OR=2.05 (1.37-3.06)	Papazoglou, 2004 [171]
USA, multiple genetic ancestry	*F5* haplotype block: *F5* rs6019/rs6022/rs2213869		PTB	300	458	Maternal	Not given (p=.025)	Hao, 2004 [180]
USA, African ancestry	*MMP8* haplotype block: *MMP8* (-799C/T)/(-381A/G)/(+17C/G) rs11225395/rs1320632/rs2155052	T/G/G	PPROM	168	216	Infant	OR=4.63 (2.01-11.94)	Wang, 2004 [181]
UK, multiple genetic ancestry	*TNFA* (-308 G/A) rs1800629		PTB	48	82	Maternal	OR=2.2 (1.0-5.0)	Moore, 2004 [142]
USA, multiple genetic ancestry	*TNFA* (-308 G/A) rs1800629	allele 2	SPTB	125	250	Maternal	OR=2.7 (1.7-4.5)	Macones, 2004 [143]
Australia, European ancestry	*IL4* (-590 C/T) rs2243250	CC	PTB	202	185	Maternal	OR=3.4 (p=0.02)	Annells, 2004 [182]
	IL10 haplotype block: *IL10* (-1082A/G)/(-819C/T)/(-592C/A) rs1800896/rs1800871/rs1800872	A/T/A					OR=2.1 (p=0.04)	

	TNFA haplotype block: *TNFA* (+488G/A)/(-238G/A)/(-308G/A)	A/G/G					OR=2.4 (p=0.04)	
Mexico, multiple genetic ancestry	*MTHFR* (677 C/T) rs1801133	T allele (CT, TT)	PTB	82	164	Maternal	OR=1.98 (0.97-4.08)	Valdez, 2004 [172]
China, East Asian ancestry	*MTHFR* (677 C/T) rs1801133	CT	PTB	250	250	Infant	OR=2.01 (1.21-3.32)	Chen, 2004 [173]
		TT					OR=1.82 (1.02-3.26)	
China, East Asian ancestry	*PON1* (Q192R) rs662	TT	PTB	80	105	Triad design	OR=3.6 (1.3-11) (only in child's genotype)	Chen, 2004 [168]
	PON2 (S311C) rs7493	CC					OR=4.6 (1.5-14) (only in child's genotype)	
USA, multiple genetic ancestry:			SPTB			Maternal		Engel, 2005 [134]
African ancestry	*IL6* (-174 C/G) rs1800795	C allele		67	238		OR=1.6 (0.7-3.8)	
European ancestry	*IL1A* (+4845 G/T) rs17561	T allele		69	336		OR=1.8 (0.9-3.7)	
	IL6 (-174 C/G) rs1800795	C allele					OR=1.8 (0.8-3.6)	
	TNFA (-308 G/A) rs1800692	A allele					OR=2.0 (0.9-4.0)	

	LTA (IVSI-82 G/C) rs746868	C allele					OR=2.6 (1.3-5.5)	
USA, multiple genetic ancestry	*IL1RN* rs2234663	*IL1RN*2*	PTB (PTB or PPROM)	95	105	Maternal	OR=3.2 (1.68-6.14)	Murtha, 2006 [153]
USA, multiple genetic ancestry	*IFNG* (+874 A/T) rs2430561	T allele	SPTB	80	80	Maternal / Infant	OR=2.3 (1.2-4.4)	Speer, 2006 [162]
USA, European ancestry	*TNFRSF1A* rs3764874	C allele	SPTB	101	321	Maternal	Not given p=.01 (allele freq.); p=.03 (genotype)	Menon, 2006 [147]
	TNFRSF1B rs653667	T allele	SPTB				Note given p=.04 (genotype)	
	Multilocus analysis: *TNFA* (3448)/ *IL6* (-7227)/ *IL6R* (33314)						OR=3.5 (2.52-4.87)	
USA, African ancestry	*IL6R* rs4845623	G allele	PTB	76	321	Maternal	Not given p=0.04 (allele freq.); p=0.05 (genotype)	Velez, 2007 [183]
Copenhagen, European ancestry	*IL1B* (-31 T/C) rs1143627	C allele	PTB	62	55	Infant	OR=6.36 (1.3-60.5)	Hollegaard, 2008 [145]
	TNFA (-857 C/T) rs1799724	T allele					OR=3.09 (1.04-10.33)	

Japan, East Asian ancestry	*IL1A* (-889 C/T) rs1800587	CT	SPTB	73	341	Maternal	OR=2.5 (1.4-4.8)	Sata, 2008 [157]
		CT, TT					OR=2.5 (1.3-4.6)	
	IL1A (+4845 G/T) rs17561	GT					OR=2.4 (1.3-4.4)	
		GT, TT					OR=2.3 (1.2-4.2)	
Germany, European ancestry	*IL13* rs1800925		PTB	43	270	Infant	Not given (p=.031)	Heinzmann, 2009 [163]
	IL13/*IL4* haplotype block: *IL13* rs1881457/rs1800925/rs20541/*IL4* rs2243250						Not given (p=.0001)	
	TLR10 haplotype block: rs4274855/rs11096955/rs10856839						Not given (p=.0011)	
Poland, European ancestry	*IL1RN* rs2234663	*IL1RN*2*	PTB	62	63	Maternal	OR=2.75 (1.02-4.13)	Kalinka, 2009 [154]
USA, multiple genetic ancestry	*IL1RAP* rs9290936	T allele	PTB	76	191	Maternal	OR= 1.97 (1.29-3.00)	Velez, 2009 [149]
	IL6R rs4553185	C allele					OR=1.69 (1.15-2.50)	
	TNFRSF1B rs5746053	A allele					OR=2.04 (1.20-3.47)	
	CD14 rs4914	G allele	PTB	65	183	Infant	OR=2.77 (1.32-5.82)	

	CTLA4 rs16840252	T allele					OR=3.21 (1.61-6.40)	
	IL2RB rs84460	C allele					OR=2.32 (1.47-3.67)	
	IL2RB rs228947	T allele					OR=2.39 (1.32-4.34)	
	IL2RB rs3218315	T allele					OR=2.37 (1.30-4.34)	
	IL2RB rs228954	G allele					OR=1.80 (1.20-2.71)	
	IL2RB rs2281094	T allele					OR=2.19 (1.18-4.06)	
	MMP2 rs243832	G allele					OR=2.93 (1.56-5.49)	
	MMP9 rs3918260	C allele					OR=2.14 (1.31-3.50)	
	PON2 rs2299267	G allele					OR=2.04 (1.20-3.47)	
USA, multiple genetic ancestry	*TIMP2* rs2277698		SPTB	223	599	Maternal	OR=1.98 (1.38-2.83)	Romero, 2010 [138]
	IL6R rs8192282		SPTB	179	628	Infant	OR=2.07 (1.42-3.02)	
USA, "Caucasian non-Hispanic"	*KCNN3* Chromosome 1:153099353	A allele	PTB / SPTB	412	100	Maternal	Not given (p=.03)	Day, 2011 [176]
	KCNN3 rs1218585	Genotypic association	SPTB				Not given (p=.04)	
	KCNN3 rs1218584	Genotypic association	SPTB				Not given (p=.05)	

Japan, East Asian ancestry	*IL6* (*-572* G/C) rs1800796		PTB	51	71	Maternal	OR=2.35 (1.20-4.64)	Sugita, 2012 [169]
	FcαR (+56 T/C) rs3816051						OR=2.19 (1.19-4.04)	
USA, multiple genetic ancestry:								Jones, 2012 [148]
"Non-Hispanic white"	*IL1RN* rs2234663	*IL1RN*2*	SPTB	93	356	Maternal	OR=2.0 (1.2-3.3)	
						Infant	OR=1.7 (1.0-2.8)	
	MMP9 (-1562 C/T) rs3918242	T allele				Maternal	OR=1.7 (1.0-2.8)	
African ancestry	*TNFRSF1B* (M196R) rs1061622	G allele		38	239	Maternal	OR=2.3 (1.1-4.6)	
Poland, European ancestry	*TNFA* (-238 G/A) rs361525	GA	PTB	150		Maternal	OR=9.43 for 28-32 gw | OR=2.62 for 32-36 gw	Drews-Pi-asecka, 2014 [184]
		A allele					OR=11.16 (*p*=.00004) for 28-32 gw | OR=2.72 (*p*=.022) for 32-36 gw	
	TNFA haplotype block: *TNFA* (-238 G/A)/(-308G/A)/(-376G/A) rs361525/rs1800629/rs1800750	GG/GG/GG					Not given (*p*=.037)	
		GA/GG/GG					Not given (*p*=.035)	

India, East Asian ancestry	*MTHFR* (677C/T) rs1801133		PTB	209	194	Maternal	OR=2.87 (1.70-4.84)	Tiwari, 2015 [174]
China, East Asian ancestry	*MTHFR* (677C/T) rs1801133	TT	PTB	108	108	Maternal	OR=3.08 (1.47-6.45)	Nan, 2015 [175]
		T allele					OR=1.85 (1.27-2.72)	
USA, African ancestry	*PRKCA* rs7225452	A allele	SPTB	77	756	Maternal	OR=1.92	Frey, 2016 [179]
		Additive model					OR=1.92	
	PRKCA rs4486944	C allele					OR=1.79	
		Additive model					OR=1.79	
	PRKCA rs6504424	A allele					OR=1.82	
		Additive model					OR=1.84	
	PRKCA rs16960070	Allele A					OR=2.45 (p=6.3^10^{-4})	
		Dominant model					OR=2.84 (p=3.2^10^{-4})	
	TIMP2 rs2277698	Recessive model					OR=8.7 (p=4.7^10^{-4})	

	IL16 rs7171517	Dominant model					OR=2.31 (p=5.8^10^{-4})	
India, East Asian ancestry	*IL1A* (-889 C/T) rs1800587	CT	PTB	559	559	Maternal	OR=1.7 (1.24-2-46)	Pandey, 2016 [156]
	IL1A (+4845 C/T) rs17561	CT					OR=1.5 (1.1-1.9)	
	IL1A haplotype block: IL1A (-889 C/T)/(+4845 C/T) rs1800587/rs17561	CT					OR=1.36 (1.14-1.62)	
	IL1B haplotype block:	CT					OR=1.29 (1.01-1.64)	
	IL1B (-511 C/T)/(-31T/C) rs16944/rs1143627	TT					OR=1.20 (1.00-1.43)	
Brazil, multiple genetic ancestry	*TLR2 rs4696480*	A allele	PPROM	63	201	Maternal	OR=2.93 (1.42-6.06)	Ramos, 2016 [139]
	IL10 (-1082A/G) rs1800896	G allele					OR=2.03 (1.06-3.92)	
	IL10 (-592 C/A) rs1800872	C allele	PTB	92	199	Infant	Note given p=.01	
	IL10 (-819C/T) rs1800871	C allele					Not given p=.026	
China, East Asian ancestry	*IL6* (-572 C/G) rs1800796	CG, GG	SPTB	569	673	Infants	OR=1.35 (1.01-1.80)	Yang, 2017 [160]
Brazil, European ancestry	*VDR* (*Fok1* T/C) rs2228570	T allele	SPTB	104	85	Maternal	OR=2.36 (p=.00014)	Javorski, 2018 [185]
		TT					OR=7.49 (2.29-3.14)	
	VDR (*Cdx2* G/A) rs11568820	A allele					OR=1.55 (p=.00466)	

		A/A					OR=3.62 (1.41-0.9.27)	
India, Multiple genetic ancestry	*MMP9* (-1562 C/T) rs3918242	CT		255	255	Maternal	OR=1.44 (0.97-2.14)	Pandey, 2020 [166]
		TT					OR=2.6 (1.46-4.69)	
Korea, East Asian ancestry	*IL10* (-592 C/A) rs1800872		PTB	115	147	Maternal	OR=1.71 (1.01-2.90)	Han, 2020 [164]
	IL10 haplotype block*:* *IL10 rs1800796/rs1800872/rs1800630*	CC/CA/CA					OR=7.43 (2.06-26.84)	
Romania, European ancestry	*FGF1* rs34003	C/C	SPTB	79	81	Infant	OR=2.59 (1.02-6.58)	Preda, 2020 [170]

Gw – gestational week; IPTB – idiopathic preterm birth; N – number of subjects; OR – odds ratio; PPROM – preterm premature rupture of membranes; PTB – preterm birth; SPTB – spontaneous preterm birth.

† OR = odds ratio for comparing the odds of CA-repeat for the specified allele compared to all others combined.

†† RR = relative risk when compared with homozygosity of the ancient allele (GG genotype) or the heterozygosity (GA genotype).

1.3.2.4 Women's ancestry underlying inflammatory response in PTB

Human genetic ancestry is fundamental to understanding diseases since differences emerge during humans' adaptation to various environments and contributions from past interbreeding [186–188]. Consequently, human ancestry is fundamental to understand PTB [189] and categorizing human sub-populations is critical to investigate genetic correlations. Herein, the terms Black and White, among others, were replaced, whenever possible, by African, European, American, and East Asian ancestries, according to Figure 1.7 (based on Li and collaborators[189]). Notwithstanding, interbreeding among different ancestries is very common, making any classification inevitably incomplete.

Regarding PTB, epidemiology studies have shown that its rates vary according to women's genetic ancestry [135,190], further indicating the role of maternal genetics [113]. However, the multifactorial traits of PTB and the genetic heterogeneity linked to multiple ethnic classifications, such as "African American", make it difficult to identify robust causal markers for PTB [113,135]. Moreover, there is an ongoing debate on the underlying reasons for the ancestry disparity in PTB. Several studies suggest socioeconomic conditions as the primary reason for variances in PTB rate, while others point to genetic ancestry [98,115,191]. A 2019 study, including African and European ancestries, found that 38% of PTB and 31% of very PTB (<32 weeks of gestation) disparities are due to maternal education, marital status, and source of delivery payment [192]. However, this study addressed disparities in PTB without considering biomarkers of genetic ancestry. In opposition, an earlier study by Zachary and colleagues (2007) indicates a PTB overrepresentation in African ancestry independently of maternal, medical, and socioeconomic factors [193]. More recently, Martin and colleagues (2020) have evidenced these disparities in PTB by the higher rate of this condition in what they referred to as non-Hispanic Black women at 14.39%, compared with the 9.26% in non-Hispanic white women, even after adjusting for maternal socioeconomic status and education [194]. This evidence is also corroborated by several other studies which reveal disparities in the inflammatory response and pregnancy outcome among women of different ancestries. In these studies, women of African ancestry are associated with a stronger inflammatory response [195,196], a markedly higher rate of PPROM, and histologic chorioamnionitis compared with women of European ancestry [197–199]. Also, Fortunato and colleagues (2004) report differences in the immune response of human fetal membranes exposed to bacterial endotoxin between African and European ancestry women, demonstrating differential expression of various mRNA associated with inflammation. The authors found that soluble forms of TNF-α receptors, which

act to bind free TNF-α and prevent its bioactivity by competitively inhibiting receptor binding, are significantly decreased in African ancestry women and unchanged in European descent. Moreover, membrane-bound TNF-α receptors were increased in African ancestry and decreased in European ancestry [200]. This data suggests a pro-inflammatory pattern that favors TNF-α biological activity among women of African ancestry. Another case-control study also found differences in predispositions for inflammatory responses between African and European ancestries. In this study, amniotic fluid samples were subjected to multiplex-based cytokine concentration measurements for IL-1β, IL-6, IL-8, IL-10, TNF-α, soluble TNF receptors, and corticotrophin-releasing hormone. Results show that IL-1β and TNF-α levels were higher in African ancestry cases, whereas IL-6 and IL-8 were elevated in European ancestry cases. Subsequent studies confirm these in vitro findings demonstrated a pronounced TNF-α response in African compared to European ancestry [196].

Candidate gene studies have also been undertaken to examine the genetic contribution to the observed disparity in PTB between women of African and European ancestries. Significant differences in allelic and genotypic frequencies of SNPs were noticed between cases and controls, and the frequencies in African and European ancestries have a 60-80% variance between them. Several single locus associations, haplotype frequencies, and *multiloci* associations in maternal and fetal DNA samples varied between the two genetic ancestries groups [116,128,147,149,155,180,193,201,202]. Finally, different genetic associations have also been found between clinical groups, even within the same geographic population [148,203–205].

In summary, several studies point out that PTB rate disparities are closely related to women's genetic background and variations in the inflammatory response, supporting the hypothesis of an involvement of inflammatory-related genes in PTB and reinforcing the importance of genetic-ancestry contribution in this condition.

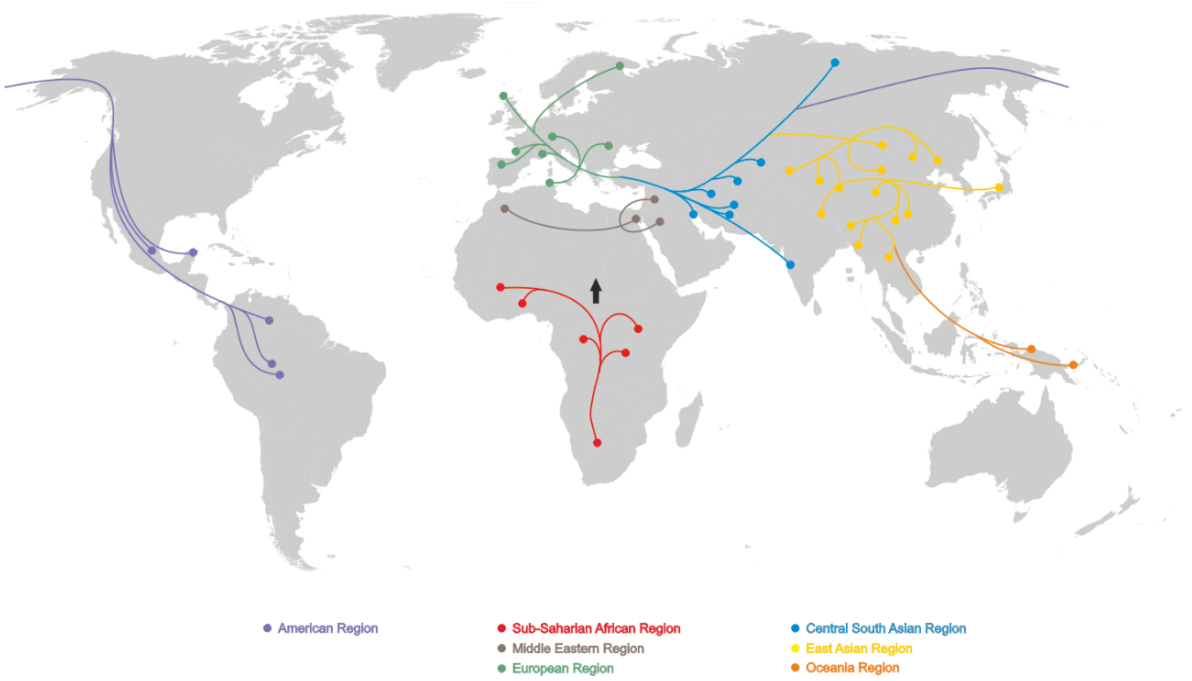

Figure 1.7. Human populations according to genetic ancestry (based on Li et al. [189]).

1.3.3 Autoimmune and inflammatory diseases related to PTB

Considering the birth process, it is not surprising that chronic inflammatory and complex immunologic disorders might be involved in the duration of pregnancy and PTB.

The prevalence of autoimmune conditions is almost twice as high in women [206], and endocrine transitions throughout their lives, such as pregnancy, can increase women's susceptibility [206,207]. Therefore, it is also expected that there is a correlation between autoimmune diseases and adverse pregnancy outcomes (APOs). The majority of the studies considered that autoimmune conditions represent a risk for the development of APOs, namely PTB [206,208]. For instance, several studies showed that the rate of PTB is higher in patients with rheumatic diseases. For example, pregnant women with systemic lupus erythematosus (SLE) have a PTB risk above the worldwide average prevalence (16-50%) [209]. Additionally, according to the literature, one-third of all SLE pregnancies are delivered preterm [210]. Also, juvenile idiopathic arthritis (JIA) presents an increased PTB risk according to population-based studies [210,211]. Rheumatoid arthritis (RA), another chronic inflammatory disease, has also been associated with higher odds of PTB [212–214], most of the cases being spontaneous (SPTB) [215]. The most recent review about autoimmune diseases and APOs is in line with previous

evidence, showing that SLE is a major risk for PTB. In this study, additional rheumatic diseases, such as RA or systemic sclerosis, were also significantly associated with PTB [206]. Other non-rheumatic immune diseases were also linked, such as type 1 diabetes mellitus, which was the most significant, increasing the chances of PTB more than fourfold [206]. Overall, it is considered that pregnant women with autoimmune diseases have a higher PTB risk, but the circumstances for this and other obstetric complications are not yet understood [212]. In fact, Scime and colleagues (2024) performed a large population-based study and found that APOs, namely SPTB, were modestly associated with elevated incidence of maternal autoimmune disease for up to 19 years following delivery [216]. Also, in 2024, a retrospective cohort study in Taiwan, involving a total of 1,385,979 births from 2004 to 2014 indicated autoimmune diseases as a risk factor for PTB [50]. This evidence suggests that the relation between autoimmune diseases and PTB may be bi-directional.

Regarding chronic inflammatory diseases, there is growing recognition that uncontrolled disease activity before conception and/or disease flares during pregnancy represent risks to mothers and infants [208,217]. Although these diseases share a perpetual dysfunction of pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6 [218–221], the mechanism underlying their association with SPTB remains unclear. One example of chronic inflammatory disease is inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC) [222]. In the time of parturition, the increased uterine contractility and cervical ripening and dilatation require a delicate interplay of inflammatory mediators, many of which are shared with the IBD inflammatory response [223]. Several studies have analyzed the relationship between IBD and APOs. Many of them reported an increased PTB risk in women with IBD [214,224–226], including big reviews [206,227].

Periodontitis (PD), another chronic inflammatory disease, has also been associated with APOs, such as hypertension, diabetes mellitus, and preeclampsia, among others [228]. Approximately 40% of pregnant women have some form of PD [229], and this condition has long been considered a risk factor for PTB [230]. As mentioned before, 30% to 50% of PTB are associated with intra-amniotic inflammation [82], many due to infection, with PD representing genuine oral infections [231]. However, the precise pathways facilitating inflammation propagation from the oral cavity to the placenta and contributing to PTB remain elusive, necessitating further research [231]. Interestingly, in women with some form of PD, pregnancy appears to worsen disease severity, suggesting that pregnancy may also influence PD status [232,233]. These findings suggest that the relationship between PD and PTB may be influenced by other

factors, such as genetic predispositions. Multiple studies try to identify the genetic variants related to PD risk, but the results are often inconclusive or contradictory. Therefore, the relationship between PD and PTB is also not yet completely understood. PD and its relationship with PTB will be further explored in section 1.4 (Relationship between PTB and PD).
In conclusion, as suggested by others [234,235], it is becoming clear that inflammation, regardless of its triggers, is one of the main PTB etiology.

1.3.3.1 Inflammatory genes associated with both PTB and inflammatory or autoimmune diseases

As mentioned previously, there is strong evidence implicating inflammatory-related genes in PTB. There is also a correlation between this condition and inflammatory and autoimmune diseases, although this is not fully understood. Both observations allow hypothesizing a genetic overlap explaining the occurrence of inflammatory and autoimmune diseases in women with PTB. Over the years, studies have identified genetic variants as risk factors for different inflammatory and autoimmune diseases, several of which are also linked to PTB. Table 1.2 presents examples of genetic variants in inflammatory-related genes simultaneously implicated in PTB and inflammatory or autoimmune diseases, analyzed alone or within haplogroups.

Table 1.2. Genetic variants related to PTB and inflammatory or autoimmune diseases.

Gene	Variant	Autoimmune rheumatic diseases			Chronic inflammatory diseases			PTB
		SLE	RA	Other	IBD	PD	Other	
IL1A	rs1800587	[236]			UC [237]	[238]		[156,157]
IL1B	rs16944	[239]	[240]		UC [237]		AR [241]	[156]
	rs1143627				[242]	[238]		[145,156]
IL1RN	rs2234663	[243–246]	[247–249]		[250] UC [251,252] CD [253]	[254,255]		[148,151,153–155]
IL6	rs1800795	[256,257]	[258,259]		CD [250,260]	[261][262][263]		[134,154]

IL10	rs1800896	[264]	JIA [265]	CD [266]			[139,182]
	rs1800872	[267]			[267,268]		[139,164,182]
TNF	rs1800629	[269]	DM [269]	[250]	[270]	AR [241]	[140–143,184]
	rs1799724	[269]	DM [269]				[145]
	rs361525					AR [241]	[184]
TLR4	rs4986790			[271,272] CD [250]	[273]		[137]
TLR10	rs4274855			CD [274]			[163]
MMP9	rs3918242	[275]			[276]		[148,166]

SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; PD, periodontitis; AR, allergic rhinitis; JIA, juvenile idiopathic arthritis; DM, adult dermatomyositis; PTB, preterm birth

As shown in Table 1.2, the cytokine genes, namely *IL1B*, *IL10* and *TNFA*, are most associated with a higher number of diseases. Regarding the specific genetic variants associated with PTB, the most frequently found were *TNFA* rs1800629 (in adult dermatomyositis (DM), IBD, PD, and allergic rhinitis (AR)), followed by *IL1B* rs16944 (in SLE, RA, UC, and AR), and the *IL1RN* VNTR rs2234663 (in SLE, RA, IBD, and PD). Table 1.2 also shows several other polymorphisms in genes involved in the inflammatory pathway of delivery, associated with both PTB and inflammatory/autoimmune diseases. Furthermore, genetic variants not yet linked to prematurity but found in these or other genes related to PTB have also been associated with the risk of inflammatory or autoimmune diseases. Examples include the genes *TLR4* [271,272], *TLR10* [274], IFN-γ protein-coding gene (*IFNG*) [277], *MMP2* [275,278], *MMP9* [279], TIMP metallopeptidase inhibitor (*TIMP*) 2, *TIMP2* [278,280], *VEGF* [281], and *AGTR2* [282].

1.3.4 Periodontitis (PD)

PD is the second most prevalent oral disease, representing over 1 billion cases in 2019. According to WHO, in 2022, severe PD has an estimated global prevalence of approximately 11%, but the prevalence in adults and elderly individuals is at a minimum of 16.9% and possibly up to

48.0% [283]. PD is a chronic inflammatory disease that affects the periodontum (i.e. the tissues supporting the teeth) and is the leading cause of tooth loss in adults [284,285]. Its principal characteristics encompass the loss of periodontal tissue support, demonstrated by clinical attachment loss (CAL), gingival bleeding, and radiographically by assessing alveolar bone loss and periodontal pockets [286]. The onset of PD results from a disruption of the homeostasis of inter-microbial and host-microbial interactions, leading to the continued accumulation of exogenous pathogens in gingival polymicrobial community biofilm. This in turn induces a persistent host immune response within the periodontum. This inflammatory process can be reversed when microbial biofilm is removed, and the inflammation is limited to the involvement of the gingival epithelium and connective tissues. However, if pathogenic biofilm accumulation persists, the inflammatory process increases, involving more profound periodontal tissue destruction and alveolar bone loss, at which point the disease progresses from gingivitis to periodontitis (Figure 1.8) [287–289]. Interestingly, the host response is the main responsible for periodontum tissue destruction [290]. The disease's risk and progression have multifactorial contributions, depending on individual habits (e.g. smoking and oral hygiene), environmental conditions (e.g. socioeconomic status and exposure to pathogens), health conditions (e.g. diabetes or stress and anxiety), and genetic background [285]. Genetic factors are highly significant since they account for up to one-third of the variance in PD within the population [291]. Animal models replicated these findings, underscoring the importance of host susceptibility in PD pathogenesis [292]. To date, more than 60 genes have been suggested to be associated with this disease [293], especially genes involved in innate and adaptive immune responses and genes related to inflammatory processes, such as cytokines, chemokines, specific receptors, and metabolic regulators [285], as further discussed in the following sub-subsection (1.3.4.1 Cytokines in PD).

Besides its high prevalence and tooth loss, this complex disease has other traits that turn it into a public health problem, such as the negative impacts on aesthetics and chewing ability, contributing to social inequality and decreasing quality of life [286]. In agreement, Patel and colleagues (2021) indicate that according to the 2019 Global Burden of Disease study, severe PD, edentulousness (complete tooth loos), and untreated dental caries cause 23.1 million disability-

adjusted life-years (DALYs[1]) worldwide [294]. Additionally, epidemiological, clinical interventional and experimental studies have demonstrated that PD adversely impacts systemic health [294,295]. A bidirectional relationship between PD and type 2 diabetes is established [296], and multiple review articles showed a possible association with other chronic diseases, such as respiratory diseases, including chronic obstructive pulmonary disease, cardiovascular disease, RA, Alzheimer's disease, and certain cancers, among other [297–299]. Lastly, PD is responsible for an important economic burden. James and colleagues (2018) indicated that the Global Burden of Disease Study 2017 estimated the global direct cost of PD at $44.28 billion and the indirect cost at $20.50 billion [300]. A more recent report estimated the economic burden of PD to be roughly $154 billion in the US and €159 billion in Europe in 2018 [301].

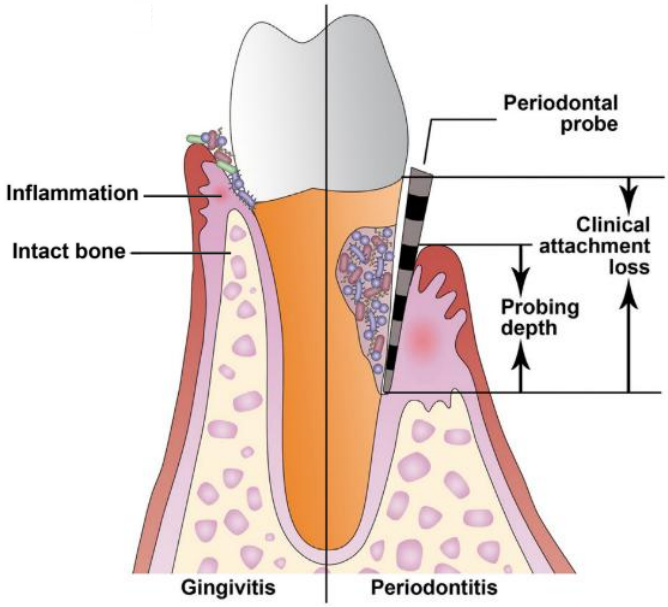

Figure 1.8. Representation of periodontal disease, which includes gingivitis and PD. Gingivitis is the presence of gingival inflammation without loss of connective tissue attachment. PD is the presence of gingival inflammation at sites where there has been apical migration of the epithelial attachment onto the root surfaces accompanied by the loss of connective tissue and alveolar bone. Clinical attachment loss is measured with a periodontal probe and is the distance from the base of the periodontal pocket to the cementoenamel junction. Probing depth is defined as the distance between the bottom of the periodontal pocket and the gingival margin. (Adapted from Ren and Du, 2017 [302]).

[1] "One DALY represents the loss of the equivalent of one year of full health. DALYs for a disease or health condition are the sum of the years of life lost to due to premature mortality (YLLs) and the years lived with a disability (YLDs) due to prevalent cases of the disease or health condition in a population" (WHO).

1.3.4.1 Cytokines in PD

Cytokines are produced by both immune and non-immune cells and play a fundamental role in regulating tissue homeostasis and activating the immune system, as previously demonstrated in pregnancy. Additionally, cytokines have a pleiotropy action, indicated for the first time by Dinarello and colleagues (2009) [303], allowing them to trigger multiple physiological responses [304]. As a result, a single cytokine can interact with different cell types, both immune and non-immune, and initiate a variety of biological functions [305]. Therefore, cytokine levels are recognized as potential modulators of immune cell activity, and multiple disorders were linked to changes in their production [305,306]. In oral mucosa, a balance between pro- and anti-inflammatory cytokines is important to maintain healthy tissue homeostasis [305]. Accordingly, high levels of pro-inflammatory cytokines were detected in gingival tissues and gingival fluid of patients diagnosed with PD [307–312]. Additionally, cytokines, namely IL-1 and TNF-α, can also control bone resorption in the periodontal area [309,313–317], and upregulate the expression of MMPs in periodontal tissues, which also contribute to connective tissue destruction [318,319]. It has already been confirmed that some cytokines play a crucial role in the pathogenesis of PD and, over the years, they have even been proposed as biomarkers for the diagnosis and monitoring of the disease, as described by Neurath and colleagues (2024) in the latest review about the subject (Figure 1.9) [305].

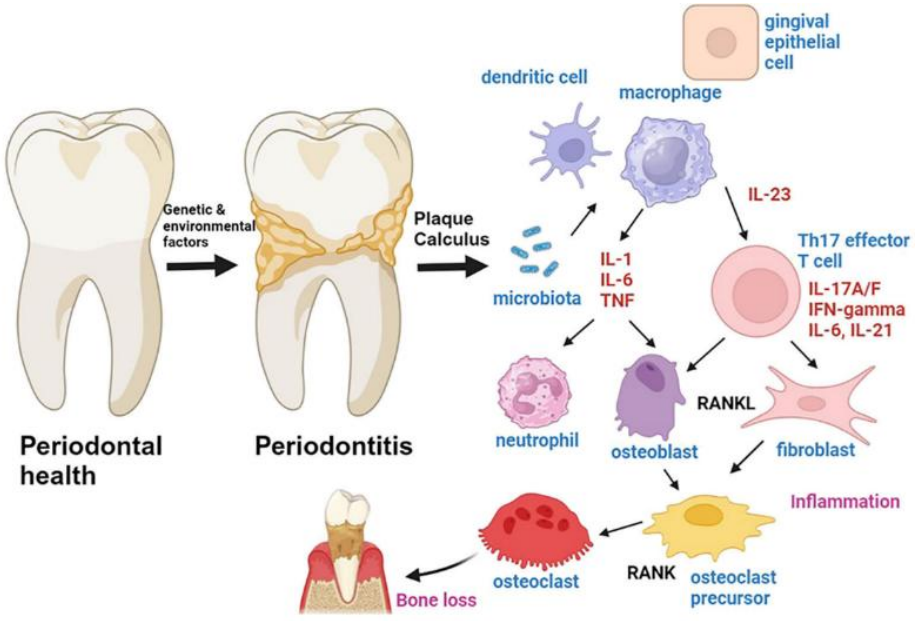

Figure 1.9. The role of cytokines in the PD pathogenesis. Genetic and environmental variables have been recognized as risk factors for PD development. This chronic inflammatory disease encompasses the activation of the immune cells by microbiota, resulting in the synthesis of pro-inflammatory cytokines, including IL-1, IL-6, TNF-α, and IL-23. Macrophages synthesize IL-1, IL-6, and TNF-α which promote inflammation though the induction of

chemotaxis and activation of neutrophils, as well as the activation of T cells and fibroblasts. IL-23, synthesized by gingival epithelial cells, and IL-1 stimulate the activation of T cells, which can produce IL-17, IFN-γ, IL-21, and IL-6. T cells induce the expression of the receptor activator of NF-κB ligand (RANKL) in osteoblasts and fibroblasts, thereby promoting osteoclastogenesis and bone resorption in PD (Neurath et. al, 2024 [305]).

In relation to the cytokines protein-coding genes, the most studied variants in PD are *IL1A* rs1800587, *IL1B* rs1143634, *IL1B* rs16944, and *TNFA* rs1800629, although the results among studies are not consistent [238,320–324]. The IL-1α, IL-1β, and IL-1RA protein-coding genes are located in a single cluster on the human chromosome two [325]. *IL1A* rs1800587 is a SNP located at position -889 (C>T) upstream of translation start (+1), which is within a transcriptional regulatory region. The TT genotype was reported to significantly increase promoter activity, producing increased *IL1A* mRNA and IL-1α levels compared with those of CC genotype [326]. Several candidate gene studies associated this *IL1A* variant with PD [255,327–329]. Notwithstanding, similar studies on the same variant reported no association with the disease [255,262,327,330,331]. Previous studies on both *IL1B* variants had the same inconsistency. *IL1B* rs1143634, also known as +3954C/T, is a silent coding sequence polymorphism located in exon 5. This silent SNP increases IL-1β serum concentration [332,333] and was associated with an increased risk of PD in some case-control studies [262,329,334], while others failed to find any correlation [255,327,335]. A recent study by Dahash and colleagues (2023) suggested that this variant could be a protective factor for PD in the Iraqi population [336]. Even the meta-analysis carried out over the years on the correlation between *IL1B* rs1143634 and the risk of PD has obtained contradictory results [238,337,338]. It is important to note that different authors have already suggested that geographical and ethnic factors may play an important role in these results since the prevalence of specific polymorphisms varies greatly depending on the population studied [238,255]. The *IL1B* rs16944, also known as -511C/T, is a SNP polymorphism within the promotor region, which also increases the IL-1β levels [339,340]. Like the other *IL1* variants described above, some studies have found a correlation between *IL1B* rs16944 and the risk for PD [328,329], but others suggested no association [262,334,341,342]. Once again, the correlation between this variant and PD seems to depend on the population being studied. For example, a study with a Brazilian population showed that the *IL1B* rs16944 T allele was positively associated with PD in what they call "black and mulatto groups" but not in the "caucasian group" [255]. The latest meta-analysis on the association between cytokine polymorphisms and PD pathogenesis by Liu and colleagues (2022), reported that *IL1A* rs1800587 T allele, the minor allele, has a favorable relationship in preventing PD. In contrast, both *IL1B*

variants, rs1143634 and rs16944, had a strong relationship with PD risk, highlighting *IL1B* as a candidate biomarker for the development and progression of the disease. However, the authors indicated that there was major asymmetry and publication bias in the results for all these polymorphisms [323]. Finally, a GWAS study showed no significant genome-wide influence of *IL1A* rs1800587 or *IL1B* rs1143634 in PD (*IL1B* rs16944 was not included in the study) [343]. Regarding TNF-α, its coding gene is located on chromosome 6 (6p21.3) [344], and the rs1800629 is one of the most studied polymorphisms in its promotor region. *TNFA* rs1800629 is a SNP, a substitution from G to A at position 308, that leads to increased transcriptional activity and consequently higher circulating TNF-α levels, as demonstrated in both in vitro and in vivo studies [345–348]. The reported results about *TNF-α* rs1800629 also do not allow the understanding of its influence on PD susceptibility unequivocally. Majumder and colleagues (2018) suggested that this variant is associated with PD in an Indian population [349]. A previous study had also positive results [350]. Conversely, different studies found no correlation between *TNFA* rs1800629 and PD susceptibility [262,351,352]. The latest case-control study accomplished with Iranian individuals also found no significant association between this variant and PD [353]. Different reviews or meta-analyses were performed with contradictory results too, even the last two published, which had some disagreements. Both correlate the *TNFA* rs1800629 polymorphism with a predisposition to PD in overall pooled analyses [320,354]. However, Li and colleagues (2020) found similar positive findings for rs1800629 polymorphism in the European ancestry population (recessive comparison) [320], while Shi and colleagues (2021) did not find any significant risk among European ancestry population for all genetic models applied [354]. Nevertheless, Li and coauthors mentioned that most eligible studies were conducted on Asians, indicating that future studies must address other populations.

Overall, none of the four polymorphisms most studied in PD has been associated with certainty in the general population, and this lack of consistency can be partly explained by ethnic heterogeneity and geographical distribution of specific gene variants [238]. Another factor that may contribute to these inconsistencies is sex dimorphism in PD. Consistent epidemiological data have highlighted higher PD prevalence in men as compared to women. This sex dimorphism is not entirely understood but must be related to the differential immune response between men and women [355]. Sex steroids [356] and X-linked genes that regulate immune function, including cytokine receptors, have been proposed to be the primary mechanisms that alter immune function [357]; together, they rationalize males' higher infection rates and

females' higher autoimmune disease prevalence [355,358]. Different studies have recommended that research into oral health and PD should be conducted with an emphasis on sex to avoid sex bias, with special consideration given to age, ancestry, and socioeconomic status. Accordingly, a meta-research study from 2022 highlighted that clinical trial reports often neglected sex, potentially hindering the comprehension of sex differences in periodontal-related studies [359].

1.4 Relationship between PD and PTB

This section delves into the current understanding of the relationship between PD and PTB, highlighting existing uncertainties and knowledge gaps while exploring the hypothesis of a bidirectional relationship. The association between PD and APOs, namely PTB, has been the subject of many clinical trials and epidemiological studies over the past two decades but has recently earned significant attention. However, the findings regarding this association remain inconsistent, and the underlying mechanisms are not yet fully understood. Overall, the factors contributing to these variations across studies include significant differences in the included populations, inclusion criteria, and sample selection [231,360–362]. Other factors can also further influence this inconsistency, such as the diverse definitions of periodontal disease and the varying criteria for its diagnosis [360–363].

1.4.1 Epidemiology results

Several epidemiological studies conducted over the years, including cross-sectional, case-control, and cohort studies, examined the link between PD and PTB. Most reported a correlation between these two conditions, the first of which was carried out in 1996 [364]. Over the years, others have reported the same positive association between PD and the incidence of PTB [365–375]. More recently, a 2022 cohort study, including 1,757,774 pregnant women from Taiwan, showed that increased PD severity was related to a higher risk of PTB [376]. In Africa, a Sudanese case-control study suggested that women with PD had a 2-fold higher chance of delivering preterm babies than women with no PD [377]. Finally, a 2024 observational cross-sectional study with Pakistani postpartum women reported that, although the extent of PD had no association with PTB (87.5% of the women had generalized PD), the severity index of PD was

significantly different between PTB and full-term birth groups [361]. Notwithstanding, Martinez-Martinez and colleagues (2016) proposed that PTB is a multifactorial condition, indicating that PD alone cannot trigger PTB [378]. Several other studies have found no association between these two conditions [169,379–386]. Furthermore, clinical investigations examining the impact of periodontal therapy on pregnancy outcomes have also produced mixed results. Some studies have suggested that periodontal treatment may reduce the risk of PTB in pregnant women with PD [387–390]. However, other studies reported the opposite, demonstrating that non-surgical periodontal treatment in patients with PD did not significantly reduce the risk of PTB [391–396]. As expected, literature and systematic reviews published over the years have also yielded inconsistent results. Some studies have demonstrated an association between PD in pregnant women and the incidence of PTB [397–399]; others have found a positive but weak association [400–402]. Still, others have reported no association between these two conditions [360,403,404]. Regarding the effect of PD treatment on reducing the risk of PTB, the trend is similar, with some reviews showing a decrease in PTB risk [398,405,406], while others have reported that this relationship is still uncertain and requires future studies [397,403,404,407,408]. Despite the numerous reviews on the impact of PD treatment in reducing the risk of PTB, there is a lack of recent original research to substantiate this discussion further. Recent analyses have already drawn attention to possible conflicts in the results. Khan and colleagues (2023) evaluated 17 systematic reviews and concluded that the evidence on this topic is inconclusive due to insufficient clinical trials, which leads to a high possibility of bias in the research. This limitation makes it challenging to evaluate specific aspects, such as the disease's diagnosis, extension, and severity [409]. Furthermore, one of the most recent umbrella reviews on the topic (2024) also reported that it is necessary to be cautious when interpreting the results of the systematic reviews since approximately 96.75% of the included primary studies overlap in multiple reviews, which could lead to repetitions in the evaluation of the same data, distorting the perception of the amount of research carried out in this area [406]. As described above, there is a large scientific consensus recommending caution when analyzing the data presented due to the significant bias inherent in these studies. In conclusion, as reported in recent studies [361], the association between PD and PTB remains unclear due to inconsistencies in the findings.

1.4.2 Biological hypothesis

Even though the relationship and mechanisms underlying PD and APOs are still not fully understood, the two proposed pathogenic mechanisms according to which PD may lead to PTB are (Figure 1.10) [302,360,403]: (i) the translocation of periodontal bacteria (or bacterial products) by the hematogenous route to the fetoplacental unit causing a metastatic infection, and (ii) the presence and intervention of inflammatory mediators, produced locally in periodontal tissues, that increase the systemic inflammatory response, which can lead to a disruption of the placental barrier homeostasis, leading to the establishment of infections in the fetal-placental unit.

The most plausible mechanism is the direct route, where pathogenic bacteria from periodontal tissues may spread to the placenta and enter the amniotic fluid and fetal circulation, activating the inflammatory signaling pathways that induce PTB [360,361,410]. Although PD has been pointed out as a risk factor for PTB since 1996 [364], this hypothesis only emerged in 2006, after Han and colleagues' study proved for the first time an oral-uterine translocation, suggesting that the oral cavity was the source of the *Bergeyella* strain found in the intrauterine infection of a PTB patient [411]. The direct route hypothesis is supported by studies that detected other periodontal bacteria, such as *Porphyromonas gingivalis* [412–416] *Fusobacterium nucleatum* (*F. nucleatum*) [413,417–419], and *Prevotella intermedia* [412,419] in PTB cases or pregnant women with a diagnosis of threatened premature labor, but not in term births. Despite these findings, there is limited evidence to support this hypothesis, first because, despite these single species findings, data on bacterial co-aggregation and biofilm formation capability in the amniotic cavity are scarce [403]; second, some studies failed to detect periodontal bacteria in the amniotic fluid of women with PD who delivered PTB, despite these pathogens being found in dental plaque [398,420,421]. On the other hand, some periodontal pathogens have also been found in the fetoplacental unit of women with normal pregnancies [360,419]. In fact, some authors proposed the existence of a unique microbiota in the fetoplacental unit, which could be most similar to the human oral microbiome, even in clinically healthy gestations [422,423]. Finally, while the direct route is the most widely accepted model for PD to induce PTB, it does not account for most PTB cases. About 60 to 70% of SPTB cases do not present PPROM [424–426] and, as previously described, sterile intra-amniotic inflammation is more common than intra-amniotic infection in women in preterm labor with intact membranes [36].

The same inconsistency occurs with the indirect hypothesis [401], which suggests that inflammatory mediators in the infected periodontal tissues, such as proinflammatory cytokines, are

released into the systemic circulation, increasing C-reactive protein levels [427,428], through an acute response in the liver of pregnant women, which can lead to APOs, namely PTB [429–431]. In a 2016 observational case-control study, Mesa and colleagues (2016) found no plasma cytokine profiles suggesting a systemic inflammatory response in pregnant women with PTB [432]. Another study indicated no direct link between increased periodontal bacteria and cytokine levels or PTB [433]. On the other hand, a 2024 review and meta-analysis showed that inflammatory mediators related to PD have been found in maternal serum, umbilical cord blood, and amniotic fluid of pregnant women who have or have not experienced APOs [360]. In short, despite the proposed hypothesis, there is no robust evidence to prove that PD can cause PTB.

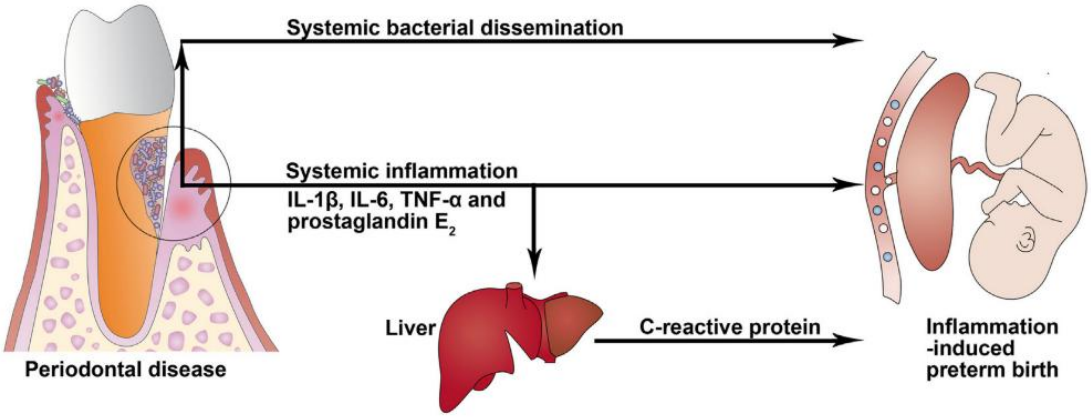

Figure 1.10. Biological hypothesis relating PD and PTB. In PD, gingival ulceration in the periodontal pockets allow for escaping and systemic bacterial dissemination, and locally produced proinflammatory mediators can enter the systemic circulation and cause an acute-phase response in the liver, such as C-reactive protein, which could then affect the fetoplacental unit (Ren et. al, 2017 [302]).

1.4.3 A bidirectional hypothesis between PD and PTB

According to the latest analysis, 50 to 70% of pregnant women develop gingivitis [406]. However, as mentioned before, the majority of studies reported that pregnancy does not cause gingivitis, only increases a pre-existing one, and that this condition resolves following parturition [434]. Nevertheless, several findings pointed out the opposite, supporting a bidirectional relationship between PTB and PD.

There is a widespread consensus that levels of sex steroid hormones in saliva increase during pregnancy [435], and this is related to more gingival inflammation, deeper probe depths and more bleeding in pregnant women compared to the general population [399,436,437]. In fact,

modest increases in gingival inflammation are observed in non-pregnant women undergoing estrogen/progesterone fluctuations associated with the menstrual cycle [438,439]. Moreover, receptors for these hormones were identified in various periodontal cell subsets [440,441], corroborating the hypothesis that periodontal tissues are a target [403]. One of the reasons for the link between an increase in the sex steroid hormones and gingivitis is related to the ability of these hormones to change the subgingival microbiota composition, enhancing periodontal pathogenic bacterium, as demonstrated since the early 80s [442–446]. Although some studies have reported no differences in the microbiome corresponding to hormonal changes [421,447], studies using DNA probes corroborate that pregnant women harbor a diverse array of pathogens that have the potential to cause gingivitis, and even PD [448,449]**.** Yokoyama and colleagues (2005) also demonstrated that female sex hormones increase the production of proinflammatory cytokines, namely VEGF, IL-6, and IL-8, by human gingival fibroblasts [442]. Other studies support the idea that hormonal changes during pregnancy induce the production and release of proinflammatory cytokines, particularly in the early stages [450]. Additionally, different reviews documented that sex hormones reduce periodontal tissue resistance to bacterial inflammation and impair repair capabilities, enhancing the inflammatory response [399,401,439,451]. Progesterone increases the synthesis of prostaglandins, particularly PGE2, which increases vascular capillarity and permeability [452–454]. It also influences connective tissue, affecting migratory cells, fibroblasts, and extracellular matrix [233]. Progesterone also increases the production of VEGF in human gingival fibroblasts [442]. Additionally, it promotes gingival capillary dilation and increases permeability by stimulating endothelial cells. This effect is mediated by inhibiting cellular antioxidants and the subsequent increase in oxidative stress [454,455]. The changes in vascular responses and connective tissue turnover in the periodontium indirectly contribute to the increased gingival inflammation [456]. Together, these factors allow the increasing of periodontal pathogenic bacteria diversity and amount, making periodontal tissue more susceptible to inflammation or aggravation of original inflammation [399,403]. Other factors, like vomiting during early pregnancy [457], changes in dietary habits, and late-pregnancy gastric reflux, which increase the acidity of saliva, are also believed to be associated with susceptibility to oral diseases, including PD, once again suggesting the possibility of a bi-directional relationship between PD and PTB [404].

Overall, there is evidence linking pregnancy with both worsening of preexisting gingival inflammation and further predisposition to new formation of gingivitis or PD, probably through

changes in periodontal microbiota and the subsequent increased and potentially dysregulated maternal inflammatory response [458].

Based on the findings explored in this subsection, a 2013 review by Armitage and colleagues suggested, for the first time, a bidirectional relationship between PD and PTB. The authors argued that, contrary to the well-established and universally accepted by the scientific community that during pregnancy, there is an increase in gingival inflammation, the effect of PD on PTB is less clear since strong biological arguments can be put forward in support of a causal link between PD and APOs, but biological plausibility by itself is not proof of causation [451]. The authors also report that there are no epidemiological studies on the putative relationship between PD and APOs including the variable of inflammation-associated gene polymorphisms, suggesting that there are probably genetic reasons why different pregnant women respond differently to similar inflammatory/infectious burdens caused by PD. Two earlier publications, an editorial [458] and a review study [400], both from 2007, already highlighted the possibility of some underlying mechanism causing both PD and APOs, such as an individual's genetic susceptibility to develop a hyperinflammatory response in the context of a bacterial challenge. However, it was only a hypothesis, not based on genetic findings. Ren and colleagues (2017), in a review of the role of maternal PD in PTB, also reported that one of the reasons for the different results in the epidemiological studies could be that the risk factors of PTB are similar to the risk factors of PD, potentially confounding the two [302]. A more recent review also considers the bidirectional relationship between these two conditions, indicating a weak but independent association between APOs and PD. The authors focused this bidirectional association on three possible mechanisms discussed above: inflammatory reaction, oral microorganisms, and immune response [401].

Evidence about possible genetic predisposition that could be related to both PD and PTB remains almost non-existent. While several previous studies explored genetic predispositions for PTB and PD separately, the only case-control study that investigated the link between the two conditions within the same population was conducted by Jeffcoat and colleagues in 2014 [459]. It was an intervention study designed to answer three questions:

(i) Is clinical evidence of periodontal treatment failure associated with SPTB?
(ii) Are specific genetic SNP associated with failure of periodontal treatment in pregnant women?
(iii) Are any of these same SNP associated with SPTB?

The study included 152 pregnant women at high risk of PTB who identified themselves as having African ancestry. All women had PD and received treatment. Several maternal tag SNP

were chosen from genes regulating the inflammatory response, from which a 1536-SNP custom chip panel was designed. The results revealed a significant relation between a specific polymorphism of the prostaglandin E receptor 3 (*PTGER3*) gene, rs2817864, and both PD treatment failure and SPTB [459]. However, this study does not appear to have performed a multiple testing correction for the results of the genotypes analyzed. The authors also highlighted that although this is a significant correlation, these results cannot be interpreted as indicating a genetic susceptibility to PD or SPTB [459]. After that, as discussed earlier in this thesis, Strauss and colleagues (2018) suggested an overlapping genetic substrate that could explain the occurrence of PD and PTB. In this clinical opinion on the advances toward the discovery of SPTB genetic predisposition, the authors based their hypothesis on the identification of two genes previously associated with PTB in a GWAS study [132] (*DEFB1* and *MBL2*), which has also been linked to PD in early research [124]. Recently, Tang and Chen (2024) conducted the first Mendelian randomization study, using a GWAS database comprising individuals of European ancestry, to explore the causal relationship between PD and APOs, and vice versa. However, the results do not support the influence of PD in APOs, nor vice versa. Notwithstanding, the authors highlighted the need for caution in causal inference due to inconsistencies in analytical methods arising from confounding factors, sample size, data quality, and statistical techniques [404].

1.5 Final considerations

This chapter is based on a review article we previously published, titled "Inflammatory factors, genetic variants, and predisposition for preterm birth" (DOI: 10.1111/cge.14001). The content of the article has been expanded, updated, and some sections were removed or added to reflect the current state of research on the subject.

The PTB section demonstrates the growing awareness of the connection between increased PTB risk and the disruption of inflammatory processes. Furthermore, numerous studies have highlighted common genomic features that intersect these processes. However, even though several data suggest a genetic influence on the gestational duration, the causality between a genetic profile and the risk for developing PTB has not been found. Also, several inflammatory and autoimmune diseases are considered risk factors for PTB. It is interesting to notice that many genes associated with the risk of developing some of these pathologies are also involved

in higher odds of PTB. The first attempt to correlate the PTB etiology with other diseases based on genetic factors was made by Strauss *and colleagues* in 2018, where it was proposed a common genetic substrate underlying PTB, IBD, and PD [124]. The study indicates that rare mutations or damaging missense variants in innate immunity genes play an essential role in PPROM risk, leading to PTB. This proposal was made based on the link of two genes, *DEFB1* and *MBL1*, in these three conditions. The data gathered from several candidate gene studies presented in this chapter significantly links thirty-two genes and PTB risk. Fourteen of these genes are also associated with at least one autoimmune or inflammatory disease – *TNFA* is the gene most often associated with PTB. Additionally, the *TNFA* polymorphism rs1800629, consistently linked to an increased PTB risk, is also associated with five autoimmune or inflammatory diseases.

Regarding the relationship between PTB and PD, current evidence remains scarce. Despite the large number of review and meta-analysis articles published recently, few new original studies have been conducted, which biases the results instead of providing further clarification on the topic. Nevertheless, the evidence explored in section 1.4 supports the hypothesis of a bidirectional relationship between PTB and PD. Individual factors may contribute to inconsistencies across different studies, and it is biologically and biochemically plausible that some of these factors are variants in inflammatory-related genes, which could predispose individuals to inflammatory dysregulation or hyperreaction. However, further research is needed to confirm this hypothesis.

In conclusion, additional studies are needed to reveal how specific genetic variants influence PTB risk and how they may contribute to the association between PTB and inflammatory processes and diseases, including PD. The data collected in this chapter supports the hypothesis of a common genetic background underlying PTB and pathologies in which the inflammatory system is compromised.

2

AIMS OF THE PROJECT

This thesis hypothesizes that both SPTB and PD are consequences of genetic predisposition for the development of inflammatory processes or a hyperinflammatory response. PD was chosen as an example of a chronic inflammatory disease that has already been related to PTB, although without conclusive findings. Furthermore, PD is also considered a public health challenge, and its diagnosis is the easiest to get among the more prevalent chronic inflammatory diseases. The main goal of this thesis is to find common genetic variants underlying SPTB and PD occurrence.

The specific aims of this thesis are:

1. to develop a screening questionnaire for the studied population to assess socio-demographic information, lifestyle habits, and health history.
2. to conduct a bibliographic review to identify candidate genes and polymorphisms previously associated with SPTB and inflammatory diseases, particularly PD.
3. to apply the questionnaire, perform a periodontal diagnosis (crucial for candidate selection and group allocation: case vs. control), and collect the biological samples.
4. to extract the DNA and genotype of the selected candidate gene variants.
5. to collect and analyze data statistically to identify polymorphisms associated with SPTB, PD, and both conditions.

To the best of our knowledge, this is the first study to simultaneously investigate multiple polymorphisms in inflammation-related genes in women with SPTB, PD or both. As both conditions have a polygenic basis, involving rare mutations or potentially deleterious genetic variants in multiple genes, the identification of new genetic variants associated with both is

crucial. A better understanding of these conditions will help the development of tools aiming to reduce their prevalence. This study also allows the first characterization of the genetic variants in inflammatory-related genes in a small cohort of the Portuguese population.

3

INFLAMMATORY-RELATED GENES ASSOCIATED TO PTB IN A PORTUGUESE POPULATION: A PILOT STUDY

Part of the content of this chapter was presented in the following conference abstract:

Couceiro J, Grosso AR, Baptista PV, Mendes JJ, Fernandes AR, Quintas A. The genetic susceptibility linking preterm birth and periodontal disease – a review. Annals of Medicine. 2021 53:sup1, S21-S22. doi: 10.1080/07853890.2021.1896890

This abstract was published in the proceedings of the Fourth International Congress of CiiEM: Health, Well-Being and Ageing in the 21st Century, held in Egas Moniz School of Health and Science in 2021.

3.1 Introduction

This chapter presents the general lines of the study's methodological framework, detailing the development of the screening questionnaire to be applied to the participants, the identification and selection of the candidate genes, and the preliminary pilot study on genetic variants associated with SPTB, carried out on a small population sample.

In 2014, the UN Secretary-General Ban Ki-moon remarked that "Intensifying our focus on prematurity will sustain gains in child survival, accelerate progress towards the Millennium Development Goals [MDGs], and help lay the groundwork for ending all preventable deaths of women and children by 2030". Notwithstanding, according to WHO (2020), PTB remains the leading cause of death among children under five years old, accounting for approximately 47% of all neonatal deaths. Thus, as discussed in chapter 1, although the PTB worldwide importance, there has been little advancement in the prevention of prematurity [65]. Additionally, it is already known that about two-thirds of PTB cases are spontaneous [56], resulting from a complex interaction of different factors, including environmental and behavioral factors, as well as genetic predispositions [76]. Since inflammation and different inflammatory diseases, notably PD, are often invoked as a risk factor for PTB [132,460,461], genetic variants associated with pathological inflammation have been of long-standing interest to scientific community. Despite the advances, many studies have failed to identify genetic variants associated with PTB, and many others have not been replicated or confirmed [65]. Therefore, a scientific effort must be conducted to understand the genetic and other molecular triggers underlying PTB. The present study will provide insights into this global need and offer tools to achieve Sustainable Development Goal 3, specifically targeting the reduction of preventable deaths of newborns and children under five years old. Moreover, since PTB is considered one of the leading health indicators of a nation [47], increasing the applicable knowledge for this public health problem in the Portuguese Health System will allow to improve the prevention of PTB, reducing the problematic outcomes to society of this condition.

3.2 Materials and methods

3.2.1 Study design

In this study, we aim to address several critical questions to understand better the etiology of PTB, such as:

1. Is SPTB a nurture or a nature condition?
2. Does social demographic status contribute to SPTB?
3. Is there a genetic predisposition for SPTB, or is it an outcome of an inflammatory susceptibility?

Hospital Garcia de Orta (HGO) is a differentiated perinatal hospital and a "Baby Friendly Hospital" (UNICEF) since 2005, receiving many women with risk of PTB from Vale do Tejo districts. Therefore, women giving birth in this hospital are predominantly from the most cosmopolitan area in the country. Thus, the search for inflammatory-related variants in SPTB mothers with heterogeneous backgrounds, will give us clues to start answering questions 2. Moreover, this search of genes also related to inflammatory response could help us clarify whether PTB is a consequence of an exposition to a pro-inflammatory environment, such as pathogenic microbiota, or of a chronic inflammatory state promoted by the host genome. This will provide clues to understanding the 1st question.

3.2.1.1 Ethics

This study was conducted in accordance with the Declaration of Helsinki and the ethical principles for medical research involving human subjects and was approved by the ethics committee of HGO (56/2019).

Prior to participation, all individuals received the 'Participant Information' document (Supplementary material A.1), which provided detailed information regarding the study objectives, methodology, potential risks, and benefits. Written informed consent was obtained from all participants before data collection and sample acquisition. Participants were informed of their right to withdraw from the study at any time without consequences. In accordance with the guidelines approved by the HGO ethics committee, all participants also received an informational leaflet from the Ordem dos Médicos Dentistas Portugueses regarding periodontitis (Supplementary material A.1).

Confidentiality and data protection measures were strictly followed. Personal identifiers were anonymized before data analysis, and all data were securely stored with restricted access limited to authorized researchers.

No financial incentives were provided for participation, and there were no conflicts of interest reported by the research team. This study did not involve vulnerable populations or invasive procedures beyond routine sample collection. Blood sample collection was carried out by authorized healthcare personnel from the HGO department.

The ethical considerations in this research underscore our commitment to maintaining high scientific and ethical standards in genetic research involving human participants.

3.2.1.2 Study population and eligibility criteria

The study population consisted of randomly selected postpartum women followed at the Puerperium of the Service of Gynecology and Obstetrics from HGO, Portugal.

Women were eligible to participate if they fulfilled the following inclusion criteria:

(i) Provided fully informed written consent, and

(ii) Completed the screening questionnaire,

Exclusion criteria included:

(i) Women with iatrogenic PTB,

(ii) Women with preeclampsia,

(iii) SPTB in multiple pregnancy,

(iv) Women with known congenital uterine and/or vaginal malformations, and

(v) Women with immunodeficiency virus.

Women with PPROM were eligible for study participation only if they labored spontaneously and delivered before 37 weeks of gestation.

Blood samples were collected up to 72 hours after delivery. A total of 45 women participated in this pilot study, including 15 women with SPTB and 30 controls (women with normal delivery at term).

3.2.1.3 Development of a Screening Questionnaire

The development of a self-reported questionnaire will be the first approach to include or exclude subjects of the study, as well as provide clues regarding the main questions/hypothesis of the project. The screening questionnaire was then subdivided into two parts, namely:

(i) A sociodemographic questionnaire for general participant information and screen out possible SPTB risk factors, such as level of education, occupation status and PTB history, and

(ii) A lifestyle questionnaire covering family history of immune or inflammatory diseases, and lifestyle habits that may promote inflammatory processes, such as dairy and cereals consumption.

3.2.1.4 Candidate gene variants

A literature review was conducted in B-on, Pubmed, and Science Direct databases using the search terms "preterm birth" or "preterm labor", "periodontal disease" or "periodontitis", "inflammation", "inflammatory disease", and "genetic variant" or "polymorphism". Only peer review papers in English published between 1999 and 2019 were included. Studies using animal cell lines, animal experiments and in silico research were excluded. Candidate genes were then selected for analysis based on biological plausibility for a role in PTB, PD, and other inflammatory conditions.

To go deeper into the search for genes potentially involved in PTB, an additional strategy was developed using STRING, a biological database and web resource of known and predicted protein-protein interactions. Specifically, this platform was used to identify protein-protein interaction networks surrounding key inflammatory genes previously associated with PTB and PD. This strategy enabled the identification of additional genes with functional relevance to inflammatory pathways, even if they had not yet been directly associated with these conditions in the published literature.

3.2.2 DNA extraction and genotyping

A 2 mL peripheral blood sample was collected in EDTA-coated tubes from all the enrolled participants (SPTB and controls) who were fully anonymized using a code by the hospital personnel so that the molecular laboratory technician was blinded to the patients' information

and the group allocation. The samples were immediately transported under refrigeration to the molecular laboratory at Egas Moniz School of Health & Science or the NOVA FCT Lab and kept at 4°C (for a maximum of 1 day) before further processing for molecular analysis.
DNA was extracted using the NZY Tissue gDNA Isolation Kit (NZYTECH) according to the manufacturer's instructions and stored at -80°C until use. Genotyping of the selected SNPs was carried out at CEGEN-FPGMX (Fundación Pública Galega de Medicina Xenómica, Santiago de Compostela, Spain) using the iPLEX Gold technology (Agena Bioscience) as a service.

3.2.2.1 iPLEX® genotyping assay

The Agena Bioscience MassARRAY® System, formerly known as the Sequenom MassARRAY, is an economic and fast technology for genotyping of specific SNPs. The iPLEX® multiplex assay workflow is summarized in Figure 3.1. Essentially, the genomic regions containing the selected SNPs are amplified by Polymerase Chain Reaction (PCR). The reaction products then undergo a shrimp alkaline phosphatase (SAP) treatment, which dephosphorylates excess unincorporated dideoxyribonucleotides (dNTPs) by cleaving their 5'-phosphate groups, thereby preventing their interference in subsequent steps. Next, the iPLEX extension reaction is performed, a highly specific method for detecting SNPs or small insertion/deletion polymorphisms in amplified DNA. Following PCR cleanup, a primer designed to anneal immediately upstream of the polymorphic site is extended by the incorporation of a single terminator nucleotide base, dideoxynucleotide (ddNTP), which is complementary to the polymorphic base. These terminator nucleotides lack a 3'-hydroxyl group, effectively preventing further elongation and ensuring a single based extension. Each ddNTP is mass-modified, allowing for the differentiation of allele-specific extension products in subsequent mass spectrometry analysis. The extension products are then analyzed using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. In this process, the DNA fragments are ionized by a laser under vacuum, and their time of flight (TOF) is measured as they travel through the mass spectrometer. The TOF is directly proportional to the mass-to-charge ratio of each ionized molecule, meaning that smaller fragments reach the detector faster, while larger fragments take longer. The mass differences between the extension products allow for precise allele discrimination. Finally, Sequenom software automatically translates the mass spectra into genotype calls. MALDI-TOF mass spectroscopy offers several characteristics that make it an exceptional platform for assessing SNPs. These encompass (i) direct detection of the analyte, (ii) high analytical precision, (iii) excellent resolution, (iv) ease of assay design, and (v) brief

analysis durations. These properties make iPLEX genotyping assay an efficient, cost-effective, and nearly infallible technique for examining genetic diversity [462–466].

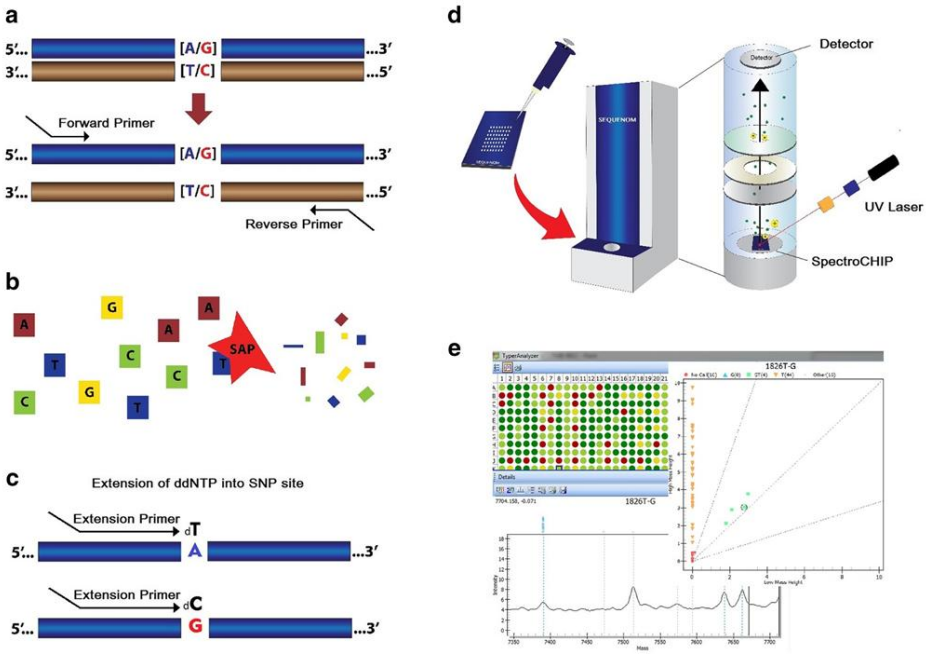

Figure 3.1. Overview of iPLEX® genotyping assay. A schematic of the genotype reaction of an A-to-G SNP. a) Locus-specific amplification reaction (PCR). b) Treatment with Shrimp Alkaline Phosphatase (SAP) enzyme to neutralize unincorporated dNTPs. c) iPLEX® extension reaction. In this reaction, an extension primer anneals immediately upstream of the polymorphic site; the primer and amplified target DNA are incubated with mass-modified ddNTPs terminators. The primer extension is made according to the sequence of the variant site. d) The products of the reaction are spotted on a spectroCHIP. The CHIP is placed into the mass spectrometer and each spot is then shot with a laser under vacuum by MALDI-TOF method. The mass of the extended primer is determined. e) The mass of the observed primers are automatically translated into a genotype by a software (Svidnicki et al, 2015 [465]).

3.2.3 Statistical analysis

Statistical analysis of the questionnaire data, including sample characterization, descriptive statistics and assessment of the differences between groups for sociodemographic and lifestyle characteristics (using chi-square/Fisher's exact test or Student's t-test) were performed using R v.4.2.1. Continuous variables are presented as mean ± standard deviation (SD), and categorical variables as frequencies (N) with percentages (%).

The chi-square test was used to evaluate SNPs within the healthy control group for potential deviations from Hardy-Weinberg equilibrium (HWE) via PLINK (1.9 version). Minor allele frequency (MAF) calculations and additional statistical analyses were also performed using PLINK. The genotype frequency distribution of the genetic variants under study in SPTB

patients and control subjects were compared using chi-square (Fischer's exact) test under genotypic, trend, dominant, recessive and allelic inheritance models. The Cochran-Armitage trend test, also known as the trend model, assesses the presence of a linear association between the binary outcome variable (case/control) and the ordinal variable (genotypes), considering individuals rather than alleles as the unit of analysis. This test evaluates the association between the genotypes and the risk of SPTB in a dose-dependent manner, reflecting the presence of minor alleles. Significant polymorphisms were further analyzed using a logistic regression model to determine their respective odds ratio (OR). Besides crude OR, logistic regression data were also adjusted for potential confounding factors – adjusted OR (aOR). The multivariable-adjusted model included ancestry, smoking habits, education level, occupation status, marital status, and dairy and cereals consumption (categorical) as covariates. To account for multiple testing in the logistic regression analysis, corrections were applied to control the family-wise error rate (FWER) and the false discovery rate (FDR). Three correction methods were used: (i) the Bonferroni (B) correction, which controls FWER by dividing the significance level (α) by the number of tests, (ii) the Benjamini-Hochberg (BH) correction, which controls FDR under the assumption of independent or positively correlated tests, and (iii) the Benjamini-Yekutieli (BY) correction, which provides a more stringent control of FDR under any dependency structure among tests. While the BH correction is less conservative than B, allowing for the detection of potentially significant associations while still controlling the FDR, the BY correction is highly conservative, leading to higher adjusted *p*-values, particularly when dependencies among tests are suspected. A significance threshold of $p < 0.05$ was applied.

3.3 Results

3.3.1 Screening self-report questionnaire

The developed self-report questionnaire, subdivided into sociodemographic and lifestyle questions, is presented below. This questionnaire was approved by the ethical committee of HGO.

Screening Questionnaire

Código do paciente para identificação da amostra: _____

Data de preenchimento: ____/_____/_____

Questionário Sócio-Demográfico

1. *Idade: _____*

2. *Nacionalidade: _____*

3. *Residência (Concelho): _____*

4. *Habilitações literárias completas:*

Sem escolaridade ☐ *4ª classe* ☐ *7º ano* ☐ *9º ano* ☐ *12º ano* ☐

Bacharelato ☐ *Licenciatura* ☐ *Mestrado* ☐ *Doutoramento* ☐

Outro _____

5. *Situação Profissional:*

Ativa ☐ *Desempregada* ☐ *Outro _____*

6. *Profissão: _____*

7. *Estado Civil:*

Solteira ☐ *Casada* ☐ *União de Facto* ☐ *Divorciada* ☐ *Viúva* ☐

8. *Filhos: _____*

9. *Teve um parto pré-termo (antes do tempo)? Sim _____ Não_____*

9.1. *Qual o tempo de gestação? _____*

9.2. *Se sim, qual o peso do bebé à nascença? _____*

9.3. *Já teve* ***algum parto pré-termo anteriormente****? Sim _____ Não_____*

9.4. *Se sim, a que idade (materna) teve o parto pré-termo? E qual o tempo de gestação?*

_____

Questionário de Hábitos de Vida

1. Consumo de tabaco

1.1. *É fumadora? Sim _____ Não_____*

1.1.1. *Se sim, por favor indique qual o seu consumo habitual de tabaco (incluindo cigarros, tabaco para cachimbo, cigarrilhas, charutos):*

	0-10 cigarros por dia
	12-20 cigarros por dia
	21 a 40 cigarros por dia
	Mais de 40 cigarros por dia
	0-20 cigarros por semana
	21-40 cigarros por semana
	Outro. Qual:_____

1.1.2. *Se sim, quando iniciou o consumo de tabaco (ano ou idade): _____*

1.2. Se não *é fumadora atualmente, já foi fumadora? Sim _____ Não_____*

1.2.1. *Se sim, quantos anos?_____ Qual a média de cigarros por dia?_____*

2. Higiene oral

2.1. *Habitualmente, qual o número de vezes que escova os dentes, por dia (assinale com um X a opção correta):*

	Uma vez por dia; De manhã ou à noite?_____
	Duas vezes por dia
	Três vezes por dia
	Quatro vezes por dia
	Outra. Qual:_____

2.2. *Habitualmente, qual o número de vezes que utiliza o fio dentário, por dia (assinale com um X a opção correta):*

	Uma vez por dia
	Duas vezes por dia
	Três vezes por dia
	Uma vez por semana
	Outra. Qual:_____

2.3. *Habitualmente, qual o número de vezes que utiliza o colutório oral (elixir para bochechar), por dia (assinale com um X a opção correta):*

	Uma vez por dia
	Duas vezes por dia
	Três vezes por dia
	Uma vez por semana
	Outra. Qual:_____

2.4. *Quando foi a última vez que foi ao dentista?*

	< 6 meses
	Entre 6 a 12 meses
	Entre 1 a 2 anos
	> 2 anos
	Nunca

2.5. *Tem alguma doença oral (selecione com um X todas as opções que se aplicam a si)?*

	Nenhuma
	Aftas
	Gengivite
	Periodontite
	Outra. Qual:_____

3. Consumo de lacticínios

3.1. *Que lacticínios é que habitualmente consome (selecione com um X todas as opções que se aplicam a si)?*

	Nenhum
	Leite de vaca (incluindo leite com redução de lactose)
	Iogurte
	Queijo
	Manteiga
	Outro. Qual:_____

3.2. *Qual a frequência de consumo de lacticínios (leite e seus derivados)? (assinale com um X a opção correta)*

	Nenhuma
	Uma vez por dia
	Duas vezes por dia
	Três vezes por dia
	Uma vez por semana
	Outra. Qual:_____

3.3. *Atualmente, tem intolerância à lactose? Sim _____ Não_____*

4. Consumo de cereais

4.1. *Que cereais é que habitualmente consome (selecione com um X todas as opções que se aplicam a si)?*

	Trigo (presente em farinhas, massas, pão, bolachas)
	Milho (presente em farinhas, massas, pão, cereais de peq. almoço)
	Arroz
	Centeio (presente em farinhas, pão)
	Cevada (presente em farinhas, bebidas)
	Aveia (presente em farinhas, bolachas, cereais de peq. almoço)
	Outro. Qual:_____

4.2. *Qual a frequência de consumo de cereais? (assinale com um X a opção correta)*

	Uma vez por dia
	Duas vezes por dia
	Três vezes por dia
	Uma vez por semana
	Outra. Qual:_____

4.3. *Atualmente tem intolerância ao glúten? Sim _____ Não_____*

4.4. *Tem ou teve o diagnóstico de doença celíaca (doença autoimune do intestino causada por reação ao glúten)? Sim _____ Não_____*

5. Doenças inflamatórias e/ou infeciosas

5.1. *Das seguintes doenças, por favor indique qual ou quais estão ou estiveram presentes na sua vida (assinale com um X todas as opções que se aplicam a si)?*

	Nenhuma
	Vírus da Imunodeficiência Humana
	Hepatite
	Doenças inflamatórias do intestino (Síndrome de Crohn, colite ulcerosa, síndrome do intestino irritável)
	Diabetes Mellitus tipo I
	Diabetes Mellitus tipo II
	Psoríase
	Artrite Reumatóide
	Hidradenite Soporativa
	Espondilite Anquilosante
	Lúpus Eritematoso Sistémico
	Esclerose Múltipla

	Asma
	Outra/s. Qual/Quais:_____

5.2. *Na sua família, algum membro familiar tem ou teve alguma das doenças acima mencionadas? Sim _____ Não_____*

5.2.1. *Se sim, qual/quais doença(s)?*

_____

5.2.2. *Se sim, quem (grau de parentesco)?*

_____

6. Amamentação

6.1. *Na sua primeira infância, como foi a sua amamentação (p.ex. leite materno; leite de vaca; leite adaptado)?*

_____

7. Medicação

7.1. *Toma medicação diariamente? Sim _____ Não_____*

7.2. *Se sim, qual/quais (assinale com um X todas as opções que se aplicam a si)?*

	Antidepressivos
	Ansiolíticos
	Analgésicos (ex. Paracetamol)
	Anti-inflamatórios
	Imunossupressores
	Diuréticos
	Anti-coagulantes (ex. Lovenox)
	Anti-hipertensores (ex. Ardomer, Nifedipino, CR30)
	Anti-parkinsónicos
	Anti-diabéticos (ex. Metilamina)
	Outro/s. Qual/Quais:_____

3.3.2 Demographic and lifestyle information of the PTB and control groups

Table 3.1 presents the demographic and lifestyle data of the women with SPTB and women with full-term pregnancy (control group). The study included 30 controls, aged 30.9 ± 6.21 years, and 15 women with SPTB, aged 30.0 ± 5.74 years. There was no significant difference in the age parameter between the two groups ($p > 0.05$). Regarding ancestry, the majority of women in both groups self-identified as being of European descent, with 86.7% in the control group (26 of 30 women) and 86.7% in the SPTB group (13 of 15 women). No significant differences were found in other demographic parameters, including education level ($p = 0.568$), occupation status ($p = 1$), marital status ($p = 0.094$), and number of children ($p = 0.221$). Similarly, lifestyle factors such as smoking habits were comparable between groups ($p = 0.699$). Most women in both groups reported never smoking – 80% in the control group and 86.7% in the SPTB group. Dietary habits related to cereal and dairy consumption also did not differ significantly between groups ($p = 0.326$ for dairy consumption and $p = 0.172$ for cereals consumption).

As expected, gestational age at delivery was significantly lower in the SPTB group compared to controls ($p < 0.001$). Within the SPTB group, 2 (13.3%) women gave birth to an extremely preterm baby (<28 weeks of gestation), 3 (20%) had a very preterm birth (28 weeks to <32 weeks), and 10 (66.7%) had a late preterm birth (32 weeks to <37 weeks).

Finally, although a higher proportion of women in the SPTB group reported a history of previous PTB (20% vs. 10%), this difference was not statistically significant ($p = 0.384$), likely due to the small sample size in this pilot study.

Table 3.1. Socio-demographic and lifestyle characteristics of SPTB and control groups

	Control (N=30)	**SPTB (N=15)**	***p* value**
Age			
Mean (SD)	30.9 (6.21)	30.0 (5.74)	0.633
Median [Min, Max]	31.0 [22.0, 43.0]	30.0 [18.0, 39.0]	
Ancestry			
African	4 (13.3%)	1 (6.7%)	0.546

	Control (N=30)	SPTB (N=15)	*p* value
Asian	0 (0%)	1 (6.7%)	
European	26 (86.7%)	13 (86.7%)	
Education level			
Uneducated	0 (0%)	1 (6.7%)	0.568
Elementary school	3 (10.0%)	2 (13.3%)	
High school	14 (46.7%)	7 (46.7%)	
Graduate	13 (43.3%)	5 (33.3%)	
Occupation status			
Unemployed	9 (30.0%)	4 (26.7%)	1
Student	1 (3.3%)	1 (6.7%)	
Employed	20 (66.7%)	10 (66.7%)	
Marital status			
Single	15 (50.0%)	8 (53.3%)	0.094
Cohabiting	6 (20.0%)	4 (26.7%)	
Married	9 (30.0%)	1 (6.7%)	
Divorced	0 (0%)	2 (13.3%)	
Number of children			
Mean (SD)	1.53 (0.819)	1.27 (0.594)	0.221
Median [Min, Max]	1.00 [1.00, 4.00]	1.00 [1.00, 3.00]	
Gestational age (weeks)			
<28	0 (0%)	2 (13.3%)	<0.001*
28-32	0 (0%)	3 (20.0%)	
32-37	0 (0%)	10 (66.7%)	
≥37	30 (100%)	0 (0%)	
Previous PTB			
No	27 (90.0%)	12 (80.0%)	0.384
Yes	3 (10.0%)	3 (20.0%)	
Smoker			
No	24 (80.0%)	13 (86.7%)	0.699
Yes	6 (20.0%)	2 (13.3%)	
Dairy consumption			
Once a week	1 (3.3%)	2 (13.3%)	0.326
Once a day	6 (20.0%)	4 (26.7%)	
Twice a day	17 (56.7%)	5 (33.3%)	

	Control (N=30)	SPTB (N=15)	*p* value
Three times a day	6 (20.0%)	4 (26.7%)	
Cereals consumption			
Occasionally	1 (3.3%)	1 (6.7%)	0.172
Once a week	1 (3.3%)	0 (0%)	
Once a day	10 (33.3%)	10 (66.7%)	
Twice a day	15 (50.0%)	4 (26.7%)	
Three times a day	3 (10.0%)	0 (0%)	

N - number of subjects; SD – standard deviation; SPTB – spontaneous preterm birth. * – Parameter with statistically significant difference between control and PTB groups ($p < 0.05$).

3.3.3 Bioinformatic analysis for candidate gene variants

All candidate genes had to be related to inflammation, directly or indirectly. Thus, genes involved in processes such as the control of immune response (i.e. pattern recognition receptors, cytokines, chemokines and their respective receptors), among others related to inflammation were considered appropriate candidates for this study. The approach using STRING to identify the protein network of each key protein of PTB and PD, allowed to include in this pilot study relevant genes to the inflammatory process that were absent in the initial search (two examples are shown in Figure 3.2 and Figure 3.3). A list of 90 variants in 40 different genes were chosen. After iPLEX mass array design, 17 variants were excluded. A panel of 73 SNPs in 36 genes were included to be typed in this pilot study (Table 3.2).

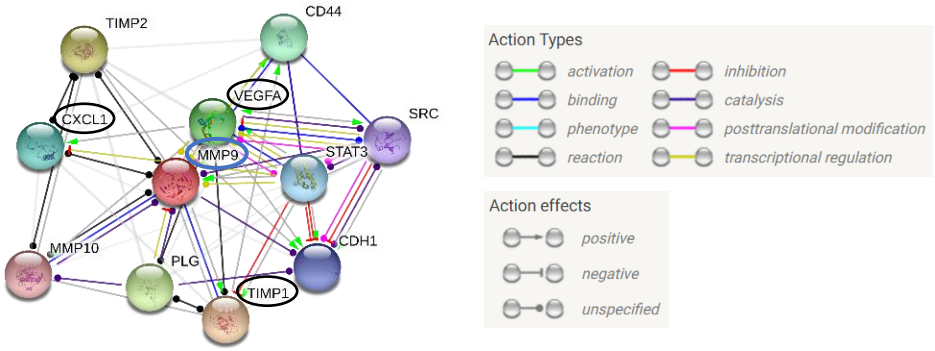

Figure 3.2. MMP-9 network based on molecular actions. Example of a protein-protein interaction network of a key protein involved in PD (STRING network based on only 10 interactions).

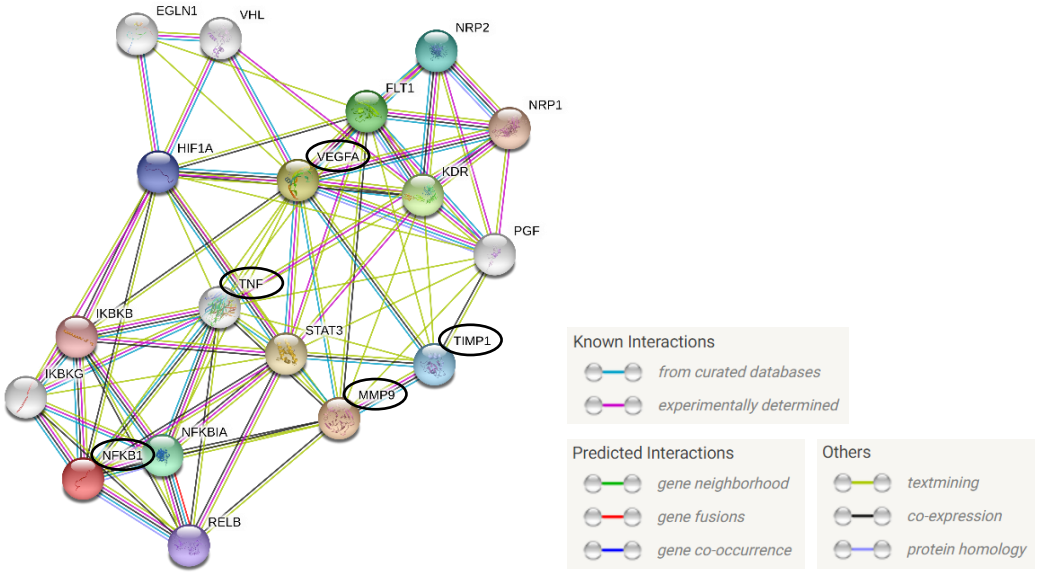

Figure 3.3. A protein-protein interaction network of some inflammatory-related proteins that may be involved in SPTB and PD (based on evidence). Example of a network generated through known and predicted protein-protein interactions that allowed the inclusion of additional genes relevant to the inflammatory process, that were absent in the initial search, such as *TIMP1*.

Table 3.2. Candidate genes and respective variants

Gene	Polymorphism	Gene	Polymorphism
IL1A	rs1800587	*MMP3*	rs679620
IL1A	rs17561	*MMP3*	rs520540
IL1B	rs1143627	*MMP8*	rs11225395
IL1B	rs16944	*MMP8*	rs2155052
IL1B	rs1143634	*MMP9*	rs17576
IL1RN	rs4251961	*TIMP1*	rs2070584
IL4	rs2243250	*TIMP1*	rs11551797
IL6	rs1800795	*TIMP1*	rs4898
IL6	rs1800796	*TIMP1*	rs5906435
IL6	rs2069827	*TIMP2*	rs2277698
IL6R	rs2228144	*TIMP2*	rs55743137

IL6R	rs4845617	*TIMP2*	rs8179090
IL8	rs2227532	*TIMP3*	rs5749511
IL8	rs4073	*TIMP4*	rs17035945
IL10	rs1800896	*NFKB1*	rs4648068
IL10	rs1800871	*CD14*	rs2569190
IL10	rs1800872	*IGF1*	rs5742637
TNFA	rs1799724	*IGF1*	rs5742612
TNFA	rs361525	*IGF1*	rs972936
TNFA	rs1800750	*IGF1R*	rs2229765
TNFA	rs1800629	*IFNG*	rs2430561
TNFA	rs1799964	*IFNGR1*	rs1327474
TLR1	rs5743595	*IFNGR1*	rs11914
TLR1	rs5743618	*IFNGR1*	rs7749390
TLR2	rs4696480	*TGFB1*	rs1800469
TLR2	rs5743708	*EBF1*	rs2946169
TLR2	rs5743700	*AGTR2*	rs5950506
TLR2	rs3804099	*KCNN3*	rs1218585
TLR4	rs4986790	*KCNN3*	rs1218584
TLR4	rs4986791	*KCNN3*	rs883319
TLR4	rs7873784	*PTGS2*	rs689466
TLR10	rs62617795	*PTGS2*	rs5275
TLR10	rs140873456	*PTGS2*	rs20417
MMP1	rs7945189	*VEGFA*	rs2010963
MMP2	rs243865	*NLRP3*	rs35829419
MMP2	rs11639960	*MBL2*	rs74754826
MMP2	rs2285053		

3.3.4 Genetic association analysis in SPTB patients and controls

DNA from all the women was genotyped for a panel of 73 selected SNPs to evaluate their putative associations with SPTB risk. Thirteen SNPs were excluded due to a MAF of less than 5%. Additionally, *IL6R* rs4845617 did not meet HWE criteria ($p > 0.05$) in the control group

and was also excluded. Consequently, 59 SNPs remained and were subjected to statistical analysis.

The genotype frequency of significant polymorphisms in SPTB patients and controls are presented in Table 3.3. The results of the genetic association tests (trend and allelic) are shown in Table 3.4. The polymorphisms rs1218585, rs1218584, rs1327474, and rs2430561 were selected for logistic regression analysis as they exhibited statistically significant differences between SPTB and control groups. Table 3.5 presents the logistic regression analysis results, including both the crude OR and the aOR. Additionally, *p*-values are provided for both the crude and adjusted models. For the adjusted analysis, *p*-values were corrected for multiple testing.

Table 3.3. Genotype distributions of significant polymorphisms in patients with SPTB and controls

SNP	Genotype	Control	SPTB	OR	95% CI	*p* value
		N (%)	N (%)			
	GG	23 (76.7)	7 (46.7)	1 (ref)		
KCNN3 rs1218585	GA	7 (23.3)	8 (53.3)	3.76	1.00 – 14.07	0.025
	AA	0 (0.0)	0 (0.0)	NA	NA	NA
	CC	23 (76.7)	6 (40.0)	1 (ref)		
KCNN3 rs1218584	CG	7 (23.3)	9 (60.0)	4.93	1.3 – 18.73	0.01
	GG	0 (0.0)	0 (0.00)	NA	NA	NA
	TT	20 (66.7)	5 (33.3)	1 (ref)		
IFNGR1 rs1327474	TC	9 (30.0)	8 (53.3)	3.56	0.91 - 13.94	0.034
	CC	1 (3.3)	2 (13.3)	8.0	0.6 - 106.94	0.05†
	TT	7 (23.3)	9 (60.0)	1 (ref)		
IFNG rs2430561	TA	15 (50.0)	5 (33.3)	0.26	0.06 – 1.07	0.031
	AA	8 (26.7)	1 (6.7)	0.1	0.0 – 0.97	0.024

CI – confidence interval; N – number of subjects; OR – odds ratio; SNP – single nucleotide polymorphism; SPTB – patients with spontaneous preterm birth; † – association at the borderline lack of statistical significance ($p = 0.05$).

Table 3.4. Genetic association test results for significant polymorphisms in SPTB patients and controls

SNP	Test	χ^2	DF	*p* value
KCNN3 rs1218585	TREND	4.05	1	0.044

SNP	Test	χ^2	DF	*p* value
KCNN3 rs1218584	TREND	5.867	1	0.015
	ALLELIC	4.599	1	0.032
IFNGR1 rs1327474	TREND	4.9	1	0.027
	ALLELIC	4.935	1	0.026
IFNG rs2430561	TREND	6.043	1	0.014
	ALLELIC	6.581	1	0.01

DF – degree of freedom; SNP – single nucleotide polymorphism; TREND – Cochran-Armitage trend test; χ^2 – Chi-square test.

As presented in Table 3.3, both *KCNN3* variants, rs1218585 and rs1218484, seem to be associated with higher odds of SPTB. There was a significant association of rs1218585 GA genotype compared to the GG reference genotype (OR = 3.76, 95% CI 1.0-14.07, $p = 0.025$). No individuals in either the control or SPTB groups carried the rs1218585 AA genotype. The association observed through the Cochran-Armitage's trend test ($p < 0.05$) supports the effect of *KCNN3* rs1218585 on SPTB (Table 3.4). Similarly, *KCNN3* rs1218584 showed significant associations, with the CG genotype conferring 4.93 times higher odds of SPTB (OR = 4.93, 95% CI 1.3-18.73, $p = 0.01$). As with rs1218585, no individuals were homozygous for the minor allele (rs1218584 GG genotype). Both the trend and allelic models provided further support for the association ($p < 0.05$). The functional SNP rs1327474 of the interferon gamma receptor 1 (*IFNGR1*) gene also exhibited a higher risk of SPTB. The TC and CC genotypes were associated with increased SPTB risk compared to the reference TT genotype, with ORs of 3.56 (95% CI 0.91-13.94, $p = 0.034$) and 8.0 (95% CI 0.6-106.94), respectively. The association of the CC genotype was borderline non-significance ($p = 0.05$). The Cochran-Armitage trend test ($p = 0.027$) and the allelic model ($p = 0.026$) confirmed this association. For the *IFNG* rs2430561, both the TA and AA genotypes were found to exhibit a lower risk of SPTB compared to the reference TT genotype (OR = 0.26, 95% CI 0.06-1.07, $p = 0.031$ and OR = 0.1, 95% CI 0.0-0.97, $p = 0.024$, respectively). The association was confirmed by the trend and allelic models ($p < 0.05$). None of the other 55 polymorphisms showed an association with SPTB.

Table 3.5. Crude and adjusted OR from logistic regression for the association of *KCNN3 rs1218585, KCNN3 rs1218584, IFNGR1 rs1327474, and IFNG rs2430561* polymorphisms with SPTB risk

SNP	OR (95% CI)	*p* value	*p* value[a]	aOR (95% CI)	*p* value	*p* value[a]
KCNN3 rs1218585	3.76 (1.00-14.07)	0.05[†]	0.05(BH)[†]	13.64 (1.75-106.4)	0.013	0.017(BH), 0.035(BY), 0.05(B)[†]
KCNN3 rs1218584	4.93 (1.3-18.73)	0.019	0.04(BH)	22.52 (2.56-197.9)	0.005	0.014(BH), 0.03(BY), 0.02(B)
IFNGR1 rs1327474	3.16 (1.08-9.24)	0.036	0.05(BH)[†]	4.63 (1.16-18.47)	0.03	0.03(BH)
IFNG rs2430561	0.29 (0.10-0.82)	0.02	0.04(BH)	0.11 (0.02-0.55)	0.007	0.014(BH), 0.03(BY), 0.028(B)

aOR – odds ratio adjusted for age, ancestry, education level, occupation status, marital status, smoking status, and dairy and cereals consumption of the subjects; B – Bonferroni correction; BH – Benjamini-Hochberg correction; BY – Benjamini-Yekutieli correction; CI – confidence interval; OR – odds ratio; SNP – single nucleotide polymorphism; [a] – adjusted *p*-value; [†] – association at the borderline lack of statistical significance ($p = 0.05$).

As shown in Table 3.5, after logistic regression analysis, the *KCNN3* rs1218585 polymorphism was found to exhibit a higher risk of SPTB, with a crude OR of 3.76 (95% CI 1.00-14.07). However, this association was at the borderline lack of statistical significance ($p = 0.05$). Nevertheless, after adjusting for ancestry, smoking habits, education level, occupation status, marital status, and dairy and cereals consumption, the association became statistically significant, with an adjusted OR (aOR) of 13.64 (95% CI 1.75-106.4) at $p = 0.013$. The adjusted p-values below 0.05 under BH and BY corrections also suggested this association. The *KCNN3* rs1218584 seems to be a strongly significant predictor for the SPTB, warranting further exploration. It was related to 4.93 times higher odds of SPTB (95% CI 1.3-18.73) with a significant association under BH correction (BH adjusted p-value < 0.05) but not under Bonferroni or BY corrections. After adjusting for demographic and lifestyle possible confounding factors (aOR), the rs1218585 showed 22.52 times higher odds of SPTB (95% CI 2.56-197.9, all adjusted p-values < 0.05). The *IFNGR1* rs1327474 polymorphism revealed a weaker association with SPTB, with 3.16 times higher odds of SPTB (95% CI 1.08-9.24) but at the borderline lack of statistical significance ($p = 0.05$) and only under BH correction. After adjusting for demographic and lifestyle possible confounding factors (aOR), this polymorphism showed a significant association with SPTB under BH correction (OR = 4.63, 95% CI 1.16-18.47, BH adjusted p-value < 0.05) but not under Bonferroni or BY corrections. Finally, the *IFNG* rs2430561 exhibit a lower risk of SPTB, with a crude OR of 0.29 (95% CI 0.10-0.82, BH adjusted p-value < 0.05). After adjusting for demographic and lifestyle characteristics, the association became strong, with an aOR of 0.11 (95% CI 0.02-0.55), statistically significant after all multiple testing corrections applied (adjusted p-values < 0.05).

3.4 Discussion

This chapter focuses on the design of the genetic association study, the selection of genetic variants, and the preliminary pilot study. The main findings and methodological considerations are discussed below.

3.4.1 Development of the questionnaire

A questionnaire was designed to collect sociodemographic and lifestyle data to control for potential confounding factors for SPTB. Previous studies have associated some of these

characteristics with PTB, including marital status [467–469], education level [467,469,470], occupation status [467], and tobacco smoking [469], among others. The pilot study analysis showed no significant differences between cases and controls regarding these variables, which could be attributed to the small sample size.

3.4.2 Selection of genetic variants

Candidate genes and polymorphisms were selected based on their biological relevance and previous associations with PTB and inflammatory diseases. The STRING database was used to explore gene interactions, ensuring that the proteins corresponding to selected genes were functionally connected to immune and inflammatory pathways. A panel of 73 SNPs was chosen.

3.4.3 Pilot study and *KCNN3* selection

The pilot study provided preliminary insights into potential genetic associations with SPTB. Among the analyzed variants, rs1218585 and rs1218584 in the *KCNN3* gene showed a significant association with this condition. Interestingly, no homozygous individuals for the minor alleles of both variants were detected. This could be due to the small sample size, low allele frequency in the studied population, or even a functional impact of these variants that might influence their distribution. Another two variants showed suggestive associations with SPTB, including *IFNGR1* rs1327474, and *IFNG* rs2430561. The *IFNG* rs2430561 was significantly associated with lower odds for SPTB even after adjustment for possible confounding factors and multiple testing corrections. The remaining 55 polymorphisms analyzed did not show significant associations in this small cohort.

The association of *KCNN3* variants rs1218585 and rs1218584 with SPTB was first reported by Day and colleagues (2011), suggesting their potential role in SPTB susceptibility [176]. The *KCNN3* gene encodes the small-conductance calcium-activated potassium channel protein 3 (SK3). SK3 channels are voltage-independent ion channels expressed in various tissues, including smooth muscle, with a crucial role in pregnancy. They maintain uterine quiescence during early gestation and are downregulated towards the end of pregnancy, contributing to the increased uterine excitability for labor onset [471–473]. Given its role in uterine excitability and labor onset, *KCNN3* represents a biologically plausible candidate gene for SPTB susceptibility. However, to the best of our knowledge, no other studies have investigated the *KCNN3* variants rs1218585 and rs1218584 in the context of SPTB. The observed association in this pilot

study, together with previous reports by Day and colleagues, reinforces its potential relevance, leading to the selection of these two SNPs for further investigation in a larger cohort.

3.4.4 Limitations and future directions

As a pilot study, the sample size may have limited the ability to detect associations, particularly for rare variants. Nonetheless, the observed findings, combined with the biological plausibility of *KCNN3* in uterine physiology, warrant further investigation. Studies with larger cohorts are essential to validate these associations, while functional analyses could provide deeper insights into how *KCNN3* variants influence uterine excitability and contribute to SPTB susceptibility (further explored in the next chapter).

4

KCNN3 RS1218585 AND RS1218584 ASSOCIATE WITH PTB IN A PORTUGUESE POPULATION: A CASE-CONTROL STUDY

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4.1 Introduction

Preterm birth (PTB), defined as birth before 37 completed weeks of gestation [45], is a major clinical and public health challenge [46]. In 2020, its estimated global prevalence was 9.9%, meaning that 1 in 10 babies were born preterm. PTB is the leading cause of death in children under five years old (18%) [52], and accounts for 70% of neonatal deaths [53] and up to 75% of neonatal morbidity [53]. Beyond its health impact, PTB imposes substantial burdens on families and healthcare systems.

The etiology of PTB remains unclear, but multiple risk factors have been identified, including socioeconomic factors, inflammatory diseases, and genetic predisposition. Twin studies suggest that up to 40% of PTB cases have a genetic component [114,117], and familial aggregation studies further support a heritable influence. However, identifying specific genetic variants has been challenging. Despite numerous genetic association studies, many reported variants have not been consistently replicated or validated, highlighting the need for further research.

Among the candidate genes investigated in PTB, *KCNN3* gene, which belongs to the *KCNN* family of potassium channels, encodes an integral membrane protein that forms the SK3. SK3 channels are voltage-independent channels expressed in various tissues, including smooth muscle. In the myometrium, these channels play a crucial role in hyperpolarizing the cell membrane, reducing contractile activity, and consequently maintaining uterine quiescence during early gestation. As pregnancy progresses, SK3 expression is downregulated, which increases uterine excitability and facilitates labor onset [471–473]. Premature SK3 downregulation may lead to excessive uterine excitability and contractions, contributing to SPTB. Genetic variations in *KCNN3* have already been linked to PTB. Within the maternal genetic characteristics, Day and colleagues (2011) were the first to investigate *KCNN3* variants in SPTB, analyzing 16 SNPs and identifying three – rs1218585 and rs1218584 among them – associated with this condition [176]. These two *KCNN3* variants have not been further explored. Actually, research on *KCNN3* polymorphisms in PTB remains scarce.

The growing recognition of SK3 channels in pregnancy and labor underscores the need for further investigation into their genetic regulation. As early as 2008, Pierce and colleagues suggested that targeting SK3 channels could help prevent or halt preterm labor progression in humans. In 2011, Day and colleagues emphasized *KCNN3* as a logical candidate for PTB research. However, few studies have explored the impact of *KCNN3* variants in this context, thus further research is needed to elucidate the role of this gene in PTB and assess its potential

as a biomarker. This study aimed to evaluate the association of the *KCNN3* variants rs1218585 and rs1218584 with SPTB.

4.2 Materials and methods

4.2.1 Study population

The study included postpartum women followed at the Puerperium of the Service of Gynecology and Obstetrics at HGO, Portugal, recruited between March 2, 2020, and May 5, 2022. The eligibility criteria, questionnaire details, and ethical considerations were described in Chapter 3 (sub-subsections 3.2.1.1 – 3.2.1.3). A total of 110 women were included in this study, comprising 32 women with SPTB and 78 term controls.

4.2.2 DNA extraction and genotyping

The DNA extraction and genotyping were conducted as described in Chapter 3, subsection 3.2.2. The two *KCNN3* (HGNC ID: 6292) variants, rs1218585 and rs1218584, identified in the pilot study (Chapter 3), were genotyped.

4.2.3 Statistical analysis

Statistical analyses were conducted similarly to the procedures described in the previous chapter (subsection 3.2.3). Data from the questionnaire were analyzed using R (v4.2.1), including sample characterization, descriptive statistics, and group comparisons for sociodemographic and lifestyle characteristics. Chi-square or Fisher's exact tests were used for categorical variables, and Student's t-test was applied for continuous variables.

HWE was assessed by chi-square tests in the control group for each SNP. The association between *KCNN3* variants (rs1218585 and rs1218584) and SPTB was initially explored through genotype-based analysis. Power calculations and computations of the required sample size to achieve the 80% power threshold were also estimated using OpenEpi. Given the relatively small sample size, the 'normal approximation with continuity correction' was applied to minimize potential bias associated with small cohorts, thereby improving the accuracy of the estimated power. This preliminary analysis allowed for the visualization of genotype

distributions and provided an initial insight into the association between *KCNN3* variants and SPTB. Subsequently, simple chi-square tests of association were conducted, using different genetic inheritance models (GENO – genotypic association; TREND – Cochran-Armitage trend; ALLELIC – allelic association; DOM – dominant model; and REC – recessive model).

For a more robust evaluation, logistic regression analysis under the additive model was performed in PLINK (v1.9), both with and without covariates, to compute crude OR and aOR. The aOR were estimated after adjusting for potential confounders, including ancestry, smoking habits, education level, occupation status, marital status, and dietary consumption of dairy and cereals (categorical covariates). To account for multiple testing in the logistic regression analysis, the corrections previously explained were applied. A significance threshold of $p < 0.05$ was used.

To evaluate the extent of linkage disequilibrium (LD) between rs1218585 and rs1218584, pairwise LD measures (r^2) were calculated using Haploview software (v4.2). The results were visualized using LD plots generated by the software. Haplotype analysis was then performed to assess the combined effect of *KCNN3* variants on SPTB risk. Initial haplotype frequencies and association statistics were calculated in PLINK using chi-square tests. To estimate OR and assess the strength of association for each haplotype, additional analysis was performed in Haploview using logistic regression-based Z-statistics. Finally, to obtain robust estimates of statistical significance, permutation tests (n = 1000) were conducted in Haploview, and empirical *p*-values were reported.

4.3 Results

4.3.1 Demographic and lifestyle information of the control group and PTB group

Table 4.1 presents the demographic and lifestyle data of women with SPTB (SPTB group) and women with full term pregnancy (control group). The study included 78 controls, aged 31.1 ± 5.71 years, and 32 women with SPTB, aged 32.0 ± 6.33 years. There was no significant difference in age between the two groups ($p > 0.05$). Regarding ancestry, most subjects in both groups self-identified as of European descent, with 87.2% in the control group and 84.4% in the SPTB group. No significant differences were found in other demographic parameters, including education level ($p = 0.735$), occupation status ($p = 0.558$), marital status ($p = 0.610$), and

number of children (p = 0.787). Additionally, smoking habits did not differ significantly between the control and SPTB groups (p = 0.232), with most women in both groups being non-smokers (88.5% in controls and 78.1% in the SPTB groups).

As expected, the gestational age at delivery was significantly lower in the SPTB group compared to the control group (p < 0.001). Among women with SPTB, 5 (15.6%) give birth extremely preterm babies (< 28 weeks of gestation), 6 (18.8%) had a very preterm birth (28 to 32 weeks), and 21 (65.6%) had a late PTB (32 to 37 weeks). Finally, consistent with the literature, the history of previous PTB was significantly more common in the SPTB group than in the control group (p = 0.013).

Table 4.1. Demographic characteristics of mothers with PTB and controls (mothers with full term babies)

	Control (N=78)	**SPTB (N=32)**	***p* value**
Age			
Mean (SD)	31.1 (5.71)	32.0 (6.33)	0.509
Median [Min, Max]	31.0 [22.0, 43.0]	32.5 [18.0, 43.0]	
Ancestry			
European	68 (87.2%)	27 (84.4%)	0.370
African	10 (12.8%)	4 (12.5%)	
Asian	0 (0%)	1 (3.1%)	
Education level			
Uneducated	1 (1.3%)	1 (3.1%)	0.735
Elementary school	11 (14.1%)	6 (18.8%)	
High school	35 (44.9%)	14 (43.8%)	
Graduate	31 (39.7%)	11 (34.4%)	
Occupation status			
Unemployed	19 (24.4%)	5 (15.6%)	0.558
Student	2 (2.6%)	1 (3.1%)	
Employed	57 (73.1%)	26 (81.3%)	
Marital status			
Single	32 (41.0%)	11 (34.4%)	0.610
Cohabiting	21 (26.9%)	11 (34.4%)	
Married	23 (29.5%)	8 (25.0%)	
Divorced	2 (2.6%)	2 (6.3%)	
Number of children			

	Control (N=78)	SPTB (N=32)	*p* value
Mean (SD)	1.50 (0.716)	1.56 (1.22)	0.787
Median [Min, Max]	1.00 [1.00, 4.00]	1.00 [1.00, 7.00]	
Gestational age (weeks)			
<28	0 (0%)	5 (15.6%)	<0.001*
28-32	0 (0%)	6 (18.8%)	
32-37	0 (0%)	21 (65.6%)	
≥37	78 (100%)	0 (0%)	
Previous PTB			
No	74 (94.9%)	25 (78.1%)	0.013*
Yes	4 (5.1%)	7 (21.9%)	
Smoker			
No	69 (88.5%)	25 (78.1%)	0.232
Yes	9 (11.5%)	7 (21.9%)	
Dairy consumption			
Once a week	1 (1.3%)	3 (9.4%)	0.075
Once a day	19 (24.4%)	10 (31.3%)	
Twice a day	43 (55.1%)	11 (34.4%)	
Three times a day	15 (19.2%)	8 (25.0%)	
Cereals consumption			
Do not consume	1 (1.3%)	0 (0%)	0.296
Occasionally	1 (1.3%)	3 (9.4%)	
Once a week	6 (7.7%)	0 (0%)	
Once a day	29 (37.2%)	13 (40.6%)	
Twice a day	31 (39.7%)	13 (40.6%)	
Three times a day	9 (11.5%)	3 (9.4%)	
Four times a day	1 (1.3%)	0 (0%)	

N – number of subjects; PTB – women with PTB; SD – standard deviation. * – Parameter with statistically significant difference between control and PTB groups ($p < 0.05$).

4.3.2 Genotypes and haplotypes distributions in SPTB patients and controls

DNA from all participants was genotyped for the two selected SNPs, *KCNN3* rs1218585 and *KCNN3* rs1218584, to assess their potential associations with SPTB risk. Both SNPs fulfilled the HWE criteria ($p > 0.05$). Table 4.2 shows the significant genotype distributions between cases and controls, along with odds ratio, corresponding 95% confidence intervals, and respective power calculations. The chi-square statistics (χ^2), degrees of freedom (DF), and *p*-values for the significant associations observed under the allelic and trend genetic models are summarized in Table 4.3. Table 4.4 presents the logistic regression analysis results under the additive genetic model, including the Wald test for crude (OR) and adjusted (aOR) odds ratio, with the latter accounting for potential confounders. The results indicate significant associations between both polymorphisms and SPTB risk, with stronger associations observed in the adjusted models after controlling for confounders.

In addition, LD analysis was performed to assess the relationship between the two *KCNN3* variants (

Figure **4.1**). A haplotype analysis was also conducted to investigate further the potential role of rs1218585 and rs1218584 in SPTB susceptibility. Table 4.5 presents the identified haplotypes and their respective frequencies in cases and controls, along with the corresponding OR.

Table 4.2. Genotype distributions of *KCNN3* rs1218585 and rs1218584 in patients with SPTB and controls and power calculations

SNP	Genotype	Control	SPTB	OR	95% CI	*p* value	Observed Power	Sample size 80% power
		N (%)	N (%)					
KCNN3 rs1218585	GG	57 (73.1)	14 (43.8)	1 (ref)				
	GA	20 (25.6)	18 (56.3)	3.67	1.54 – 8.7	0.002	81.03%	NA
KCNN3 rs1218584	CC	52 (66.7)	13 (40.6)	1 (ref)				
	CG	24 (30.8)	18 (56.3)	3.00	1.27 – 7.10	0.006	62.3%	140

CI – confidence interval; N – number of subjects; OR – odds ratio; SPTB – patients with spontaneous preterm birth.

Table 4.3. Association test results for *KCNN3* rs1218585 and rs1218584 under genetic inheritance models (allelic and trend tests)

SNP	Test	χ^2	DF	*p* value
KCNN3 rs1218585	TREND	7.151	1	0.007
	ALLELIC	5.998	1	0.014
KCNN3 rs1218584	TREND	5.344	1	0.021
	ALLELIC	4.707	1	0.030

DF – degree of freedom; SNP – single nucleotide polymorphism; TREND – Cochran-Armitage trend test; χ^2 – Chi-square test.

Table 4.4. Crude and adjusted OR from logistic regression under the additive model for the association of *KCNN3* rs1218585 and rs1218584 polymorphisms with SPTB risk

SNP	OR (95% CI)	*p* value	*p* value[a]	aOR (95% CI)	*p* value	*p* value[a]
KCNN3 rs1218585	2.97 (1.31-6.76)	0.009	0.019(BH), 0.028(BY), 0.02(B)	4.17 (100.58-11)	0.004	0.008(BH), 0.012(BY), 0.008(B)
KCNN3 rs1218584	2.37 (1.38-1.12)	0.024	0.024(BH), 0.036(BY), 0.045(B)	2.86 (1.23-6.7)	0.015	0.015(BH), 0.023(BY), 0.030(B)

aOR – odds ratio adjusted for age, ancestry, education level, occupation status, marital status, smoking status, and dairy and cereals consumption of the subjects; B – Bonferroni correction; BH – Benjamini-Hochberg correction; BY – Benjamini-Yekutieli correction; CI – confidence interval; OR – odds ratio; SNP – single nucleotide polymorphism; [a] – adjusted *p*-value.

As presented in Table 4.2, both *KCNN3* variants (rs1218585 and rs1218584) were associated with significant differences in genotype distributions between SPTB patients and controls. For *KCNN3* rs1218585, the GA genotype was significantly associated with an increased risk of SPTB compared to the GG reference genotype, with an OR of 3.67 (95% CI 1.54-8.70, $p = 0.002$). No individuals in the SPTB group carried the AA genotype for rs1218585, whereas one individual in the control group had the AA genotype. Consequently, the analysis for this genotype was not performed. For *KCNN3* rs1218584, the CG genotype conferred a 3-fold increased odds of SPTB (OR = 3.00, 95% CI 1.27-7.10, $p = 0.006$). Again, the number of individuals homozygotes for the minor allele (GG) was too low to allow for a meaningful analysis (only one in the SPTB group and two in the control group). Thus, these results were excluded from the results presentation. These findings suggest that both SNPs may influence SPTB risk. In the trend and allelic models (Table 4.3), both variants also demonstrated significant association with SPTB. The Cochran-Armitage trend test showed a significant association for both rs1218585 (trend $p = 0.007$) and rs1218584 (trend $p = 0.021$). The allelic model for both SNPs also showed significant associations (allelic $p = 0.014$ for rs1218585 and $p = 0.030$ for rs1218584), providing further support for the involvement of these variants in SPTB risk.

Logistic regression analysis (Table 4.4) confirmed that the *KCNN3* rs1218585 and rs1218584 variants were associated with SPTB risk under the additive model, the most biologically plausible inheritance model in this context. For rs1218585, the rude OR was 2.97 (95% CI 1.31-6.76, $p = 0.009$). After adjusting for potential confounders, including age, ancestry, education level, occupation status, marital status, smoking status, and dairy and cereals consumption, the aOR increased to 4.17 (95% CI 1.00-11.0, $p = 0.004$), remaining statistically significant after B, BH, and BY corrections. For rs1218584, the crude OR was 2.37 (95% CI 1.38-4.12, $p = 0.024$). The adjusted OR was 2.86 (95% CI 1.23-6.70, $p = 0.015$), also remaining statistically significant after the same corrections (BH-adjusted $p = 0.023$, BY-adjusted $p = 0.030$, B-adjusted $p = 0.045$). These results indicate that both *KCNN3* variants contribute to SPTB risk, with the adjusted models further strengthening these associations.

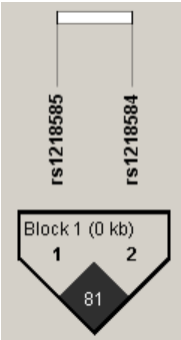

Figure 4.1. Linkage disequilibrium (LD) pattern among *KCNN3* variants. The two SNPs, rs1218585 and rs1218584, are in LD ($r^2 = 0.814$), forming a haplotype block (Block 1).

Table 4.5. Haplotype frequencies of *KCNN3* rs1218585-rs1218584 in patients with SPTB and controls

Haplotype (rs1218585-rs1218584)	Control (%)	SPTB (%)	OR	*p* value	Empirical *p* value[a]
AG	14.1	28.1	2.97	0.009	0.038
GC	82.1	68.8	0.42	0.024	0.046
GG	3.8	3.1	0.84	0.795[†]	NA

OR – odds ratio; SPTB – spontaneous preterm birth; [a] – empirical *p*-value from permutation testing; [†] – non-significant association.

Linkage disequilibrium analysis revealed that rs1218585 and rs1218584 were in strong LD ($r^2 = 0.814$), forming a haplotype block (

Figure **4.1**). Haplotype analysis identified three possible haplotypes in the study population: AG, GC, and GG. The AG haplotype, formed by the minor alleles of both SNPs, was significantly more frequent in SPTB patients (28.1%) compared to controls (14.1%) and was associated with increased risk of SPTB (OR = 2.97, $p = 0.009$). The GC haplotype was more frequent in controls (82.1%) than in SPTB cases (68.8%) and was associated with a decreased risk of SPTB (OR = 0.42, $p = 0.0242$). The GG haplotype showed no significant association ($p > 0.05$). Permutation testing confirmed the significance of the AG haplotype (empirical $p = 0.038$) and

the GC haplotype (empirical $p = 0.046$), suggesting that these *KCNN3* haplotypes may influence SPTB (Table 4.5).

4.4 Discussion

PTB remains a major global health concern [46], contributing to infant mortality [52] [53], morbidity [53], and long-term developmental complications [55–61]. Genetic factors play a crucial role in susceptibility to PTB, particularly genes involved in inflammation and uterine physiology [38,134]. The present study investigated the association between two variants in the *KCNN3* gene (rs1218585 and rs1218584) and the risk of SPTB, providing further evidence of the involvement of *KCNN3* in pregnancy outcomes.

The main findings of this study were the association of *KCNN3* rs1218585 and rs1218584 with SPTB. Both SNPs showed a strongly significant association with this condition in all performed analysis.

As previously described, these findings align with the established role of SK3 channels, encoded by *KCNN3*, in uterine physiology. In myometrial smooth muscle cells, potassium channels play a crucial role in maintaining gestation and triggering parturition [471–474]. Their expression and density changes dynamically throughout pregnancy [473,475,476]. Small-conductance calcium-activated potassium (SK) channels are constitutively associated with calmodulin, which regulates their activity by binding intracellular calcium, thus enabling channel opening [477]. This allows for the voltage-independent transmembrane transfer of potassium ions across the cell membrane, generating a hyperpolarization current that promotes smooth muscle relaxation and reducing contractile activity [478]. Alterations in SK expression or activity may impair proper repolarization, potentially leading to aberrant uterine contractility [478]. Among SK family members, the SK3 channels appears to play a particularly significant role in gestation. Studies in both humans and mice support its unique and particularly significant contribution to gestation [471–474]. During pregnancy, SK3 activity is paramount for uterine quiescence, maintaining pregnancy and prevent premature contractions [473,479]. From mid-to-late gestation, its downregulation increases uterine excitability, facilitating labor onset [471,473,475,480]. Consequently, SK3 overexpression may weaken uterine contractility and delay or cause cessation of parturition [481], while premature SK3 dysfunction or reduced expression could enhance uterine excitability and increase SPTB risk [176]. Beyond uterine contractility, SK3 channels are involved in vascular remodeling during pregnancy, regulating

placental blood flow and ensuring proper fetal development [482,483]. These findings highlight the multifaceted role of these channels in pregnancy, suggesting that dysregulations of SK3 activity may impact both uterine activity and gestational hemodynamics.

Genetic variants affecting SK3 expression or function may influence uterine contractility patterns. Within maternal genetic characteristics, Day and colleagues (2011) were the first to investigate *KCNN3* in the context of SPTB. They analyzed 16 SNPs and identified rs1218585 and rs1218584 as associated with this condition, along with another newly discovered SNP [176]. The authors found that rs1218585 is in the intronic region between exons 1 and 2. This region is highly conserved among mammalian species and includes the promotor and alternate exon 1c. This exon encodes an inhibitory SK3 isoform, described as a dominant-negative suppressor of the native SK3 channel activity at the cell surface [484]. This finding supports the hypothesis that *KCNN3* variants influence SPTB pathophysiology [176]. Additionally, another *KCNN3* variant identified in the same study, rs883319, has been linked to PTB in subsequent studies [177,178], reinforcing the potential role of this gene in pregnancy outcomes. However, the SNPs rs1218585 and rs1218584 have not been independently investigated or confirmed in later investigations. As described, the study by Day et al. (2011) was the first to report an association between rs1218585 and rs1218584 and SPTB. However, some limitations should be considered. First, the authors did not provide raw genotype or allele frequency data, making it difficult to fully assess the robustness of their findings. Additionally, their maternal DNA analysis appears to rely solely on genotype distribution comparisons between groups, without a clear explanation of the statistical tests applied. Another key limitation is that, although initial associations were identified, they did not remain significant after correction for multiple comparisons.

In the present study, we re-examined these SNPs in maternal DNA using a case-control design and a more comprehensive statistical approach. Our findings reveal a strong and statistically robust association between both *KCNN3* rs1218585 and rs1218584 and SPTB in all the analysis performed, including within the logistic regression framework, even under the application of stringent significance corrections using the BH, BY, and Bonferroni methods.

As in the pilot study presented in the previous chapter, we did not observe any homozygous individuals for the minor allele of rs1218585 or rs1218584 in maternal DNA. According to data from the 1000 Genomes Project, the expected frequency of homozygotes for these SNPs is approximately 12% and 16%, respectively, in the overall population and about 23% for both SNPs in the European population. However, they were completely absent in our cohort of pregnant

women. This finding raises the possibility that minor allele in homozygosity could have a deleterious effect on pregnancy, potentially reducing the likelihood of carrying a pregnancy to term. Unfortunately, Day and colleagues do not provide raw genotype frequencies in their maternal DNA analysis, leaving it unclear whether they observed a similar pattern, or if it is related to our small sample size. Although our study does not establish a direct causal link, the observed genotype distribution suggests that *KCNN3* polymorphisms may influence pregnancy viability. Further investigations using larger cohorts with longitudinal follow-up are needed to clarify potential effects on fetal survival and pregnancy outcomes.

It is important to notice that several studies have demonstrated that sociodemographic factors are associated with PTB, namely maternal education [470,485–487] and marital status [467,488]. However, in our study, none of the analyzed sociodemographic or lifestyle characteristics showed significant differences between cases and controls. To account for potential confounding, we performed logistic regression analyses adjusting for these variables, and the associations between *KCNN3* polymorphisms and SPTB remained statistically significant. These findings suggest that the observed genetic associations are independent of the evaluated demographic and lifestyle factors.

Additionally, LD analysis revealed a strong correlation between rs1218585 and rs1218584, suggesting the formation of a haplotype block. Notably, the haplotype composed of the minor alleles of both SNPs was also significantly associated with SPTB, further supporting the potential role of these *KCNN3* variants in pregnancy outcomes.

The main limitation of this study, despite the significant associations observed, is the limited sample size (N = 110), which may affect the robustness of our findings and precludes further stratified analyses, such as assessing potential differences among PTB subtypes. This is the first independent study to replicate and confirm the association of rs1218585 and rs1218584 with SPTB risk. However, larger studies with diverse cohorts are needed to confirm these associations and explore the potential biological mechanisms involved, which could pave the way for novel therapeutic strategies to prevent SPTB.

4.5 Final considerations

The primary limitation of this study is the relatively small sample size, which may have reduced the power to detect subtle genetic effects, particularly for rare variants. The absence of

homozygotes for the minor alleles may also affect the interpretation of the results, as this reduces the ability to assess the full spectrum of genetic variation. Notwithstanding, if the minor allele has a deleterious effect, the lack of homozygotes may reflect a selection bias or a severe impact on pregnancy. Larger studies with increased sample sizes are necessary to validate these findings and confirm the role of *KCNN3* in PTB susceptibility. Additionally, functional analyses are necessary to investigate the mechanistic basis for the observed associations. Understanding how *KCNN3* variants modulate SK3 channels activity in uterine smooth muscle could provide valuable insights into the pathophysiology of SPTB and guide future therapeutic strategies. Furthermore, studies involving diverse populations are essential to explore the potential interactions between genetic variants and environmental factors that may influence SPTB risk.

In conclusion, this Chapter highlights the potential role of *KCNN3* variants rs1218585 and rs1218584 in SPTB susceptibility. Building upon the hypotheses proposed in the pilot study presented in Chapter 2, this is the first independent study to replicate the association of these variants with SPTB risk. These findings reinforce the hypothesis that genetic variations in genes involved in uterine excitability, such as *KCNN3*, play a role in the onset of preterm labor. This work lays the foundation for further research with larger cohorts and functional analyses to confirm these results and elucidate the mechanistic pathways through which these variants may influence pregnancy outcomes.

5

ASSOCIATION OF INTERLEUKIN-1 RECEPTOR ANTAGONIST (*IL1RN*) RS4251961 WITH RISK OF PERIODONTITIS IN A PORTUGUESE POPULATION

Submitted for publication:

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5.1 Introduction

According to the latest WHO global oral health report, oral diseases represent a significant public health issue for countries and populations worldwide despite often not being publicly recognized. The global number of cases of oral diseases is estimated to be 1 billion higher than the combined cases of all five main noncommunicable diseases [283]. PD is the second most prevalent oral disease, representing over 1 billion cases in 2019. The estimated global prevalence of severe periodontitis in adults and elderly individuals is at least 16.9% [283], and the 2019 Global Burden of Disease study revealed that oral diseases, including severe PD, cause 23.1 million disability-adjusted life-years worldwide [294]. Moreover, epidemiological, clinical interventional, and experimental studies have demonstrated that PD adversely impacts systemic health, connecting this disease to several chronic conditions, such as type 2 diabetes, cardiovascular disease, Alzheimer's disease, and certain cancers, among others [297–299]. PD is a chronic inflammatory disease that affects the integrity of the tissues supporting the teeth and results from a complex interplay between microbiome equilibrium disruption and the host immune response [289]. Despite the many risk factors associated with PD, rates of disease progression vary from individual to individual with similar environments and habits, indicating a genetic influence. It is already known that up to one-third of PD variance is attributed to genetic factors, and in the case of higher disease severity, heritability has an increased weight [291]. Among the genes already suggested to be associated with PD, those related to inflammatory pathways are predominant; however, the results of the studies are inconsistent. The IL-1 and TNF-α are the most studied inflammatory cytokine families in PD [489,490]. TNF-α is a pro-inflammatory cytokine associated with the pathogenesis of PD [313,491]. It is ubiquitously present in inflammatory responses and plays a critical role in the expression of many chemokines and other cytokines [492]. IL-1α, IL-1β and IL-1RA are ligand members of the IL-1 family, produced and secreted by many cells, playing a crucial role in activating and controlling inflammatory responses [493]. IL-1α and IL-1β are pro-inflammatory cytokines [489,494], while IL-1RA is the principal endogenous IL-1 antagonist, competing with the IL-1 receptor, reducing IL-1α and IL-1β bioactivity, thus acting as an inflammatory control protein [495,496]. The pro-inflammatory response is initiated and persists or ends depending on the relative levels of IL-1RA and IL-1 [497]. Higher levels of IL-1RA reduce the severity of the inflammation [498–500]. Supporting these findings, IL-1RA knockout mice reveal excessive inflammatory responses and develop different spontaneous inflammatory diseases [500–502].

Finally, the clinical relevance of IL-1RA is also shown by the FDA approval of Anakinra, a recombinant human IL-1RA, for the treatment of rheumatoid arthritis, cryopyrin-associated cyclic syndrome, and systemic juvenile idiopathic adult-onset arthritis [503].

The most studied polymorphisms in cytokine-encoding genes related to PD are the *IL1A* rs1800587 (-899), *IL1B* rs1143634 (+3953/4), *IL1B* rs16944 (-511), and *TNFA* rs1800629 (-308) [238,322–324]. However, the results of the different studies are inconsistent. Several candidate gene studies associated the *IL1A* rs1800587 with PD [329–331,504–506], while others reported no association [255,327,328,507]. Both *IL1B* polymorphisms revealed the same trend, as some studies showed a relation between *IL1B* rs1143634 [329,508,509] or *IL1B* rs16944 [328,329,510] and PD, while others failed to demonstrate a significant correlation [255,327,342,511,512]. Like *IL1A* and *IL1B*, case-control studies examining the association between *TNFA* rs1800629 and PD susceptibility have shown inconsistencies [349,353]. The authors explain contradictory results by variations in the applied criteria and partially due to the geographic distribution of specific gene variants [238,293] and sex dimorphism in PD, which is not entirely understood but may be related to the differences in immune response between men and women [355].

Although IL-1RA plays a crucial role in defending against PD [489], variants in its encoding gene, *IL1RN*, have been poorly addressed in PD, and *IL1RN* rs4251961 has never been studied in this context. *IL1RN* rs4251961 reduces IL-1RA expression, increasing pro-inflammatory activities of IL-1α and IL-1β [500,513]. Thus, this variant is likely to be present in PD patients.

The present pilot study aimed to identify possible associations between *IL1A* rs1800587, *IL1B* rs1143634, *IL1B* rs16944, *IL1RN* rs4251961, and *TNFA* rs1800629 and the risk of PD. It addresses issues previously reported by narrowing down origin and sex dimorphism. Thus, this study was conducted in a Portuguese women cohort. The study found a significant association between *IL1RN* rs4251961 and PD and a moderately significant association for *TNFA* rs1800629, underscoring the importance of these variants as potential genetic markers for the disease. Underlying biochemical mechanisms support both associations.

5.2 Materials and methods

5.2.1 Study population

The study population consisted of postpartum women followed at the Puerperium of the Service of Gynecology and Obstetrics from HGO, Portugal, recruited between March 2, 2020, and

May 5, 2022. The study protocol was approved by the HGO ethics committee (56/2019). All the women provided fully informed written consent to participate and completed the questionnaire, which included questions regarding social-demographic and lifestyle characteristics, such as level of education, occupation status, smoking habits, toothbrush frequency, and floss usage. Clinical diagnosis and blood sample collection were carried out up to 72 hours after delivery. All experimental methods throughout the study were conducted in accordance with the Declaration of Helsinki and the ethical principles for medical research involving human subjects.

5.2.2 Selection of cases and control subjects

A full-mouth periodontal examination was performed for PD diagnosis. No radiographic examination was performed. All fully erupted teeth, except for the third molars, dental implants, and retained roots, were evaluated via a manual periodontal North Carolina probe (Hu-Friedy, Chicago, Illinois, USA). The number of missing teeth, plaque index, gingival recession (REC), periodontal probing depth (PPD), and bleeding on probing were recorded at six sites per tooth. PPD was measured as the distance from the free gingival margin to the bottom of the periodontal pocket, and REC was defined as the distance from the cementoenamel junction to the free gingival margin. The clinical attachment level was calculated as the algebraic sum of the REC and PPD measurements. Furcation involvement was assessed using a 2 N probe (Hu-Friedy; Chicago, Illinois, USA) in molars and upper first premolars. PD cases were defined according to the American Association of Periodontology consensus [514,515]. Following examination, each woman was informed of their periodontal status and, if diagnosed with periodontal disease (either gingivitis or periodontitis), was referred to the Egas Moniz Dental Clinic for treatment without cost. Among the 95 subjects, 33 women had PD. The remaining subjects were eligible for use as healthy controls, but seven women were excluded due to missing data on the variables under study; thus, 55 women were enrolled as control subjects. Immediately after PD diagnostics, a 2 mL peripheral blood sample was collected in EDTA-coated tubes from all the enrolled participants (patients and controls) who were fully anonymized using a code by the hospital personnel so that the molecular laboratory technician was blinded to the patients' information and the group allocation. The samples were immediately transported under refrigeration to the molecular laboratory at Egas Moniz School of Health & Science or the NOVA FCT Lab and kept at 4°C (for a maximum of 1 day) before further processing for molecular analysis.

5.2.3 DNA extraction and genotyping

DNA was extracted using the NZY Tissue gDNA Isolation Kit (NZYTECH) according to the manufacturer's instructions and stored at -80°C until use. Genotyping was carried out at CEGEN-FPGMX (Fundación Pública Galega de Medicina Xenómica, Santiago de Compostela, Spain) using the iPLEX Gold technology (Agena Bioscience) as a service. Five single nucleotide polymorphisms (SNPs) in *IL1A* (HGNC ID: 5991), *IL1B* (HGNC ID: 5992), *TNFA* (HGNC ID: 11892), and *IL1RN* (HGNC ID: 6000) genes were typed, namely, *IL1A* rs1800587 (-889), *IL1B* rs1143634 (+3954), *IL1B* rs16944 (-511), *IL1RN* rs4251961 (-1129), and *TNFA* rs1800629 (-308).

5.2.4 Statistical analysis

Statistical analysis of the questionnaire data, including sample characterization, descriptive statistics and assessment of the differences between groups for sociodemographic and lifestyle characteristics (using chi-square/Fisher's exact test or Student's t-test) were performed using R v.4.2.1. The chi-square test was used to evaluate SNPs within the healthy control group for potential deviations from Hardy-Weinberg equilibrium. Additionally, logistic regression models were used to assess the role of genetic predictors in explaining the presence or absence of PD. Firstly, the likelihood ratio test was applied to estimate the global effect of the predictors on PD. Secondly, whenever such effect was significant, the Wald test was applied to determine whether the odds ratio for each predictor category was significant relative to a reference category. Adjustments to the significance of the results in each model were implemented using multiple correction methods, to control the FWER and the FDR in view of the number of simultaneously tested hypotheses. A total of ten logistic models were constructed, involving the five genetic variants under study – *IL1RN* rs4251961, *TNFA* rs1800629, *IL1A* rs1143634, *IL1B* rs16944, and *IL1B* rs1800587. For each gene, two models were considered, namely (i) the full models, with all three genotypic categories, and (ii) the 'collapsed models', in which the two possible genotypes with the minor allele collapsed into one category, with the homozygote for the major allele being the alternative genotype (reference genotype). All procedures in the logistic regression analysis of the data were implemented using IBM SPSS Statistics (version 29).

Given the simultaneous testing of 10 models, three correction methods were applied to control the FWER and the FDR: Bonferroni, Benjamini-Hochberg, and Benjamini-Yekutieli. For the results interpretation, predictors were considered 'strongly significant' if the significance is

demonstrated by adjusted p-values below 0.05 under BH and Bonferroni corrections and between 0.05 and 0.1 under BY. Predictors were considered 'moderately significant' if they showed significance after BH correction, but not under Bonferroni or BY corrections, suggesting a potential association with PD that warrants further exploration. Finally, predictors that failed to achieve significance after adjustment were considered 'non-significant'. Observed power calculations and computations of the required sample size to achieve the 80% power threshold were performed with the GPower 3.1.9.6 software.

5.3 Results

5.3.1 Demographic and lifestyle information of the control group and PD group

Table 5.1 shows the demographic and lifestyle data of periodontitis patients and healthy subjects. The study included 55 controls, aged 31.9±5.97 years, and 33 patients with PD, aged 30.2±5.8 years. There were no significant differences in the age parameter between the two groups ($p > 0.05$). In addition, the numbers of women who smoke in the control and PD groups were six (10.9%) and five (15.2%), respectively. There were also no significant differences in smoking status between the two groups. Concerning the individuals' ancestry, most of the subjects identified themselves as European descent in both groups, 83.6% in the control group and 87.9% in the PD group. No significant differences were found in any demographic or lifestyle parameters, namely, level of education, occupation status, marital status, daily frequency of tooth brushing, or usage of floss.

Table 5.1. Demographic and lifestyle characteristics of patients with PD and healthy controls

	Control (N=55)	**PD (N=33)**	***p* value**
Age			
Mean (SD)	31.9 (5.97)	30.2 (5.80)	0.206
Median [Min, Max]	32.0 [18.0, 43.0]	30.0 [22.0, 43.0]	
Ancestry			
European	46 (83.6%)	29 (87.9%)	0.320
Education level			

	Control (N=55)	PD (N=33)	*p* value
Uneducated	1 (1.8%)	1 (3.0%)	0.807
Elementary school	7 (12.7%)	4 (12.1%)	
High school	25 (45.5%)	18 (54.5%)	
Graduate	22 (40.0%)	10 (30.3%)	
Occupation status			
Student	2 (3.6%)	1 (3.0%)	1.000
Active	40 (72.7%)	24 (72.7%)	
Unemployed	13 (23.6%)	8 (24.2%)	
Marital status			
Single	21 (38.2%)	16 (48.5%)	0.774
Cohabiting	14 (25.5%)	8 (24.2%)	
Married	17 (30.9%)	8 (24.2%)	
Divorced	3 (5.5%)	1 (3.0%)	
Smoker			
Yes	6 (10.9%)	5 (15.2%)	0.741
Daily tooth brushing frequency			
Once or less	5 (9.1%)	2 (6.1%)	0.725
Twice	37 (67.3%)	20 (60.6%)	
Three or more	13 (23.6%)	10 (30.3%)	
Missing	0 (0%)	1 (3.0%)	
Floss usage			
No	13 (23.6%)	12 (36.4%)	0.213
Occasionally	4 (7.3%)	4 (12.1%)	
One to three times a week	16 (29.1%)	4 (12.1%)	
Everyday	22 (40.0%)	12 (36.4%)	
Missing	0 (0%)	1 (3.0%)	

N – number of subjects; PD – patients with periodontitis; SD – standard deviation.

5.3.2 Genotype frequencies in PD patients and healthy controls

DNA from all subjects was genotyped for five selected SNPs (*IL1A* rs1800587, *IL1B* rs1143634, *IL1B* rs16944, *IL1RN* rs4251961, and *TNFA* rs1800629) to evaluate their putative associations with PD risk. The five SNPs in the control group fulfilled the HWE criteria ($p > 0.05$). Table 5.2

shows significances and their adjustments of the results in each of the models applied to assess the global effect of the genetic predictors. Significant associations for specific genetic variants were detected under the logistic regression framework, particularly for *IL1RN* rs4251961, and more moderately for *TNFA* rs1800629. The genotype frequencies of the two significant SNPs in the PD patients and healthy controls are presented in Table 5.3, which also shows the results of the Wald test to the odds ratio (OR) in the corresponding models.

Table 5.2. Results of likelihood tests of global effects of genetic predictors: ordered *p*-values and their adjustments for the 10 logistic models

Model	*p* value	BH Adjusted *p*	BY Adjusted *p*	B Adjusted *p*
rs4251961_coll	0.003	0.025*	0.073	0.030*
rs4251961	0.005	0.025*	0.073	0.050*
rs1800629_coll	0.014	0.047*	0.137	0.140
rs1800629	0.029	0.073	0.212	0.290
rs1143634	0.046	0.092	0.269	0.460
rs16944_coll	0.194	0.323	0.947	1.000
rs16944	0.417	0.573	1.000	1.000
rs1143634_coll	0.543	0.573	1.000	1.000
rs1800587	0.559	0.573	1.000	1.000
rs1800587_coll	0.573	0.573	1.000	1.000

B – Bonferroni; BH – Benjamini-Hochberg; BY – Benjamini-Yekutieli corrections; coll – collapsed model; * Polymorphisms with statistically significant associations with PD (adjusted $p < 0.05$).

Table 5.3. Genotype distributions of *IL1RN* rs4251961 and *TNFA* rs1800629 in patients with PD and controls, significances of the Wald test results, and power calculations

SNP	Genotype	Control	PD	OR	95% CI	*p* value	*p* value[a]	Observed Power	Sample size 80% power
		N (%)	N (%)						
Il1RN rs4251961	TT	27 (49.1)	6 (18.2)	1 (ref)					
	TC	23 (41.8)	18 (54.6)	3.52	1.20 - 10.35	0.022	0.022(BH), 0.033(BY), 0.044(B)	0.493	154
	CC	5 (9.1)	9 (27.3)	8.10	1.98 - 33.05	0.004	0.008(BH), 0.012(BY), 0.008(B)	0.758	97
	TC + CC	28	27	4.34	1.55 - 12.16	0.005	0.005(NA)	0.520	138
TNFA rs1800629	GG	45 (81.8)	19 (57.6)	1 (ref)					
	GA + AA	10	14	3.32	1.25 – 8.77	0.016	0.016(NA)	0.494	161

B – Bonferroni correction; BH – Benjamini-Hochberg correction; BY – Benjamini-Yekutieli correction; CI – confidence interval; N – number of subjects; OR – odds ratio; PD – patients with periodontitis; ref – reference genotype; SNP – single nucleotide polymorphism; [a] – Adjusted *p*-value.

The *IL1RN* rs4251961 is a strongly significant predictor for the PD, as the significance of both the full and the collapsed model for this SNP is demonstrated by adjusted *p*-values below 0.05 under BH and Bonferroni corrections. The adjusted *p*-value above 0.05 but below 0.1 under BY corrections also suggested the effect of the predictor (Table 5.2). As shown in Table 5.3, the IL1RN/TC genotype was associated with significantly higher odds of PD (OR = 3.52, 95% CI 1.20-10.35, all adjusted *p*-values < 0.05), compared with reference genotype TT. The IL1RN/CC genotype showed 8.10 times higher odds of PD (95% CI 1.98-33.05, all adjusted *p*-values < 0.05). Additionally, the collapsed model (TC+CC vs TT) showed 4.34 times higher odds of PD (95% CI 1.55-12.16, *p*-value < 0.05). The distribution of IL1RN rs4251961 genotypes in controls and PD patients is depicted in Figure 14, to illustrate the significant differences in genotype frequencies between cases and controls. The *TNFA* rs1800629 showed a significant association with PD in the 'collapse' model (GA+AA vs GG) under BH correction, but not under Bonferroni or BY corrections, suggesting a potential association with PD that warrants further exploration. Nevertheless, the results suggest 3.32 higher odds of PD for the collapsed model, compared with the reference genotype *TNFA* /GG (OR = 3,32, 95% CI 1.25 – 8.77, BH adjusted *p*-value < 0.05). Despite the significant associations, the observed statistical power was lower than the desired 80% (Figure 5.1). Based on these results, the estimated sample size required to strengthen the robustness of these results is 161 subjects.

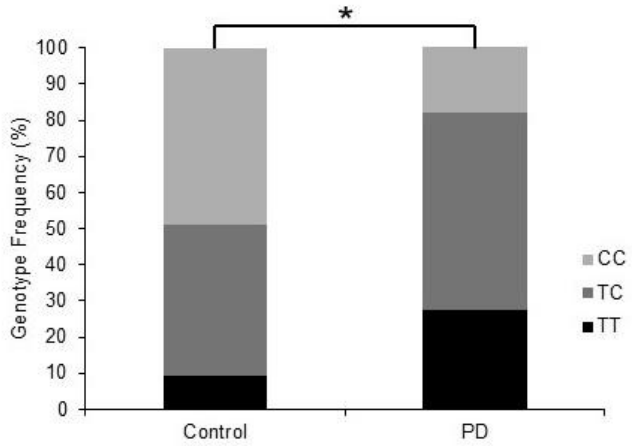

Figure 5.1. The genotype distributions of *IL1RN* rs4251961 in healthy controls and patients with PD are summarized. * Adjusted *p*-value ≤ 0.05

5.4 Discussion

PD is a significant public health issue due to its high prevalence, leading to disability and tooth loss. It also adversely affects general health, contributing to social inequality and decreasing quality of life [286]. PD results from a complex interplay between the disruption of microbiome equilibrium and the host immune response [289,516], influenced by the individual's genetic background [291,517]. As previously described, studies suggest that some variations in cytokines and related genes modulate PD susceptibility, although this association remains unclear for many of the genes studied. These inconsistencies are partially due to the geographic distribution of specific gene variants and sex dimorphism in PD [238,293,355]. Accordingly, in the present pilot study, we investigated the potential associations of *IL1A* rs1800587, *IL1B* rs1143634 and rs16944, *TNF-α* rs1800629, and *IL1RN* rs4251961 with PD in a Portuguese women cohort.

The main findings of the present study were the associations of the polymorphisms in *TNFA* and *IL1RN* with PD. *TNFA* rs1800629 showed a moderately significant association with PD, while *IL1RN* rs4251961 revealed a strongly significant association with the disease for both the full and the collapsed models, even under BH and Bonferroni corrections. It is noteworthy to mention that this is the first study with the *IL1RN* rs4251961 variant in the PD context.

TNF-*α* is one of the earliest and most important mediators of the inflammatory response and a pro-inflammatory cytokine involved in bone turnover. Higher levels of TNF-*α* increase osteoclast formation, which is responsible for dissolving old and damaged bone tissue [518]. Thus, genetic variants in its encoding gene that lead to overexpression or produce a higher activity protein contribute to bone loss. The genetic variant analyzed in this study (rs1800629/-308) is a promotor polymorphism linked to enhanced *TNFA* expression [346,347], potentially contributing to the propensity for PD. The most recent meta-analysis of 24 studies also showed a positive association between this *TNFA* variant and PD in the collapsed model (AA+GA vs GG) in overall analyses (OR = 1.46, 95% CI 1.12-1.89). However, after controlling for ancestry, the association between *TNFA* rs1800629 and PD was found to be non-significant in populations of European descent across all tested models [354]. Interestingly, Li and colleagues (2020), in a meta-analysis of 36 studies, registered that most of the eligible studies were conducted on Asian populations, with a significant lack of research on other groups, reporting that future genetic association studies should also explore the relationship between *TNFA* variants and PD in other populations [519]. *IL1RN* rs4251961 has never been studied previously

in the context of PD; however, the correlation found was not surprising due to (i) IL-1RA role in inflammation and (ii) the previous association of *IL1RN* rs2234663 with PD. Regarding the first point, IL-1RA controls both IL-1 pro-inflammatory actions [500,513] and bone remodeling [520]. The IL-1RA levels increase late during the development of an inflammatory event, allowing an induced acute inflammation to resolve and preventing it from becoming chronic and damaging healthy cells [499,500]. An imbalance between IL-1 and IL-1RA predisposes to local inflammatory diseases, such as inflammatory arthritis [521], arteritis [521], and inflammatory bowel disease [522]. Additionally, as IL-1 stimulates osteoblastic activity and osteoclastic differentiation [314,315], a decrease in IL-1RA function leads to higher levels of IL-1β, increasing the risk of bone loss [523], which in turn heightens susceptibility to PD [505], marginal bone loss around dental implants [524], and female osteoporosis [525]. Addressing the second point, the most studied variant of *IL1RN*, an 86-bp VNTR (rs2234663), which decreases the IL-1RA expression, has been linked with several inflammatory and autoimmune diseases, including ulcerative colitis [250,526], systemic lupus erythematosus [527], Graves' disease [528], and even PD [254,255,334]. Remarkably, the *IL1RN* variant investigated in this pilot study, rs4251961, is a SNP that is associated with lower concentrations of serum IL-1RA than those previously recorded with *IL1RN* VNTR [529–531]. Although *IL1RN* genetic variants were not fully explored in PD, it is well known that IL-1RA plays a defense role in the disease, and its deficiency contributes to severe periodontal tissue destruction [489,532].

TNFA and *IL1RN* genetic variants exhibit significant variability across different ancestries and between sexes, influencing health outcomes and inflammatory response [255,334,526,533–535]. Our pilot study considered the previous concerns on the frequency of genetic variants across different ancestries, explicitly addressing the lack of research on the *TNFA* rs1800629 variant in European descent populations. Additionally, we considered sex differences, focusing exclusively on women. Our findings reveal a significant association between the *IL1RN* rs4251961 variant and PD within the logistic regression framework. Notably, despite the application of stringent significance corrections using the Benjamini-Hochberg and Bonferroni methods, the effect of this genetic variant on disease susceptibility remained robust. These findings underscore the importance of this polymorphism as a potential genetic marker for the disease. Moreover, moderately significant results for *TNFA* rs1800629 also suggest its relevance for PD. The three other studied variants, *IL1A* rs1800587, and *IL1B* rs1143634 and rs16944, were non-significant predictors. All failed to achieve significance after adjustment, reflecting weaker associations or insufficient evidence of an effect on PD.

As expected, the main limitation of our study is the sample size (N = 88), which may be insufficient for detecting moderate effect sizes, especially given the binary nature of the outcome. The power was further reduced by multiple testing adjustments, with Bonferroni, Benjamini-Hochberg, and Benjamini-Yekutieli corrections lowering the significance threshold, increasing the likelihood of Type II errors. Additionally, the effect size, while notable (e.g., OR = 3.52 for the *IL1RN*/TC genotype), is not large enough to compensate for the sample size constraints, particularly if the distribution of subjects across genotype categories is unbalanced. The wide confidence intervals, especially for the *IL1RN*/CC genotype, further suggest instability in the estimates due to the limited number of events.

5.5 Conclusion

Overall, this study successfully detects an effect but future studies with larger sample sizes are needed to confirm and validate the relationship between the *IL1RN* rs4251961 and PD in European ancestries. Notwithstanding, the present study enriches the scientific knowledge on PD susceptibility background associating a new variant, *IL1RN* rs4251961, and contributes to understanding the weight of sex dimorphism and ancestry to genetic association studies. In conclusion, our findings underscore the importance of *IL1RN* rs4261961 as a new potential genetic marker for PD.

6

TOWARDS UNDERSTANDING OF GENETIC SUSCEPTIBILITY LINKING PRETERM BIRTH AND PERIODONTITIS

6.1 Introduction

This chapter focuses on possible common genetic variants underlying SPTB and PD. These are two clinically distinct conditions that share a strong inflammatory component. As discussed in Chapter 1, the relationship between these conditions has been extensively explored over the past few decades, with epidemiological studies reporting both positive associations [364–377] and null findings [169,379–386]. These inconsistencies have been largely attributed to variations in study design, population characteristics [231,360–362], diagnostic criteria, and methodological approaches [360–363]. Despite the discrepancies, the biological plausibility of a connection between these two conditions is widely recognized, and PD has been considered a risk factor for PTB for a long time. Two main mechanisms have been proposed to explain how PD might contribute to PTB [302,360,403]: (i) translocation of periodontal pathogens or their products to the fetal-placental unit and (ii) the initiation of a systemic inflammatory cascade originating in periodontal tissues. Although the direct translocation hypothesis is more widely accepted, inconsistencies in detecting oral pathogens in the fetal compartment [398,420,421] and the presence of oral bacteria in healthy pregnancies [360,419] have led some authors to propose the presence of a placental microbiome, which is similar to the oral microbial ecosystem [422,423]. These conflictual findings mirror the broader uncertainty regarding the relationship between PD and PTB. Similarly, studies assessing the effect of PD treatment during pregnancy have yielded mixed results: while some recent reviews report a decrease in PTB risk following treatment [398,405,406], others conclude that the evidence remains insufficient and that further studies are needed to clarify this relationship [397,398,403,404,406–408].

Given the immune system's central role in PD and PTB, genetic predisposition to an exaggerated inflammatory response has been hypothesized as a potential unifying factor [400,451,458]. It is possible that groups of individuals are genetically primed to mount stronger inflammatory reactions, thereby increasing their vulnerability to both conditions in the presence of some challenge [400,458]. Although genetic studies have investigated PTB and PD separately, some authors have theoretically suggested an overlapping genetic substrate, such as variants in innate immunity genes (e.g., *DEFB1*, *MBL2*), which have been associated with both conditions in different studies [124]. However, no study has yet tested this hypothesis within the same population.

Building on this rationale, the present chapter investigates the role of selected inflammation-related gene variants' contribution to a general inflammatory susceptibility phenotype.

Specifically, we analyzed four candidate SNPs – *IL1RN* rs4251961, *TLR1* rs5743618, *IL6* rs2069827, and *IL6R* rs4845617—based on their functional relevance and previous associations with inflammation in preliminary analysis. The main goal was to determine whether these variants are linked to a generalized proinflammatory profile.

6.2 Materials and methods

6.2.1 Study design

This chapter presents an integrative analysis based on the 73 candidate SNPs selected in Chapter 3, aiming to evaluate whether a shared genetic predisposition to inflammation can explain susceptibility to either SPTB or PD. To test this hypothesis, a combined phenotype ("inflammation") was defined as the presence of at least one of the two conditions. Statistical analyses were applied using this composite outcome, focusing on identifying variants associated with generalized inflammatory susceptibility rather than condition-specific associations.

6.2.1.1 Definition of variables

Participants were categorized as follows:

- **Inflammation group**: women diagnosed with at least one of the following inflammatory conditions: SPTB and/or PD.
- **Control group**: women with neither diagnosis.

6.2.1.2 Sample population

The sample consisted of 126 postpartum women recruited from the Puerperium Service of the Gynecology and Obstetrics Department at HGO, Portugal, between March 2, 2020, and February 6, 2023. These women were the same participants included in the previous studies, with slight variations in sample sizes due to the continued progression of data collection. Of the total sample, 59 women had at least one inflammatory condition (SPTB and/or PD), while 67 had neither diagnosis (control group). The distribution of participants by group and clinical condition is summarized in Table 6.1. Further details regarding eligibility criteria, clinical diagnostic, and sample processing are described in chapter 3 (sub-subsections 3.2.1.1-3.2.1.3). Due to missing data in some variables (i.e., SNP genotypes or covariates), the sample size

varied slightly between analyses. The final sample size used in each statistical model is indicated in the respective results' tables.

Table 6.1 Distribution of participants by group and conditions

Group	N	SPTB	PD	Both conditions
Inflammation	59	21†	26	12
Control	67	0	0	0

N – number of subjects; PD – periodontitis; SPTB – spontaneous preterm birth; † – partial missing data in PD diagnosis for 10 SPTB cases

6.2.2 Genotype and variants selection

Genotyping was conducted using iPLEX Gold technology (Agena Bioscience). From the initial panel of 73 SNPs related to inflammatory pathways, variants were excluded if they deviated from HWE ($p < 0.05$) in the control group or if they presented a MAF lower than 5%. These filtering steps were conducted using PLINK (v1.9).

To explore their potential contribution to inflammatory susceptibility, the 73-SNP panel was screened using PLINK through genotypic association test (comparable to a chi-square test for allele frequency differences), and Fisher's exact test. This preliminary analysis aimed to identify variants nominally associated with inflammation ($p < 0.05$). Based on these results and biological plausibility, four SNPs—*IL1RN* rs4251961, *TLR1* rs5743618, *IL6* rs2069827, and *IL6R* rs4845617—were selected for further analysis.

6.2.3 Statistical analysis

Descriptive statistics and comparisons between groups for sociodemographic and lifestyle characteristics were performed using R (v4.2.1). Categorical variables were compared using chi-square or Fisher's exact test, and continuous variables using Student's t-test.

Genetic association analyses were conducted using IBM SPSS Statistics (v29.0). For the primary analysis, logistic regression models were applied to test associations between each SNP and the inflammation phenotype (defined as SPTB and/or PD presence). An initial set of

models without covariates was used to estimate the unadjusted OR, providing baseline correlations between genotypes and the outcome (under the codominant model).

Subsequently, adjusted logistic regression models were implemented, including covariates identified as confounders through prior chi-square analyses. Multiple inheritance models – additive, dominant, and recessive – were also explored to better understand potential genotype-phenotype relationships.

Multiple testing corrections were applied using B, BH, and BY methods for significant associations under the codominant model. Observed statistical power and the sample size required to achieve 80% power were calculated using G*Power (v3.1.9.6). These calculations were also not extended to exploration analyses under alternative genetic models.

Finally, to evaluate whether genetic associations persisted independently of the co-occurrence of SPTB and PD, an interaction term (SPTBxPD) was computed and included as a covariate in a secondary adjusted model. This approach aimed to isolate variants related to the broader inflammatory phenotype, regardless of the most severe clinical expression (i.e., women affected by both conditions).

6.3 Results

6.3.1 Demographic and lifestyle information of the control group and the Inflammation group

Table 6.2 presents the demographic and lifestyle data of women in the inflammation group and the control group. The study included 67 controls, aged 31.5 ± 5.53 years, and 59 patients with inflammation, aged 30.9 ± 6.02 years. There were no significant differences in the age parameter between the two groups ($p > 0.05$). In addition, no significant differences were observed between groups for ancestry, education level, occupation status, marital status, number of children, smoking habits, or dietary patterns. Gestational age at delivery was significantly lower in the inflammation group compared to control ($p < 0.001$), and floss usage also differed significantly between groups ($p = 0.02$).

Table 6.2 Demographic and lifestyle characteristics of patients with PD and healthy controls

	Control (N=67)	Inflammation (N=59)	*p* value
Age			
Mean (SD)	31.5 (5.53)	30.9 (6.02)	0.579
Median [Min, Max]	32.0 [19.0, 43.0]	31.0 [18.0, 43.0]	
European Ancestry			
No	11 (16.4%)	7 (11.9%)	0.611
Yes	56 (83.6%)	52 (88.1%)	
Education Level			
Less than secondary graduation	11 (16.4%)	12 (20.3%)	0.533
Secondary graduation	28 (41.8%)	28 (47.5%)	
Higher education	28 (41.8%)	19 (32.2%)	
Occupation Status			
Unemployed	18 (26.9%)	13 (22.0%)	0.649
Student	1 (1.5%)	2 (3.4%)	
Employed	48 (71.6%)	44 (74.6%)	
Occupation			
No income	12 (17.9%)	7 (11.9%)	0.135
Low income	31 (46.3%)	38 (64.4%)	
High income	24 (35.8%)	14 (23.7%)	
Marital Status			
Unpartnered	25 (37.3%)	29 (49.2%)	0.381
Cohabiting	20 (29.9%)	16 (27.1%)	
Married	22 (32.8%)	14 (23.7%)	
Gestational Age			
<28	0 (0%)	5 (8.5%)	< 0.001*
28-31	0 (0%)	6 (10.2%)	
32-36	0 (0%)	21 (35.6%)	
≥37	67 (100%)	27 (45.8%)	
Previous PTB			
No	63 (94.0%)	52 (88.1%)	0.345
Yes	4 (6.0%)	7 (11.9%)	
Smoker			
No	61 (91.0%)	47 (79.7%)	0.122
Yes	6 (9.0%)	11 (18.6%)	

	Control (N=67)	Inflammation (N=59)	*p* value
Missing	0 (0%)	1 (1.7%)	
Ex-smoker			
No	51 (76.1%)	36 (61.0%)	0.119
Yes	16 (23.9%)	22 (37.3%)	
Missing	0 (0%)	1 (1.7%)	
Daily Tooth Brushing Frequency			
Once	7 (10.4%)	8 (13.6%)	0.798
Twice	44 (65.7%)	38 (64.4%)	
Three or more	16 (23.9%)	12 (20.3%)	
Missing	0 (0%)	1 (1.7%)	
Floss Usage			
Never	15 (22.4%)	19 (32.2%)	0.018*
Occasionally	2 (3.0%)	8 (13.6%)	
One to three times a week	23 (34.3%)	9 (15.3%)	
Everyday	27 (40.3%)	22 (37.3%)	
Missing	0 (0%)	1 (1.7%)	
Dairy Consumption			
Once a week	1 (1.5%)	4 (6.8%)	0.513
Once a day	18 (26.9%)	13 (22.0%)	
Twice a day	34 (50.7%)	29 (49.2%)	
Three times a day	14 (20.9%)	13 (22.0%)	
Cereals Consumption			
Occasionally	2 (3.0%)	3 (5.1%)	0.107
Once a week	7 (10.4%)	0 (0%)	
Once a day	25 (37.3%)	22 (37.3%)	
Twice a day	23 (34.3%)	25 (42.4%)	
Three or more times a day	10 (14.9%)	9 (15.3%)	
Other Autoimmune/Chronic Inflammatory Diseases			
No	61 (91.0%)	52 (88.1%)	1
Yes	6 (9.0%)	6 (10.2%)	
Missing	0 (0%)	1 (1.7%)	
Family History of Chronic Diseases – First Degree Relatives			

	Control (N=67)	Inflammation (N=59)	*p* value
No	47 (70.1%)	44 (74.6%)	0.691
Yes	20 (29.9%)	15 (25.4%)	
Early Childhood Feeding			
Unknown	3 (4.5%)	5 (8.5%)	0.692
Formula-fed	12 (17.9%)	11 (18.6%)	
Breast feeding	52 (77.6%)	43 (72.9%)	

N – number of subjects; SD – standard deviation. * – Parameter with statistically significant difference between control and PTB groups ($p < 0.05$).

6.3.2 Variants filtering and selection

The *PTGS2* gene polymorphism rs20417 was excluded from the analysis due to genotype failure. Three additional SNPs (rs2228144, rs5743595 and rs5950506) were excluded for deviating from HWE ($p < 0.05$) in the control group. Thirteen variants were excluded due to MAF being below 5%. From the initial 73-panel, 56 SNPs remained for statistical analysis. A preliminary analysis of these 56 SNPs was performed using PLINK to screen for nominal associations ($p < 0.05$) with the inflammation phenotype. Both the genotypic association test and Fisher's exact test were applied, yielding highly similar results; thus, only the association test outputs are presented in Table S. 1. Based on these results and biological plausibility, four candidate SNPs—*IL1RN* rs4251961, *TLR1* rs5743618, *IL6* rs2069827, and *IL6R* rs4845617—were selected for further analysis.

6.3.3 Genotypes distribution in inflammation group and controls

Genotype distributions and association results under the codominant model for *IL1RN* rs4251961, *TLR1* rs5743618, *IL6* rs2069827, and *IL6R* rs4845617 are presented below. Table 6.3 shows the unadjusted logistic regression results, while Table 6.4 presents the analyses adjusted for gestational age and floss usage, identified as confounding variables through preliminary chi-square analyses of crosstabulations (Table S. 2). Secondary exploration analyses under additive, dominant, and recessive inheritance models were also performed and are summarized in Table 6.5.

Table 6.3 Genotype distributions and logistic regression results for *IL1RN* rs4251961, *TLR1* rs5743618, *IL6* rs2069827, and *IL6R* rs4845617 in women with inflammation and controls. Only *p*-values < 0.05 were considered for multiple testing correction and power estimation

SNP	Genotype	Control	Inflammation	OR	95% CI	*p* value	*p* value[a]	Observed power	Sample size 80% power
		N (%)	N (%)						
	TT	31 (45.0)	21 (36.2)	1(ref)					
Il1RN rs4251961	TC	29 (43.9)	24 (41.4)	1.20	0.50-2.85	0.688			
	CC	6 (9.0)	13 (22.4)	3.38	0.99-11.5	0.051[†]			
TLR1 rs5743618	AA	29 (43.9)	20 (34.5)	1(ref)					
	AC	29 (43.9)	18 (31.0)	0.84	0.34-2.04	0.693			
	CC	8 (12.1)	20 (34.5)	3.65	1.24-10.7	**0.018**	0.144(B), 0.072(BH), 0.196(BY)	0.849	
Il6 rs2069827	GG	54 (81.8)	55 (94.8)	1(ref)					
	GT	11 (16.7)	3 (5.2)	0.28	0.07-1.17	0.081			
	TT	1 (1.5)	0 (0.0)	NA		NA			
Il6R rs4845617	GG	21 (31.8)	27 (46.6)	1(ref)					
	GA	34 (51.5)	29 (50.0)	0.66	0.29-1.50	0.323			
	AA	11 (16.7)	2 (3.4)	0.12	0.02-0.66	**0.015**	0.12(B), 0.072(BH), 0.326(BY)	0.685	160

Note: N = 124 women (58 with inflammation, 66 controls) included in this analysis after excluding two cases with missing genotype data.

B – Bonferroni correction; BH – Benjamini-Hochberg correction; BY – Benjamini-Yekutieli correction; CI – confidence interval; N – number of subjects; OR – odds ratio; ref – reference genotype; SNP – single nucleotide polymorphism; a – Adjusted *p*-value; † – association at the borderline lack of statistical significance ($p = 0.051$); The *p*-value in bold indicates statistical significance.

Table 6.4 Genotype distributions and logistic regression results for the association between *IL1RN* rs4251961, *TLR1* rs5743618, *IL6* rs2069827, and *IL6R* rs4845617 with inflammation, adjusted for confounding factors. Only *p*-values < 0.05 were considered for multiple testing correction and power estimation.

SNP	Genotype	Control	Inflammation	aOR	95% CI	*p* value	*p* value[a]	Observed power	Sample size 80% power
		N (%)	N (%)						
	TT	31 (45.0)	20 (35.1)	1(ref)					
Il1RN rs4251961	TC	29 (43.9)	24 (42.1)	1.45	0.44-4.78	0.542			
	CC	6 (9.0)	13 (22.8)	5.53	1.24-24.74	**0.025**	0.2(B), 0.196(BH), 0.679(BY)	0.561	220
TLR1 rs5743618	AA	29 (43.9)	20 (35.1)	1(ref)					
	AC	29 (43.9)	18 (31.6)	1.21	0.37-3.99	0.751			
	CC	8 (12.1)	19 (33.3)	4.09	1.00-16.69	**0.049**	0.392(B), 0.196(BH), 0.666(BY)	0.811	
Il6 rs2069827	GG	54 (81.8)	54 (94.7)	1(ref)					
	GT	11 (16.7)	3 (5.3)	0.23	0.02-2.11	0.192			
	TT	1 (1.5)	0 (0.0)	NA	NA	NA	NA		
Il6R rs4845617	GG	21 (31.8)	26 (45.6)	1(ref)					
	GA	34 (51.5)	29 (50.9)	1.41	0.46-4.34	0.550			
	AA	11 (16.7)	2 (3.5)	0.20	0.02-1.97	0.167			

Note: N = 123 women (57 with inflammation, 66 controls) included in this analysis after excluding cases with missing data (two genotype; one covariate).

aOR – odds ratio adjusted for gestational age and floss usage; B – Bonferroni correction; BH – Benjamini-Hochberg correction; BY – Benjamini-Yekutieli correction; CI – confidence interval; N – number of subjects; ref – reference genotype; SNP – single nucleotide polymorphism; a – Adjusted *p*-value; The *p*-value in bold indicates statistical significance.

Table 6.5 Exploratory analysis of genetic associations in women with inflammation and controls, under additive, dominant, and recessive models, adjusted for confounding factors. Only *p*-values < 0.05 were considered for multiple testing correction

SNP	Model	aOR	95% CI	*p* value	*p* value[a]
	Additive	2.02	0.99-4.10	0.053	
Il1RN rs4251961	Dominant	2.32	0.81-6.62	0.118	
	Recessive	5.09	1.33-19.52	**0.018**	0.216(B), 0.108(BH), 0.503(BY)
TLR1 rs5743618	Additive	1.82	0.94-3.52	0.074	
	Dominant	1.47	0.53-4.13	0.463	
	Recessive	4.45	1.36-14.58	**0.014**	0.168(B), 0.108(BH), 0.782(BY)
Il6 rs2069827	Additive	0.20	0.02-1.66	0.135	
	Dominant	0.17	0.02-1.43	0.104	
	Recessive	NA	NA	NA	
Il6R rs4845617	Additive	0.71	0.33-1.55	0.390	
	Dominant	1.09	0.38-3.09	0.878	
	Recessive	0.17	0.02-1.48	0.107	

Note: N = 123 women (57 with inflammation, 66 controls) included in this analysis after excluding cases with missing data (two genotype; one covariate).

aOR – odds ratio adjusted for gestational age and floss usage; B – Bonferroni correction; BH – Benjamini-Hochberg correction; BY – Benjamini-Yekutieli correction; CI – confidence interval; SNP – single nucleotide polymorphism; a – Adjusted *p*-value; The *p*-value in bold indicates statistical significance.

Unadjusted logistic regression results (Table 6.3) showed that the *TLR1* rs5743618/CC genotype was significantly associated with higher odds of inflammation compared to the AA reference group (OR = 3.65, 95%CI 1.24-10.7, $p = 0.018$). For *IL6R* rs4845617, the AA genotype was associated with reduced odds of inflammation (OR = 0.12, 95%CI 0.02–0.66, $p = 0.015$). Although neither association remained statistically significant after correction for multiple comparisons, the observed statistical power for the *TLR1* rs5743618 association was 84.9%. The observed power for *IL6R* rs4845617 was 68.5%, and the estimated sample size required to confirm this result with adequate power (>80%) is 160 participants. The *IL1RN* rs4251961/CC genotype showed an association with higher odds of inflammation (OR = 3.38, 95%CI 0.99-11.5) at the borderline lack of statistical significance ($p = 0.051$). No significant associations were observed for *IL6* rs2069827.

After adjusting for gestational age and floss usage (Table 6.4), the *TLR1* rs5743618/CC genotype remained significantly associated with inflammation (aOR 4.09, 95% CI 1.00-16.69, $p = 0.049$), consistent with the findings from the unadjusted analysis. In contrast, the *IL6R* variant did not remain statistically linked to inflammation. Additionally, the *IL1RN* rs4251961/CC genotype had significantly increased odds of inflammation compared with the TT reference group (aOR 5.53, 95% CI 1.24–24.74, $p = 0.025$). These associations did not remain statistically significant after correction for multiple testing; however, consistent with the first analysis, the observed statistical power for *TLR1* rs5743618 was higher than 80% (81.1%). The observed power for *IL1RN* rs4251961 was 67.9%, and the estimated sample size required to strengthen the robustness of this result is 220 subjects.

Exploratory analyses under alternative inheritance models (Table 6.5) revealed significant associations under the recessive model for *IL1RN* rs4251961 (aOR = 5.09, 95%CI 1.33-19.52, $p = 0.018$) and *TLR1* rs5743618 (aOR = 4.45, 95%CI 1.36-14.58, $p = 0.014$).

6.3.3.1 Genetic association with inflammation-adjusted model including SPTBxPD interaction

To test whether specific genetic variants remained associated with the inflammation phenotype independently of the presence of SPTB and PD, we performed a logistic regression analysis adjusted for the interaction term SPTB×PD (Table 6.6 and Table 6.7). This model allowed us to control for the possible confounding effect of the most severe clinical manifestation —

individuals presenting both conditions — and evaluate genetic contributions to a shared inflammatory susceptibility.

Table 6.6 Logistic regression results for the association between *IL1RN* rs4251961, *TLR1* rs5743618, *IL6* rs2069827, and *IL6R* rs4845617 with inflammation, adjusted for the SPTBxPD interaction term. Only *p*-values < 0.05 were considered for multiple testing correction and power estimation

SNP	Genotype	Control	Inflammation	aOR	95% CI	*p* value	*p* value[a]	Observed power	Sample size 80% power
		N (%)	N (%)						
	TT	31 (47.0)	16 (33.3)	1(ref)					
Il1RN rs4251961	TC	29 (43.9)	21 (43.8)	0.75	0.27-2.06	0.576			
	CC	6 (9.1)	11 (22.9)	2.89	0.75-11.11	0.122			
TLR1 rs5743618	AA	29 (43.9)	16 (33.3)	1(ref)					
	AC	29 (43.9)	16 (33.3)	1.20	0.43-3.37	0.723			
	CC	8 (12.1)	16 (33.3)	4.46	1.28-15.58	**0.019**	0.152(B), 0.152(BH), 0.465(BY)	0.778	122
Il6 rs2069827	GG	54 (81.8)	46 (95.8)	1(ref)					
	GT	11 (16.7)	2 (4.2)	0.38	0.07-1.98	0.251			
	TT	1 (1.5)	0 (0.0)	NA	NA	NA	NA		
Il6R rs4845617	GG	21 (31.8)	22 (45.8)	1(ref)					
	GA	34 (51.5)	25 (52.1)	0.86	0.33-2.19	0.746			
	AA	11 (16.7)	1 (2.1)	0.10	0.01-0.95	**0.044**	0.352(B), 0.176(BH), 0.538(BY)	0.736	124

Note: N = 114 women (48 with inflammation, 66 controls) included in this analysis after excluding cases with missing data (two genotype; 10 SPTB cases without PD diagnosis).

aOR – odds ratio adjusted for SPTBxPD interaction term (women with both SPTB and PD); B – Bonferroni correction; BH – Benjamini-Hochberg correction; BY – Benjamini-Yekutieli correction; CI – confidence interval; N – number of subjects; ref – reference genotype; SNP – single nucleotide polymorphism; a – Adjusted *p*-value; The *p*-value in bold indicates statistical significance.

Table 6.7 Exploratory analysis of genetic associations in women with inflammation and controls, under additive and recessive models, with SPTBxPD as covariate. Only *p*-values < 0.05 were considered for multiple testing correction

SNP	Model	aOR	95% CI	*p* value	*p* value[a]
Il1RN rs4251961	Additive	1.38	0.75-2.54	0.304	
	Recessive	3.74	1.06-13.21	**0.041**	0.328(B), 0.096(BH), 0.501(BY)
TLR1 rs5743618	Additive	1.74	0.98-3.10	0.060†	
	Recessive	4.14	1.40-12.21	**0.010**	0.080(B), 0.080(BH), 0.245(BY)
Il6 rs2069827	Additive	0.27	0.06-1.28	0.098	
	Recessive	NA	NA	NA	
Il6R rs4845617	Additive	0.50	0.25-1.01	0.054†	
	Recessive	0.12	0.01-1.03	0.053†	

Note: N = 114 women (48 with inflammation, 66 controls) included in this analysis after excluding cases with missing data (two genotype; 10 SPTB cases without PD diagnosis).

aOR – odds ratio adjusted for SPTBxPD interaction term (women with both SPTB and PD); B – Bonferroni correction; BH – Benjamini-Hochberg correction; BY – Benjamini-Yekutieli correction; CI – confidence interval; SNP – single nucleotide polymorphism; a – Adjusted *p*-value; † – Association at the borderline lack of statistical significance ($p = 0.051$); The *p*-value in bold indicates statistical significance.

Table 6.6 presents the logistic regression results under the codominant model for the four selected SNPs, with SPTBxPD as the covariate. Two variants showed a significant correlation with inflammation - The *TLR1* rs5743618 CC genotype was significantly associated with an increased odds of inflammation compared to the AA reference (OR = 4.46, 95%CI 1.28–15.58, $p = 0.019$). The *IL6R* rs4845617 AA genotype showed a protective effect (OR = 0.10, 95%CI 0.01–0.95, $p = 0.044$). Despite the significant associations, the observed statistical power was lower than the desired 80%. Based on these results, the estimated sample size required to strengthen the robustness of these results is 124 subjects. No significant associations were observed for *IL1RN* rs4251961 or *IL6* rs2069827 in the codominant model.

Exploratory analysis under additive and recessive models (Table 6.7) revealed that *TLR1* rs5743618 remained significant in both additive (OR = 1.74, 95%CI 0.98–3.10, $p = 0.060$) and recessive (OR = 4.14, 95%CI 1.40–12.21; $p = 0.010$) models. *IL1RN* rs4251961 was also significantly associated with inflammation under the recessive model (OR = 3.74, 95%CI = 1.06–13.21, $p = 0.041$). None of the associations remained significant after multiple testing corrections.

These results reinforce the possible role of *TLR1* and *IL1RN* variants in predisposing individuals to a heightened inflammatory state, independent of the simultaneous presence of both clinical conditions.

6.4 Discussion

This chapter explored whether specific genetic variants in inflammatory pathway genes may contribute to a shared predisposition to SPTB and PD through a common inflammatory phenotype. From the broader panel of SNPs analyzed, four variants were prioritized for integrative analysis based on their statistical significance and biological plausibility – *IL1RN* rs4251961, *TLR1* rs5743618, *IL6* rs2069827, and *IL6R* rs4845617.

As previously described, inflammation was used as the central phenotype for case definition (SPTB and/or PD), assuming that an exacerbated inflammatory response may underly both conditions. In the primary regression analysis, *TLR1* rs5743618 and *IL6R* rs4845617 showed nominal associations with inflammation. Although neither remained significant after corrections for multiple comparisons, the observed statistical power for the *TLR1* variant was high (84.9%), suggesting a potential signal worthy of further exploration. Given the multifactorial nature of both SPTB and PD, several potential confounders described in the literature and collected through a structured questionnaire were tested. Gestation age and floss usage

emerged as significant confounders in preliminary chi-square analyses and were included in the adjusted logistic regression models. The inclusion of gestation age was expected, as many women classified as "inflammatory" had SPTB. After adjustment for these covariates, *TLR1* rs5743618 remained significantly associated, with homozygosity for the minor allele representing 4-times greater odds of developing inflammation. The observed statistical power exceeded 80% again. The *IL1RN* rs4251961, which had previously shown a borderline association, reached statistical significance, also related to higher odds of inflammation. In the exploratory analysis, both variants reached statistical significance under the recessive model.

Importantly, we introduced a novel approach by adjusting models for the interaction term SPTBxPD, aiming to control for individuals with the most severe manifestation – both conditions simultaneously – and to isolate SNPs robustly associated with the inflammation phenotype. The results were similar to those obtained before adjusting for the conditions: *IL1RN* rs4251961 and *IL6R* rs4845617 showed moderate associations with inflammation, whereas *TLR1* rs5743618 retained the strongest and most consistent signal.

The *IL1RN* rs4251961 was significantly associated with inflammation only under the recessive model. *IL1RN* encodes IL-1RA, a naturally occurring anti-inflammatory cytokine that competitively inhibits the binding of IL-1α and IL-1β to IL-1 receptors, thereby regulating the extent and duration of inflammatory responses [495,496]. As previously described, IL-1RA plays a central role in controlling acute inflammation and preventing excessive tissue damage [497]. Its critical role was demonstrated by the reduced severity of the inflammation in the presence of higher levels of IL-1RA, the development of different spontaneous inflammatory diseases in IL-1RA knockout mice, and finally, the FDA approval of Anakinra, a recombinant human IL-1RA, for the treatment of different inflammatory and autoimmune diseases [503].

Among *IL1RN* variants, the most extensively studied is the VNTR variant rs2234663, which has been associated with reduced IL-1RA expression and increased susceptibility to several inflammatory and autoimmune conditions, including UC [250,526], SLE [527], Graves' disease [528], and PD [254,255,334]. In pregnancy, this VNTR has also been linked to a higher risk of PTB, in maternal [148,153,154] and fetal genomes [148,151,155].

The variant investigated in this study, rs4251961, is located in the *IL1RN* promotor region and, similarly to rs2234663, has been associated with decreased circulating levels of IL-1RA, thereby increasing proinflammatory cytokines IL-1 [500,513]. Although rs4251961 has not been previously studied in PD, its biological effect is consistent with a proinflammatory phenotype. In our study, this SNP was significantly associated with the inflammation phenotype under the

recessive model. Additionally, *IL1RN* rs4251961 was also associated for the first time with higher odds of PD, discussed in Chapter 5. These findings are coherent with the established role of IL-1RA in periodontal defense – reduced IL-1RA contributes to severe periodontal tissue destruction [489,532].

In the context of pregnancy, IL-1β is a critical mediator of labor onset, and IL-1RA serves a protective role by antagonizing excessive IL-1 signaling [536]. Studies in animal models have shown that IL-1RA administration prevents IL-1β-induced PTB, and elevated IL-1RA levels in human amniotic fluid have been linked to fetal responses to intrauterine infection [537]. As discussed above, genetic variants that reduce IL-1RA expression – such as rs2234663 and potentially rs4251961 – may impair this protective buffering mechanism, increasing the risk of APOs.

Taken together, although *IL1RN* rs4251961 has not been extensively investigated in the context of SPTB or PD, its known functional consequences, its association with PD and inflammation in our study, and the broader evidence on *IL1RN* variants support the potential of rs4251961 in modulating systemic inflammatory susceptibility. Although the association in this study was observed only under the recessive model, this may reflect a genetic mechanism in which two copies of the risk allele are required to elicit a detectable inflammatory response. This pattern has been reported for other IL1-related variants and is biologically plausible. The absence of significance in codominant or additive models may also reflect limited power to detect more subtle genotype-phenotype gradients in this small cohort.

The *IL6R* rs4845617 was also weakly associated with inflammation since significance was only obtained under the codominant model and only for individuals homozygous for the minor allele. With the result obtained under this model, we expected to find a correlation, at least for the recessive model, but for both this and the additive model, the results were only borderline in terms of significance, with *p*-values very close to 0.05. The *IL6R* rs4845617 is located in the 5' untranslated region (5' UTR) of the *IL6R* gene. This variant is involved in transcriptional regulation and has been linked to increased *IL6R* expression, particularly of the soluble form (sIL-6R), which facilitates IL-6 trans-signaling [538] – a pathway linked to chronic inflammation. Previous studies have reported an association between the rs4845617 variant and psoriasis [539], and increased plasma levels of sIL-6R have been linked to other chronic inflammatory conditions, including Crohn's disease [540] and adverse pregnancy outcomes [541]. Moreover, a recent study by Farias-Jofre and colleagues (2023) showed that IL-6R blockage can prevent SPTB [542]. In our study, the minor allele of *IL6R* rs4845617 was associated with decreased

odds of inflammation, suggesting a protective effect. Interestingly, the protective effect contrasts with the findings of Farias-Jofre and colleagues, which were obtained in a model of sterile inflammation. In our cohort, infection status was not directly assessed; however, the observed association between lack of floss usage and inflammation in this population suggests that microbial dysbiosis may be contributing to the inflammatory phenotype. This raises the possibility that the rs4845617 variant effect is context-dependent and may confer a beneficial effect in settings where microbial-associated inflammation is present. This interpretation gains support from findings in preeclampsia, where sIL-6R levels were found to be reduced [541], probably a compensatory response to inflammation and IL-6 overexpression. This observation reinforces the notion that IL-6R signaling is tightly regulated and may exhibit distinct roles depending on tissue environment or type of inflammatory challenge. This hypothesis requires further investigation, and future studies in larger cohorts and diverse populations will be essential to confirm and clarify the role of *IL6R* rs4845617 in inflammatory susceptibility.

Lastly, *TLR1* rs5743618 emerged as the most consistent finding. The rs5743618/CC genotype was associated with higher odds of inflammation even after adjustment for SPTBxPD interaction term, with nominal significance observed in codominant model. Although the observed power in this final model dropped slightly below 80%, reflecting the smaller sample size, the strength and consistency of the association remain noteworthy. Furthermore, this result was replicated in both the additive model – considered biologically the most plausible for complex traits – and the recessive model. Notably, this SNP did not reach significance under the dominant model, reinforcing the likelihood of a dose-dependent effect of the minor allele. This pattern supports a recessive or additive model of inheritance, consistent with functional studies reporting a reduction in TLR1-mediated immune responses among homozygous carriers of the minor allele [44,45].

TLRs are key components of the innate immune system, acting as pattern recognition receptors that detect conserved microbial motifs, PAMPs, and endogenous danger signals, DAMPs. Genetic variants in TLR genes have been associated with various chronic inflammatory and autoimmune conditions, including IBD [543] and RA [544], underscoring their broader role in immune regulation beyond pathogen recognition. The *TLR1* variant rs5743618 (I602S) is a non-synonymous SNP located in exon 4, resulting in a substitution of isoleucine for serine at position 602. This polymorphism does not appear to alter the overall expression of the TLR1 protein but affects its signaling capacity, potentially through altered receptor trafficking or conformation. Johnson and colleagues (2007) demonstrated that monocytes from 602S

homozygous individuals exhibit reduced surface expression of TLR1 without a deficiency in total cellular levels, suggesting an intracellular accumulation. This impaired trafficking leads to reduced inflammatory signaling [545]. Conversely, although Hawn and colleagues (2007) reported that individuals with the 602S variant exhibit reduced NF-κB activation and IL-6 secretion in response to bacterial lipopeptides, they did not detect differences in protein expression or localization in their transfected HEK293 cell model [546]. The authors, therefore, proposed that signaling impairment may result from structural alterations within the transmembrane domain that impact receptor activation. Interestingly, another study suggested that although the *TLR1* rs5743618 variant may influence TLR1 membrane localization, its effect is modulated by other polymorphisms, emphasizing that the 602S allele is not alone responsible for surface deficiency and function [547]. In contrast to most reports, Whitmore and colleagues (2016) observed increased TLR1 surface levels in neutrophils from individuals carrying the 602S allele, proposing alternative mechanisms involving interaction with the gp96 chaperone [548]. This immunogenic complexity may help explain why this *TLR1* variant is associated with opposing clinical phenotypes: while the 602S allele has been associated with protection against certain conditions, including leprosy [545,549], it has also been linked to increased risk for other diseases, such as tuberculosis [550] or candidemia [551]. Some authors suggest that evolutionary trade-offs help explain why both alleles persist in the human population, as each may confer selective advantages under different infectious or inflammatory contexts [552]. However, the frequency of the G allele (602S) varies across populations and is considerably higher in Europeans [553–555]. According to the 1000 Genomes Project, it reaches 67% in European populations and approximately 50% in Iberian sub-population.

Even though the exact mechanism remains partially unresolved, there is robust evidence that rs5743618 modulates the intensity of TLR1-mediated immune responses, likely through impaired surface trafficking or conformational changes that affect signaling pathways. Additionally, rs5743618 also modulates the expression of an extensive gene network (432 genes), including the downregulation of inflammatory response genes [555].

In PD context, the primary effect of this variant is likely mediated by its direct impairment of microbial recognition, leading to altered host-pathogen interactions and promoting chronic inflammation. Supporting this hypothesis, an exploratory study identified an association between *TLR1* rs5743618 and increased subgingival levels of *Treponema denticola*, a key periodontopathogen, in a cohort of HIV-positive individuals with PD [556].

Within the PTB context, during pregnancy, TLRs are highly expressed in the placenta, decidua, and fetal membranes playing a critical role in immune surveillance at the maternal-fetal interface. The vaginal and placental environments are not sterile; instead, as discussed previously, they harbor specific and relatively stable microbial communities, even in healthy pregnancies [422,423,557]. Like other mucosal epithelial tissues, these surfaces are continuously exposed to microbial ligands (PAMPs), and epithelial TLRs must strike a delicate balance between immune activation and tolerance [558]. In this context, genetic variants such as *TLR1* rs5743618 may impair this balance. A reduced receptor function due to the rs5743618 variant could lead to inadequate microbial recognition, followed by compensatory and potentially exaggerated inflammatory responses. This dysregulation may be particularly relevant in tissues like the decidua and fetal membranes, where controlled TLRs activation is required to maintain immunological homeostasis throughout gestation.

Notwithstanding, beyond microbial detection, TLR signaling pathways regulate cell proliferation and survival, promote compensatory tissue regeneration, and can suppress apoptosis [559]. Disruption of these regulatory functions also impairs inflammation resolution and prolongs immune activation. In pregnancy, controlled trophoblast apoptosis is an essential physiological process involved in placental development, immune tolerance, and tissue remodeling. As reviewed by Straszewski-Chavez and colleagues (2005), trophoblast cell turnover is tightly regulated by apoptosis throughout gestation, contributing to spiral artery remodeling, trophoblast renewal, and maternal-fetal tolerance [560]. Failure to regulate this balance – either by excessive apoptosis or insufficient apoptotic clearance – has been linked to adverse outcomes such as preeclampsia and intrauterine growth restriction [560,561]. Therefore, genetic variants that alter apoptotic dynamics or prolong unresolved inflammation may increase susceptibility to inflammation-driven PTB. Supporting this possibility, Kinkead and colleagues found that the 602S allele of *TLR1* rs5743618 change neutrophil apoptosis in the context of *Francisella tularensis* infection [552]. Although this SNP has never been studied in the PTB context, other variants in TLR genes, including TLR4 [11,562], TLR1 (in infants) [138], and TLR2 (in mothers) [138,139] have also been reported, suggesting broader involvement of these receptors in pregnancy-related inflammation.

Taken together, these findings reinforce the biological plausibility of our results, as TLR1 plays a multifaceted role in regulating inflammation. Beyond microbial recognition, its function extends to tissue remodeling, epithelial repair, and the resolution of immune responses – processes equally relevant in pregnancy, in periodontitis, and in other chronic inflammatory

contexts. Indeed, chronic diseases such as PD involve not only host-pathogen interactions but also sustain epithelial stress and apoptosis. As highlighted by Huebener and colleagues (2013), failure to properly resolve epithelial inflammation can have profound consequences, as illustrated by disorders like Crohn's disease or atopic dermatitis [558]. In line with this, Kamdar and colleagues (2016) demonstrated that individuals with deficient TLR1 signaling – including carriers of the 602S variant – can develop persistent inflammation and anti-commensal immunity even after the resolution of acute gastrointestinal infection, reinforcing the link between this variant and chronic inflammatory states [563]. Moreover, the contribution of TLR1 to inflammation may be modulated by additional genetic factors. The rs5743618 variant, in particular, has been shown to interact with other SNPs such as *TLR1* rs4833095 [547] or to form risk haplotypes with *TLR2* and *TLR6* variants [564], potentially amplifying its effect on immune susceptibility. While the precise mechanisms remain to be elucidated, it is plausible that, in individuals with a genetic or environmental predisposition to inflammation, the 602S variant promotes an exaggerated or unresolved immune response [548]. Further studies are warranted to clarify the context-dependent effects of this variant and to better define its role in the pathogenesis of inflammatory diseases.

6.5 Conclusion

In this chapter it was explored whether variants in inflammatory genes could help explain a shared susceptibility to SPTB and PD through a common inflammatory phenotype. This integrative approach allows the identification of genetic contributions to an intermediate inflammatory endophenotype, which is potentially involved in the pathophysiology of both conditions.

Our findings support the hypothesis that variants in genes modulating innate immune functions, such as *TLR1*, *IL1RN*, and *IL6R*, may underlie susceptibility to complex conditions where inflammation plays a central role. Notably, the *TLR1* rs5743618 variant remained significantly associated with inflammation even after adjusting for the interaction between SPTB and PD, a variable introduced explicitly to control for the most severe clinical presentation. This suggests that the observed association reflects not merely disease co-occurrence but an independent genetic contribution to the inflammatory phenotype. The fact that this association persisted despite sample size limitations and correction for key confounders reinforces the

biological and statistical robustness of this result. These findings align with prior studies reporting the involvement of *TLR* gene variants in overstated or unresolved inflammatory responses. The role of rs5743618 in epithelial inflammation, microbial sensing, and apoptotic regulation provides a coherent mechanistic link to both periodontal and pregnancy-related inflammatory contexts. Furthermore, our results illustrate the added value of interaction-adjusted regression models in uncovering independent genetic effects in the presence of overlapping clinical conditions.

Despite the exploratory nature of this study and the limited sample size warrant caution, these results highlight promising targets for future investigation. Larger, population-based studies integrating genetic, clinical, and inflammatory biomarker data will be essential to validate and expand on these associations.

7

General Discussion and Final Considerations

The present thesis aimed to investigate the potential existence of a shared genetic predisposition for SPTB and PD, hypothesizing that both conditions may emerge from an exaggerated inflammatory response rather than a causal relationship between them. PD was selected as a model of chronic inflammatory disease due to its prevalence, established but inconclusive link with SPTB, and its relatively easy diagnosis. The empirical approach of this research focused on exploring whether variants in genes involved in inflammatory processes could underlie susceptibility to both conditions rather than one disease being causally linked to the other. The study was conducted in a Portuguese cohort using a case-control design and structured in sequential analyses. Initially, associations were tested separately for SPTB and PD, and later, the inflammatory phenotype was defined as the presence of either or both conditions. This final integrative approach allowed the exploration of potential shared genetic contributions to inflammatory dysregulation, accounting for overlapping features between the two conditions.
Chapter 1 provided a comprehensive review of the literature on the association between inflammation-related genes and the increased risk for PTB. It is highlighted that several specific variants in inflammatory-related genes previously associated with PTB have also been associated with other inflammatory or autoimmune conditions. For instance, the *IL1RN* VNTR variant rs2234663, linked to PTB in both maternal and fetal genomes, has also been associated with SLE [243–246], RA [247–249], IBD [250–253], and PD [254,255]. Likewise, *TLR4 rs4986790*, another variant previously associated with PTB, has been implicated in IBD [250,271,272] and PD [273]. These overlapping associations underscore a potential shared genetic susceptibility to inflammatory-driven pathologies.
Chapter 3 presented the study design, including the development of a socio-demographic and lifestyle questionnaire and the selection of SNPs based primarily on literature associating specific genetic variants with PTB. Given the hypothesis of a shared genetic predisposition to inflammation, the gene panel was further expanded to include variants previously implicated in PD and other chronic inflammatory or autoimmune conditions. In addition, a complementary strategy was implemented using STRING, allowing the identification of further candidate genes with functional links to inflammation, which had not emerged in the initial literature search due to limited or absent prior evidence of association with SPTB or PD. By exploring interaction networks around well-established inflammatory mediators, it was possible to highlight other genes involved in immune regulation, tissue repair, or host-pathogen responses, thereby strengthening the biological rationale for their inclusion in this study. A list of 90 variants in 40 genes was initially selected. After iPLEX mass array design, a final panel of 73 SNPs

in 36 genes were included for genotyping in a pilot case-control study focusing on SPTB. The purpose of this preliminary study was to evaluate the feasibility of the approach and to identify preliminary potential associations with SPTB. Based on the results of the pilot study and considering the limited sample size, it was not feasible to pursue comprehensive analyses for the full SNP panel. Therefore, a more focused strategy was adopted, prioritizing the most promising findings. Two variants in the *KCNN3* gene – rs1218585 and rs1218584 – were selected for further investigation, supported by the preliminary association signals with SPTB and a strong biological plausibility. As previously written in this thesis, *KCNN3* encodes the SK3 potassium channel, which plays a crucial role in regulating uterine contractility. Disruption in the expression or function of these channels may compromise uterine stability, leading to premature uterine contractions and, ultimately, to PTB. An additional criterion for selecting these SNPs was the limited prior literature – although one earlier study had identified a potential association between *KCNN3* variants rs1218585 and rs1218584 and SPTB [176], the findings lacked statistical robustness after correction for multiple testing and had not been independently replicated. Altogether, these factors justified a more in-depth analysis of this gene in a subsequent chapter.

Chapter 4, therefore, aimed to investigate the association between *KCNN3* rs1218585 and rs1218584 and SPTB. The results confirmed a significant association of both SNPs with this condition, including a haplotype comprising both minor alleles. These associations remained significant even after multiple testing corrections. The strong and consistent association of *KCNN3* rs1218585 and rs1218584 with SPTB supports previous findings by Day and colleagues (2011)[176] and reinforces the importance of this gene in pregnancy outcomes. Notably, to the best of our knowledge, no other study has replicated the association between these SNPs and SPTB. In addition to the individual associations of these two *KCNN3* variants, this study is the first to identify and analyze a haplotype comprising the minor alleles of both variants in relation to the SPTB risk. This haplotype analysis revealed a statistically significant association with SPTB, supported by empirical *p*-values obtained through permutation testing. The identification of this associated haplotype reinforces the hypothesis that *KCNN3* variants may play a critical role in uterine physiology and SPTB susceptibility. This novel finding not only strengthens the evidence for the involvement of *KCNN3* in pregnancy outcomes but also highlights the importance of considering variant interactions through haplotype analysis in genetic association studies of complex traits, namely SPTB.

Chapter 5 extended the investigation to PD and identified, for the first time, *IL1RN* rs4251961 as significantly associated with increased odds for this condition. This chapter aimed to investigate the role of inflammatory gene variants in susceptibility to PD in our cohort. The SNPs chosen from the initial panel included those most frequently studied in the PD context, particularly *IL1A*, *IL1B*, and *TNFA*, due to their central roles in cytokine-mediated inflammatory responses. However, despite the well-established involvement of these cytokines in PD pathophysiology, genetic association studies have yielded inconsistent results across populations. This inconsistency highlights an important gap in the knowledge of the genetic basis of PD, which this study helps to address. *IL1RN* rs4251961 was also included, given the pivotal regulatory role of IL-1RA in modulating IL-1-mediated inflammation and bone resorption. In this analysis, *TNFA* rs1800629 showed a modest association with PD, whereas *IL1RN* rs4251961 demonstrated a robust and consistent association across models, remaining significant even after Bonferroni and Benjamini-Hochberg corrections for multiple comparisons. Although rs4251961 had not been previously analyzed in PD, its strong association in our study is consistent with the known regulatory role of IL-1RA in periodontal inflammation. This finding, along with previous evidence linking IL-1RA and *IL1RN* variants to other chronic inflammatory diseases, supports the potential of rs4251961 as a novel genetic marker for PD.

Chapter 6 concluded the investigation by testing the central hypothesis of a shared genetic predisposition to inflammation underlying both SPTB and PD. In this final analysis, a composite inflammatory phenotype was defined, and a subset of four SNPs from the original 73-SNP panel – *IL1RN* rs4251961, *TLR1* rs5743618, *IL6* rs2069827, and *IL6R* rs4845617 – was selected for logistic regression modelling based on preliminary associations. Of these, *TLR1* rs5743618 consistently emerged as the strongest signal across all analytic approaches, with the CC genotype showing an increased inflammation in both unadjusted and adjusted models (including those incorporating relevant confounders and the interaction term SPTBxPD). Despite not retaining statistical significance after multiple testing corrections – a common limitation in studies with relatively small sample sizes and multiple comparisons – the association with the *TLR1* variant showed consistently high statistical power, exceeding 80% in the unadjusted and confounder-adjusted models and remaining close to that threshold even in the final model incorporating the interaction term (SPTBxPD). The *IL1RN* and *IL6R* variants demonstrated more modest and model-dependent associations. In the final analysis, controlling for SPTBxPD, *IL1RN* rs4251961 reached significance only under the recessive model, whereas IL6R rs4845617 was significant only under the codominant model. Both findings are biologically

plausible, given the established roles of IL-1RA and IL-6R in immune regulation and tissue inflammation.

Taken together, the findings from Chapter 6 support the central hypothesis of this thesis – that SPTB and PD may not be causally linked *per se* but instead represent distinct clinical manifestations of a shared genetic predisposition to inflammatory dysregulation. This view challenges the traditional assumption that PD is a direct risk factor for SPTB, aligning instead with more recent evidence suggesting a bidirectional relationship and extending it by proposing a common genetic foundation underlying both conditions. Within this framework, additional genetic and environmental factors likely determine which condition (or set of conditions) ultimately manifests in each individual. Overall, the results strengthen the biological and statistical plausibility of *TLR1* rs5743618 – and, to a lesser extent, *IL1RN* rs4251961 and *IL6R* rs4845617 – as contributors to a systemic inflammatory predisposition. *TLR1* rs5743618, in particular, emerged as the most robust finding across all analytical approaches, with consistently elevated odds of inflammation, reinforcing its potential relevance as a marker of inflammatory susceptibility and justifying further investigation in larger, more integrative studies that combine genetic, immunological, and microbial data.

The main limitation of this study lies in the relatively small sample size, which may have compromised the detection of genetic associations, particularly those involving variants with moderate or subtle effects. Genetic association studies typically require large cohorts to achieve adequate statistical power, especially when multiple genetic variants are tested. The application of multiple testing corrections – although necessary to control for Type I error – further reduces statistical power, increasing the likelihood of Type II error, whereby true associations may remain undetected. In addition, classifying conditions as binary traits further reduced the sensitivity to detect subtle associations, particularly in complex traits such as inflammation-related conditions. Nevertheless, the binary definition was necessary for the consistency of case-control comparisons and aligned with common clinical diagnostic criteria used in genetic studies. The small sample size also amplified the impact of genotype distribution imbalances between groups, which may have compromised the precision of effect estimates, as evidenced by wide confidence intervals in some models.

Furthermore, although participants were recruited at HGO – a reference perinatal Hospital that receives women from various regions, including areas beyond the Lisbon metropolitan area – the study population cannot be considered fully representative of the broader Portuguese or European populations. It is well established that many genes involved in immune

and inflammatory responses show population-specific variation, which may influence both allele frequencies and the strength or direction of genetic associations. Therefore, caution is warranted when generalizing these findings. Replication in independent and larger cohorts is essential to validate the observed associations and confirm their relevance.

Despite these limitations, the findings of this study make a meaningful contribution to addressing the core research questions initially posed at the outset of this research (subsection 3.2.1, study design), which aimed to understand better the origins and mechanisms underlying SPTB. These questions included (i) whether SPTB is primarily driven by environmental or genetic factors ("nurture or nature"), (ii) whether socio-demographic and lifestyle characteristics influence the risk of SPTB in this population, and (iii) whether SPTB reflects a condition-specific genetic predisposition or rather a broader genetic susceptibility to inflammation-related diseases. The results presented throughout this thesis offer partial but meaningful answers to each of these points.

First, concerning the question of whether genetic or environmental factors primarily drive SPTB, the present findings reinforce the significant role of genetic predisposition. While environmental and socio-demographic factors have been linked to SPTB in several studies, a substantial heritable component is recognized as a contributor to this condition. In this study, a notable proportion of women with SPTB reported a previous episode of PTB, consistent with the idea of familial recurrence. Moreover, a robust association was identified between two variants in the *KCNN3* gene (rs1218585 and rs1218584) and SPTB. These findings support the hypothesis that susceptibility to complex traits, particularly SPTB, may result from the combined effect of multiple genetic variants. The role of the *KCNN3* gene in uterine contractility further reinforces its relevance in the etiology of SPTB and justifies its inclusion in future genetic studies on the condition.

Second, regarding the potential influence of socio-demographic and lifestyle characteristics on SPTB risk, the analyses performed in this cohort suggest that most variables commonly reported as potential confounders – including marital status, education level, and economic status – did not significantly affect the association between genetic variants and SPTB. Among all variables tested, only floss usage emerged as a relevant covariate. This result was unexpected, particularly given that all cases of PTB in this study were classified as spontaneous and were not associated with any clinically diagnosed infection.

Although exploratory, this observation raises important questions about the role of immune regulation in maintaining a balanced microbial environment during pregnancy. In this

context, the involvement of the *TLR1* variant rs5743618 – the strongest genetic signal identified – becomes particularly relevant, as it is known to modulate host responses to microbial components. While no causal inference can be drawn, the relation between floss usage and SPTB, along with the involvement of an immune receptor gene, suggests that subtle disruptions in host-microbiome interactions may contribute to the inflammatory process underlying SPTB. These factors should be further explored in future studies that integrate genetic, immunological, and microbiological data.

Third, addressing whether SPTB reflects a condition-specific genetic predisposition or a broader susceptibility to inflammation-related diseases, the findings of this thesis support the latter. The consistent association of the *TLR1* rs5743618 variant with the composite inflammatory phenotype – encompassing both SPTB and PD – suggests a shared genetic underpinning. This variant, known to modulate innate immune responses to microbial components, has been implicated in various inflammatory and infectious conditions, highlighting its role in systemic inflammatory regulation. The association between *TLR1 rs5743618* and the combined phenotype of SPTBxPD suggests that individuals carrying this variant may have an inherent predisposition to exaggerated inflammatory responses to various stimuli. This finding aligns with the central hypothesis of this thesis, which posits that certain genetic factors contribute to a heightened inflammatory state, manifesting in various clinical conditions depending on additional environmental or genetic influences.

In sum, the results of this thesis underscore the importance of considering systemic inflammatory susceptibility in the pathogenesis of complex inflammatory diseases, particularly in SPTB and PD. These results also highlight the need for further research into the role of TLR-1 and related pathways in mediating inflammatory responses during pregnancy. Understanding these mechanisms may open new avenues for targeted interventions aimed at modulating inflammatory responses in susceptible individuals.

Overall, the present work contributes to the current knowledge in the following ways:

First, it provides original data to address the long-standing and unresolved question of whether PTB and PD are mechanistically linked or share common underlying risk factors. While many reviews and meta-analyses have attempted to evaluate this relationship, a closer inspection reveals that most recent publications are not based on new original data but rather on overlapping primary studies. This has led to substantial redundancy, potential bias in the evidence base, and inconsistent conclusions, often unsupported by robust biological data. By adopting a hypothesis-driven, genetics-based approach, the present study makes a novel

contribution to this debate and supports the hypothesis of a shared inflammatory predisposition underlying these conditions.

Second, this study reinforces the scientific value of targeted, hypothesis-driven genetic association studies. While studies using big data, such as GWAS, have revolutionized the study of complex traits, they are often limited in their ability to detect modest or context-specific effects in smaller or more homogenous populations. In contrast, candidate gene association studies enable the careful definition of phenotype, control of confounders, and integration of biological plausibility, making them well-suited for the early-stage discovery of genetic markers in multifactorial conditions such as SPTB. This study demonstrated that, even with a modest small sample size, such approaches can yield biologically meaningful and statistically robust findings – particularly when guided by a clear hypothesis and well-justified SNP selection.

Third, by focusing on inflammation as a unifying biological mechanism between clinically distinct conditions, this work makes a conceptual contribution to the field of complex disease research. Rather than treating SPTB and PD as isolated entities, this thesis proposes that they may represent different clinical manifestations of a shared susceptibility to inflammatory dysregulation. This framework promotes an integrative approach to disease research and underscores the potential for cross-disciplinary approaches to uncover common genetic pathways across seemingly unrelated conditions.

In conclusion, this thesis advances the understanding of how genetic factors may contribute to inflammation-driven conditions by identifying shared molecular signatures in two clinically distinct yet biologically connected phenotypes. Adopting a focused, hypothesis-driven approach not only yields new data but also provides a conceptual model that encourages integrated thinking in the study of complex diseases. As scientific and technological capacities continue to evolve, studies like this one – grounded in biological plausibility and committed to uncovering systemic patterns – will remain essential for translating genetic insights into meaningful clinical and public health impact.

Finally, looking ahead, future research should prioritize the replication of these findings in larger and more diverse cohorts, as well as the functional characterization of the implicated variants to clarify their mechanistic roles in inflammatory diseases, particularly in SPTB. Such efforts will be essential to move from association to understanding – and, ultimately, to intervention. Inflammatory conditions like SPTB and PD carry not only a significant burden for public health systems but also profound consequences for affected families. By contributing to the identification of early genetic markers and potential pathways for targeted prevention,

this line of investigation holds promise for improving maternal and child health outcomes in both clinical and societal contexts.

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A

SUPPLEMENTARY MATERIAL

A.1 Ethics documents

Informação ao Participante

1. ***Identificação do estudo:*** *Este estudo trata-se do desenvolvimento de um projeto de doutoramento, sendo a investigadora responsável a Joana Morais Couceiro da Costa.*

2. ***Objetivo e metodologia:*** *Este trabalho tem como objetivo identificar alterações genéticas envolvidas com a propensão para o parto pré-termo e a periodontite. Caso aceite fazer parte deste estudo, será realizado gratuitamente um diagnóstico de periodontite, por membros investigadores colaboradores, devidamente calibrados. Adicionalmente será colhido sangue por punção venosa, em contexto hospitalar e por pessoal autorizado, para análise genética de polimorfismos específicos das duas condições em estudo. Serão pesquisados um total de 90 variantes genéticas já suspeitos de estarem associados e uma das condições em estudo em 40 genes envolvidos no sistema inflamatório (este número pode diminuir após otimização das técnicas a utilizar). Caso não se encontre qualquer associação com os polimorfismos escolhidos, faz parte do plano de contingência do estudo, a escolha de genes alvo, dentro da lista escolhida inicialmente, para uma análise do gene completo ou ainda uma análise do exoma completo.*
   Relativamente aos dados que serão colhidos de cada sujeito, além das informações que permitam aplicar os critérios de inclusão e exclusão (como idade, recurso a técnicas de reprodução assistida, partos prematuros anteriores ou problemas congénitos, como malformações vaginais), apenas teremos como informação extra se os sujeitos têm alguma doença inflamatória diagnosticada. Estes dados serão fornecidos pelo Hospital Garcia de Orta pelo que, da mesma forma que as amostras, serão imediatamente anonimizados. Assim, todas as amostras e respetivos dados informativos chegarão ao Laboratório de Patologia Molecular e Bioquímica Forense da Egas Moniz com um número de código. A preparação da amostra e extração de DNA será realizada no laboratório referido anteriormente pela investigadora Joana Couceiro da Costa. Após análise das variantes genéticas, as amostras de sangue que sobrarem serão armazenadas na Egas Moniz e destruídas no final do projeto.

   <u>Benefícios esperados e riscos possíveis:</u> Este estudo é observacional, não estando previsto nenhum benefício para as pacientes que dele fizerem parte. Da mesma forma, não estão previstos quaisquer riscos uma vez que apenas será recolhida uma pequena amostra de sangue, por pessoal do Hospital. Não haverá assim qualquer consulta ou exame extra, bem como nenhuma deslocação adicional ao Hospital por participar no estudo.
   Relativamente ao diagnóstico da periodontite é um método não invasivo, pelo que o único incómodo causado às participantes do estudo será a observação da sua cavidade oral.
   Ainda em relação ao estudo da cavidade oral, todas as participantes que integrarem o projeto serão convidadas a realizar uma triagem, de forma gratuita, na Clínica Dentária Egas Moniz Cooperativa de Ensino Superior. Nesta consulta será realizado o rastreio de doenças da cavidade

oral, como por exemplo cáries dentárias (estará também incluído o raio X – ortopantomografia). Após triagem, a paciente é convidada a realizar uma destartarização, também esta gratuita. Nas participantes com doença periodontal em fase ativa será possibilitado o tratamento gratuito da doença. Neste estarão incluídos os seguintes atos clínicos: status diagnóstico (raio X de diagnóstico periodontal); alisamentos radiculares nos locais onde a doente apresenta doença periodontal; 6 a 8 semanas após o último tratamento de alisamento radicular, uma reavaliação periodontal. A Egas Moniz possibilitará a todas as participantes deste estudo a sensibilização para a doença periodontal, que é silenciosa na fase inicial – será entregue um folheto educativo da Ordem dos Médicos Dentistas – assim como o tratamento da doença, em caso de diagnóstico de doença ativa.

3. *Todas as participantes serão voluntárias, sendo que será uma decisão pessoal, fazer parte ou não do estudo. Em qualquer momento poderá recusar continuar a fazer parte do estudo, sem que isso comprometa o relacionamento com o médico ou o respeito pelo direito à assistência que lhe é devida.*

4. *Terá tempo disponível para refletir sobre o pedido de participação, inclusive pode pedir a opinião de familiares e/ou amigos.*

5. *É garantida a privacidade e confidencialidade dos resultados. Como referido acima, todas as amostras recolhidas em contexto hospitalar serão imediatamente anonimizadas. Não será avaliado ou analisado nenhum parâmetro extra que não esteja definido neste documento.*

6. *Por fim, informo que o desenvolvimento deste estudo foi aprovado pela Comissão de Ética do Hospital Garcia de Orta.*

A OMD ACONSELHA A VISITA REGULAR AO MÉDICO DENTISTA

EM CASO DE DÚVIDA CERTIFIQUE-SE QUE O SEU MÉDICO DENTISTA É CREDENCIADO

Este é o modelo de cédula profissional dos Médicos Dentistas inscritos na Ordem dos Médicos Dentistas e por isso legalmente habilitados ao exercício da Medicina Dentária em Portugal.Inclui diversos elementos de segurança, entre os quais um holograma, para evitar possíveis falsificações.

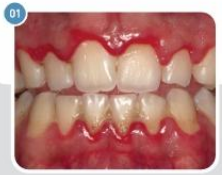
MAIS INFORMAÇÕES EM: www.omd.pt

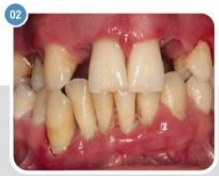
FOLHETOS EDUCATIVOS
ORDEM DOS MÉDICOS DENTISTAS

PERIODONTOLOGIA

01 O QUE SÃO DOENÇAS PERIODONTAIS?

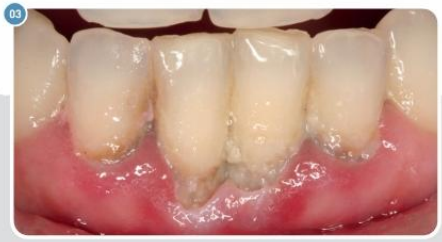
São doenças que afectam os tecidos que envolvem e suportam os dentes – periodonto – que incluem, para além da gengiva, o osso alveolar e outras estruturas responsáveis por manter os dentes firmemente implantados nos maxilares.
As doenças periodontais dividem-se em dois grandes grupos: as gengivites e as periodontites. Nas gengivites há uma inflamação superficial da gengiva, sendo facilmente tratadas, com recuperação total dos tecidos. Nas periodontites há uma destruição das estruturas mais profundas, com reabsorção do osso, e se não tratadas, podem levar à perda do dente. Geralmente não causam dores, mesmo nos casos mais avançados.





02 QUAL A CAUSA DAS DOENÇAS PERIODONTAIS?

A causa mais frequente das doenças periodontais são bactérias. Na boca existem mais de 300 tipos diferentes e muitas delas são potencialmente lesivas para a gengiva. As bactérias que vivem na boca acumulam-se na superfície dos dentes e no sulco gengival, constituindo a placa bacteriana. Quando as bactérias crescem em número ultrapassando um certo nível, produzem as doenças periodontais.



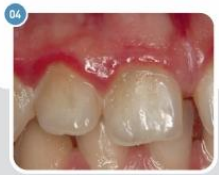
03 AS DOENÇAS PERIODONTAIS SÃO HEREDITÁRIAS?

Para ter periodontite é necessária a presença de bactérias. No entanto, a gravidade das lesões depende da susceptibilidade individual que é uma característica geneticamente determinada. Tendo em conta que hoje em dia pouco podemos fazer para modificar a predisposição genética, a forma de prevenir e tratar as periodontites é através do controlo da placa bacteriana. Frequentemente, as pessoas que têm periodontite, sobretudo as formas mais graves, conhecem parentes próximos que também são afectados, o que representa o carácter familiar-hereditário.

04 COM QUE IDADE SE INICIAM AS DOENÇAS PERIODONTAIS?

As formas mais frequentes aparecem nos adultos, iniciando-se em idades jovens à volta dos 30 anos. Geralmente quanto mais jovem é a pessoa, maior a probabilidade de aparecimento de uma forma grave de periodontite e que necessita de mais cuidados. Raramente as doenças periodontais afectam crianças, mas quando aparecem são formas graves que ameaçam de forma séria a dentição e inclusive a própria saúde geral.
As doenças periodontais estão entre as mais frequentes da raça humana. A gengivite afecta quase a totalidade da população, tanto infantil como adulta. A periodontite afecta quase um em cada dois adultos com mais de 35 anos.

04



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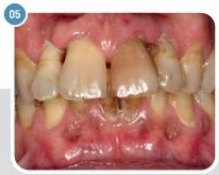


05 COMO SEI QUE A MINHA GENGIVA ESTÁ DOENTE?

Os sintomas são o sangramento espontâneo ou durante a escovagem, o aparecimento de pus na gengiva, mau sabor ou mau hálito, gengiva muito vermelha, retracção da gengiva, alteração da posição dos dentes e até mobilidade dentária. O diagnóstico só pode ser feito pelo médico dentista pelo, que se apresentar qualquer destes sinais, deverá consultá-lo para que o seu caso seja avaliado.

06 É NORMAL QUE A GENGIVA SANGRE?

O sinal que mais precocemente nos avisa da existência de problemas na gengiva é a ocorrência de sangramento gengival espontâneo ou após a escovagem. Uma gengiva que sangra pode apresentar uma gengivite (forma menos grave) ou uma periodontite (forma mais grave). Em casos particulares, como quando associado à toma de certos medicamentos ou em certas doenças, pode haver uma maior tendência ao sangramento gengival.

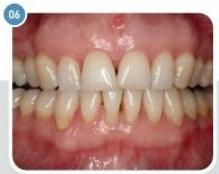
07 A MOBILIDADE DENTÁRIA ASSOCIADA ÀS DOENÇAS GENGIVAIS É REVERSÍVEL?

Nos casos em que a mobilidade não é muito acentuada, pode diminuir após o tratamento. Contudo, pode não desaparecer totalmente porque, geralmente, a quantidade de osso não aumenta após o tratamento da periodontite. Esta é uma das razões que justifica a necessidade de fazer o diagnóstico e tratamento precoce das doenças periodontais.

08 A DOENÇA PERIODONTAL TEM CURA?

O tratamento das doenças periodontais consegue suster o avanço da doença, mas não consegue, na maioria dos casos, curar no sentido de repor os tecidos perdidos. Por outro lado, a periodontite é uma doença crónica, pelo que é fundamental frequentar as consultas regulares de manutenção. Caso contrário, a doença volta a reactivar-se. Nas formas mais agressivas, como nos casos que aparecem em crianças ou adultos jovens, em fumadores de mais de 20 cigarros por dia, ou em algumas doenças sistémicas, como a diabetes não controlada, o tratamento pode não resultar no controlo total da doença, mas no seu avanço mais lento.

06



09 COMO SE TRATAM AS DOENÇAS PERIODONTAIS?

O tratamento tem por objectivo a eliminação das bactérias responsáveis e o controlo dos factores que aumentam a susceptibilidade às doenças periodontais, como o tabaco e algumas doenças sistémicas, criando condições para que a doença se possa manter controlada a longo prazo.
Nas gengivites, é suficiente uma melhoria da higiene oral pelo doente e um tratamento de profilaxia, rápido e fácil. Nas periodontites, o tratamento desenrola-se em várias fases. Em primeiro lugar, realiza-se um estudo periodontal clínico e radiológico para avaliar a situação, fazer um diagnóstico e traçar um plano de acção. Seguidamente, efectua-se a fase básica do tratamento para remoção da placa bacteriana da bolsa periodontal, que se designa como raspagem e alisamento radicular. Em bolsas profundas é necessário fazer, adicionalmente, uma pequena cirurgia periodontal correctiva. Após a doença estar controlada, inicia-se a fase de tratamento de manutenção.

10 A ESCOVAGEM NORMAL É SUFICIENTE PARA PREVENIR A DOENÇA PERIODONTAL?

Não. Como a escova não alcança os espaços interdentários, é necessário usar o fio dentário ou os escovilhões interdentários para manter esses espaços livres de placa bacteriana. Contudo, a utilização destes instrumentos, no início, é difícil e morosa, requerendo alguma aprendizagem e prática. Com o ganho de destreza manual é possível passar o fio e/ou o escovilhão em toda a boca em poucos minutos.
Se ao aplicar o fio dentário sentir mau hálito, significa que esse espaço contém bactérias responsáveis pelas alterações da sua gengiva. Esse cheiro será pior quanto mais tempo passar entre cada higienização interdentária.

O diagnóstico precoce das doenças periodontais é essencial e só o seu médico dentista o poderá fazer correctamente.

Consulte o seu Médico Dentista 2 vezes por ano.

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A.2 Tables

Table S. 1 Minor allele frequencies and association results for 56 SNPs tested for association with the inflammation phenotype, using PLINK genotypic association test (chi-square)

SNP	Minor allele	Control	Inflammation	OR	95% CI	*p* value
		Freq (%)	Freq (%)			
***IL6R* rs4845617**	**A**	**0.42**	**0.30**	**0.59**	**0.35-0.99**	**0.045***
KCNN3 rs883319	T	0.18	0.23	1.36	0.73-2.52	0.327
KCNN3 rs1218585	A	0.15	0.24	1.77	0.94-3.35	0.076
KCNN3 rs1218584	G	0.19	0.25	1.42	0.78-2.57	0.251
PTGS2 rs5275	G	0.37	0.41	1.19	0.72-1.98	0.503
PTGS2 rs689466	C	0.16	0.17	1.1	0.56-2.15	0.784
IL10 rs1800872	T	0.34	0.34	1.01	0.6-1.71	0.958
IL10 rs1800871	A	0.34	0.34	1.01	0.6-1.71	0.958
IL10 rs1800896	C	0.37	0.34	0.86	0.51-1.45	0.572
IL1A rs17561	A	0.34	0.33	0.98	0.58-1.65	0.929
IL1A rs1800587	A	0.38	0.36	0.93	0.56-1.56	0.791
IL1B rs1143634	A	0.25	0.24	0.92	0.51-1.63	0.762
IL1B rs1143627	G	0.36	0.47	1.56	0.94-2.59	0.082
IL1B rs16944	A	0.34	0.43	1.46	0.87-2.42	0.148
***IL1RN* rs4251961**	**C**	**0.31**	**0.43**	**1.73**	**1.03-2.9**	**0.038***
TIMP4 rs17035945	T	0.18	0.14	0.77	0.39-1.52	0.452
***TLR1* rs5743618**	**C**	**0.34**	**0.50**	**1.93**	**1.16-3.23**	**0.011***
CXCL8 rs4073	A	0.50	0.49	0.97	0.59-1.59	0.893
NFKB1 rs4648068	G	0.25	0.24	0.92	0.51-1.63	0.762
TLR2 rs4696480	A	0.52	0.48	0.85	0.52-1.4	0.523
TLR2 rs3804099	C	0.48	0.42	0.8	0.49-1.32	0.391
TLR2 rs5743700	T	0.07	0.04	0.61	0.2-1.89	0.391
IL4 rs2243250	T	0.21	0.28	1.47	0.82-2.62	0.191
CD14 rs2569190	A	0.46	0.48	1.08	0.66-1.78	0.759
EBF1 rs2946169	T	0.30	0.31	1.07	0.63-1.84	0.796
TNFA rs1799964	C	0.22	0.25	1.23	0.69-2.21	0.479
TNFA rs1799724	T	0.10	0.09	0.96	0.41-2.22	0.918

SNP	Minor allele	Control	Inflammation	OR	95% CI	*p* value
		Freq (%)	Freq (%)			
TNFA rs1800629	A	0.10	0.19	1.96	0.95-4.04	0.064
TNFA rs361525	A	0.06	0.10	1.78	0.7-4.52	0.218
VEGFA rs2010963	C	0.33	0.34	1.05	0.62-1.77	0.858
IFNGR1 rs11914	C	0.10	0.12	1.15	0.53-2.53	0.721
IFNGR1 rs7749390	G	0.49	0.47	0.93	0.56-1.54	0.767
IFNGR1 rs1327474	C	0.27	0.31	1.2	0.69-2.07	0.523
***IL6* rs2069827**	**T**	**0.10**	**0.03**	**0.24**	**0.07-0.87**	**0.02***
IL6 rs1800796	C	0.10	0.15	1.68	0.78-3.59	0.18
IL6 rs1800795	C	0.30	0.26	0.84	0.48-1.46	0.528
TLR4 rs4986790	G	0.05	0.06	1.34	0.44-4.12	0.602
TLR4 rs7873784	C	0.17	0.18	1.04	0.54-2.0	0.895
MMP8 rs2155052	C	0.15	0.10	0.65	0.3-1.38	0.258
MMP8 rs11225395	A	0.41	0.42	1.04	0.62-1.75	0.869
MMP1 rs7945189	T	0.10	0.07	0.62	0.25-1.54	0.303
MMP3 rs520540	A	0.48	0.37	0.65	0.39-1.08	0.094
MMP3 rs679620	T	0.46	0.36	0.66	0.4-1.1	0.11
IFNG rs2430561	A	0.37	0.36	0.93	0.56-1.55	0.777
IGF1 rs972936	T	0.28	0.26	0.9	0.52-1.57	0.711
IGF1R rs2229765	A	0.42	0.37	0.83	0.5-1.38	0.466
MMP2 rs243865	T	0.19	0.15	0.75	0.39-1.45	0.387
MMP2 rs2285053	T	0.16	0.15	0.92	0.46-1.81	0.801
MMP2 rs11639960	G	0.30	0.20	0.57	0.32-1.02	0.058
TIMP2 rs2277698	T	0.12	0.14	1.24	0.6-2.58	0.562
TIMP2 rs55743137	G	0.22	0.23	1.07	0.59-1.95	0.813
TGFB1 rs1800469	A	0.34	0.27	0.72	0.42-1.25	0.24
MMP9 rs17576	G	0.37	0.25	0.59	0.34-1.02	0.057
TIMP1 rs4898	C	0.40	0.43	1.16	0.7-1.92	0.555
TIMP1 rs2070584	G	0.37	0.43	1.28	0.77-2.12	0.34
TIMP1 rs5906435	T	0.23	0.27	1.24	0.7-2.19	0.466

CI – confidence interval; Freq – minor allele frequency; OR – odds ratio; SNP – single nucleotide polymorphism; * – polymorphism with statistically significant difference between control and inflammation groups ($p < 0.05$); Rows in bold indicates the four SNPs selected for further analysis

Table S. 2 Chi-square test results for confounding variables significantly associated with the inflammation phenotype

Variable	Categories	Control	Inflammation	*p* value
		N (%)	N (%)	
Gestation Age	<28 weeks	0 (0.0)	5 (8.5)	
	28-31 weeks	0 (0.0)	6 (10.2)	
	32-36 weeks	0 (0.0)	21 (35.6)	
	≥37 weeks	67 (100)	27 (45.8)	
	Total	**59 (100)**	**67 (100)**	**<0.001**
Floss Usage (4 categories)	Never	15 (22.4)	19 (32.8)	
	Occasionally	2 (3.0)	8 (13.8)	
	1-3 times/week	23 (34.3)	9 (15.5)	
	Everyday	27 (40.3)	22 (37.9)	
	Total	**67 (100)**	**58 (100)**	**0.018**
Floss Usage (binary)	Rare			
	Regular	50 (74.6)	31 (53.4)	
	Total	**67 (100)**	**58 (100)**	**0.013**

Note: both the original four-category floss usage variable and a binary version were tested. The binary version grouped 'Never' and 'Occasionally' vs '1-3 times/week' and 'Everyday' to increase statistical power given the limited sample size

2025

JOANA MORAIS COUCEIRO DA COSTA

TOWARDS UNDERSTANDING OF GENETIC SUSCEPTIBILITY LINKING PRETERM BIRTH AND PERIODONTITIS