

INSTITUTO UNIVERSITÁRIO EGAS MONIZ

3º CICLO DE ESTUDOS CONDUCENTE AO GRAU DE DOUTOR EM CIÊNCIAS BIOMÉDICAS

ASSOCIAÇÃO DOS POLIMORFISMOS DOS GENES DA IL-1 E DO IL-1RA COM A PERI-IMPLANTITE

Trabalho submetido por
José Maria Correia Gonçalves Neves Cardoso
para a obtenção do grau de Doutor em Ciências Biomédicas

janeiro de 2024

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Trabalho orientado por
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RESUMO

Os implantes dentários são atualmente uma modalidade cada vez mais previsível e eficaz para a reabilitação de pacientes parcial ou totalmente edêntulos, contribuindo para a qualidade de vida dos pacientes. Este tipo de reabilitação apresenta elevadas taxas de sucesso e sobrevivência, no entanto, podem surgir complicações biológicas. Estas complicações referem-se principalmente a condições inflamatórias associadas à agressão bacteriana, podendo distinguir-se duas variantes clínicas: a mucosite peri-implantar e a peri-implantite. Embora a presença de uma lesão inflamatória seja uma característica comum a ambas as condições, apenas a última está associada à perda de osso de suporte. Existe evidência científica que a mucosite precede a peri-implantite.

Com o enorme aumento do número de pacientes com implantes dentários, a prevalência de condições inflamatórias em torno dos implantes está também a intensificar-se. A peri-implantite está a tornar-se um flagelo e um grave problema de saúde pública, sendo o seu controlo um desafio e estando associada a uma morbilidade significativa. O tratamento da peri-implantite envolve custos elevados para a comunidade e muitas vezes não consegue controlar a progressão da doença, levando à perda do implante. Essa situação de perda do implante está associada a perda óssea o que pode requerer, em alguns casos, procedimentos complexos de regeneração óssea para uma nova colocação de implante ou em último caso inviabilizar de todo uma nova colocação de implante. A impossibilidade de reposição de um implante traz consequências para o paciente a nível de saúde, de função e de estética. Atualmente, não existe nenhum teste que permita determinar a suscetibilidade de um paciente, que vai receber um implante dentário, para a peri-implantite.

No final do século passado, surgiu a hipótese de que a condição genética de um indivíduo podia modular a gravidade e o carácter progressivo das inflamações orais, através da expressão de citocinas e dos seus moduladores. A interleucina-1 (IL-1) é uma citocina multifuncional que desempenha um papel fulcral na patogénese das doenças inflamatórias, com um grande número de atividades e funções diversas na resposta imunitária, inflamação, degradação e homeostasia dos tecidos. A família da IL-1 inclui 11 citocinas, cujos genes

codificadores estão localizados no braço longo do cromossoma 2q. Os dois membros mais extensamente estudados são os genes *IL-1A* e *IL-1B*, que codificam a IL-1 α e a IL-1 β , respetivamente. Dentro da família de proteínas IL-1, existe também o antagonista do recetor da IL-1 (IL-1Ra), que é codificado pelo gene *IL-1RN*. O IL-1Ra desempenha um papel anti-inflamatório, impedindo a transmissão de sinais pró-inflamatórios e prevenindo a resposta imunitária ao utilizar o mesmo recetor que a IL-1 pró-inflamatória.

A evidência científica indica que as complicações biológicas nos implantes dentários tendem a concentrar-se num subconjunto de indivíduos, o que parece implicar que a resposta do hospedeiro pode desempenhar um papel determinante no sucesso implantar. Mesmo quando expostos a fatores de risco semelhantes, nem todos os pacientes desenvolvem peri-implantite, situação que realça a importância da predisposição genética e das diferenças entre populações.

Dado o papel crucial da IL-1 e da IL-1Ra na inflamação, os genes *IL-1A*, *IL-1B* e *IL-1RN* são potenciais candidatos a marcadores genéticos da peri-implantite. No entanto, estudos que analisaram a relação entre esses polimorfismos e a peri-implantite mostraram resultados contraditórios. Assim, antes do trabalho experimental foi realizada uma revisão sistemática e meta-análise para avaliar a associação dos polimorfismos funcionais comuns nos genes *IL-1* e *IL-1RN* com a suscetibilidade à peri-implantite, sintetizando quantitativamente os estudos existentes (**Capítulo 2**). Esta revisão tentou ultrapassar as limitações existentes nas revisões sistemáticas e meta-análises publicadas.

Não é possível retirar conclusões sólidas da literatura atual acerca da possível ligação entre as variações genéticas e a ocorrência de peri-implantite. As diferenças ao nível do desenho dos estudos exigem a realização de mais estudos, com amostras populacionais maiores e de diferentes grupos étnicos. Os estudos têm relatado que a frequência de polimorfismos genéticos associados à suscetibilidade genética a uma determinada doença pode ser influenciada por variações étnicas e raciais. Existem poucos estudos que tentam avaliar esta possível relação no sul da Europa. Neste sentido, o nosso principal objetivo foi investigar se as três variantes genéticas (*IL-1A* - 889C/T, *IL-1B* +

3954C/T e *IL-1RN* ("Variable Number of Tandem Repeats (VNTR)") estavam associadas à ocorrência de peri-implantite numa população Caucasiana Portuguesa.

Antes do estudo principal, foi efetuado um estudo piloto com vinte participantes (**Capítulo 4**) para verificar a exequibilidade do estudo e permitir o cálculo do tamanho da amostra.

Posteriormente, foi efetuado um estudo analítico transversal (**Capítulo 5**), envolvendo um total de 102 indivíduos Portugueses Caucasianos. Os participantes foram divididos em dois grupos: 59 com implantes saudáveis e 43 indivíduos com peri-implantite. Foram obtidas através de esfregaço amostras da mucosa bucal e efetuada a análise genética no Laboratório de Biologia Molecular da Egas Moniz School of Health and Science, pela técnica de *polymerase chain reaction* (PCR) em tempo real para o *IL-1A* e *IL-1B* e através de PCR para o *IL-1RN*. O polimorfismo *IL-1A* -889 C/T mostrou uma maior prevalência do alelo alterado (alelo T) nos casos de peri-implantite do que nos casos saudáveis (27,9% vs 16,9%, respetivamente), embora sem diferenças estatisticamente significativas ($p = 0,060$). Relativamente aos polimorfismos *IL-1B* +3954 C/T e *IL-1RN* (VNTR) a análise revelou que as frequências dos diferentes alelos e dos diferentes genótipos não diferiram significativamente entre os grupos.

Apesar de existirem estudos que tentam relacionar a existência de determinados polimorfismos genéticos e a presença de peri-implantite, com resultados contraditórios, a evidência científica acerca da possível relação das alterações genéticas com alterações a nível bioquímico no fluido crevicular peri-implantar (FCP) é muito escassa.

A possível associação entre estes polimorfismos genéticos e a peri-implantite deve ser baseada numa alteração na resposta imuno-inflamatória do indivíduo. Desta forma, o objetivo da nossa investigação foi também avaliar os níveis dos marcadores da resposta inflamatória IL-1 β , PGE2 e TNF- α no FCP de pacientes com implantes dentários saudáveis e indivíduos com peri-implantite, tendo em consideração os genótipos individuais da IL-1 e do *IL-1RN* dos pacientes. A análise bioquímica foi efetuada na Universidad Complutense de Madrid em 51 dos 102 pacientes que foram submetidos à análise genética (**Capítulo 6**). Para a análise bioquímica foram analisadas as concentrações dos

mediadores inflamatórios no FCP da IL-1 β e TNF- α através de imunoensaios à base de esferas fluorescentes multiplexadas e da PGE2 por *enzyme-linked immunosorbent assay* (ELISA). Nos pacientes em que foi detetada a presença do polimorfismo no gene *IL-1RN*, verificou-se um aumento da concentração dos três mediadores inflamatórios, com diferenças estatisticamente significativas para os valores médios de TNF- α e PGE2, independentemente do estado de saúde peri-implantar ($p = 0,002$ e $p = 0,049$, respetivamente).

Com base nos resultados obtidos neste trabalho, pode-se concluir que não houve correlação entre os polimorfismos *IL-1A* -889, *IL-1B* +3954 e *IL-1RN* e a peri-implantite, na população estudada. Por outro lado, verificou-se que a prevalência destes polimorfismos na população avaliada foi menor do que a observada noutros países europeus. Esta situação poderá hipoteticamente explicar a ausência de diferenças entre os grupos relativamente ao papel destes polimorfismos na ocorrência de peri-implantite. Esta situação realça a importância de desenvolver este tipo de estudos em diferentes grupos étnicos.

Observou-se que indivíduos portadores do polimorfismo do gene *IL-1RN*, independentemente do estado de saúde peri-implantar, apresentam níveis mais elevados de TNF- α e PGE2 no FCP, quando comparados com pacientes sem o polimorfismo, o que poderá colocá-los em maior risco de complicações inflamatórias peri-implantares. Estes resultados devem ser confirmados em estudos com amostras maiores e idealmente com um carácter longitudinal, pois isso permite um melhor controlo dos vários fatores que podem influenciar o início e progressão de uma doença com uma etiologia multifatorial, como é a peri-implantite. Entre os pacientes com genótipo composto positivo (pelo menos um alelo com a sequência de nucleótidos alterada nos genes *IL-1A* e *IL-1B*), observou-se que aqueles com peri-implantite apresentavam maior volume de FCP e maior concentração de PGE2.

Palavras-chave: implantes dentários, peri-implantite, interleucina-1, antagonista do recetor da interleucina-1, polimorfismos genéticos, fluido crevicular peri-implantar

ABSTRACT

Dental implants are currently an increasingly predictable and effective modality for the rehabilitation of partially or totally edentulous patients, contributing to the patient's quality of life. This type of rehabilitation has high success and survival rates, however biological complications may arise. Such complications mainly refer to inflammatory conditions associated with bacterial challenge, and two clinical variants can be distinguished: peri-implant mucositis and peri-implantitis. While the presence of an inflammatory lesion is a common feature of both conditions, only the latter is associated with loss of supporting bone. Mucositis is thought to precede peri-implantitis.

With the massive increase in the number of patients with dental implants, the prevalence of inflammatory conditions around implants is also intensifying. Peri-implantitis is becoming a scourge and a serious public health problem, being their management challenging, and being associated with significant morbidity. The treatment of peri-implantitis involves high costs for the community and often fails to control the progression of the disease, leading to loss of the implant. This situation of implant loss is associated with bone loss, which in some cases may require complex bone grafting procedures for a new implant placement or, as a last resort, may make a new implant placement impossible. The impossibility of replacing an implant has consequences for the patient in terms of health, function and esthetics. There is currently no test that can determine the susceptibility of a patient to peri-implantitis who will receive a dental implant.

At the end of the last century, the hypothesis was generated that the genetic background of an individual may modulate the severity and progressive character of oral inflammations through expression of cytokines and their modulators. Interleukin-1 (IL-1) is a multifunctional cytokine playing a key role in the pathogenesis of inflammatory diseases with a large number of diverse activities and functions in immune response, inflammation, tissue breakdown, and tissue homeostasis. The IL-1 family includes 11 cytokines, whose coding genes are located on the long arm of chromosome 2q. The two most extensively studied members are the *IL-1A*, *IL-1B* genes, which encode IL-1 α and IL-1 β , respectively. Within the IL-1 family of proteins, there is also the IL-1 receptor antagonist (IL-1Ra), which is encoded by the *IL-1RN* gene. IL-1Ra plays an anti-

inflammatory role by preventing the transmission of proinflammatory signals and preventing the immune response by using the same receptor as proinflammatory IL-1.

Scientific evidence indicates that biological complications in dental implants tend to be concentrated in a subset of individuals, which seems to imply that the host response may play a determining role in implant success. Even when exposed to similar risk factors, not all patients develop peri-implantitis, a situation that highlights the importance of genetic predisposition and differences between populations.

Given the crucial role of IL-1 and IL-1Ra in inflammation, the genes *IL-1A*, *IL-1B* and *IL1RN* are potential candidates for genetic markers in peri-implantitis. However, studies that analyzed the relationship between these polymorphisms and peri-implantitis have shown conflicting results. Therefore, a systematic review and meta-analysis was performed, before the experimental work, to quantitatively synthesize existing studies to evaluate the association of common functional polymorphisms in the *IL-1* and *IL-1RN* genes with susceptibility to peri-implantitis (**Chapter 2**). This review attempted to overcome the existing limitations of published systematic reviews and meta-analyses.

No robust conclusions could be drawn from the current literature about the possible link between genetic variations and the occurrence of peri-implantitis. The inequality of the design of the studies requires more studies to be carried out using larger population samples and from different ethnic groups. Studies have reported that the frequency of gene polymorphisms associated with genetic susceptibility to a particular disease can be influenced by ethnic and racial variations. There are few studies that try to evaluate this possible relationship in southern Europe. In this sense our main purpose was to investigate whether the three genetic variants (*IL-1A* - 889C/T, *IL-1B* + 3954C/T and *IL-1RN* (Variable Number of Tandem Repeats (VNTR))) were associated with the occurrence of peri-implantitis in a Portuguese Caucasian population.

Prior to the main study, a pilot study was conducted with twenty participants (**Chapter 4**) to test the feasibility of the study and to calculate the sample size.

Subsequently, a cross-sectional analytical study was carried out (**Chapter 5**), involving a total of 102 Portuguese Caucasian individuals. Participants were

categorized into two groups: 59 with healthy implants and 43 individuals with peri-implantitis. Buccal mucosa samples were swabbed and genetic analysis was performed at the Molecular Biology Laboratory of the Egas Moniz School of Health and Science, using the real-time polymerase chain reaction (PCR) technique for *IL-1A* and *IL-1B* and using PCR for *IL-1RN*. The *IL-1A* -889 C/T polymorphism showed a higher prevalence of the altered allele (T allele) in cases of peri-implantitis than in healthy cases (27.9% vs 16.9% respectively), although without statistically significant differences ($p = 0.060$). For the *IL-1B* +3954 C/T and *IL-1RN* (VNTR) polymorphisms, the analysis revealed that the frequencies of the different alleles and different genotypes did not differ significantly between the groups.

Although there are studies that attempt to link the existence of certain genetic polymorphisms and the presence of peri-implantitis, with contradictory results, the scientific evidence on the possible relationship between genetic alterations and biochemical alterations in the peri-implant crevicular fluid (PICF) is very scarce.

The possible association between genetic polymorphisms and peri-implantitis must be based on a change in the individual's immune-inflammatory. In this way the aim of our investigation was also to evaluate the levels of the inflammatory response markers IL-1 β , PGE2 and TNF- α in the PICF of patients with healthy dental implants and individuals with peri-implantitis, under consideration of the patients' individual *IL-1* and *IL-1RN* genotypes. Biochemical analysis was performed on 51 of the 102 patients who underwent genetic analysis (**Chapter 6**). For the biochemical analysis, the concentrations of PICF inflammatory mediators IL-1 β and TNF- α were analyzed using multiplexed fluorescent sphere immunoassays and PGE2 by enzyme-linked immunosorbent assay (ELISA). In patients where the presence of the polymorphism in the *IL-1RN* was detected, there was an increase in the concentration of the three inflammatory mediators with statistically significant differences for TNF- α and PGE2 mean values, regardless of peri-implant health status ($p = 0.002$ and $p = 0.049$, respectively).

Based on the results obtained in this work, it could be concluded that there was no correlation between *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* polymorphisms and peri-implantitis, on the studied population. On the other hand, it was observed

that the prevalence of these polymorphisms in the population evaluated was lower than in other European countries. This situation may hypothetically explain the absence of differences between groups regarding the role of these polymorphisms in the occurrence of peri-implantitis. This situation emphasizes how important it is to develop this type of studies in different ethnic groups.

It was observed that individuals with the *IL-1RN* gene polymorphism, regardless of their peri-implant health status, have higher levels of TNF- α and PGE2 in the PICF compared to patients without the polymorphism, which could put them at greater risk of peri-implant inflammatory complications. These results should be confirmed in studies with larger samples and ideally with a longitudinal character, as this will allow better control of the various factors that may influence the onset and progression of a disease with a multifactorial etiology such as peri-implantitis. Among the patients with the positive composite genotype (at least one allele with nucleotide sequence changed in *IL-1A* and *IL-1B* genes), it was observed that those with peri-implantitis had a greater volume of PICF and a higher concentration of PGE2.

Keywords: dental implants, peri-implantitis, interleukin-1, interleukin-1 receptor antagonist, genetic polymorphisms, peri-implant crevicular fluid

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ABBREVIATIONS LIST

A: adenine

AAP: American Academy of Periodontology

AINE: non-steroid anti-inflammatory

BL: bone loss

BOP: bleeding on probing

bp: base pairs

C: cytosine

CD14: cluster of differentiation 14

CI: Confidence Interval

CSF-1: colony-stimulating factor-1

dATP: deoxyadenosine triphosphate

dCTP: deoxycytidine triphosphate

ddATP: dideoxyadenosine triphosphate

ddCTP: dideoxycytidine triphosphate

ddGTP: dideoxyguanosine triphosphate

ddNTPs: dideoxynucleotide triphosphates

ddTTP: dideoxythymidine triphosphate

dGTP: deoxyguanosine triphosphate

dNTPs: deoxynucleotide triphosphates

dTTP: deoxythymidine triphosphate

DNA: deoxyribonucleic acid

EFP: European Federation of Periodontology

ELISA: enzyme-linked immunosorbent assay

EMDC: Egas Moniz Dental Clinic

FMPS: full-mouth plaque score

G: guanine

GCF: gingival crevicular fluid

GI: gingival index

HbA1c: glycosylated hemoglobin

HWE: Hardy-Weinberg Equilibrium

IL: interleukin

IL-1RI: interleukin-1 receptor type I

IL-1RII: interleukin-1 receptor type II

IL-1Ra: interleukin-1 receptor antagonist

JB: Joanna Brigs Institute

KDa: kilodaltons

KM: keratinized Mucosa

LPS: lipopolysaccharides

MCP-1: monocyte chemoattractant protein-1

M-CSF: macrophage colony stimulating factor

MIP-1 α : macrophage inflammatory protein-1 alpha

MMP: matrix metalloproteinase

ND: non-defined

OPG: osteoprotegerin

OR: odds ratio

PAI-2: plasminogen activator inhibitor type 2

PA system: plasminogen activator system

PCR: polymerase chain reaction

PD: probing depth

PGE2: prostaglandin E2

PI: plaque index

PICF: peri-implant crevicular fluid

PID: peri-Implant disease

PIG: peri-implantitis group

PMNs: polymorphonuclear neutrophils

PPD: probing pocket depth

RANK: receptor activator of nuclear factor-kappa B

RANKL: receptor activator of nuclear factor-kappa B ligand

RCT: randomized clinical trial

RFLP: restriction fragment length polymorphism

Runx2: runt-related transcription factor-2

SD: standard deviation

SDGs: sustainable development goals

SLA: acid-etched implant surface

SNP: single nucleotide polymorphism

STR: short tandem repeat

SUP: suppuration

T: thymine

Th: T helper

TLRs: toll-like receptors

TNF- α : tumor necrosis factor alfa

TPS: titanium plasma-spraying

VNTR: variable number of tandem repeats

1. INTRODUCTION

1.1. Dental Implants

1.1.1 Dental Implants as a rehabilitation option

The use of dental implants to support fixed and removable protheses is a widely accepted treatment modality with high success and predictability [1–3]. Dental implant therapy is a standard treatment to replace missing teeth. Systematic reviews are currently reporting implant survival rates of about 95% up to 10 years, both for single-tooth restorations and fixed dental protheses [4,5]. Moreover, dental implants improve the physical appearance of a person and provide comfort and convenience, unlike removable dentures. Every year, around 5 million implants are placed in the United States, according to the American Dental Association.

However, dental implants can lose supportive bone, even in cases of successful osseointegration. The main cause of this loss of crestal bone surrounding an implant is local inflammation during the course of peri-implant diseases [6].

1.1.2 Current definitions of peri-implantitis and mucositis

Biological complications affecting osseointegrated implants are a topic of major interest in contemporary dentistry. Such complications mainly refer to inflammatory conditions associated with a bacterial challenge [7,8] Within these complications, two clinical situations can be distinguished: peri-implant mucositis and peri-implantitis. Although the presence of an inflammatory lesion is a feature that both conditions have in common, only the latter form presents loss of supporting bone [9].

According to the most recent classification of periodontal and peri-implant diseases and conditions, peri-implantitis is a pathological condition occurring in tissues around dental implants, characterized by inflammation in the peri-implant mucosa and progressive loss of supporting bone [10]. In the clinical setting, soft

tissue inflammation is detected by probing (bleeding on probing (BOP)), while progressive bone loss is identified on radiographs [11].

It should be noted that the definition of peri-implantitis has varied considerably in different studies, over the years. Peri-implant diseases, including peri-implant mucositis and periimplantitis, were first defined and described at the First European Workshop on Periodontology in Ittingen in 1993 [12]. After that, numerous workshops were held addressing the definition, prevalence and treatment of these diseases [9,13].

1.1.3 Conversion from peri-implant mucositis to peri-implantitis

Mirroring the progression of gingivitis to periodontitis, peri-implant mucositis is assumed to precede peri-implantitis [8]. Both peri-implant diseases are infectious in nature and are caused by bacteria from dental biofilms [7]. Although the presence of bacterial pathogens represents the initial step of the disease process, the ensuing local inflammatory response and the misbalance in the host–pathogen interaction seem to be key factors in the pathogenesis of the tissue destruction in this disease [14].

The peri-implant soft tissue reactions to plaque formation and accumulation have been extensively evaluated in both animal [15–17] and human studies [18,19]. Thus, plaque formation consistently resulted in an inflammation of the peri-implant soft tissues, associated with clinical signs of inflammation, such as redness and edema [17].

According to current knowledge, there is strong evidence, from both animal and human studies, that plaque is the main etiological factor for peri-implant mucositis. There is evidence from experimental human studies that peri-implant mucositis can resolve. Resolution of the clinical signs of inflammation may take more than 3 weeks following reinstitution of plaque/biofilm control [10].

Zitzmann et al. (2001) examined human biopsies after a 21-day period of plaque formation [18]. The histologic analysis revealed the establishment of a B and T cell-dominated inflammatory cell infiltrate in the soft tissue lateral to the barrier epithelium, occupying an area of approximately 0.14 mm² [18]. Similar

findings were observed in animal studies, presenting with a varying apical extension of the inflammatory lesion [15,17]. At most of the implant sites investigated, the lesion was located lateral to the barrier epithelium and separated from the crestal bone by a zone of healthy connective tissue.

The exact histopathologic mechanisms that result in an apical extension of the inflammatory cell infiltrate and associated loss of crestal bone have not been identified [10].

1.1.4 Onset and pattern of disease progression

The so-called “ligature model” is often used to study experimental peri-implantitis in animals [20]. The protocol comprises a phase of active tissue breakdown around osseointegrated implants, including plaque formation and placement of ligatures in a submucosal position [21]. The ligature breaks the mucosal seal to the implant and promotes submucosal bacterial biofilm formation. The ensuing inflammatory lesion initiates tissue destruction, including bone loss. Also, after the removal of the ligatures and under continuous plaque formation, progression of disease may occur [22]. This model mimics naturally occurring peri-implantitis. When compared to experimentally induced periodontitis, lesions associated with experimental peri-implantitis demonstrate larger inflammatory cell infiltrates and more rapid and pronounced bone loss [23]. After a period of several weeks of plaque formation subsequent to ligature removal, spontaneous progression of peri-implantitis was associated with severe inflammation and tissue destruction [22]. Disease progression was influenced by implant surface characteristics with more pronounced breakdown at implants with modified than with non-modified surfaces [22].

Retrospective observational studies that estimated the onset and progression of peri-implantitis associated with bone loss, found that the pattern of bone loss was not linear, demonstrating an acceleration and increase over time that was attributed to individual heterogeneity [24,25]. It is suggested that peri-implantitis progresses in a non-linear and accelerated pattern and that the onset occurs, usually, within 3 years of function [25,26].

Clinical studies have shown that peri-implantitis can occur in the early stages during follow-up implant treatment and that the progression of the disease seems to be faster than that observed in periodontitis [27–29].

It is important to distinguish between initial physiological bone remodeling and progressive crestal peri-implant bone loss, with the latter implying that a pathological process is ongoing. The initial remodeling of the crestal bone is considered a physiological process following implant placement [7]. This process is influenced by a variety of biological (mucosal thickness [30]), technical (prosthetic connections [31]) and surgical (implant positioning [17]) factors. In the classic implant success criteria, the acceptable bone loss threshold is dynamic (<1.5mm bone loss during the first year of loading and bone loss < 0.2mm annually after the first year) [32]. The literature supports that the marginal bone surrounding the implant remodels after functional loading to establish a biological width between the implant platform and crestal bone [33,34].

1.1.5 Characteristics of peri-implantitis

1.1.5.1 Histopathologic characteristics of peri-implantitis

To understand the histopathological characteristics of peri-implantitis, it is necessary to previously understand the histopathological characteristics of locations with peri-implant health. The histological characteristics of a healthy peri-implant site are derived mainly from animals studies [35,36]. The healthy peri-implant mucosa averages 3 to 4 mm in height and is covered by either a keratinized (masticatory mucosa) or non-keratinized epithelium (lining mucosa). The portion of the peri-implant mucosa facing the implant/abutment contains a “coronal” portion that is lined by a sulcular epithelium and a thin junctional epithelium, and a more “apical” segment in which the connective tissue is in direct contact with the implant surface. The connective tissue lateral to the sulcular epithelium harbors a small infiltrate of inflammatory cells. Most of the intrabony part of the implant is in contact with mineralized bone, while the remaining portion faces bone marrow, vascular structures or fibrous tissue [37].

Compared to the periodontium, the peri-implant tissues do not have cementum and periodontal ligament. The peri-implant epithelium is often longer and in the connective tissue zone there are no inserting fibers into the implant surface. The peri-implant tissues are less vascularized in the zone between the bone crest and the junctional epithelium when compared to the connective tissue zone of the periodontium [10].

It was observed that inflammatory lesions from sites with peri-implant mucositis were dominated by T cells and had an apical extension that was restricted to the epithelial barrier [18].

When compared with peri-implant mucositis, peri-implantitis sites (case definition: BOP+, suppuration, radiographic bone loss) harbor more neutrophil granulocytes and larger proportions of B cells (CD19+) [38]. Similar to periodontitis, peri-implantitis sites are also dominated by plasma cells and lymphocytes [39], but characterized by larger proportions of polymorphonuclear leukocytes and macrophages [40].

Moreover, peri-implantitis lesions are characterized by larger area proportions, numbers and densities of plasma cells, macrophages and neutrophils, as well as a higher density of vascular structures outside and lateral to the cell infiltrate [41]. Immunohistochemical analysis of harvested soft tissue biopsies showed that interleukin (IL)-1 α was a dominant osteoclast activating cytokine at peri-implantitis sites [42].

1.1.5.2 Microbiologic characteristics of peri-implantitis

A review concluded that the microbiota associated with peri-implant diseases has a mixed anaerobic composition, with a profile similar to that of the subgingival microbiota of chronic periodontitis, although some cases of peri-implant disease may be specifically associated with other bacterial species, such as *Peptostreptococcus* spp. or *Staphylococcus* spp [43].

When compared with healthy implant sites alone, peri-implantitis was associated with higher counts of 19 bacterial species, including *Porphyromonas*

gingivalis and *Tannerella forsythia* [44]. Moreover, observational studies have indicated that peri-implantitis was more frequently linked with opportunistic pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* [43], fungal organisms (e.g. *Candida albicans*, *Candida boidinii*, *Penicillium spp.*, *Rhadorula laryngis*, *Paelicomycetes spp.*) [45] and viruses (i.e. human cytomegalovirus, Epstein-Barr virus) [46], pointing to a rather complex and heterogenous infection [47].

1.1.5.3 Immunologic characteristics of peri-implantitis

The pathogenesis of peri-implantitis is similar to that of periodontitis [48]. Microbes in plaque adhered to implant surfaces release chemotactic peptides that attract neutrophils toward the peri-implant pockets. Moreover, cytokines are released into the peri-implant crevicular fluid as a result of microbial damage to epithelial cells, which attract more leukocytes (predominantly neutrophils) toward the affected site [49]. If neutrophils become overloaded with microbes, they degranulate and the toxic enzymes released from neutrophils cause tissue damage and gingival inflammation [48]. If the inflammation persists, it may progress to the marginal gingiva and ultimately cause bone loss, a classical feature of peri-implantitis [50]. Stromal cells (such as granulation tissue fibroblasts) may also participate in the pathogenesis of peri-implantitis by upregulating vascularity and matrix breakdown, thereby promoting migration/maintenance of infiltrates (proinflammatory cytokines) into the inflamed site [51].

Most systematic reviews have focused on the correlations between various cytokines (i.e. proinflammatory/ anti-inflammatory/ osteoclastogenesis-related) and chemokines measured in the peri-implant crevicular fluid (PICF) and the clinical condition at implant sites [52,53]. Most of the included studies focused on the assessment of IL-1 β and tumor necrosis factor alpha (TNF- α).

Based on a meta-analysis by Faot et al. (2015), the release of IL-1 β was reported to be significantly increased at mucositis and peri-implantitis sites, when compared with healthy implant sites. However, no significant difference in IL-1 β

levels was noted between peri-implant mucositis and peri-implantitis sites. Peri-implantitis sites were also associated with a significant increase in TNF- α levels over healthy implant sites [52]. In contrast, most of studies failed to identify any significant differences in the levels of either IL-4, IL-10 or osteoclastogenesis-related (receptor activator of nuclear factor-Kappa B ligand (RANKL)) cytokines between healthy and peri-implantitis sites [53]. Accordingly, the systematic reviews indicated that the assessment of proinflammatory cytokines (mainly IL-1 β) in the PICF might be of beneficial value to differentiate between peri-implant health and disease, but inappropriate to determine the onset of peri-implantitis [52,54].

1.1.5.4 Clinical characteristics of peri-implantitis

The definition and clinical characteristics associated with peri-implantitis have varied between studies, but usually include the presence of BOP, deep values of probing pocket depth (PPD) and bone loss, although using different threshold values [14]. PPD values are always greater than 3 mm [14] and vary between 1 and 5 mm for bone loss, using the implant platform or baseline bone level radiographic as a reference [55].

Clinical signs of inflammation including redness, edema, mucosal enlargement, presence of BOP with or without suppuration along with increases in values of PPD and radiographic bone loss are commonly used in case definitions for peri-implantitis [39–41].

Implant sites diagnosed with peri-implantitis commonly show increased PPD. In a study evaluating 588 patients and 2,277 implants after a function time of 9 years, PPD ≥ 6 mm was recorded at 59% of all implants presenting with moderate/severe peri-implantitis (case definition: BOP+ and bone loss >2 mm) [10]. Out of the implants classified as healthy (case definition: BOP-) or diagnosed with mucositis (case definition: BOP+ but no bone loss >0.5 mm), 3% and 16% showed PPD ≥ 6 mm, respectively. It was also noted that the frequency of implants demonstrating PPD ≥ 6 mm increased with increasing severity of peri-

implantitis. In peri-implantitis locations, PPD is related to bone loss and is, therefore, an indicator of disease severity [10].

In a cross-sectional analysis, Schwarz et al. (2017) evaluated a total of 238 patients (n = 512 implants) after a median function time of 23 months (1 to 80 months). Diseased implant sites were associated with higher frequencies of 4 to 6 mm PPD than implants with a healthy peri-implant mucosa, with an equal distribution between mucositis and peri-implantitis sites. PPD values of ≥ 7 mm were only observed at one implant diagnosed with peri-implantitis [56].

In this context, the determination of what constitutes a physiological probing depth (PD) at implant sites is difficult [11]. Fuchigami et al. (2017) described a high degree of variation in the vertical mucosal thickness measured at healthy implant sites, ranging from 1.6 to 7.0 mm (i.e. mucosal margin to the crestal bone level) [57].

Clinically, in healthy situations, there are no visual differences between peri-implant and periodontal tissues. However, the probing depths are usually greater at implant versus tooth sites [10]. In implants is not possible to define a range of probing depths compatible with health; of more importance are the clinical signs of inflammation [10].

Clinical studies also reported on the configuration of peri-implantitis defects [58,59]. In 79% of all sites investigated, naturally occurring peri-implantitis lesions featured a combined supra- (Class II) and intrabony (Class I) defect configuration [60]. More frequently (55%), the intrabony component exhibited circumferential bone loss with maintenance of the buccal and lingual contours of the supporting crestal bone (i.e. Class Ie). This was followed by buccal dehiscence-type defects revealing a semicircular defect to the middle of the implant body (i.e. Class Ib) (16%), and buccal dehiscence-type defects with circular bone resorption in the presence (i.e. Class Ic) (13%), or absence (i.e. Class Id) (10%) of the lingual bone plate. The lowest frequency was noted for isolated buccal dehiscence-type defects (i.e. Class Ia) (5%) [60]. Similar intraoperative findings were also reported by Serino et al. (2013) [59]. The majority (66%) of the implants investigated (n = 59) exhibited a uniform bone loss at all four aspects [59].

Based on the above studies, it is assumed that peri-implantitis lesions commonly progress circumferentially around the affected implants [11].

It is recommended that the clinician obtain baseline radiographic and probing measurements following the completion of the implant-supported prosthesis. An additional radiograph after a loading period should be taken to establish a bone level reference following physiological remodeling [10]. If the patient presents for the first time with an implant-supported prosthesis the clinician should try to obtain clinical records and previous radiographs in order to assess changes in bone levels. According to the new classification of periodontal and peri-implant diseases and conditions, a case of peri-implantitis is defined as follows [10]:

- Presence of bleeding and/or suppuration on gentle probing.
- Increased probing depth compared to previous examinations.
- Presence of bone loss beyond crestal bone level changes resulting from initial bone remodeling.

In the absence of previous examination data diagnosis of peri-implantitis can be based on the combination of:

- Presence of bleeding and/or suppuration on gentle probing.
- Probing depths of ≥ 6 mm.
- Bone levels ≥ 3 mm apical of the most coronal portion of the intraosseous part of the implant.

1.1.6 Risk factors/ indicators for peri-implantitis

Interventional studies with a longitudinal design are needed to identify the true risk factors for the disease. Observational studies, cross-sectional or retrospective in nature, can only describe risk indicators.

Next, risk factors/indicators with substantial evidence will be addressed in each section, while factors with limited evidence will be summarized as “Areas for future research”.

1.1.6.1 Poor plaque control/ lack of regular maintenance therapy

As demonstrated in classic studies on periodontitis, lack of regular maintenance therapy is associated with tooth loss and loss of periodontal attachment [61,62]. These observations highlighted the importance of infection control measures performed by both the patient and the clinician in preventing periodontal disease.

Results of a longitudinal study including patients diagnosed with peri-implant mucositis demonstrated the importance of plaque control in the prevention of peri-implantitis [63]. The analysis showed that the incidence of peri-implantitis over a 5-year period was lower in patients undergoing regular maintenance (18%) compared to patients without supportive therapy (44%). These results are in agreement with those of Roccuzzo et al. (2012) [64]. The authors observed that patients who, over a 10-year period, failed the recommended regular maintenance therapy required substantially more treatment for peri-implantitis (41%) than those who had regular follow-up visits (27%). These results are in line with the observations of a cross-sectional study carried out by Monje et al. (2017) where it was observed that patients who underwent regular maintenance therapy, during an average observation period of 3.8 years, were less likely to be diagnosed with peri-implantitis compared to those who did not adhere to supportive therapy (odds ratio (OR) 0.14) [65].

Several cross-sectional studies that evaluated the effect of appropriate plaque control measures, performed by patients, on the development of peri-implantitis showed a strong correlation between both. According to several authors, poor plaque control at the final examination was the strongest statistical predictor for the development of peri-implantitis with an OR ranging from 5 to 14. [66,67].

Other factors related to implants oral hygiene measures must also be considered. Souza et al. (2016) demonstrated that brushing in implant areas with an amount of keratinized mucosa (KM) < 2 mm was associated with considerably greater discomfort compared to brushing in locations with KM $\geq$ 2 mm [68]. The authors also observed higher levels of plaque accumulation and bleeding at locations with a reduced amount of KM. Serino and Strom (2009) evaluated the

accessibility of implant-supported rehabilitations for oral hygiene measures in patients diagnosed with peri-implantitis [69]. The authors observed that only a few locations accessible to oral hygiene were affected by peri-implantitis (18%), while 65% of the areas that were not accessible had peri-implantitis. Consequently, oral hygiene instructions must be individually tailored to patients undergoing implant treatment. Furthermore, whenever possible, the margins of implant-supported rehabilitations should be placed at or above the margin of the peri-implant mucosa in order to facilitate access for bacterial biofilm control [70]. Implant-supported rehabilitations that do not allow access for biofilm removal should be adjusted or replaced with hygienic rehabilitations [70].

Nowadays, there is evidence that inadequate plaque control and lack of regular maintenance are risk factors for peri-implantitis.

1.1.6.2 History of periodontitis

Periodontitis is considered a common disease. Its severe form is considered the 6th most prevalent disease [71]. In a survey developed in the United States of America, Eke et al. (2015) reported that 50% of the adult population (age ≥ 30 years) had periodontitis [72]. In individuals aged over 65 years, this value corresponded to 68%.

Rocuzzo et al. (2010, 2012) followed 101 patients treated with dental implants, categorized in 3 groups according with periodontal status: 1) no periodontal compromise, 2) moderate periodontal compromise, 3) severe periodontal compromise [64,73]. The authors observed that the frequency of peri-implant locations demonstrating PPD ≥ 6 mm (2%, 16%, 27%, respectively) and bone loss ≥ 3 mm (5%, 11%, 15%, respectively) differed significantly between groups. The results also showed that the time taken to treat peri-implantitis was greater in patients with a history of periodontitis.

In a longitudinal study, including 80 patients who had mucositis at baseline, the incidence of peri-implantitis after 5 years was assessed by Costa et al. (2012) [63]. The authors observed that the incidence of peri-implantitis was 30%. Patients who had periodontitis at the final assessment also had a higher risk of

developing peri-implantitis when compared with patients without periodontitis (OR = 9). In another study that included 216 patients who were evaluated over a period of 9 to 14 years after implant placement, Ross-Jansaker et al. (2006) reported that implants placed in patients with a history of periodontitis had a significantly higher risk (OR = 5) for peri-implantitis compared to implants placed in patients without periodontitis [74,75]. Several subsequent studies confirmed this relationship with different degrees of association [76,77].

Although most studies agree on the association between periodontitis and peri-implantitis, it should be noted that there are studies that do not observe such an association [66,78]. This disagreement may be explained by differences in case definitions of (history of) periodontitis and peri-implantitis between studies.

It can therefore be concluded that there is strong evidence that a history of periodontitis is a risk factor for peri-implantitis.

1.1.6.3 Smoking

Tobacco has been strongly associated with chronic periodontitis, attachment loss as well as tooth loss [79,80].

Lindquist et al. (1996) observed that smokers had substantially more peri-implant crestal bone loss than non-smokers [81]. Several subsequent studies verified a strong association between smoking habits and peri-implantitis. In a 10-year cohort study, Karoussis et al. (2003) found that 18% of all implants placed in smokers developed peri-implantitis, while only 6% of implants placed in non-smokers were affected by the disease [82]. Several studies confirmed these observations, reporting odds ratios of 32 [83], 3 [84] and 5 [74], respectively.

In a systematic review and meta-analysis performed by Lee et al. (2017), the percentage of smokers was positively associated with the prevalence of implant-based peri-implantitis [55]. However, there are other publications that do not consider tobacco as an indicator/risk factor for peri-implantitis. Aguirre-Zorzano et al. (2015) examined 239 individuals who had implants after a mean follow-up time of 5 years and observed that the prevalence of peri-implantitis was 15% [67]. They found that smokers did not have a higher risk for peri-implantitis.

The results of other cross-sectional studies confirmed these observations [78,85]. It should also be noted that some studies report an association between tobacco and peri-implantitis in an initial univariate analysis [76,77]. However, in subsequent analysis with adjustments for confounding and interaction variables (multivariate analysis), tobacco does not remain a relevant predictor factor for peri-implantitis. This means that tobacco can be confused with other variables, namely a history of periodontitis.

The reasons for these contradictory results are currently not fully understood but may be related to the categorization between smokers and non-smokers. The criteria for the factor “smoking habits” varied considerably between studies. In addition, most studies rely solely on patient-reported information to determine smoking status.

The available evidence is inconclusive in establishing whether quitting smoking reduces the risk of peri-implant diseases. Additionally, there is limited evidence to support the claim that e-cigarette use or the habit of water pipe smoking is associated with a reduced risk of peri-implant diseases compared to traditional smoking [86]. However, according to the systematic review published by Carra et al. (2023), validated smoking cessation interventions are recommended to reduce the risk of peri-implant diseases in patients with healthy peri-implant tissues [87].

1.1.6.4 Diabetes

Diabetes is on the rise worldwide, with a global prevalence in adults in 2017 of 8.8% of the world's population [88] This disease has been identified as a risk factor for periodontitis [89]. There are studies that have shown that patients with diabetes were at a higher risk for developing peri-implantitis. Ferreira et al. (2006) found peri-implantitis in 24% of patients, who were either medicated for glycemic control or who had a fasting blood glucose value > 126 mg/dl at the final examination. In contrast, only 7% of non-diabetic patients were diagnosed with peri-implantitis. The authors reported an OR of 1.9 [90]. Findings from a study involving 96 patients with 225 implants demonstrated that, after a median period

of 11 years, there was a 3-fold increased risk (at the implant level) of peri-implantitis in patients who had been diagnosed with diabetes at the time of placement of the implants [91]. This analysis, however, was not adjusted for potential confounders. Tawil et al. (2008) followed 45 patients with diabetes for a median period of 42 months (range 1 to 12 years) [92]. In patients with an average value of glycosylated hemoglobin (HbA1c) $\leq 7\%$, there were no implants with peri-implantitis. In patients with HbA1c values between 7 and 9%, 6 out of 141 implants developed peri-implantitis.

On the other hand, there are several studies that failed to identify diabetes as a risk factor/indicator for peri-implantitis. In a retrospective study by Costa et al. (2012) patients with diabetes diagnosed with mucositis did not have a higher risk of developing peri-implantitis compared to non-diabetics [63]. Similarly, a lack of association between peri-implantitis and diabetes was observed in most available cross-sectional studies [25,66,74,77,78].

It should also be noted that the assessment of diabetes in most studies is based solely on patient-reported information.

The available evidence is inconclusive regarding the role of diabetes as an indicator/risk factor for peri-implantitis.

1.1.6.5 Areas of future research

Dimension of keratinized mucosa

The effect of the dimension of peri-implant keratinized mucosa as a risk factor for peri-implant mucositis has been investigated in several human studies. While some studies report higher rates of peri-implant mucositis in implants that do not have or have an inadequate width (< 2 mm) of KM [93,94], other studies found no association [95,96]. Collectively, the evidence for the need of the presence or minimum width of KM around implants to maintain peri-implant soft tissue health and stability remains controversial [70].

Some systematic reviews indicate that locations with KM < 2 mm are associated with greater plaque accumulation and inflammation of peri-implant

tissues compared to implants with $KM \geq 2\text{mm}$ [97,98]. In particular, the meta-analytical evaluation demonstrated statistically significant differences in terms of plaque index, modified gingival index, mucosal recession and attachment loss in favor of sites with greater amount of KM [98]. However, the evidence for the need for KM to maintain peri-implant health is still limited [96,97].

In clinical situations, in which adequate biofilm removal measures are performed by the patient, the presence or a graft of keratinized mucosa for the maintenance of peri-implant health does not seem to be an essential factor [70].

Although studies suggest that the absence or a reduced width of KM can negatively affect the oral hygiene measures performed by the patient, there is limited evidence that this condition can be a risk factor for peri-implantitis.

Although the level of evidence is low, for patients with dental implants who have no or insufficient keratinized/attached mucosa and experience discomfort while brushing, it may be worth considering increasing the width of peri-implant keratinized/attached mucosa as a way to preserve peri-implant health [86].

Excess cement

Several observational studies have found a correlation between excess cement and the prevalence of peri-implant disease. By employing a variety of different case definitions, it was suggested that the presence of excess cement was closely linked to the occurrence of both mucositis and peri-implantitis [99,100]. However, the proportion of sites with disease, in sites that had excess cement, varied considerably between studies (9 to 81%). Thus, several locations that had excess cement did not exhibit peri-implant disease [99,100]. On the other hand, implants with cemented restorations did not present a greater risk of peri-implantitis compared to implants with screw-retained rehabilitations [101,102].

Thus, it is suggested that excess cement may be a potential indicator/risk factor for peri-implantitis.

Implant surface characteristics and/ or implant material

Presently, dental implants commonly consist of commercially pure titanium or titanium alloy, zirconia or a combination of these materials. The endo-osseous portion of the implants typically features a minimally to moderately rough surface, as observed in most available implants [103,104]. Notably, recent studies indicate that newer implant designs with modified surfaces tend to exhibit higher survival rates in low-density bone compared to earlier implants with non-modified (turned) surfaces [105]. Theoretically, implant surfaces with rougher textures tend to accumulate more biofilm compared to smoother surfaces [106]. Both preclinical *in vivo* experiments and clinical studies have indicated that certain modified implant surfaces may have a detrimental impact on the occurrence and/or progression of peri-implantitis [107,108]. However, it is important to note that not all studies support this notion, and a previous systematic review on the subject could not reach definitive conclusions due to the limited data available [109]. In a more recent systematic literature review about the influence of implant surface characteristics and/ or implant material on the incidence and progression of periimplantitis, the authors concluded that implant surface characteristics do not seem to have a significant impact on peri-implantitis incidence [110]. However, no assumptions can be made regarding disease progression once peri-implantitis has occurred, due to lack of information [110]. In fact, preclinical *in vivo* experiments have suggested that the surface characteristics of modified implants can potentially have a notable adverse effect on the progression of peri-implantitis. However, due to ethical reasons, it is not possible to study progression of untreated peri-implantitis in humans. Clinical studies do not provide support for the idea that there is a discrepancy in the incidence of peri-implantitis among different types of implant surfaces. No assumptions can be made regarding the possible impact of implant material on incidence and/ or peri-implantitis progression due to limited information. In this systematic review, a non-modified implant surface refers to a minimally rough surface (e.g., turned or polished), while a modified implant surface refers to a moderately rough or rough implant surface [110]. According to the summary and consensus statements of group 3 of the 6th EAO Consensus Conference 2021 based on the current available

literature, there is no specific implant surface or material that has been demonstrated to effectively decrease the risk of peri-implantitis [111].

Other systemic conditions

The association of systemic conditions, other than diabetes, with peri-implantitis has rarely been studied and therefore its relationship with the disease is unclear. A cross-sectional study observed an increased risk for peri-implantitis in patients diagnosed with cardiovascular disease (OR = 9) and rheumatoid arthritis (OR = 7) [77]. Roos-Jansaker et al. (2006); Casado et al. (2013) and Canullo et al. (2016) combined different systemic diseases into a single parameter and did not find an increased risk for peri-implantitis in their respective analyses [74,85,112]. Other studies have considered osteoporosis [113], thyroid disease [114,115], hepatitis [78], body mass index [113] as well as radiation and chemotherapy [76]. There was no association with peri-implantitis in these evaluations. There is limited scientific evidence supporting the association between peri-implantitis and other systemic conditions.

Iatrogenic factors and bone augmentation procedures

In the consensus of the 7th European Workshop on Periodontology, it was recognized that the onset and progression of peri-implantitis can be influenced by iatrogenic factors such as: inadequate adaptation of the abutment, overcontouring of prosthetic rehabilitations and bad position of implants [7]. The position of the implant and the design of the prosthetic superstructure must facilitate access for plaque control measurements by both the patient and the professional [8]. However, there are few studies that assess the influence of iatrogenic factors on the development of peri-implant diseases.

In a retrospective analysis, it was suggested that peri-implantitis was associated with implant malposition and bone augmentation procedures [116]. The potential association between bone augmentation procedures and peri-implantitis was observed in two cross-sectional studies [116,117]. In one of these studies, the impact of the result of guided bone regeneration, in dehiscence-type

bone defects, on peri-implant health was evaluated [117]. The height of the residual defect was evaluated 4 months after the grafting procedure. After 4 years of follow-up, it was observed that implants with residual defects > 1mm presented a higher risk for the development of peri-implant disease.

In the absence of sufficient data, it seems reasonable to suggest that the position and design of the prosthetic superstructure may influence the access to the plaque removal measures by either the patient or the professional.

1.1.7 Prevalence of peri-implant mucositis and peri-implantitis

The prevalence of peri-implant diseases has been reported in several studies [5,13,118]. However, there is a considerable variation between studies. In a systematic review by Berglundh et al. (2002), the technical and biological complications of dental implant therapy were summarized by reviewing a large number of longitudinal prospective studies. It was found that 6.47% of the implants included in this review had peri-implantitis, according to the definition proposed by Albrektsson and Isidor [119]. In contrast, Zitzmann and Berglundh (2008) showed that the frequency of peri-implantitis varied between 28% and ≥ 56% of individuals and in 12% and 43% of implant sites [7]. The causes for the discrepancy in the reported results can be attributed to the lack of standardized criteria for diagnosing peri-implant mucositis and peri-implantitis, the different implant systems used or the differences in the observation periods. The multifaceted etiology and diverse characteristics of the disease result in a lack of consensus on the definition of peri-implantitis from a clinical perspective.

In 2012, Atieh et al. performed a systematic review and meta-analysis, including 9 studies, with a follow-up time of at least 5 years, obtaining a sample of 1497 participants and 6283 implants. The estimated frequency for peri-implant mucositis was 63.4% at the individual level and 30.7% at the implant level. It was observed that 18.8% of participants and 9.6% of implants had peri-implantitis. In smokers, a higher frequency of occurrence of peri-implantitis was observed with an estimated value of 36.3%. However, the implementation of supportive

periodontal/peri-implant therapy appeared to reduce the rate of occurrence of peri-implant disease [120].

At the XI European Workshop in Periodontology (2014), which focused on the effective prevention of periodontal and peri-implant diseases, a systematic review was commissioned to examine the prevalence of peri-implant diseases. Eleven studies were selected for inclusion, and through meta-analyses, it was found that the estimated prevalence at patient-level for peri-implant mucositis was 43%, while for peri-implantitis it was 22% [121].

In 2017 Lee et al., published a systematic review and meta-analysis that included 47 studies with a mean follow-up time of at least 3 years. The estimated prevalence of peri-implantitis based on implant and subject were 9.25% and 19.83%, respectively. For mucositis, implant-based and individual-based prevalence were 29.48% and 46.83%, respectively. They also observed that the time in function and the ratio implants *per* individual positively influenced the prevalence of peri-implantitis based on the subject, not affecting the prevalence of peri-implant mucositis [55].

The use of participants *versus* implants as a statistical unit of analysis remains a subject of considerable impact, limiting data comparisons between some systematic reviews. Most authors consider that the choice of analysis unit should depend on the result to be evaluated. Thus, when comparing treatment protocols or morphological features of implant designs and surfaces, the implant could be used as the unit of analysis, whereas the participant would be a more appropriate unit of analysis in studies evaluating demographic, compliance issues or systemic complications of implant therapy [120]. It is apparent that in studies assessing the frequency of peri-implant disease, data based on implants rather than participants as a unit of analysis may underestimate the true prevalence of this condition [122]. This is because each implant is not an independent unit, and intra-participant correlation among implants needs to be accounted for [123]. In addition, the use of an implant as a statistical unit may result in a statistically invalid estimation particularly in high-risk groups, where the difference between an implant and a participant unit is more apparent.

It is essential to determine the true prevalence of peri-implant diseases to allow the execution of adequate informed consents and in order to create rigid support programs for high-risk groups.

1.1.8 Treatment of peri-implantitis

1.1.8.1 Peri-implantitis nonsurgical therapy

The basis of non-surgical peri-implant therapy is infection control through decontamination of the implant surface, with the aim of removing adhered biofilm and reducing the bacterial load to levels below the threshold level that can cause disease [124]. While in periodontitis treatment, end points of therapy have been well established, and success of steps 1 (successful removal of supragingival dental biofilm/ risk factor control) and 2 (reducing/eliminating the subgingival biofilm and calculus) of treatment is a reasonable expectation [125], comparable evidence for the treatment of peri-implantitis is still scarce [86].

Mechanical decontamination around implants must consider some specific characteristics such as: absence of periodontal ligament, variable roughness of the implant surface and different types of abutment connection. These factors can jeopardize not only professional therapy, but also the plaque control carried out by the patient, since these characteristics can facilitate the formation of biofilm when they become exposed to the oral environment [109]. In order to overcome these limitations, different protocols combining adjuvant treatments have been proposed [14]. These protocols usually involve mechanical decontamination of the implant surface using curettes [126]; ultrasonic instruments [126]; abrasive air devices [127] or lasers [128], alone or in combination with some kind of chemical action essentially based on local antibiotics [128] or antiseptics such as chlorhexidine [127]. According to a systematic review carried out by Barbato et al. (2023) which included 16 randomized clinical trials (RCTs), non-surgical treatment, with or without additional methods, has the potential to decrease PPD and BOP, although complete resolution of the pocket cannot be guaranteed [129]. Among several adjuvant methods (local application of antimicrobials, adjunctive use of lasers, systemic antibiotics, antimicrobial photodynamic therapy), only

systemic antibiotics seem to offer additional benefits; however, its use should be approached with caution. In addition, this treatment approach does not result in changes in bone levels. On the other hand, it is important to consider that most of the studies that analyze these protocols have limited follow-up times, small samples and lack of a defined control group. According to the available evidence, there is no specific professional mechanical plaque removal regimen that is more effective in reducing the risk of recurrent peri-implantitis. However, based on the periodontal literature and indirect evidence, the following approaches to biofilm removal from dental implants can be used alone or in combination: titanium area-specific curettes, ultrasonic/sonic instruments, rubber cup or brushes, air polishing devices with powdered glycine or erythritol alone or in combination [86].

There are studies on the treatment of peri-implantitis that reveal that anti-infection treatment strategies are successful in decreasing soft tissue inflammation and slowing the progression of the disease [10]. However, based on the available data, it seems that the nonsurgical therapy of peri-implantitis is not effective in disease resolution because only limited improvements in the main clinical parameters have been reported and there is a clear tendency for disease recurrence [14]. It is therefore recommended to consider advanced therapies, such as surgical interventions, when nonsurgical peri-implant surgery is unable to achieve significant improvements in the clinical parameters.

1.1.8.2 Peri-implantitis surgical therapy

According to the European Federation of Periodontology (EFP) practice guideline, published in 2023, in peri-implantitis patients in whom end points of non-surgical therapy ($PD \leq 5$ mm and ≤ 1 point of BOP) have not been achieved, it is recommend to perform surgical therapy [86].

The surgical approach to peri-implantitis should ideally aim to regenerate bone and achieve re-osseointegration. The question of whether true re-osseointegration may occur following treatment of peri-implantitis is controversial. The rationale of the surgical treatment of peri-implantitis is therefore twofold: to

improve implant surface cleanability; and to modify the anatomy of soft and hard peri-implant tissues in order to obtain re-osseointegration [14].

Various surgical techniques have been recommended, depending on the final objective of the surgical intervention: (1) access for cleaning and decontamination of the implant surface (access flaps); (2) access for cleaning and decontamination plus exposure of the affected surfaces for cleaning (apically repositioned flaps); and (3) access for cleaning plus aiming for bone regeneration and re-osseointegration (regenerative techniques).

When comparing apical repositioning flaps, with and without implantoplasty after 24 months of follow-up, it was observed that the groups treated with implantoplasty showed significant improvements in PPD values and clinical attachment levels [130]. Another study, compared decontamination using air abrasive powder or CO2 laser, during resective or reconstructive surgery (with a mixture of beta-tricalcium phosphate and autogenous bone) [131]. In a short-term evaluation, no significant differences were observed between the groups. However, after longer follow-up periods, resective surgery with laser decontamination was the approach with better results. When the reconstructive approach was performed, no differences were observed between the application of the abrasive air powder and the laser. Schwarz et al. (2011), carried out a study comparing two methods of implant surface decontamination: plastic curettes with saline solution and Er:YAG laser, in bone defects combined with an intra- and supra-osseous component [132]. Surgical treatment consisted of implantoplasty and treatment of the intraosseous component using bovine xenograft and collagen membrane. At 6 months, there were no differences in clinical attachment level and in BOP between groups. According to the EFP clinical practice guideline it is recommended not to use air-polishing or Er:YAG laser for implant surface decontamination during surgical treatment of peri-implantitis. Titanium brushes may be considered as an alternative/adjunct to standard decontamination and there is insufficient evidence to make any recommendation regarding the use of implantoplasty [86].

Reconstructive procedures aim to regenerate the bony defect, achieve re-osseointegration and limit peri-implant soft tissue recession [133]. Reconstructive therapy of peri-implant bone defects can include the use of bone grafts, bone

replacement grafts, barrier membranes, bioactive agents (growth factors, autologous platelet concentrates and amelogenins) or combinations of these. In the reconstructive approach, an important conclusion obtained from some studies is the absence of differences regardless of the use or not of a resorbable membrane. In a study carried out by Khoury & Buchmann, 2001 it was observed that after 3 years of follow-up, the differences between the 3 treatment protocols (surgery with autologous graft only, surgery with autologous graft and non-resorbable membrane, surgery with autologous graft and resorbable membrane) did not affect treatment results [134]. Roos-Jansakervet al. (2007) in a prospective study where two different treatment approaches were evaluated (surgery with bone substitute with or without a resorbable membrane), observed that after 1 year there was no BOP in 75% of the implants in both groups [135]. It was also observed that the reduction in PPD, the gain of clinical attachment and the filling of the defect were similar between the groups. A 3-year observation confirmed these results [136]. There are a limited number of controlled studies evaluating the surgical treatment of peri-implantitis, and many of them have small sample sizes, limited follow-up time and a high risk of bias [137]. In addition, the assessment of its effectiveness is affected by the absence of a standard mode of therapy, and therefore there is no specific control therapy. Also, few studies provide information related to the true end results, i.e., resolution of peri-implantitis [14]. There are systematic reviews that have evaluated the effectiveness of regeneration procedures, with the objective of complete elimination of peri-implant infected tissues together with a reconstructive bone filling of the defects. However, the long-term effectiveness of regeneration procedures appears limited, especially in assessing peri-implantitis recurrence and implant failure. [138].

The characteristics of peri-implant bone defects caused by peri-implantitis are very important in the selection of the most appropriate surgical approach. A classification was proposed to categorize the morphology of peri-implant defects, and based on this morphology, the development of a decision tree for the recommended surgical intervention [60]. In the presence of circumferential bone defects with intact bone walls, the use of a regenerative surgical approach provided better results compared to treatment of bone defects with some degree

of vestibular dehiscence [139]. Thus, regenerative surgical techniques should be used in the presence of circumferential and intraosseous defects. On the other hand, when defects have a predominant supraosseous component, an apically repositioned flap should be the option of choice in non-aesthetic areas [14]. Although there is not much evidence to support the use of access surgeries, this type of procedure may be indicated in shallow defects or in aesthetic areas, after an unsuccessful non-surgical treatment, which have no indication for a regenerative approach [14].

In terms of surface decontamination, the literature does not clearly indicate superiority of a specific decontamination protocol [14]. There is also no evidence to recommend a specific regenerative surgical technique, such as the use of autogenous grafts, xenografts or bone substitutes, although it seems clear that the use of membranes as barriers does not provide a clear added value [134,136]. A recent systematic review regarding the treatment of peri-implantitis concluded that there is no superiority of a specific biomaterial or product in terms of long-term clinical benefits [140].

1.1.9 Peri-implantitis recurrence after treatment

A 5-year clinical study observed that regenerative treatment of peri-implantitis results in a stable clinical and radiographic situation over 1 year after treatment, however, a tendency towards disease recurrence is observed for longer periods of observation [141]. In 2020 Roccuzzo et al., observed that regenerative treatment led to significant improvements in interproximal bone levels one year after treatment. However, recurrence was observed in some implants at 7 and 10 years of follow-up. In the first year of follow-up, no implant failed, however, after 7 and 10 years of observation it was observed that 31% and 53% of the implants failed, respectively [142,143].

In 2021, Martins et al. performed a systematic review and meta-analysis with the aim of evaluating disease recurrence and implant failure after at least 6 months of surgical treatment of peri-implantitis [144]. Surgical approaches were divided into access surgery, regenerative surgery, resective surgery,

implantoplasty and combined surgery. Thirteen RCTs were selected for quantitative analysis. The rate of recurrence and implant failure was 3% and 4%, respectively, for short follow-up periods (3-12 months). For long follow-up periods (>36 months) the implant loss rate was 36%. There were insufficient data for assessments of the rate of recurrence and implant loss at other follow-up periods. The combined approach was associated with the highest recurrence rate (7%) and regenerative surgery with the lowest (2%). Access surgery demonstrated the highest rate of implant loss (15%) and the regenerative approach the lowest (3%).

Although the role of the implant surface in re-osseointegration has been investigated in animal studies [145,146] with some controversial results, a human case report made reference to this possibility [147]. According to a prospective study carried out by Roccuzzo et al. (2020) implant surface characteristics affect the decision regarding the treatment of peri-implantitis [143]. In this study, 6 patients with a titanium plasma-spraying (TPS) implant surface (rough surface) and 2 with an acid-etched implant surface (SLA) surface (moderately rough) had additional antibiotic and/or surgical treatment during the 10-year follow-up after peri-implantitis treatment. Five TPS and two SLA implants had to be removed due to recurrent infections. In a long-term retrospective study (11 years), Berglundh et al. (2018) obtained a better PPD reduction in implants with non-modified surfaces compared to those with modified surfaces [148].

Replacing one implant with another is always associated with an additional cost and the need for another surgical intervention. Replacing an implant due to infectious causes, loss of osseointegration or other causes is often a challenge for the clinician and has lower survival rates than reported rates for previous implant placement attempts [149,150]. These lower survival rates suggest the influence of site-specific or patient-specific risk factors, and clinicians should always bear in mind that replacement of an implant is usually subject to, at least, all the initial factors that led to implant failure [150].

Regardless of the surgical approach, an adequate support program has proved to be crucial in maintaining the post-surgical results obtained. In a systematic review carried out by Roccuzzo et al. (2018) the authors concluded that the majority of patients who were included in regular maintenance therapy after surgical treatment of peri-implantitis did not lose their implants [151].

1.2 Inflammatory cytokines

Inflammatory cytokines are cell signaling molecules with a peptide structure, water soluble, vary between 8 and 30 kilodaltons (kDa) and are mainly produced by macrophages, however other nucleated cells, such as monocytes, fibroblasts and epithelial cells may also be at their origin [152,153]. Pro-inflammatory cytokines are proteins secreted by innate immunity cells (mainly polymorphonuclear neutrophils (PMNs)), monocytes/macrophages and dendritic cells) and adaptive immunity cells (mainly lymphocytes) involved in the progression of peri-implantitis and periodontitis, being considered biomarkers of this process [154,155].

These molecules bind to specific receptors on other cells, trigger cellular responses and thus not only influence the activity, differentiation and proliferation of immune cells, but also regulate the production and activity of other inflammatory cytokines [156,157]. In this sense, cytokines act as modulators of the inflammatory response by mediating innate immune responses and thus allowing the regulation of the host response to microbial infection [156,158]. This host regulation involves the identification of bacteria through Toll-like receptors (TLRs), which establish a link with specific molecular patterns associated with pathogens, such as bacterial lipopolysaccharides (LPS) of Gram-negative bacteria and virulence factors, culminating in the production of inflammatory cytokines [159,160]. The key pro-inflammatory cytokines are IL-1, IL-6 and TNF- α [161].

The anti-inflammatory cytokines are immunoregulatory molecules that control the pro-inflammatory cytokine response. Cytokines act in concert with specific cytokine inhibitors and soluble cytokine receptors to regulate the human immune response. Major anti-inflammatory cytokines include IL-1 receptor antagonist (IL-1Ra), IL-4, IL-10, IL-11 and IL-13 [161].

1.2.1 Interleukin-1

IL-1 is synthesized by various cells such as macrophages, lymphocytes, monocytes, vascular cells, brain cells, epithelial cells and fibroblasts [162].

PMNs are leukocytes recruited from peripheral blood vessels by chemotactic factors, more specifically by chemokines, and predominate in the initial acute inflammatory response. They are responsible for producing numerous cytokines, such as IL-1 β , TNF- α and IL-6 [163]. On the other hand, macrophages are produced from the differentiation of monocytes in tissues. Monocytes/macrophages can have an inflammatory or provascularization phenotype, known as M1 and M2, with M1 macrophages responsible for the production of cytokines such as IL-1 β , TNF- α and RANKL, while M2 macrophages produce anti-inflammatory mediators, for example, IL-10 and IL-4 [164].

The main cells of adaptive immunity are B and T lymphocytes. B lymphocytes play a role in the expression of TNF- α , IL-6 and RANKL, stimulating osteoclastogenesis. In contrast, T lymphocytes can convert into T helper 1 cells (Th1), T helper 2 cells (Th2) and T helper 17 cells (Th17), which regulate bone resorption. Th1-type cells produce IL-1 and TNF- α [165].

IL-1 is a multifunctional cytokine with a central role in the pathogenesis of chronic diseases characterized by bone resorption processes such as periodontitis and peri-implantitis, presenting several actions, namely in terms of the immune response, inflammation, homeostasis and tissue degradation [166]. IL-1 is a pivotal mediator of the immune-inflammatory response that acts both in response to bacterial infection and in bone metabolism [166]. IL-1 has several functions, including activation of T and B cells, ability to stimulate connective tissue catabolism as well as bone resorption, activating chemotaxis of macrophages, lymphocytes and neutrophils, facilitating their migration, and it also stimulates the production of other cytokines, such as TNF- α , prostaglandin E2 (PGE2) and matrix metalloproteinases (MMPs) [162,167].

IL-1 family has at least 11 cytokines; clustered on the long arm of chromosome 2q, and the three most studied members are *IL-1A*, *IL-1B* and *IL-1RN* genes, which encode the agonistic proteins IL-1 α , IL-1 β and IL-1Ra, respectively [168]. IL-1 α and IL-1 β exert an identical biological activity by binding to the active IL-1 receptor type I (IL-1RI). They may also bind to the IL-1 receptor type II (IL-1RII), however without involvement in signal transduction [158,169]. IL-1 β is considered more potent and pathogenic.

Unlike IL-1 α , IL-1 β is not bioactive, being produced as an inactive precursor (pro-IL-1 β). Conversion to its biologically active form first requires its cleavage by an enzyme, caspase-1, and then activation by multiprotein complexes called inflammasomes [158].

IL-1 α is considered a mediator of local inflammation and is mainly produced by keratinocytes from the junctional epithelium or present in the periodontal pocket. In contrast, IL-1 β is predominantly produced and released by fibroblasts and macrophages that are active and acts at the extracellular level [170]. In the presence of a peri-implant infection, it is produced locally, together with other factors of the local immune response, in order to regulate inflammatory reactions. It also controls the extracellular matrix degradation activity by the plasminogen activator system (PA system) during inflammation and wound healing, as well as the production of PGE2 [171,172]. Plasmin, the active enzyme resulting from the plasminogen activator system, activates IL-1 β [173].

1.2.2 IL-1 receptor antagonist

IL-1Ra is secreted by various types of cells including immune cells, epithelial cells and adipocytes. IL-1Ra is an anti-inflammatory cytokine that works as a competitive inhibitor of interleukin-1, inhibiting its bioactivity [174]. It has an anti-inflammatory role, preventing the transmission of pro-inflammatory signals and preventing the immune response. This cytokine has the ability to bind to the host cell surface using the same receptor as pro-inflammatory IL-1, thereby inhibiting signal transduction by blocking the receptor [175]. The effect of IL-1 is determined by the balance between IL-1 α , IL-1 β and IL-1Ra through competitive binding of IL-1Ra to the IL-1 receptor.

IL-1Ra is a protein encoded by the *IL-1RN* gene and has the only known function of preventing the activation of target molecules [176].

1.2.3 Tumor necrosis factor alpha

TNF- α is considered a pro-inflammatory cytokine with properties similar to those described for IL-1 β , with a central role in the pathogenesis of peri-implantitis. In addition, it is also the main mediator in the response to Gram-negative bacteria and its concentration reflects the amount of bacteria present in the inflammatory state of the disease [177,178].

Regarding its origin, TNF- α is mainly produced by activated macrophages and other types of cells, such as activated T lymphocytes, neutrophils, epithelial cells, endothelial cells, fibroblasts and bone lining cells, including osteoblasts [154].

TNF- α expression promotes osteoclastogenesis, through increased expression of the colony-stimulating factor-1 receptor (CSF-1 receptor), as well as induction of RANKL signaling mechanisms, responsible for activating osteoclasts. Additionally, TNF- α stimulates osteoclast precursors and stromal cells to produce osteoclastogenic cytokines such as IL-1, RANKL and human macrophage colony stimulating factor (M-CSF) [154].

However, there is a lack of agreement on the possible induction of osteoclastogenesis from TNF- α , regardless of the action of RANKL. Schett (2011) stated that it is likely that TNF- α provides a synergistic increase in osteoclast-mediated bone resorption, only when interacting with RANKL [179]. However, Xiao et al. (2016) argued that TNF- α is capable of inducing RANKL and stimulating osteoclastogenesis directly and independently of RANKL [154]. On the other hand, TNF- α inhibits the differentiation of precursor cells into new osteoblasts and the role of osteoblasts in bone formation [154].

In addition to promoting bone resorption, Schett (2011) emphasizes the fundamental role of TNF- α in inflammation, through the activation of the expression of cytokines, chemokines and endothelial cell adhesion molecules [179].

TNF- α also stimulates fibroblast apoptosis, which results in limited repair of the periodontium and increases local secretion and activity of metalloproteinases, which degrade connective tissue. This cytokine also acts in

the promotion of angiogenesis, in the inhibition of the activity of regulatory T cells and, finally, in the sensitization to pain [52].

Through the synergistic action of TNF- α and IL-1 β , several mechanisms involved in the degradation of peri-implant tissues are triggered, as shown in Figure 1.1 [177,178,180].

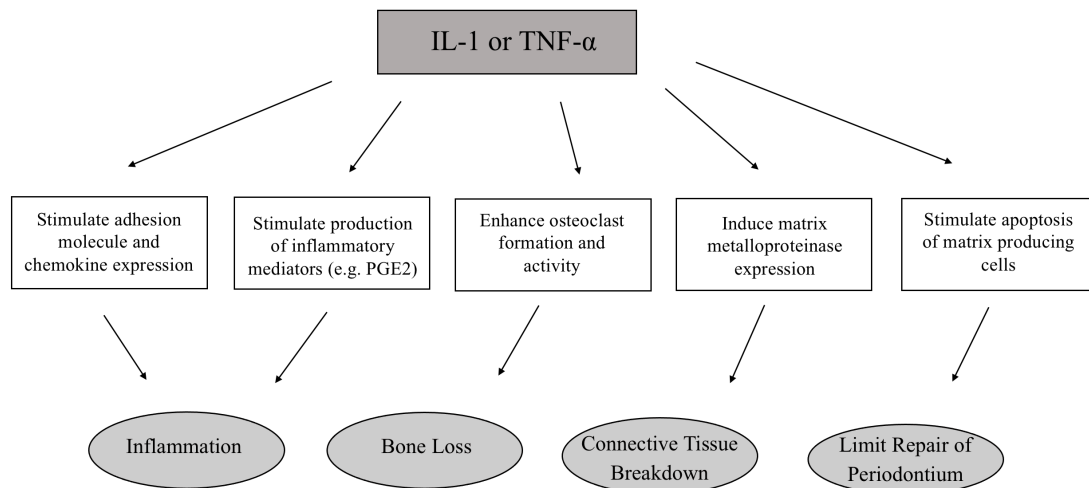


Figure 1.1 - Mechanisms by which IL-1 or TNF- α could contribute to the loss of periodontal tissues. Adapted from Graves & Cochran (2003) [177].

1.3 Prostaglandin E2

PGE2 is a metabolite of arachidonic acid that has several pro-inflammatory effects in peri-implant and periodontal tissues [181]. The production of this inflammatory mediator is attributed to different types of cells, mainly macrophages, but also fibroblasts and gingival epithelial cells [182].

According to Nasry et al. (2018) when PGE2 binds to one of its four receptors (EP1, EP2, EP3 and EP4) it triggers physiological processes related to the host immune response and blood pressure [183]. Thus, as described by Alkan et al. (2016) [182], Basegmez et al. (2012) [184] and Tatli et al. (2013) [185], PGE2 works as a vasodilator that enhances vascular permeability at sites of inflammation, in addition to contributing to the release of collagen, activation of osteoclasts and mediation of bone resorption. PGE2 is associated with osteoclast-mediated bone resorption and is found in high concentrations in sites

with periodontitis and peri-implantitis. On the other hand, by promoting angiogenesis it contributes to the healing process in chronic inflammation [183].

However, Minamizaki et al. (2009) consider that although PGE2 promotes the formation of osteoclasts through RANKL, it may also influence osteogenesis [186]. This fact is based on the ability of PGE2 to alter the expression of molecules that are crucial for osteoblast development and function, including Runt-related transcription factor-2 (Runx2) and Transforming Growth Factor- β Type III Receptor [186].

1.4 Gingival Crevicular fluid and Peri-implant Crevicular Fluid

In the middle of the last century, many researchers dedicated themselves to the study of the anatomy and physiology of the periodontal sulcus, with the aim of determining the composition and function of the gingival crevicular fluid (GCF) [187,188]. The analysis of GCF was proposed for the quantitative analysis of the inflammatory status of periodontal tissues [189]. Since then, interest in the potential of the GCF as an auxiliary mean of diagnosis has been increasing and continues, over the years, to be a relevant topic of study [190].

However, although the importance of the GCF has been recognized over the years, its origin and function are a controversial topic. This is due to the disagreement between studies, regarding the physiological or pathological origin of the fluid, which results in a discrepancy in its characterization according to the different definitions attributed by various authors [191]. Originally, most investigators defined GCF as an inflammatory exudate [192]. However, other evidences indicated that the GCF from clinically healthy tissue constitutes a serum transudate, whose change to an inflammatory exudate only occurs in the presence of clinical disease [193]. Recently, this statement was refuted considering the discovery of the migration of neutrophils to the sulcus even under healthy conditions. This fact calls into question the characterization of the GCF as an inflammatory exudate versus a physiological transudate [191]. Thus, according to most authors, GCF is considered a physiological fluid and an inflammatory exudate, formed in the dentogingival plexus, located in the connective tissue, underlying the epithelium of the gingival sulcus [191,194].

According to Khurshid et al. (2017), GCF consists essentially of cellular components, organic ions, inorganic ions, enzymes and bacterial products [195]. Among the cells of the immune system of the GCF, 91 to 97% are neutrophils and other polymorphonuclear leukocytes, which quantitatively prevail among all the leukocytes present in the fluid. Only 2 to 3% are monocytes/macrophages and 1 to 6% lymphocytes, of which 29% are T lymphocytes and 71% are B lymphocytes [196].

According to some investigators, during the course of the inflammatory state, the composition of the GCF changes and the levels of proteins obtained from the affected locations increase significantly, presenting values similar to the concentrations of proteins in the blood serum [188]. Barros et al. (2016) [191] and Rahnama et al. (2014) [196] confirmed the release of numerous inflammatory cells and mediators in the GCF, such as cytokines, proteinases and phosphatases, during inflammation (Table 1.1). Thus, its composition can be used to detect subclinical changes in tissue metabolism, inflammatory cell recruitment and connective tissue remodeling [194].

Table 1.1 - Inflammatory mediators present in the gingival crevicular fluid. Adapted from Rahnema et al. (2014) [196].

The mediators of the immune response	Total and subgroups of immunoglobulin IgG The mediators of the inflammatory response Arachidonic acid derivatives such as PGE2 Cytokines, such as IL-1, IL-2, IL-4, IL-6, TNF α	
Enzymes and enzyme inhibitors	Proteolytic enzymes	Collagenase Elastase Cathepsin-B Cathepsin-G Cathepsin-D Typtase
	Hydrolytic enzymes	Arylsulfatase β -glucuronidase Alkaline phosphatase Acid phosphatase Myeloperoxidase Lactoferrin Lysozyme
Bone-specific proteins	Osteonectin Phosphoprotein (N-propeptide) Osteocalcin Telopectide of type I collagen Tissue breakdown products	
Components	Fibronectin Collagen Proteoglycans Glycosaminoglycans	
The product breakdown	Split components of fibronectin Hydroxyproline	
The final peptides	Glycosaminoglycans Chondroitin-4-sulphate Chondroitin-6-sulphate	

The peri-implant crevicular fluid (PICF) is considered an osmotically mediated inflammatory exudate. Its composition, similar to that of GCF, is rich in biomarkers useful in the diagnosis of peri-implant disease, as they are considered measurable indicators of its severity or progression [197]. In the early stage of peri-implant disease, although clinically latent, PICF volume and the expression of certain biomarkers increase in response to tissue inflammation [198]. In addition, higher flow levels are also recorded at the PICF level, depending on the severity of gingival inflammation [190].

1.4.1 Biomarkers in Peri-implant Crevicular Fluid

Researchers and clinicians continually try to find methods and techniques that help in the early diagnosis of peri-implantitis. Therefore, the measurement of biomarker levels has aroused interest as a potential tool for the early detection of this disease [199]. A biomarker, or biological marker, is considered “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention” [200]. Among the main advantages of biomarkers is the repeatability and non-invasive nature of sample collection [199].

The bacterial infection of peri-implantitis promotes the immunological and biochemical response of the host tissues and results in the release of several cytokines [201]. Thus, these soluble mediators of immune reactions, present in PICF, are produced as a result of the physiological interaction of the gingival epithelium and local leukocytes with bacterial plaque and oral microbiota. In periodontology and implantology, the effect of cytokines is crucial in tissue homeostasis, through the alteration and repair that they promote in situations of inflammation [178].

With regard to the most studied biomarkers in PICF, the following stand out: inflammatory mediators, including PGE₂, pro-inflammatory cytokines such as IL-1 β , TNF- α , IL-6, IL-12, IL-17, IL-8 and IL-23, in addition to anti-inflammatory cytokines, namely IL-4 and IL-10; bone markers, such as RANKL, receptor activator of nuclear factor-Kappa B (RANK), and osteoprotegerin (OPG); and

enzymes, particularly MMP-1, MMP-8, MMP-9 and MMP-13 [194].

Most systematic reviews and meta-analyses highlight the role of pro-inflammatory cytokines IL-1 β and TNF- α , compared to other biomarkers, in the early diagnosis of peri-implantitis [194,199]. This fact is due to the significant statistical differences observed when comparing the concentrations of these inflammatory mediators in the PICF of locations affected by the disease, compared to healthy locations [180].

Zani et al. (2016) carried out an investigation whose approach comprises the use of multiple biomarkers to assess the ability of these PICF analytes to differentiate health from peri-implant disease [202]. Consequently, logistic regression was applied to determine the biomarker model that reveals superior diagnostic properties compared to isolated biomarkers. This model consisted of a combination of six biomarkers, including TNF- α . This study also proved that the locations diagnosed with peri-implantitis exhibit higher levels of IL-1 β , TNF- α , IL-2, IL-6, IL-10, IL-12 and IL-17. However, there are other studies that contradict the change in the concentration of IL-1 β in diseased locations, compared to healthy ones.

The cross-sectional study carried out by Gürlek et al. (2017) analyzed cytokine levels in PICF, GCF, and submucosal/subgingival plaque samples from clinically healthy and diseased locations in the same subjects [203]. Regarding IL-1 β levels, these were significantly lower in health situations, contrary to peri-implantitis and mucositis [203]. On the other hand, when comparing dental implants with natural teeth with similar health status, higher IL-1 β concentrations were recorded in the group of healthy dental implants in comparison with natural teeth.

The results of an investigation published in 2016 suggest that higher levels of biomarkers (IL-1 β) in the PICF of areas of tissue inflammation, matrix degradation and bone resorption, combined with specific microbial profiles, have potential for peri-implantitis prediction [198].

Javed et al. (2011) in a systematic review, concluded that increased levels of pro-inflammatory cytokines are evidenced in the PICF of patients with peri-implantitis [204]. As a result, monitoring cytokine concentrations makes it

possible to detect inflammatory conditions early, even if they are not yet clinically detectable. Accordingly, 4 and 6 studies, out of the 15 analyzed in this review, validated the association between IL-1 β and TNF- α , respectively, with peri-implantitis.

Dursun & Tözüm (2016) observed, through a systematic review that pro-inflammatory cytokines such as IL-1 β and TNF- α are up to date, the most promising proteins to be used as markers in PICF for differentiation between peri-implantitis and healthy implants [199]. Of the 19 studies that assessed IL-1 β , 13 showed higher levels of IL-1 β in PICF of locations with inflammation than healthy implant sites. Of the 10 studies that assessed TNF- α , 7 studies showed significant relationship with peri-implant inflammation, while 3 of them showed no relationship.

A systematic review and meta-analysis published by Faot et al. (2015) concluded that TNF- α and IL-1 β present in the crevicular fluid of peri-implant pockets can be used to diagnose peri-implantitis earlier [52]. All 19 included studies showed that the values of the pro-inflammatory cytokines of the PICF, especially IL-1 β and TNF- α , increased in sites affected by mucositis and peri-implantitis, compared to healthy sites. However, after the onset of the inflammatory process, no significant differences were detected between the concentrations of cytokines in peri-implantitis and mucositis. Therefore, peri-implant therapy should be initiated at an early stage of disease progression, in order to modulate the host's immune response [52].

Conversely, Hultin et al. (2002) reported no statistically significant differences regarding IL-1 β levels in peri-implant affected and unaffected locations [205]. According to the narrative review by Alassy et al. (2019) the assessment of the levels of pro-inflammatory cytokines IL-1 β and TNF- α in PICF may be relevant in the diagnosis of peri-implantitis and peri-implant mucositis [194]. However, more controlled longitudinal studies are needed to understand the role of these cytokines in predicting peri-implantitis progression. The results of a systematic review carried out by Duarte et al. (2016) presented moderate levels of evidence regarding the association between elevated concentrations of pro-inflammatory cytokines in PICF and peri-implantitis [53]. On the other hand, the evidence is limited regarding the concentrations of other biomarkers, namely

anti-inflammatory cytokines, cytokines related to osteoclastogenesis and chemokines, as possible indicators of peri-implantitis.

The role played by PGE2 in osteoclast-mediated bone resorption has aroused the interest of several researchers, who have carried out retrospective and cross-sectional studies with the aim of investigating the association of this mediator with the clinical parameters of healthy peri-implant tissues and of implants that show signs of peri-implant disease [184,185]. The first studies that evaluated the association between the concentrations of this inflammatory mediator and peri-implantitis were published in the last century. However, while the study by Salcetti et al. (1997) [206] detected an association between elevated levels of PGE2 and a higher frequency of *P. nigrescens* and *P. micros*, providing an additional diagnostic method in implants with peri-implantitis, the study by Aboyoussef et al. (1998) reported that PGE2 concentrations did not differ significantly between healthy and peri-implantitis-affected locations [207].

Subsequently, Yalçın et al. (2005), in a cross-sectional study, considered the possibility of using biochemical tests, particularly the detection of PGE2 levels in the PICF, as an appropriate means of diagnosing and controlling functional implants [181]. This study presented some limitations, including a small sample and a limited number of cases with deep pockets. Therefore, a greater number of longitudinal studies are needed to confirm the relevance of PGE2 levels in detecting bone and tissue destruction around implants.

In an investigation carried out by Ketabi et al. (2017) four biomarkers were simultaneously analyzed in the GCF and PICF of 41 patients in 4 different groups (healthy teeth, healthy implants, periodontitis and peri-implantitis) during a period of 6 months [208]. According to the results of the study, PGE2 was found in higher levels in the PICF of the peri-implantitis group compared to the PICF of healthy implants.

In a prospective study by Basegmez, et al. (2012) compared the levels of PGE2 and MMP-8 in PICF after osseointegration and rehabilitation of implants with fixed or partial prostheses [184]. After 12 months, an increase in the PGE2 level was observed, which reached its peak at the 18th month. PGE2 and MMP-8 demonstrated positive correlations with gingival index and PD. It was concluded that the detection of PGE2 and MMP-8 in PICF can be useful to monitor the

progression of peri-implant disease.

According to Dursun & Tözüm (2016), although one of the studies included in the systematic review reveals a positive correlation between the gingival index and the PD with PGE2, two other studies did not report differences between peri-implantitis and peri-implant health, in relation to concentrations of this biomarker in the PICF [199].

According to the evidence presented, it can be concluded that although certain studies do not find a relationship between IL-1 β , TNF- α , PGE2 and peri-implantitis, most of them, as evidenced in the referenced systematic reviews, demonstrate an association between high concentrations of these biomarkers (mainly IL-1 β and TNF- α) in the PICF of diseased versus healthy locations.

The conflicting results of the above studies may be due to differences in study design, material and methods used, such as sample collection, processing and assay sensitivity. Meta-analyses related to PICF biomarkers are scarce due to heterogeneity between studies in peri-implantitis diagnosis criteria. With the new classification of periodontal and peri-implant diseases (American Academy of Periodontology (AAP) and EFP 2018) it will be easier to standardize mucositis and peri-implantitis diagnostic criteria among the different studies [10,209]. The definition of peri-implantitis varied over time, mainly from more permissive to stricter inclusion criteria. Such misdiagnosis of peri-implantitis and inclusion of cases of peri-implant mucositis may affect the results of studies on PICF. In addition, the majority of PICF studies assessed only a few cytokines, enzymes or pathogens to correlate them with peri-implant diseases.

Most studies lacked data on the sensitivity and specificity of certain biomarkers in PICF; hence, the probability of false positive or false negative results could not be calculated. PICF biomarkers analyzed individually have shown mixed results or low sensitivity and high specificity values, which may weaken the biomarker's predictive value. Selected PICF-derived biomarkers of peri-implant tissue inflammation, matrix degradation/regulation and alveolar bone turnover/resorptive molecules combined with a site-specific microbial profile may be used to diagnose peri-implant diseases [198].

Some reports failed to discuss important data, such as the general

periodontal health in their subjects, smoking habits, systemic confounders and other related criteria which may influence the levels of cytokines in PICF. Smoking may be an important confounder because it may affect the PICF volume and cytokine levels. Only a few studies excluded smokers.

Standardization of PICF sampling is relevant due to the atypical morphology of the implant prosthesis. The insertion of different paper strips, cones, membranes or other devices would be technique sensitive and may offer misleading results. Biomarker concentration in the collected PICF should be adjusted for the collected volume due to the higher volume in inflamed tissues. This controversial confounder was rarely discussed in PICF studies.

The techniques for analyzing biomarkers also vary between studies and include Enzyme-Linked Immunosorbent Assay (ELISA), Luminex, Time-resolved immunofluorimetric assay and quantitative immunoblot, Modified Urokinase Assay, Spectrophotometric assay and Radioimmunoassay.

Biomarkers are not necessarily present in a single moment of PICF collection due to several systemic or local factors. Most studies were cross-sectional which leads to another limitation. Due to a cyclic progression of peri-implant diseases, the immune-inflammatory event biomarkers responsible for tissue breakdown may not always be active in cross-sectional studies with a single moment of fluid collection. Thus, studies on bone markers are often inconclusive despite bone loss being one of the main features of peri-implantitis. Longitudinal studies may confirm the concentrations of biomarkers at specific sites and would theoretically show the shifts of such concentrations in diseased implants over time. These longitudinal investigations may aid in presenting biomarkers that predict the shift from health to disease or predicting the deterioration of a diseased implant.

1.4.2 Means for collection and quantification of Peri-Implant Crevicular Fluid or Gingival crevicular fluid Volumes

The process involving the collection of the PICF is considered a non-invasive, painless, and relatively simple process, compared to, for example, a biopsy of gingival tissue [210]. Several methods can be used to obtain PICF,

namely the intracrevicular washing technique, in which the sulcus is irrigated with an isotonic solution and the fluid obtained consists of a dilution of the crevicular fluid, composed of cells and soluble components. Another option is capillary tubes or micropipettes of known sizes, which are inserted superficially into the gingival sulcus after isolation and drying of the location, in order to collect and quantify the volume. Absorbent paper strips, which is an easy, quick and non-traumatic technique, can also be used as a method of collecting gingival crevicular fluid. The strips are inserted into the sulcus, or gingival pocket for a certain period of time, allowing the collection of the fluid [194,211].

The most common method of collection is by using filter paper strips. Originally, these were pieces of laboratory filter paper of various types cut to size by researchers. It has been reported that recovery of proteins in GCF samples varies considerably according to the type of filter paper on which the GCF sample is collected [212], but this problem is likely to be less of an issue now as most researchers appear to use Periopapers (Oraflow Inc.). These are presterilized filter paper strips of standard size that can absorb fluid volumes of up to approximately 1.2 μ l (larger Periocol paper strips are also available for collecting larger volumes of up to 2.0 μ l). Typically, Periopapers are placed just into the entrance of the sulcus/pocket until mild resistance is felt (i.e., utilizing the intracrevicular method of placement) [213]. Attempts to position Periopapers to the known depth of a particular periodontal/ peri-implant pocket are usually unsuccessful because the paper has a tendency to crumple up under the pressure of insertion as it becomes increasingly saturated with fluid. Furthermore, contamination with blood is also very common if trying to position a Periopaper to the full depth of a pocket. Therefore, the Periopapers are typically inserted no more than 1–2 mm into the sulcus/ pocket to permit absorption of GCF from the site, with gentle placement until mild resistance is felt. The length of time that the Periopaper is held in place is an important consideration. Some authors have advocated holding the Periopaper in place until it is 'visibly wet', but, more commonly, the periopaper is held in place for a fixed period of time (typically 30 s) [210,214,215]. Many researchers report that samples visibly contaminated with blood should be rejected. However, there are occasions (particularly at inflamed sites) in which a very small amount of light-pink/reddish-brown discoloration might

be noted just at the very tip or corner of the Periopaper, beyond which there is visible wetting, indicating that gingival crevicular fluid has been collected; in this scenario it may be perfectly acceptable to retain this Periopaper for analysis. Plaque deposits can also influence recorded GCF volumes [216], and therefore large, visible supragingival plaque deposits should be carefully removed with a curette before sampling [213]. The area where the sample will be taken must be dry and isolated to avoid contamination of the sample with saliva.

The volume of GCF absorbed by Periopaper strips can be quantified using a Periotron device (Periotron Ide-interstate, NY, USA). The current Periotron model is the Periotron 8000. The Periotron is an electronic instrument that measures the effect of wetness of filter paper strips on the capacitance between the 'jaws' of the device, between which the filter paper is placed after the sample has been collected. The Periotron electrodes contain opposite charges, thus creating an electric field that consequently induces the polarity of the molecules, reducing the potential difference between the plates and increasing the capacitance. The greater the number of polar molecules positioned between the electrodes, the greater the capacitance and, therefore, the greater the values recorded by the Periotron. To calculate volume from the Periotron read-out, the device must be calibrated with known volumes of fluid pipetted onto Periopaper strips to generate a standard curve. Various types of regression models have been proposed to solve the equation for the calibration curve that is generated [217–219], and the 8000 series is supplied with software for calculating GCF volumes using a 4th order polynomial regression.

1.4.3 Analysis of the Immunological Content of the Fluid - ELISA and Luminex Techniques

Due to the low levels of cytokines and small sampling volumes, sensitive methods are required for accurate quantification of mediators present in gingival/peri-implant crevicular fluid. Therefore, techniques such as bioassay, radioimmunoassay, ELISA as well as multiplex assays for the simultaneous quantification of multiple cytokines have been applied with the aim of analyzing

the content of the PICF [210]. Only the techniques used in this work will be described.

The most recent investigations evaluating GCF mediators mostly use the ELISA technique, due to its specificity and sensitivity in antigen-antibody interactions [210,220]. One of the advantages of the ELISA is the possibility of using it in different types of samples, whether saliva, GCF or even blood [210]. The ELISA technique consists of capturing antigens by antibodies linked to enzymatic markers in microplate wells, also called microtiter plates, resulting in a conjugate with immunological and enzymatic activity. By using a colorless substrate of this enzyme linked to a chromogen and after the enzymatic reaction, the result is an observable and measurable colored final product. The intensity of the color in the wells can be measured by reading the absorbance and the amount of antigen is determined by comparing it to a standard curve [210,221]. In laboratory practice, it is common to perform the immunoassay in a microplate with about 48 to 96 sample wells [220].

Regarding the disadvantages presented by the technique it can be highlighted the demanding manipulation of the samples, the high consumption of reagents with an equally high cost and the prolonged incubation time. Another limitation is the fact that this technology allows studying only one analytic in each assay. So, it is very useful when the number of parameters that are going to be studied is not high but if a larger study is required another technique must be selected [210,220].

Thus, multiplex immunoassays, such as the Luminex technique, were developed to quantify multiple cytokines in a single sample. The Luminex® 100/200™ system can analyze 100 different analytes, in the same sample volume simultaneously, guided by the principles of flow cytometry [210,222]. The Luminex 100/200™ technique utilizes polystyrene microspheres as a surface for anchoring various reagents such as antibodies, oligonucleotides and substrates. Each microsphere emits a unique spectrum, enabling simultaneous identification of all microspheres involved in the reaction or test. To establish a ratio of two fluorophores on each microsphere, they are labeled in a specific manner. This labeling scheme allows for the perfect identification of 100 microspheres within the reaction tube. Following incubations, during which fluorophores attach to

specific reagents on the surface of the microspheres (such as antibodies, oligonucleotides and substrates), the test plate is read using the Luminex system 100/200™. In this system, the microspheres flow through a stream and are analyzed.

Both techniques are suitable when dealing with high sample volumes. However, if the sample volume is restricted, for instance, in cases of cerebrospinal fluid samples, GCF, synovial fluid, tears and similar situations, the Luminex 100/200™ system offers the advantage of analyzing multiple parameters using only a very small sample volume. When analyzing a single parameter, both techniques require the same amount of time. However, if the objective is to analyze multiple parameters, the Luminex system significantly reduces the time required as it enables simultaneous analysis of all the parameters. The Luminex system offers a time-saving advantage when conducting multi-parameter analyses.

1.5 Genetic Polymorphisms

Genetic polymorphisms correspond to changes in the deoxyribonucleic acid (DNA) sequence [223]. Compared to genetic mutations, they are more prevalent and affect at least 1% of the population [224]. Their prevalence varies between populations and ethnic groups, and they are considered a normal variant in the population [223,225].

Changes that occur at the nucleotide sequence level may have no effect, when there is no change in the amino acids and encoded proteins, or else have relevant biological effects, when an amino acid sequence of a codon is altered, and, consequently, there is a change in the structure of the encoded protein, as well as its function. This alteration may lead to a protein with a severely altered function, resulting in a dysfunctional protein, or only slightly altered. In the latter case, a change in physiological functioning, influenced by certain environmental factors (gene-environment interactions) may result, directly or indirectly, for example, in changes in innate and adaptive immunity. In addition, it can be a preventive factor for the appearance of a disease or, on the contrary, increase

the risk of its appearance. In this case, it may cause phenotypic differences in the individual's inflammatory response [225,226].

Genetic polymorphisms can have a variety of effects, and their impact on individuals can vary from subtle to significant. Genetic polymorphisms can contribute to the natural variation observed in traits between individuals. Certain genetic polymorphisms are associated with a greater or lesser risk of developing certain diseases. They may also influence an individual's response to medication. Genetic polymorphisms in genes encoding enzymes can affect enzyme activity. Polymorphisms in genes related to the immune system can affect how the body responds to infections and environmental factors. Genetic polymorphisms play a role in the evolutionary process. It is important to recognize that the consequences of genetic polymorphisms are often context-dependent and influenced by factors such as gene-gene interactions, environmental factors and individual lifestyle. Genetic research continues to explore the relationships between specific polymorphisms and their functional consequences to better understand their role in health and disease [224].

The presence of polymorphisms in the human genome varies and may be present in the following regions: promoter or 5' flanking region, exons or coding regions, introns or in the 3' UTR region (3'-untranslated region) [227]. Genetic polymorphisms can be subdivided into several categories, namely single nucleotide polymorphism (SNP), tandem repeat polymorphisms, which includes variable number of tandem repeats (VNTR) and short tandem repeat (STR), and insertion or deletion polymorphisms [224].

1.5.1 Single-nucleotide polymorphism (SNP)

Most polymorphisms that occur in the human genome correspond SNPs. It consists of a genetic variation of a single base pair, involving only one nucleotide, so it only allows combinations with two alleles [228]. It constitutes about 90% of the variations present in the human genome and occurs every 300 nucleotides, originating a total of several million SNP's throughout the individual's genome [224,229].

Considering that only 3-5% of DNA sequences encode proteins, it is more frequent that this polymorphism is present in non-coding regions [230]. SNP's have been linked to increased susceptibility to disease by stimulating the gene responsible for it [160]. In addition, these polymorphisms may be useful biological markers in identifying a disease, in the possible proximity to the genes responsible for it, as well as for carrying out studies related to genetic predictions.

1.5.2 Tandem Repeat polymorphism

Tandem repeat polymorphisms include a variable number of tandem repeats (VNTRs) minisatellites and short tandem repeats (STRs) microsatellites. The distinction between mini and microsatellites depends on the length of the repeated blocks. In microsatellites, the order repeat base composes between 2 and 9 units; while mini-satellites composes between 9 and 100 units [231]. As VNTRs can be located both in coding regions, untranslated regions or UTR's and regulatory regions close to a gene, the variation in the length of this sequence may show a significant impact on its functionality [232].

1.5.3 Insertion/deletion polymorphisms

Insertion/deletion polymorphisms, also called "indel", consist of a type of genetic variation in which a specific nucleotide sequence is present or absent, characterized as insertion or deletion, respectively.

They are not as common as SNPs, however, they are found widely spread throughout the genome and constitute a total of 3 million of the 15 million known genetic variants [224].

1.6 Laboratory methods for polymorphisms analysis

The common genotyping methods used in genomics studies, include polymerase chain reaction (PCR) - restriction fragment length polymorphism

(RFLP) analysis, TaqMan, pyrosequencing, Sanger sequencing, mass spectrometry, DNA microarray chip and denaturing high-performance liquid chromatography. Since there is a wide variety of molecular biology techniques for genotyping, only the methods used in this work for genotyping will be reviewed.

DNA sample collection

Genomic DNA can be obtained from any nucleated cell, which includes all cells in the body except red blood cells and platelets. The most common sources for genomic DNA isolation are blood lymphocytes and buccal cells. Collection of whole blood via phlebotomy is considered the gold standard for sample collection due to the large yield of genetic material. In general, 1 mL of whole blood yields approximately 20–30 µg of genomic DNA [233]. Since genotyping procedures require, on average, 10–100 ng of genomic DNA, whole blood collection of approximately 5 mL yields sufficient DNA for thousands of genotyping reactions. White blood cells are the main source of DNA for gene analysis. DNA isolation from anti-coagulated blood has, however, some disadvantages: donation is painful, medical supervision is needed and there is a risk of exposure to blood borne pathogens [175].

Buccal cell samples are commonly collected via one of two methods: cheek swab or the mouthwash method [234]. Cheek swabs are relatively easy to perform but this technique may result in low yields of DNA and higher rates of nonhuman-DNA contamination (e.g., oral flora) [235]. In the mouthwash method, patients are asked to vigorously swish a mouthful (10–30ml) of commercially available mouthwash for approximately one minute and then expectorate into a sterile container. The mouthwash technique offers the advantages of being easier to perform (compared with intravenous blood draws), being inexpensive and yielding higher amounts of DNA with less nonhuman genomic DNA collection than buccal swabs. In general, a 15–30-mL mouthwash sample yields a median of 25 µg of DNA, enough for over 250 genotyping reactions [236].

DNA isolation

Once DNA sample is collected, the genomic DNA must be isolated from other cellular material. DNA isolation methods differ based on the source of the sample and the type of commercially available kit used to isolate the DNA. Efficient isolation of DNA from a sample is the basis for successful forensic DNA profiling. There are many DNA extraction methods available and they vary in their ability to efficiently extract the DNA, as well as in processing time, operator intervention, contamination risk and ease of use [237].

Generally, the first step of DNA isolation from whole blood is to lyse the red blood cells. During this process, the red blood cells are broken down while the white blood cells stay intact. The intact white blood cells are collected in a pellet after centrifugation. The white blood cells are then lysed to break down cellular membranes and free the DNA into solution. Protein remnants are collected in a pellet, and the supernatant containing the genomic DNA is collected. The genomic DNA in the supernatant is then precipitated and hydrated, producing a DNA stock solution that can be used in subsequent DNA amplification and genotyping procedures [238].

The DNA isolation from buccal cells obtained by the mouthwash method differs slightly from the process of isolating DNA from whole blood. The first step of DNA isolation from buccal cells is centrifugation of the sample to concentrate the cells. After centrifugation, the supernatant is discarded, and a visible pellet (containing the buccal cells and other material) is obtained. The pellet is resuspended, and cell lysis is performed to release the DNA from the buccal cells. Like DNA isolation from blood, protein in the buccal cell sample is usually separated from DNA through a protein precipitation step, yielding a supernatant (which contains the DNA) and a protein pellet. A DNA stock solution is produced after precipitation and hydration of the supernatant [239].

Another option is to use a technique that combines the selective binding properties of a silica-based membrane with flexible elution volumes of between 20 μ l and 100 μ l. In this technique, the sample is lysed under denaturing conditions with proteinase K, the DNA binds to the membrane and the contaminants pass through. The residual contaminants are then washed away

and the pure, concentrated DNA is eluted from the membrane. The procedure is suitable for a wide range of forensic and human identity sample materials [237].

DNA amplification via PCR

As the human genome contains approximately 3 billion base pairs, proper identification, isolation and amplification of a specific region of interest (also called a target sequence) are crucial. The target sequence is a segment of genomic DNA that contains the polymorphic region to be tested. Amplification of a target sequence containing the polymorphic region to be tested is accomplished by PCR. PCR has revolutionized molecular biology and is one of the most common techniques used in genomic analyses [240].

PCR is based on the inherent physical and biochemical properties of DNA. DNA is a double-stranded molecule, with the two strands bound together in antiparallel form ($5' \rightarrow 3'$ and $3' \rightarrow 5'$) [241]. The two strands are bound complementary to each other by hydrogen bonds between nucleotide bases (adenine (A) binds with thymine (T), and cytosine (C) binds with guanine (G)). During PCR, the DNA molecule is denatured, or separated into two single strands.

To isolate the target sequence of interest, two single-stranded primers (oligonucleotides) are designed to flank the region of interest. Each primer is designed to bind specifically to a complementary sequence of nucleotides on the single-stranded DNA molecule. Consequently, following DNA denaturation, one primer will bind complementary to one strand of DNA in the forward direction, and the second primer will bind complementary to the second strand of DNA in the reverse direction. Generally, PCR primers are 17–30 base pairs in length and designed based on the known sequence of interest using primer design software programs [242]. The primers are placed in a reaction mixture that contains deoxynucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), buffer (which usually contains Tris hydrochloric acid [pH 8.3], magnesium chloride, and potassium chloride), genomic DNA and DNA polymerase [243]. DNA polymerase is an enzyme that catalyzes DNA strand synthesis in the $5'$ to $3'$ direction. The

most common DNA polymerase is Taq polymerase, a heat-stable DNA polymerase isolated from the bacterium *Thermus aquaticus* [244].

To amplify the region of interest, PCR requires a series of temperature cycles that each serve a distinct purpose. The first step of the cycling process is to denature the double-stranded DNA molecule into two single-stranded templates at approximately 95 °C. Following strand separation, the reaction is cooled to allow the PCR primers to anneal to their complementary strands. The annealing temperature, which generally ranges from 40 to 70 °C, is highly dependent on the template of interest and the designed primers. After primer annealing, the temperature is increased to 72 °C to allow for strand extension. During the extension step, deoxynucleotides are added to the 3' end of the hybridized primer by the action of DNA polymerase. Thus, the single-stranded product is built up to form a double-stranded replicate. This series of cycles (denaturation, annealing and extension) is repeated 30–40 times, and the number of replicates exponentially increases, producing millions of copies of the target sequence.

1.6.1 Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments

PCR coupled with RFLP analysis is a conventional SNP genotyping method. This method does not rely on automated technology and is practical for laboratories that genotype a limited number of samples. RFLP analysis uses restriction enzymes to discriminate between variant and nonvariant alleles. There are a large number of restriction enzymes available, and each recognizes a specific sequence of nucleotides, called restriction sites. Restriction sites are generally four to eight base pairs in length and are found throughout the genome. Once the restriction enzyme recognizes the restriction site, it cuts the DNA molecule at a precise point within that particular sequence. For genotyping purposes, RFLP analysis is used when the polymorphism of interest results in an allele that either possesses or lacks a specific restriction site [245]. The specific restriction enzyme used for a given genotyping assay is selected *a priori* based on the known alleles at the polymorphic site. Ideally, when the restriction enzyme

is incubated with the PCR product (approximately 5 μ L), it will recognize only one allele (either the wild type or variant) and hydrolyze the DNA molecule at that site. Depending on which alleles a person carries, different fragment patterns are produced as a result of the restriction digest. These fragments are resolved using gel-based methods, and genotypes are visually assigned based on different restriction fragment size patterns [246]. Gel electrophoresis is often performed to analyze the presence of polymorphisms. A small sample of the product obtained with the restriction process is mixed with a dye and loaded into an agarose gel, along with a DNA standard (or "ladder"). The gel is placed into an electrophoresis chamber and an electric current is passed through the chamber. The negatively charged DNA migrates through the gel toward the positive electrode. Because of the porous nature of the agarose gel, the speed at which the PCR product migrates down the gel is based on particle size: large pieces of DNA tend to move slowly through the gel, while smaller pieces move faster [247]. Because the PCR product is not visible, it is mixed with a fluorescent dye so that it can be identified when electrophoresis is complete, using ultraviolet light. Once the electrophoresis is complete, the positions of the fragments produced by the PCR-RFLP product are compared with the positions of the bands of the DNA ladder, allowing the identification of the presence of the polymorphism.

PCR-RFLP has been a traditional genotyping method in many genomic analyses because of its relative ease of use and the inexpensive equipment costs. However, the labor costs for RFLP can be expensive, particularly when analyzing a large number of samples. In addition, the time needed to digest and run each sample on gels limits the genotyping speed and throughput. Furthermore, as genotype determinations are made visually, this can make analysis difficult, particularly with assays that result in complex fragment patterns. For these reasons, RFLP is considered a low-throughput genotyping method and is most suitable for small genomic investigations [248].

1.6.2 Real-time PCR using TaqMan analysis

TaqMan is a commercial platform that uses a non-gel-based genotyping method [249]. Unlike pyrosequencing and RFLP, where PCR amplification and

allele discrimination occur in different steps, the TaqMan method is unique because PCR amplification and allele discrimination are conducted in the same step [250]. TaqMan PCR is a type of real-time PCR and uses a nucleic acid probe complementary to an internal segment of the target DNA. The TaqMan assay also differs from traditional RFLP and pyrosequencing genotyping methods in that it relies on the use of fluorescence-labeled probes (oligonucleotides), in addition to PCR primers, in the reaction mixture. These fluorescence-labeled probes are the basis for an allele specific hybridization reaction. For biallelic polymorphisms, one probe is designed to complementarily bind to the wild-type allele, while the other probe is designed to bind to the variant allele. Each probe is fluorescently labeled on the 5' end with a reporter dye, which releases a fluorescent signal, and on the 3' end with a quencher dye, which neutralizes or absorbs the fluorescent signal. Due to the closeness of the reporter and quencher dyes when the probes are intact, little fluorescence is released [251]. The amount of DNA used in the reaction can vary from 5 to 25 ng, depending on the quality of DNA.

During target amplification, both the PCR primers and the fluorescence-labeled probes bind complementary to the target sequence during annealing. The fluorescence-labeled probe that perfectly matches the target sequence (i.e., binds to the DNA without any nucleotide mismatches) forms a stable duplex, while the fluorescence labeled probe that contains a mismatch does not form a stable duplex [252]. Subsequently, DNA polymerase extends the strand during the extension step from the 3' end of the PCR primer. When DNA polymerase encounters a probe that is bound securely to the strand, it cleaves the probe at the 5' end. The cleavage of the probe separates the 5' reporter dye from the 3' quencher dye. Because the reporter and quencher dyes are no longer close together, there is an increase in fluorescence from the reporter dye. Genotypes are determined by measuring the relative amounts of fluorescence emitted during the reaction. For a biallelic polymorphism and a group of samples that are representative of the three possible genotypes (i.e., wildtype homozygotes, heterozygotes and variant homozygotes), three distinct fluorescence clusters will be present. Subsequently, software is used to process the fluorescence data and clustering pattern to make genotype determinations [250]. The TaqMan assay is

a particularly attractive genotyping method because it incorporates both PCR amplification and genotype determination into a single step [253]. Thus, it does not require post-PCR sample processing or gel electrophoresis. The TaqMan assay requires the use of a real-time PCR machine.

1.6.3 Sanger sequencing

Sanger sequencing is a targeted sequencing technique that uses oligonucleotide primers to determine specific DNA regions. Sanger sequencing begins with denaturation of the double-stranded DNA. The single-stranded DNA is then annealed to oligonucleotide primers and elongated using a mixture of deoxynucleotide Triphosphates (dNTPs), which provide the needed arginine (A), cytosine (C), tyrosine (T) and guanine (G) nucleotides to build the new double-stranded structure. In addition, a small quantity of chain-terminating dideoxynucleotide Triphosphates (ddNTPs) for each nucleotide is included. The sequence will continue to extend with dNTPs until a ddNTP attaches. As the dNTPs and ddNTPs have an equal chance of attaching to the sequence, each sequence will terminate at varying lengths.

Each ddNTP (ddATP, ddGTP, ddCTP, ddTTP) also includes a fluorescent marker. When a ddNTP is attached to the elongating sequence, the base will fluoresce based on the associated nucleotide. By convention, A is indicated by green fluorescence, T by red, G by black and C by blue. A laser within the automated machine used to read the sequence detects a fluorescent intensity that is translated into a “peak”. When a heterozygous variant occurs within a sequence, *loci* will be captured by two fluorescent dyes of equal intensity. When a homozygous variant is present, the expected fluorescent color is replaced completely by the new base pair’s color [254].

Sanger sequencing is a robust testing strategy able to determine whether a point mutation or small deletion/duplication is present. It has been widely used for several decades in many settings, including defining the mutational spectrum of a tumor as well as identifying a constitutional variant in diagnostic testing. Primers can be created to cover several regions (amplicons) to cover any size region of interest.

Although one could use individual Sanger sequencing reactions to cover any desired region, this testing approach can be costly when compared with other multiplex testing systems. Therefore, most currently available Sanger sequencing tests are gene-specific or analyze a small subset of genes.

Sanger sequencing is a “first-generation” DNA sequencing method. Despite the advantages of next-generation sequencing techniques, where throughput is orders of magnitude higher, Sanger sequencing retains an essential place in clinical genomics [255].

1.7 Hardy-Weinberg equilibrium

Identification of genes underlying human traits including diseases is crucial to our understanding of their etiology and is an important prerequisite for clinical diagnostics and prophylaxis [256]. One common strategy in identifying such genes has been the candidate gene association approach. Although this approach requires knowledge of prioritizing genes for screening, it benefits from simplicity in design and has thus attracted the attention of many investigators. In this approach, case-control analysis, compared with familial transmission disequilibrium test, has been by far the most commonly employed design [257]. This design aims to detect *loci*, at the population level, for which allelic or genotype status correlates with disease outcome by comparing unrelated cases and controls. In practical terms, it is relatively easy to implement. For instance, recruiting large number of unrelated participants is relatively easier than family-based sampling and results in increased statistical power [258]. However, with this comes certain drawbacks of which subject selection in creating a control group, to compare with the case group, is quite challenging [259]. The control group should represent the general population of the region where the patients emanate from, and be free of the disease present in case-group individuals. Clinical assessment of the control group can somewhat eliminate the possibility of disease presence, however, fulfilling the former criterion is not easily established and may result in biased inferences. Moreover, population stratification can also lead to spurious associations [260] when the control group represents more than one ethnic group with varying allele frequencies. One way

to address both representativeness and homogeneity (i.e., lack of significant population stratification) of the control group is to ensure that observed genotype frequencies are compatible with the Hardy-Weinberg equilibrium (HWE) predictions [260].

The Hardy Weinberg law, which is the basis of population genetics, states, in part, that in a large random mating population at equilibrium (i.e. no selection, migration or genetic drift), genotype frequencies are functions of allele frequencies and the former can be predicted from the latter [261]. HWE is the state of the genotype frequency of two alleles of one gene *locus* after one generation of random mating in an indefinitely large population with discrete generations, in the absence of mutation and selection: if the alleles are A and a with frequencies p and q ($q = 1-p$), then the equilibrium gene frequencies are just p and q and the equilibrium genotype frequencies for AA, Aa and aa are p^2 , $2pq$ and q^2 . Thus, there is equilibrium at both the allelic and the genotype level.

If such stability is the rule, then it provides the basis for the detection and estimation of the effects on the population, including natural and artificial selection, mutation, assortative mating, migration, inbreeding and random sampling (through finite population size).

As well as demonstrating how HWE must arise in the diallelic case, Weinberg also considered multiple alleles and multiple independent genes [262].

1.8 The importance of the chosen topic in the field of current implantology

Considering that more than 5 million dental implants are placed annually worldwide, peri-implant disease can affect more than 1 million of them each year. With the massive increase in the number of patients with dental implants, the prevalence of inflammatory conditions around implants is also intensifying [120]. Peri-implantitis is becoming a scourge and a serious public health problem, being their management challenging, and being associated with significant morbidity. The treatment of peri-implantitis involves high costs for the community and often fails to control the progression of the disease. In fact, there are several risk factors

that are not always considered by clinicians and that can, later, negatively influence the health of the peri-implant tissues. However, these risk factors are not always obvious and, only after a few years of implant placement, peri-implantitis processes can be triggered. There is currently no test that can determine the susceptibility of a patient to peri-implantitis who will receive a dental implant. While the treatment of periodontitis is established and effective, the treatment of peri-implantitis is poorly understood and very unreliable.

At the end of the last century, the hypothesis was generated that the genetic background of an individual may modulate the severity and progressive character of oral inflammations through expression of cytokines and their modulators [156,263,264]. Over the past 25 years, numerous SNPs have been associated with an increased incidence or severe course of periodontal inflammation. Current evidence suggests that the variability in periodontitis traits in humans may be attributable to genetic factors [265]. The polymorphisms that have been most evaluated are the *IL-1A* -889 and *IL-1B* +3954. Meta-analyses seem to show an association between these polymorphisms and the periodontal condition [266–268]. *IL-1A* -889 and *IL-1B* +3954 polymorphisms were reported as noteworthy biomarkers for chronic periodontitis susceptibility in the evaluation of eight meta-analyses, by means of a Bayesian approach [269]. Case–control studies, however, when examined separately, show inconsistent outcomes. This may result from the variety of ethnic groups examined, but seems to suggest that evaluation of a single SNP may have little or no prognostic/ diagnostic value for clinicians. Evaluation of specific genetic patterns, consisting of two, three or more genes, may more accurately indicate individuals with greater risk of periodontal inflammation [156,270]. The results of published studies focusing on *IL-1RN* gene (VNTR) polymorphism in periodontitis susceptibility are often conflicting. However, a meta-analysis suggested that *IL-1RN* (VNTR) polymorphism might contribute to an increased risk on chronic periodontitis [271]. The sequence of immunopathological events in peri-implant infections has some similarities to periodontal infections [272]. However, when compared to experimentally induced periodontitis, lesions associated with experimental periimplantitis demonstrate larger inflammatory cell infiltrates and more rapid and pronounced bone loss [23]. Due to the similarity between periodontal disease and peri-implantitis, the

hypothesis arose that patients with *IL-1A* -889, *IL-1B* +3954 or *IL-1RN* (VNTR) polymorphisms also presented a higher risk for peri-implantitis. Furthermore, scientific evidence indicates that biological complications in implants tend to be concentrated in a subset of individuals, which seems to imply that the host response may play a determining role in implant success. Even when exposed to similar risk factors, not all patients develop peri-implantitis, a situation that highlights the importance of genetic predisposition and differences between populations.

Some studies have investigated different SNPs in the *IL-1* genes as a risk factor for biological complications in dental implants [264,273–279]. Among them, the *IL-1A* -889 C/T (rs1800587) and *IL-1B* +3954 C/T (rs1143634) polymorphisms have been mostly investigated [280]. These polymorphisms are characterized by the substitution of cytosine with thymine in the DNA sequence [281]. The *IL-1A* -889 polymorphism is situated in the promoter region of the *IL-1A* gene. The presence of the T allele has been linked to an increase in gene transcriptional activity. In contrast, individuals with the CC genotype have shown reduced transcriptional activity, resulting in lower levels of IL-1 α compared to those with the TT genotype. *IL-1B* +3954 polymorphism is located in exon 5 of the *IL-1B* gene, and the replacement of the C allele by the T allele is associated with an increase in IL-1 β production [278,282].

In the *IL-1RN* gene, there is a genetic polymorphism located in intron 2 which is composed of a VNTR of 86 base pairs (bp) length. This sequence can be repeated two, three, four, five or six consecutive times, providing five possible alleles: 1, 2, 3, 4 and 5 [283]. The presence of *IL-1RN* allele 2 has been linked to a reduction in the production of IL-1Ra protein [284]. Additionally, *in vitro* studies have demonstrated that mononuclear cells from carriers of *IL-1RN* allele 2 produce higher levels of IL-1 β [285]. It is possible that *IL-1RN* allele 2 mainly increases the level of IL-1 β and therefore results in an imbalance in the IL-1 β /IL-1Ra ratio leading to an increased susceptibility or more severe outcome of inflammatory diseases.

Given the crucial role of IL-1 and IL-1Ra in inflammation, the genes *IL-1A*, *IL-1B* and *IL1RN* are potential candidates for genetic markers in peri-implantitis [286]. However, studies that analyzed the relationship between these

polymorphisms and peri-implantitis have shown conflicting results. There are studies that found an association between the presence of these polymorphisms and peri-implantitis [276–279,286], while others did not [176,264,287,288]. In 2008, Huynh-Ba et al. carried out a systematic review [289] where two articles were included [273,275], and the authors concluded that there is not enough evidence to support or refute an association between the IL-1 genotype status and peri-implantitis. It should be mentioned that the articles included in this review evaluated peri-implantitis based on bone loss [273,289]. In 2011, Dereka et al., published a systematic review in which the authors observed that, in two of the three studies that evaluated peri-implantitis in relation to IL-1 genotype, the *IL-1A* -899, *IL-1B* +3954 and *IL-1RN* (VNTR) polymorphisms were correlated to increased peri-implant tissue infection and destruction [290]. In a study by Liao et al. (2014), a meta-analysis was conducted to investigate the potential relationship between IL-1 polymorphisms (specifically, *IL-1A* -889 and *IL-1B* +3954) and the occurrence of complications or failure in dental implants [280]. Among the 13 studies analyzed, only four provided sufficient data to explore the possible connection between these genetic variations and peri-implantitis. The results of the meta-analysis indicated that exists a borderline significant association among Caucasian Europeans, between the positive composite genotype of *IL-1A* -889 and *IL-1B* +3954 and peri-implantitis (OR 1.65, 95 % CI 1.00–2.73). The positive composite genotype is the presence of at least one altered allele in the *IL-1A* -889 C/T and *IL-1B* +3954 C/T genes. More recently, Mohammadi et al. (2021), performed a meta-analysis that evaluated the association between the *IL-1A* -889, *IL-1B* -511, *IL-1B* +3954 and *IL-1RN* (VNTR) polymorphisms and the risk of peri-implant disease (PID) [291]. The meta-analysis included 16 studies and observed that the positive composite genotype of *IL-1A* -889 and *IL-1B* +3954 and the T allele and the CT genotype of the *IL-1B* +3954 polymorphism were associated with an increased risk of PID. PID included peri-implantitis, marginal bone loss and implant failure. This study also carried out a subgroup analysis and found no association between the polymorphisms evaluated and peri-implantitis.

To date, no consensus exists on whether genetic alterations of the *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* substantially contribute to an increased

susceptibility to peri-implantitis. Therefore, in the following chapter a systematic review and meta-analysis, quantitatively synthesizing existing studies, will be presented, to evaluate the association of common functional polymorphisms in the *IL-1* and *IL-1RN* genes with susceptibility to peri-implantitis. In this evaluation, an attempt was made to overcome the existing limitations of published systematic reviews and meta-analyses.

It is imperative to establish a genetic profile that can determine a patient's susceptibility *a priori* to the placement of an implant. A clarification of the genetic basis associated with peri-implant pathology could be used to predict peri-implantitis occurrence and to improve treatment and monitoring of patients with dental implants. The possible association between genetic polymorphisms and peri-implantitis must be based on a change in the individual's immune-inflammatory response.

The development of this work will contribute to the implementation of the following Sustainable Development Goals (SDGs): SDG 3. Good Health and Well-being; SDG 4. Quality Education and SDG 9. Industry, Innovation and Infrastructure.

SGD 3. Good Health and Well-being - realizing the implication of genetics in the etiopathogenesis of peri-implantitis will allow an earlier diagnosis of peri-implantitis as well as preventing disease development through closer monitoring of susceptible patients. Some of the objectives that belong to SGD3 are: to reduce dental implant failures due to peri-implantitis; to increase the success rates associated with the placement of dental implants; to adjust prevention measures in patients who are more susceptible to biological complications in dental implants; to substantially reduce the prevalence of peri-implantitis, as well as its recurrence after treatment; to reduce the number of treatments for peri-implantitis and to increase the predictability of peri-implantitis treatment.

SDG 4. Quality Education - this study will support the training of postgraduate and undergraduate students, since there is a lack of scientific evidence on the risk factors for peri-implantitis and the predictability of its treatment.

SDG 9. Industry, Innovation and Infrastructure - some of the objectives

that belong to SDG 9 are: developing a point-of-care test to help clinicians monitor patients with dental implants and predict the risk of peri-implant pathology; helping the implant industry to produce implants with specific characteristics, to be used in patients more susceptible to biological complications and helping the implant industry to produce implants with surfaces more resistant to bacterial contamination.

1.9 References

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2. SCIENTIFIC EVIDENCE OF THE RELATION BETWEEN GENETIC POLYMORPHISMS AND PERI-IMPLANTITIS

This chapter was based on the published work:

Paper I - Cardoso JM, Duarte S, Ribeiro AC, Mascarenhas P, Noronha S, Alves RC. Association between *IL-1A*, *IL-1B* and *IL-1RN* Polymorphisms and Peri-Implantitis: A Systematic Review and Meta-Analysis. Appl. Sci. 2022 Jul; 12 (14):6958. doi:<https://doi.org/10.3390/app12146958>.

2.1. Association between *IL-1A*, *IL-1B* and *IL-1RN* Polymorphisms and Peri-Implantitis: A Systematic Review and Meta-Analysis

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Feature Application: The evaluation of genetic polymorphisms may have great clinical relevance since they can be measured before the onset of the disease and may be of great benefit for treatment planning and prognosis at an early stage.

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Abstract:

Recent studies report that individuals with polymorphisms in the genes that encode for interleukin (IL)-1 α and IL-1 β (*IL-1A* and *IL1B*, respectively) and for IL-1 receptor antagonist (*IL-1RN*) may be more susceptible in developing peri-implantitis. Therefore, the current systematic review evaluates what is reported

about the role of genetics, more specifically of single nucleotide polymorphisms (SNP) on *IL-1* and variable number of tandem repeats (VNTR) on *IL-1RN*, in the development of peri-implantitis. This systematic review was carried out by screening PubMed, B-on, Cochrane and Scopus databases, for articles English, Spanish, and Portuguese, with no limit regarding the publication year. Eight articles were selected for systematic review and four for meta-analytic syntheses. Our results show that although there is a lack of consensus in the literature, there seems to be an association between *IL-1A*, *IL-1B* and *IL-1RN* polymorphisms and peri-implantitis. The results of the meta-analysis showed that patients who have the polymorphic allele at position +3954 of the *IL-1B* gene have on average almost twice the risk of developing peri-implantitis (odds ratio = 1.986, 95% confidence interval).

Keywords: genetics; peri-implantitis; interleukin-1; interleukin-1 receptor antagonist; interleukin-1 genotype; genetic polymorphisms; peri-implant disease

2.1.1 Introduction

Dental implants are now considered as an effective and predictable treatment modality for the functional and aesthetic rehabilitation of either partially or completely edentulous patients [1,2]. This rehabilitation method has a success rate of more than 90% for implants in function for more than five years [3]. However, despite the high success rates associated with implant rehabilitation, biological complications may arise in the peri-implant soft tissues, such as peri-implantitis, which can compromise the permanence of the implant [4]. Peri-implantitis is a plaque-associated pathological condition occurring in tissues around dental implants, characterized by inflammation in the peri-implant mucosa and progressive loss of supporting bone [5,6]. This definition is in accordance with the more recent classification of periodontal and peri-implant diseases (American Academy of Periodontology (AAP)/ European Federation of Periodontology (EFP) 2018) [6].

According with the Consensus report of workgroup 4 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions, there is strong evidence from animal and human experimental studies that plaque is the etiological factor for peri-implant mucositis, which is

assumed to precede peri-implantitis [6]. Data indicate that patients diagnosed with peri-implant mucositis may develop peri-implantitis, especially in the absence of regular maintenance care. However, the features or conditions characterizing the progression from peri-implant mucositis to peri-implantitis in susceptible patients have not been identified [6].

With a growing number of dental implants inserted, the potential number of sites for implant-associated diseases increases [7]. But the actual value of the incidence/prevalence of this disease is uncertain since the method of classifying peri-implantitis has varied between authors over the years and, in addition, few studies follow up and evaluate the sample for several years [8]. The characteristics of the populations included also vary between studies, which may influence the results [8]. In a systematic review carried out by Atieh et al. (2013), the prevalence of peri-implantitis obtained per patient was 18.8% while the prevalence per implant was 9.6% [9]. Lee et al. (2017) conducted a systematic review and meta-analysis, that included forty-seven articles, and concluded that the mean prevalence of peri-implantitis, at implant and subject level was 9.25% and 19.83%, respectively [10].

There are some similar features in the sequence of immunopathological events in peri-implant and periodontal infections [11]. Both are initiated primarily by Gram-negative anaerobic bacteria, while the inflammatory process goes faster and deeper around implants than around natural teeth and thus is a more significant problem for patients with dental implants [12]. However, Becker et al. (2014) in a study in which the transcriptome profiling using mRNA from patients suffering from either peri-implantitis or periodontitis was compared, the authors observed that these two pathologies react in a different way [13]. These differences may be explained by the anatomy, which is very different comparing the scar tissue in peri-implantitis with the specialized fibers inserting the surface of the teeth. In peri-implantitis tissue, transcripts associated to innate immune responses, and defense responses were dominating, while in periodontitis tissues, bacterial response systems prevailed [13].

Research evidence indicates that implant complications tend to be clustered in a subset of individuals rather than being randomly distributed in the

population implying that patient's host response might play a role for the implant success [14,15].

Interleukin-1 (IL-1) is the pivotal mediator of the immune-inflammatory response that acts both in response to bacterial infection and in bone metabolism [16]. IL-1 family has at least 11 cytokines; clustered on the long arm of chromosome 2q, and the three most studied members are *IL-1A*, *IL-1B*, and *IL-1RN* genes, which encode the agonistic proteins IL-1 α and IL-1 β , and IL-1 receptor antagonist (IL-1Ra), respectively [17]. The effect of IL-1 is determined by the balance between IL-1 α , IL-1 β , and IL-1Ra through competitive binding of IL-1Ra to the IL-1 receptor to block the activity of IL-1 α or IL-1 β . IL1 is strongly induced by lipopolysaccharides from the cell walls of Gram-negative bacteria and acts either directly or indirectly to initiate and amplify inflammatory responses through inducing expression of a substrate of effectors including cytokines/chemokines and matrix metalloproteinases [18].

The variations of IL-1 gene cluster, including *IL-1A* and *IL-1B* genes, and the variations of *IL-1 RN* are the most commonly studied functional polymorphisms for peri-implantitis.

Many studies have investigated different single nucleotide polymorphisms (SNPs) in the IL-1 genes as a risk factor for peri-implantitis. Among them, the *IL-1A* -889 C/T (rs1800587) and *IL-1B* +3954 C/T (rs1143634) have been mostly investigated [19]. The *IL-1B* -511 (rs16944) is also studied in some studies. These polymorphisms are characterized by the substitution of cytosine with thymine in the DNA sequence, which has been demonstrated to be associated with directly changed levels of gene expression and secreted cytokines, respectively [20]. In the *IL-1RN* gene, there is a genetic polymorphism located in intron 2 which is composed of a variable number of tandem repeats (VNTR) of 86 base pairs length. Several studies have analyzed the relationship between these polymorphisms and peri-implantitis. However, studies have yielded conflicting results on this issue [21–26].

A clarification of the genetic basis associated with peri-implant pathology could be used to predict peri-implantitis occurrence and to improve treatment and monitoring of patients with dental implants [21].

Most of these studies had a relatively small sample size and thus had insufficient statistical power to detect the genetic associations. Some of them do not refer to confounding variables such as periodontal condition, ethnicity and smoking habits. Furthermore, many studies, including published systematic reviews, assess the relationship of these polymorphisms with the peri-implant disease, pooling in the same group patients with bone loss, implant loss, and peri-implantitis [27–29]. However, peri-implant tissue health can exist around implants with variable levels of bone support [6]. In addition, an implant can fail without having an associated chronic inflammatory reaction as occurs in peri-implantitis.

Therefore, we performed a systematic review and meta-analysis, quantitatively synthesizing previous studies, to evaluate the association of common functional polymorphisms in the *IL-1* and *IL-1RN* genes with susceptibility to peri-implantitis.

2.1.2 Material and Methods

Study Design

The guidelines of PRISMA were followed while reporting this systematic review and meta-analysis.

The research question used for this systematic review was: “What is the importance of the interleukin-1 genotype (*IL-1A* -889 (rs1800587), *IL-1B* -511 (rs16944) and *IL-1B* +3954 (rs1143634)) and the IL-1 receptor antagonist genotype (*IL-1 RN* (rs2234663)) in the development of peri-implantitis, in adults, smokers or not, after at least one year of the implant in function?”. In addition, the PECO nomenclature was also used:

P (Population)—Adult patients

E (Exposure)—Genotype including selected polymorphisms of interleukin-1 and its antagonist

C (Control)—Genotype not including selected polymorphisms of interleukin-1 and its antagonist

O (Outcomes/Outcome)—Development of peri-implantitis

A search protocol was specified in advance and registered at PROSPERO (International Prospective Register of Systematic Reviews ID 322662).

Search Strategy

Two authors (J.M.C.) (S.D.) extracted the specific studies from the databases, and the same authors removed duplicates and irrelevant studies. Discrepancies, if occurred, were resolved by a third researcher (P.M.).

Systematic searches were performed on the PubMed, B-on, Cochrane and Scopus literature databases for studies published until January 2022.

The MESH terms and other keywords were used in combination, and Boolean operators such as AND and/or OR and/or NOT were added to obtain more relevant studies regarding the topic in question [30].

We used a specific search strategy with the following focused key terms: (“dental implants” or “oral implants”) and (“polymorphism” or “interleukins” or “interleukin-1”); (“peri-implantitis”) and (“interleukin-1” or “interleukins”) and (“gene polymorphism” OR “genotype”) not animal.

Inclusion and Exclusion Criteria

The inclusion criteria were: (1) Human case-control studies; (2) peri-implantitis as the outcome of interest in functional implants with at least one year follow up; (3) studies reporting *IL-1A* -889, *IL-1B* -511, *IL-1B* +3954 and *IL-1RN* (VNTR), and composite genotype of *IL-1A* -889/ *IL-1B* +3954 polymorphisms; (4) articles written in English, Spanish or Portuguese.

The exclusion criteria were: (1) Studies that included patients with uncontrolled systemic diseases; (2) studies in which patients with peri-implantitis were included in a general category disease group of peri-implant diseases or

other conditions (presence of suppuration, development of fistula, radiographic bone loss or implant loss).

The author (S.D.) screened all the titles and abstracts based on the eligibility criteria and included/excluded studies for full-text review. Another author (J.M.C.) rechecked relevant articles. Discrepancies, if occurred, were resolved by a third researcher (P.M.).

Data Extraction

One author (S.D.) independently extracted the information or data from each study and another author (J.M.C.) rechecked them.

Risk of Bias Assessment

Two authors (S.D. and P.M.) evaluated the quality of included articles using the Joanna Briggs Institute (JBI) checklist [31]. This tool evaluates “cross-sectional analytical” studies regarding eight domains. These domains evaluate if the criteria in the sample were clearly defined, if the study subjects and the setting were described in detail, if the confounding factors were identified and strategies to deal with, and if the outcomes were measured in a valid and reliable way.

Statistical Analysis

Statistical analysis was performed using the Open Meta [Analyst] for Windows 8 (built 04/06/2015) software, from Center for Evidence Synthesis in Health (Brown University, Providence, RI, USA), which allowed us to obtain all meta-analysis and meta-regression plots, which are described later [32]. Allele frequencies against peri-implantitis incidence were converted in odds ratios effect size and associated 95% confidence intervals in a binomial model framework. Model parameters were estimated applying the restricted maximum likelihood method.

Heterogeneity was assessed using the I^2 index and it was considered high if it was above 50%. A high I^2 value means that the authors of the different articles analyzed are not in consensus. To counteract this value, covariates can be added [33].

We used 95% confidence intervals and considered the p -value test results lower than 0.05 to correspond to a statically significant result.

A subgroup analysis was done in relation of the odds ratio (OR) of the ethnicity in the difference in the frequency of the mutated allele between the disease and control group.

Meta-regressions were carried out for longitude and latitude of the sample's provenance, the mean age, the percentage of males and females, the representative ratio of the mutated allele, sample size and year of publication.

2.1.3 Results

Study Selection

Initially, 324 articles were obtained from all databases. After excluding the duplicates, 218 articles remained, of which the titles and abstracts were read. Since 199 were not included in the theme of this systematic review, they were excluded, leaving only 19. These were read in full and, as eleven articles did not meet the inclusion and exclusion criteria, only eight articles remained, which were included in this systematic review [21–26,34,35]. However, only four of these articles contained eligible quantitative data, and were included in the meta-analysis [21,23,24,26].

A PRISMA flowchart was carried out to systematize the selected information throughout the different research phases (Figure 2.1).

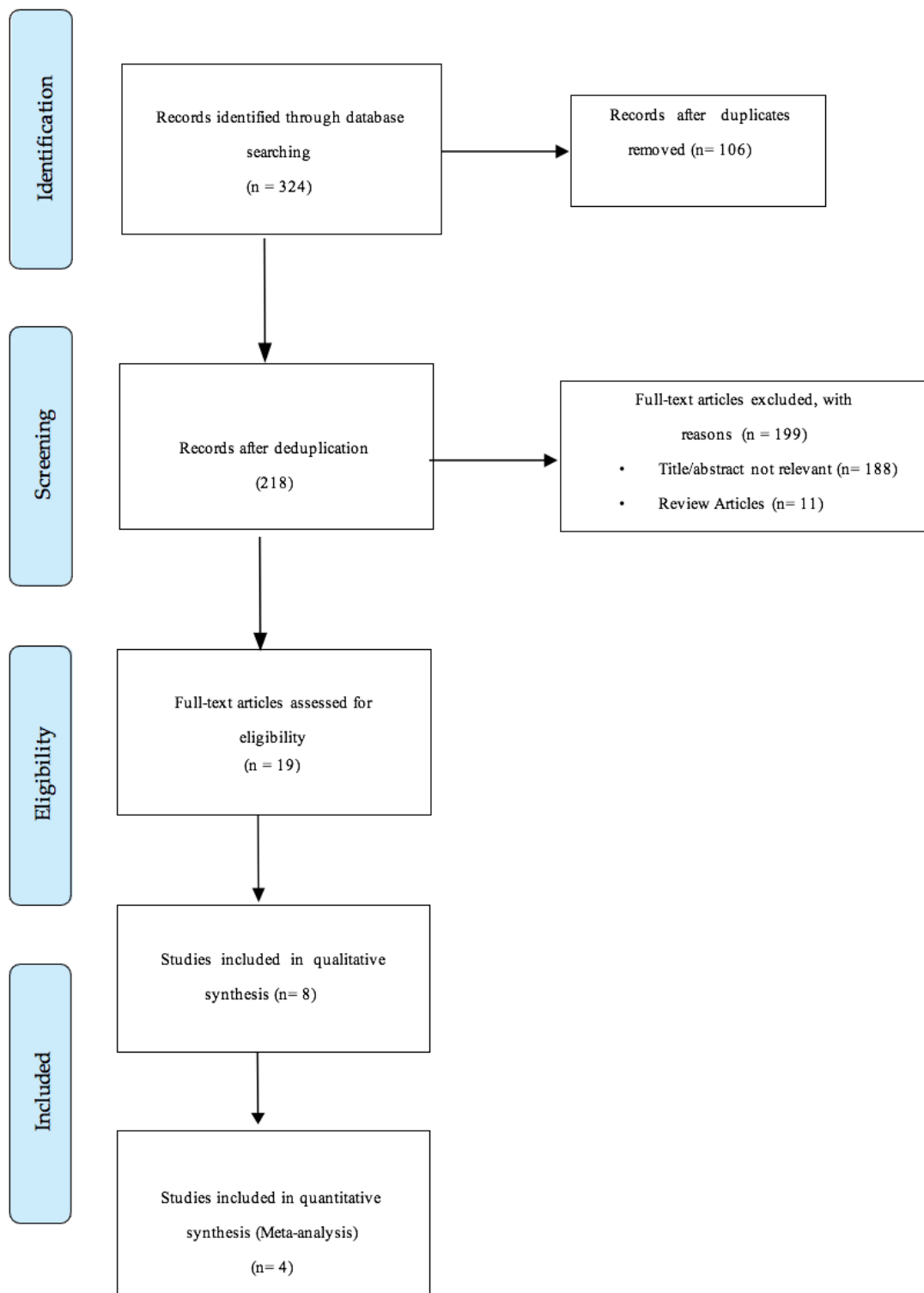


Figure 2.1 - Flow chart of the study selection.

Results of the Bias Risk Assessment

In order to know the risk of bias in the articles, by completing the JBI checklist, a Traffic Plot-type graph was obtained (Figure A1), where it is possible to clearly observe the risk of bias for each article selected for this systematic review. None of the articles had three or more high risk judgments and they were all included in the review.

Characteristics of the Studies

The Table 2.1 provides the characteristics of eight articles [21–26,34,35] included in the systematic review. Four articles included individuals from Europe [21,25,34,35], two from Asia [24,26], one from North Africa [22] and one from South America [23]. Five studies reported *IL-1A* -889 polymorphism [21,22,24,25,34], two *IL-1B* -511 [21,23], seven *IL1B* +3954 polymorphism [21–26,34], two *IL-1RN* (VNTR) polymorphism [21,35] and five composite genotype of *IL-1A* -889 and *IL-1B* +3954 polymorphisms [21,22,24,25,34].

The table A1 provides the percentage of men and women, from which it is possible to conclude that there is a predominance of female individuals in five of the eight articles [21, 23, 25, 26, 34]. The mean age varies between 41 and 67 years. The mean age of the sample of each study was considered, since between the group with peri-implantitis and the control group there was no difference in mean age of more than five years.

The mean percentage of the mutated allele in the sample are indicated in four of the eight articles [21, 23, 24, 26]. Some articles only indicated the genotype of the individuals, without referring to whether they were homozygous or heterozygous for the mutated allele, which could influence the results.

Table 2.1 - Systematic review table of the 8 articles included in the present study.

Year & Author	Type of study	Polymorphisms evaluated	Geographic region	Inclusion criteria	Exclusion criteria	Smoking habits	Time of implant in function (months)	Outcome
Garcia-Delaney, 2015	Case-control	<i>IL-1A</i> -889 <i>IL-1B</i> +3954 <i>IL-1RN</i> +2018	Spain	Systemically healthy patients; Peri-implantitis group: BOP or SUP (+); BL > 2mm Control group: BOP (-); SUP (-); BL < 2 mm	- Cases with incomplete data or with dubious diagnosis	All smokers	≥18	<i>IL-1</i> genotypes do not seem to be good predictors of peri-implantitis in the great majority of smoking patients. Furthermore, no synergic effect was found between <i>IL-1</i> genotypes and heavy smokers. Patients with a previous history of periodontitis were more prone to peri-implantitis.
Hamdy, 2011	Case-control	<i>IL-1A</i> -889 <i>IL-1B</i> +3954	Egypt	Systematically healthy patients; Peri-implantitis group: BOP (+); PPD > 4mm; BL (+)	- Smokers; - History of antibiotic intake or periodontal treatment in previous 6 months	Non-smokers	≥36 (implant placement)	The combination of the polymorphism in <i>IL-1A</i> -889 and <i>IL-1B</i> +3954, in patients with inflamed periodontal or peri-implant tissues, may act as a risk factor that increases tissue destruction. <i>IL-1</i> gene polymorphism (<i>IL-1A</i> -889 and <i>IL-1B</i> +3954) may have a negative effect on treatment outcomes of peri-implantitis.
He, 2019	Case-control	<i>IL-1A</i> -889 <i>IL-1B</i> +3954 <i>TNFA</i> -308	China	Peri-implantitis group: PPD ≥ 4mm; BOP (+); GI (+); BL involving ≥ 2 threads compared to prosthetic placement Control group: Healthy peri-implant tissue; PPD < 3mm; BOP (-); BL (-)	- Smokers; - Pregnant or lactation; - General health problems (diabetes mellitus, HIV infection); - Intake of any antibiotics and anti-inflammatories in the last 3 months.	Non-smokers	≥ 24	The <i>IL-1A</i> -889 or <i>IL-1B</i> +3954 genetic polymorphisms were associated with the risk of peri-implantitis and periodontal status
Lachmann, 2007	Case-control	<i>IL-1A</i> -889 <i>IL-1B</i> +3954	Germany	- Systemic health in general; - Absence of medical conditions that compromise the immune system.	ND	ND	≥ 12	The composite <i>IL-1A</i> -889 and <i>IL-1B</i> +3954 genotype investigated exerted only little influence on the peri-implant crevicular immune response, and this influence appeared to be of limited impact

				Peri-implantitis group - PPD > 4mm; BOP (+); BL (+)				in sites with established peri- implantitis lesions.
Laine, 2006	Case-control	<i>IL-1A</i> -889 <i>IL-1B</i> +3954 <i>IL-1B</i> -511 <i>IL-1RN</i> (VNTR)	Sweden	Peri-implantitis group: BL involving ≥ 3 threads; BOP and/or SUP (+)	ND	Peri-implantitis group - 76% smokers Control group - 49% smokers	≥24	<i>IL-1RN</i> gene polymorphism is associated with peri-implantitis and may represent a risk factor for this disease.
Melo, 2011	Case-control	<i>IL-1B</i> +3954 <i>IL-1B</i> -511 <i>IL-6</i> -174	Brazil	- No medical history of chronic illness; - No history of antibiotic therapy or use of steroidal or AINE medications in the 6 months prior to the study. Control group: no mucosal bleeding, PD ≤4 mm, BOP and SUP (-)	- Smokers; - Pregnant or lactation; - Periodontitis	Non-smokers	ND	There was no correlation between the concentration of IL- 1β and IL-6 in the crevicular sulcular fluid present in healthy or diseased osseointegrated implants in comparison with healthy teeth. The studied genetic polymorphisms had no influence on peri-implant disease.
Petkovic-Curcin, 2017	Case-control	<i>IL-6</i> -174 <i>IL-10</i> -1082 <i>TNF-α</i> -308 <i>CD14</i> -159 <i>IL-1RN</i> (VNTR)	Serbia	Peri-implantitis group: PPD ≥ 4 mm, BOP +, GI (+); PI (+) and BL involving ≥ 2 threads compared to prosthetic replacement Control group: Healthy peri-implant tissue, BOP (- , PPD < 4 mm, BL (-)	ND	C - 42% smokers PI -71% smoker	≥24	Smoking and the presence of <i>TNFα</i> -308 polymorphism may increase the risk for peri- implantitis, while <i>CD14</i> -159 polymorphism decreases the risk. The results also indicate significant association of <i>CD14</i> - 159, <i>TNFα</i> -308, and <i>IL-6</i> -174 genotypes and clinical parameters in the Serbian population.
Saremi, 2021	Case-control	<i>IL-1B</i> +3954 <i>IL-10</i> -819 <i>IL-10</i> -592 <i>TNF-α</i> -308 <i>TNF-α</i> -857	Iran	- No history of periodontitis Peri-implantitis group: PPD > 5 mm; BOP (+) with or without SUP; BL ≥ 2 mm Control group: PPD< 4 mm; BL (-)	- Oral and periodontal diseases (except caries), - Current orthodontic treatment; - History of systemic diseases or any complication that compromises the immune system (diabetes, HIV, hepatitis, chemotherapy); - Pregnant or lactation	ND	≥12	Genetic polymorphisms of <i>IL-10</i> - 819, <i>IL-10</i> - 592 and <i>IL-1B</i> +3954 may play a role in the pathogenesis of peri-implantitis and increase its risk of occurrence.
(+) - presence, (-) -absent, BL – bone loss, BOP – bleeding on probing, SUP – suppuration, GI – gingival index, ND – non-defined, PPD – probing pocket depth, PI – plaque index, VNTR - Variable number of tandem repeats, AINE – non-steroid anti-inflammatory.								

Meta-Analysis

To carry out the meta-analysis, only four articles were used, as explained above [21, 23, 24, 26]. However, due to the lack of information and the different types of polymorphisms analyzed by each article, it was only possible to perform the meta-analytic study for the *IL-1B* +3954 polymorphism, which was the only common polymorphism in the previously selected articles. In addition, these four articles contained information about the amount of mutated alleles in the sample.

Figure 2.2 shows the odds ratio (OR) meta-analysis as a forest plot. The OR values extracted from each study are graphically illustrated. This graph performs an analysis of heterogeneity that is expressed in the form of I^2 , in %, and presents the mean value of the ORs and the respective uncertainty in the form of a 95% confidence interval. On the left, the authors and the year of the included studies are indicated, followed by the corresponding analytical values obtained.

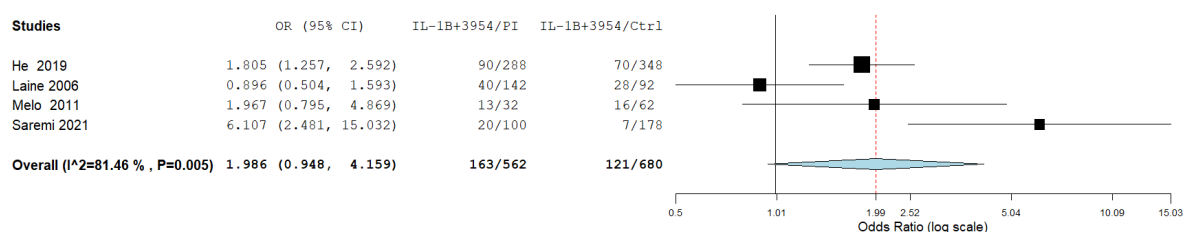


Figure 2.2 - Meta-analysis Forest Plot. (OR- odds ratio, CI – confidence interval, PI – peri-implantitis group, Ctrl – control group).

The odds ratio of these studies is 1.986 (chi square test, $p < 0.001$), which means that this polymorphism increases the risk of carriers of the mutated allele in the *IL-1B* +3954 to have peri-implantitis, by almost twice, as compared to the control group, patients that don't have this polymorphism.

The I^2 in this case is quite high, which may indicate that the authors are not in agreement with the results they present.

Subgroup Analysis

The subgroup analyses (based on the ethnicity) of the association between the frequency of the mutated allele in the *IL-1B* +3954 gene and the risk of peri-implantitis is shown in Figure 2.3. The results showed that the ethnicity, Asian, is one factor that affects the difference in frequencies of mutated allele in the *IL-1B* +3954 gene between the disease and the control groups (OR = 3.088, chi square test, $p < 0.001$). The ethnicity, Caucasian, does not affect the difference in frequencies of the mutated allele in the *IL-1B* +3954 gene between the disease and the control groups (OR = 0.896, chi square test, $p = 0.709$). Regarding mixed ethnicity, further studies are needed to understand its influence in the difference in frequencies of the mutated allele in the *IL-1B* +3954 gene between peri-implantitis and control groups (OR = 1.967, chi square test, $p = 0.140$).

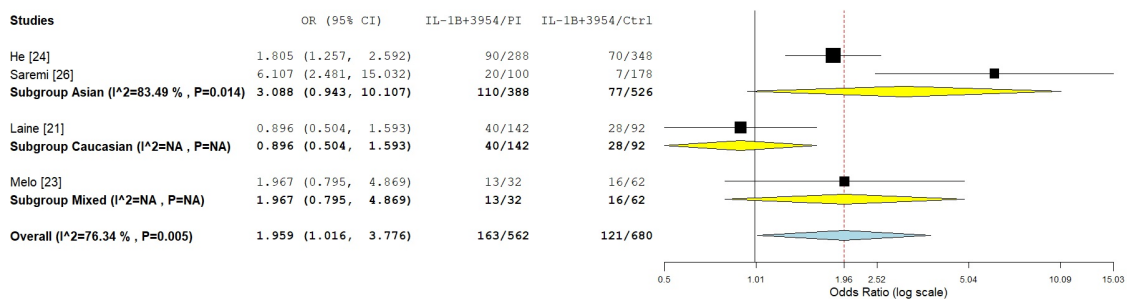


Figure 2.3 - The subgroup analyses (based on the ethnicity) of the association between the frequency of the mutated allele in the *IL-1B* +3954 gene and the risk of peri-implantitis. (OR- odds ratio, CI – confidence interval, PI – peri-implantitis group, Ctrl – control group).

Meta-Regression

Regarding meta-regressions, in all there is a limitation, since there are only four points represented in the graphs, only four articles were analyzed. There is, therefore, a limitation to being able to extrapolate this trend to other populations or other geographic regions. Therefore, further studies are recommended in order to confirm this trend.

The size of the circles in all figures represents the weight that this article contributed to the average. Since the OR logarithm is proportional to the OR, with

negative coefficient, as the latitude increases, there is a tendency for the OR to decrease, and in this case the trend is statistically supported/significant since the p -value obtained was 0.025 (Figure 2.4). The result of the coefficient statistical test in the longitude-relative meta-regression presents a value of $p = 0.295$, so it is not statistically significant (Figure A2).

Regarding the mean age, the p -value in this meta-regression has a value of 0.005, thereby it is statistically significant (Figure 2.5).

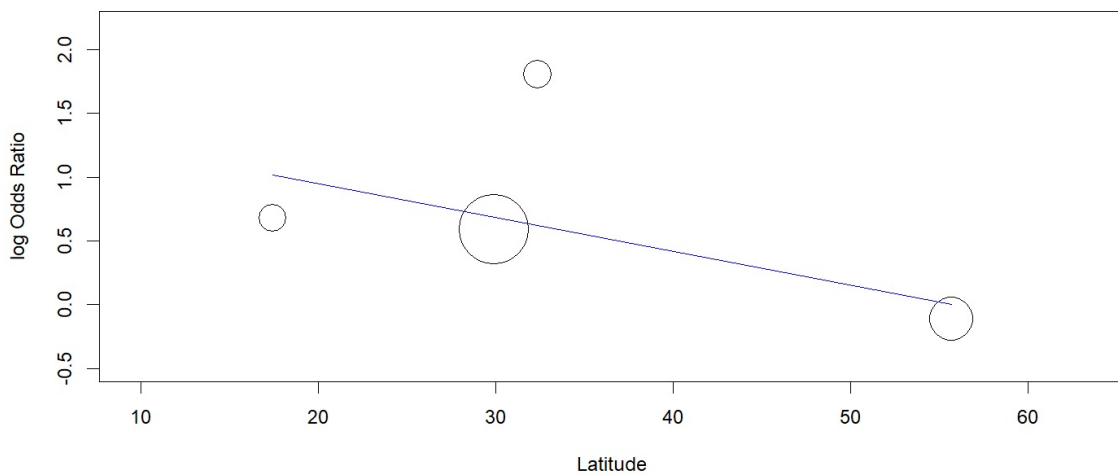


Figure 2.4 - Log OR meta-regression for *IL-1B* +3954 polymorphism as a function of latitude covariate (p -value = 0.025 Coefficient = -0.026).

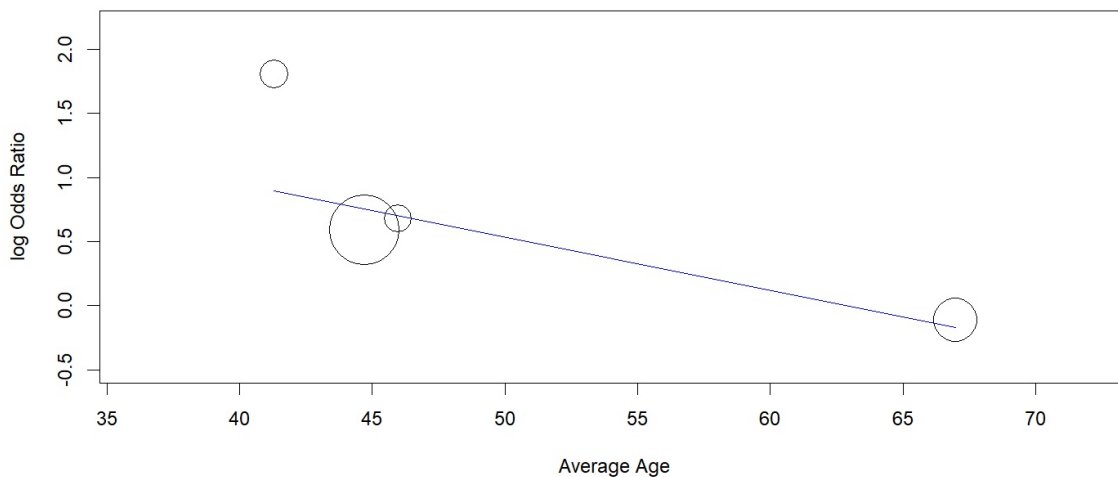


Figure 2.5 - Log OR meta-regression for the *IL-1B* +3954 polymorphism as a function of the mean age covariate (p -value = 0.005, Coefficient = -0.041).

Analyzing the meta-regression analysis regarding the percentage of male subjects, it is possible to conclude that, as the proportion of male individuals increases, there is an increase in the OR, therefore, there is an increase in risk, however, it is not statistically significant (Figure A3).

We can also observe that as the percentage of the mutated allele increases, the OR decreases. It is necessary to consider that the value of the mutated allele corresponds to the entire sample, both for control patients and patients with peri-implantitis. The effect of the lower percentage of the mutated allele on the risk for peri-implantitis is statistically significant (p -value < 0.05) (Figure 2.6).

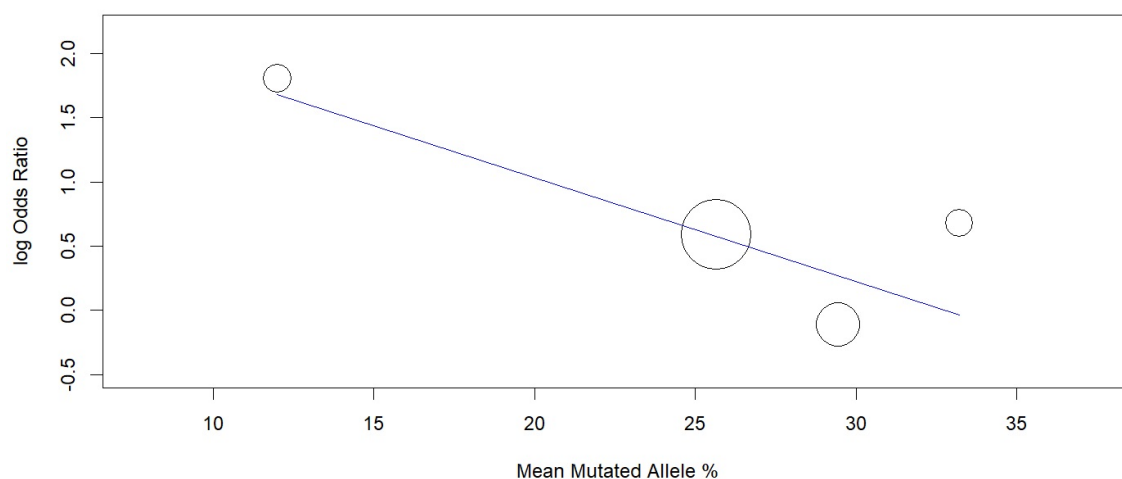


Figure 2.6 - Log OR meta-regression for the *IL-1B* +3954 polymorphism as a function of the covariate percentage of the mutated allele in the sample (p -value = 0.003, Coefficient = -0.081).

Performing a meta-regression analysis regarding the year of publication (Figure 2.7) and the sample size (Figure A4) of the included studies, we can conclude that the effect of year of publication is statistically significant while the effect of the sample size is not statistically significant. We can observe that as the year of publication increases, the risk for peri-implantitis also increases.

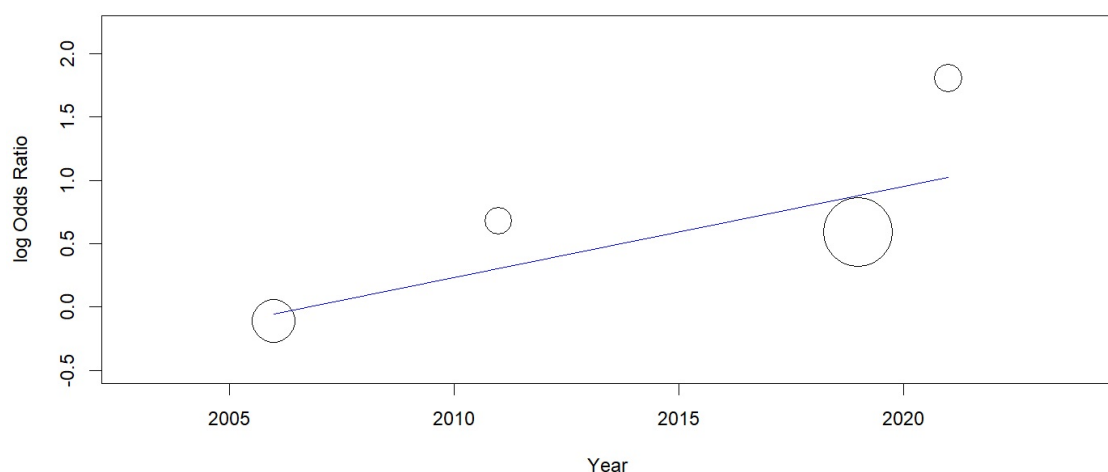


Figure 2.7 - Log OR meta-regression for the *IL-1B* +3954 polymorphism as a function of the year of publication (p -value = 0.014, Coefficient = 0.067).

Through the interpretation of this meta-analysis, we were able to analyze the historical perspective from the oldest to the most recent article. From the Figure 2.8, we concluded that, with these successive assessments, there is an increased risk of carriers of the *IL-1B* +3954 polymorphism to have peri-implantitis.

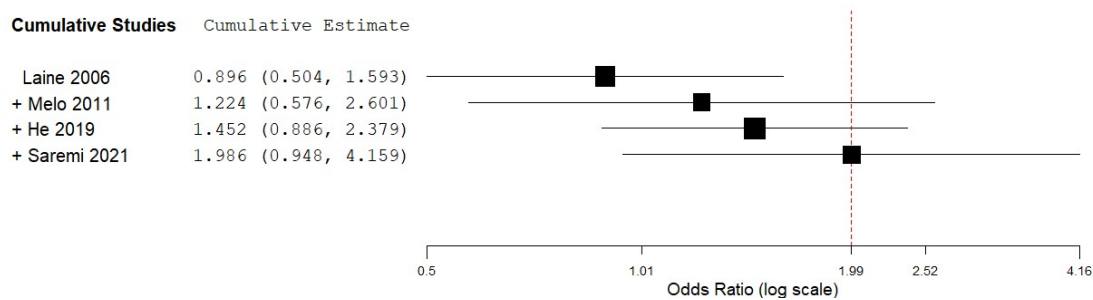


Figure 2.8 - Forest Plot of the cumulative meta-analysis for the *IL-1B* +3954 polymorphism.

Sensitivity Analysis

Both “leave-one-out” and “cumulative analysis” were performed within the sensitivity analysis for the *IL-1B* +3954 polymorphism study effects sizes. Results are illustrated in Figure A5. All studies seem to have had a balanced contribution to the pooled results although recent studies seem to overestimate the effect in comparison with previous ones.

2.1.4 Discussion

This study aimed to verify if there is an association between the polymorphisms of the *IL-1A*, *IL-1B* and *IL-1RN* genes and peri-implantitis. It is a relevant topic since, if this association is confirmed, it is possible to identify patients who have this polymorphism and the physician’s approach can be adjusted in order to prevent the onset of this disease, considered a public health problem. Genetic polymorphisms are constant and can be measured before disease onset, thus it could be of great benefit for treatment planning and

prognosis in an early stage. Despite the fact that peri-implantitis etiology and pathology are complex, the identification of genetic biomarkers associated with peri-implantitis risk could be a valuable tool in daily clinical practice. They could be used for early identification of individuals predisposed to increased peri-implantitis risk, and this would help practitioners (after estimating dental implants prognosis) in individualizing their treatment plan.

The future of disease diagnosis must involve not only identifying more susceptible patients but also identifying disease-associated biomarkers. Biomarkers are biological indicators with high prognostic and predictive value that can be related to the onset or development of a pathology. Frequently they must be able to predict the presence of a disease or its progression. A recent paper that has focused on five promising host derived biomarkers as candidate for early diagnosis of periodontitis, IL-1 β was one of them [36].

After a systematic search, which is described in the flowchart in Figure 1, eight articles were obtained for the present systematic review [21–26,34,35].

Table 1 shows the general conclusions of each of the eight articles. Four of these articles concluded that individuals with the IL-1 gene polymorphisms had a higher risk for the development of peri-implantitis [21,22,24,26]. These results are in agreement with what is found in the systematic review of Dereka et al. (2012) [27]. In this systematic review, the authors observed that in two of the three studies which evaluated peri-implantitis in relation to IL-1 genotype, the findings indicate that *IL-1RN*, *IL-1A* -899, *IL-1B* +3954 gene polymorphisms were correlated to increased peri-implant tissue infection and destruction [27]. In 2008, Huynh-Ba et al. carried out a systematic review [37] where two articles were included [38,39], and they concluded that there is not enough evidence to support or refute an association between the IL-1 genotype status and peri-implantitis. It should be mentioned that the articles included in this review evaluated peri-implantitis based on bone loss [37,38].

The number of studies in the literature evaluating this association is limited and some of these do not refer to confounding variables such as periodontal condition, ethnicity and smoking status. These factors may explain the differences found between the different studies. The ethnicity of the populations

included in the studies varies and there are studies that include patients who smoke and others who do not. On the other hand, the definition of peri-implantitis varies between studies, which makes comparisons between studies difficult. In our systematic review, we only included articles in which periimplantitis was the evaluated outcome. From 2018, with the new Classification of Periodontal and Peri-implant Diseases and Conditions, it will be easier to standardize the definition of peri-implantitis between studies [6]. This new classification is the product of the World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions organized jointly by the American Academy of Periodontology (AAP) and the European Federation of Periodontology (EFP) to create a consensus knowledge base for a new classification to be promoted globally.

Furthermore, some aspects such as the surface and position of the implants (anterior or posterior), the type of prosthetic restoration used (removable or fixed), type of retention (screw or cemented), the need for guided bone regeneration before or at the time of implant placement, general oral cavity health conditions were not referred or varied between studies. The techniques of DNA isolation also differ between studies, with some studies obtaining DNA from oral mucosal cells using swabs [22–25,34] and others using mouthwashes [21]. Other authors obtain DNA from blood [26,35].

Within the limits of this systematic review, it might be concluded that there is no obvious association between specific genetic polymorphism of *IL-1* and *IL-1RN* and periimplantitis, although a tendency should be underlined showing the potential link between *IL-1* genotype and peri-implantitis.

For the meta-analysis, it was only possible to include four articles [21,23,24,26] since, in order to obtain a more real result on the true influence of the polymorphism in the disease, allele frequencies were evaluated. The remaining studies were excluded as they did not mention whether the patients were homozygous or heterozygous for the mutated allele, which may influence the interpretation of the results. The common polymorphism in these four studies was *IL-1B* +3954, which was evaluated.

In the present study, it was found that individuals who had the polymorphism in the *IL-1B* +3954 had an almost twice higher risk of developing peri-implantitis (OR 1.986). This result is in agreement with a meta-analysis performed by Jin et al. (2021) where the association between *IL-1A* -889, *IL-1B* +3954, *IL-1B* -511, composite genotype of *IL-1A* -889 and *IL-1B* +3954, and *TNF- α* -308 and risk of peri-implant disease (PID) was evaluated [29]. The PID included implant failure/loss, marginal bone loss and peri-implantitis. When a subgroup analysis is performed, it is observed that only *IL-1B* +3954 is associated with a higher risk of peri-implantitis (OR 1.87).

In a meta-analysis carried out by Liao et al. (2014), that included four articles regarding the relationship of IL-1 polymorphisms and peri-implantitis, the composite genotype of *IL-1A* -889 and *IL-1B* +3954 was associated with increased risk of peri-implantitis (OR 2.34) [19]. The inclusion criteria differed from the present study, which allowed the authors to assess the relationship between the composite genotype and peri-implantitis.

In a recent meta-analysis, which evaluated association between interleukin-1 polymorphisms and susceptibility to PID, the authors observed that there was no association between *IL-1A* -889, *IL-1B* -511 and *IL-1RN* (VNTR) polymorphisms and the risk of PID [28]. In contrast, an association was observed between the composite genotype of *IL-1A* -889 and *IL-1B* +3954 and PID. In addition, the T allele and CT genotype of *IL-1B* +3954 polymorphism were also associated with an elevated risk of PID. In subgroup analysis no association was found between the polymorphisms evaluated and the risk of peri-implantitis [28]. However, the results of the study have to be interpreted with caution because the authors included in the same group of PID, patients with peri-implantitis, patients with marginal bone loss and patients with implant failure. However, bone loss can occur with or without signs of infection, whereas peri-implantitis is an inflammatory lesion associated with loss of supporting bone around implant [5]. On the other hand, not all implant failures are caused by peri-implantitis and not all peri-implantitis lead to implant loss.

Regarding meta-regressions carried out in our meta-analysis, we observed that as the latitude increases, there is a tendency for the OR to decrease, so with increasing latitude, the risk for peri-implantitis decreases. The

result of the coefficient statistical test in the longitude-relative meta-regression is not statistically significant. The influence of ethnic and racial variations in the frequency of gene polymorphisms in terms of the genetic susceptibility to a specific disease has been reported [40,41]. It has been demonstrated that there is low prevalence of the periodontitis-associated *IL-1A* +4845 and *IL-1B* +3954 gene polymorphisms in Chinese (2.3%) [41] compared with that reported for Caucasians (36%) [40]. The *IL-1A* +4845 polymorphism is more than 99% in linkage disequilibrium with the *IL-1A* -889 polymorphism (if one is present, the other usually is present) [42]. In our study, it was observed that in populations where the allele frequency of the mutated allele is higher, the risk of peri-implantitis is lower. Furthermore, in a subgroup analysis we also found that the Asian population was at a greater risk for the disease than the Mixed or Caucasian subgroup. This is not in agreement with the information that exists regarding certain geographic areas, such as the Asian continent. In these areas, there is a low prevalence of the mutated allele in the population and a lower risk for inflammatory diseases such as periodontitis, compared to other geographic areas. We were only able to evaluate four articles in our meta-analysis so we can't extrapolate these results.

Through the interpretation of our meta-analysis, we were able to analyze the historical perspective from the oldest to the most recent article. We concluded that with these successive assessments, there is an increased risk of carriers of the *IL-1B* +3954 polymorphism to have peri-implantitis. This finding may be related to a more adequate definition of peri-implantitis over the years in the various published studies.

In carrying out this work, there were some limitations. Not all articles presented information about the follow-up period, the inclusion and exclusion criteria were not similar between the various articles and there were parameters that some articles evaluated while others did not. In addition, there were also factors, such as age, gender and ethnicity, which were not analyzed in the same way in all studies. These factors contributed to the heterogeneity obtained in this work.

The definition of peri-implantitis itself was not the same in all studies. Another limitation is the fact that some of the articles referred only to the genotype

of the individuals and in this study the allele frequency was studied, since it is more specific.

The number of studies in the literature evaluating this association is limited, had small samples and some of them do not refer to confounding variables such as periodontal condition, ethnicity and smoking status. The inequality of these study designs necessitates the conduction of further studies using proper methodologies and from different ethnic groups. A clarification of the genetic basis associated with peri-implant pathology could be used to predict peri-implantitis occurrence and to improve treatment and monitoring of patients with dental implants.

2.1.5 Conclusions

It is possible to conclude that there are still no studies with an adequate sample size or sufficient studies to reach robust conclusions about the influence of interleukin 1 and interleukin 1 receptor antagonist polymorphisms on the development of peri-implantitis. However, the available evidence, while constrained by the above mentioned issues, shows that there seems to be a possible influence of these polymorphisms on the development of the disease. Regarding the meta-analysis performed, it was observed that individuals with the polymorphism in the *IL-1B* +3954 gene have a higher risk for the development of peri-implantitis.

Nevertheless, there is a need for further studies, with larger samples and in different ethnic groups, to increase scientific evidence about the possible role of these polymorphisms in the development of peri-implantitis.

Appendix A

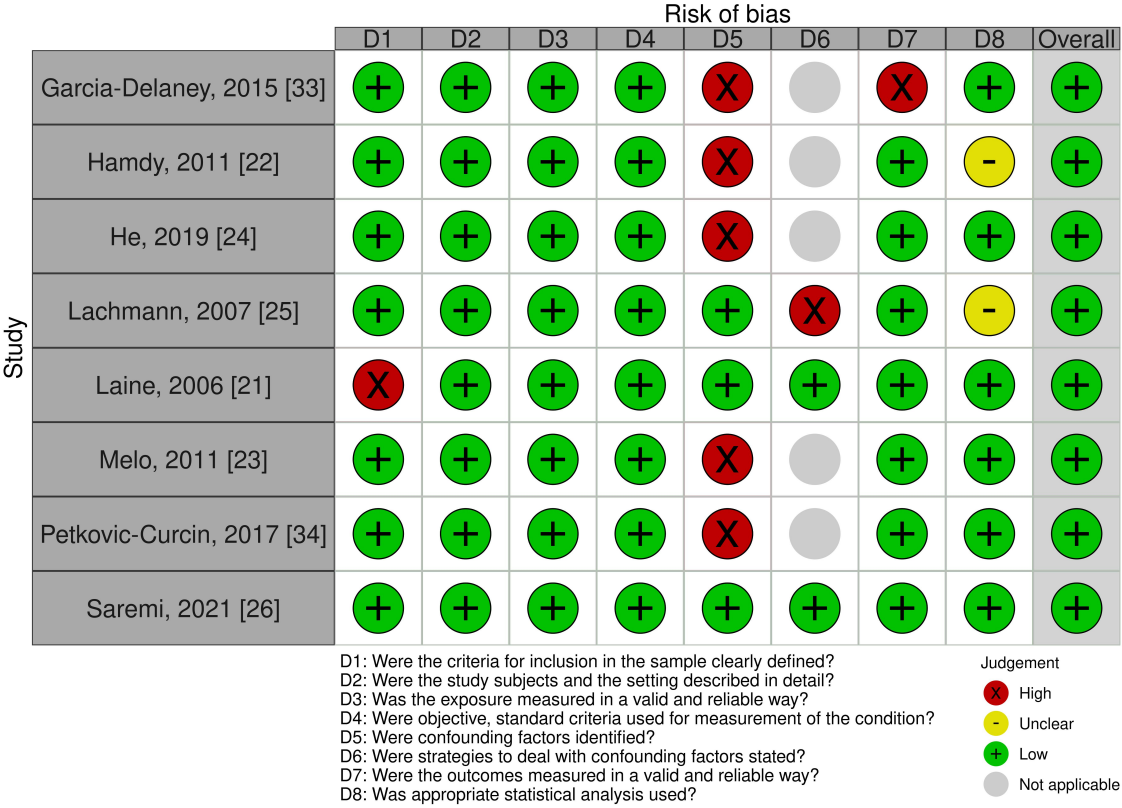


Figure A1 - Risk of bias assessment of the included studies.

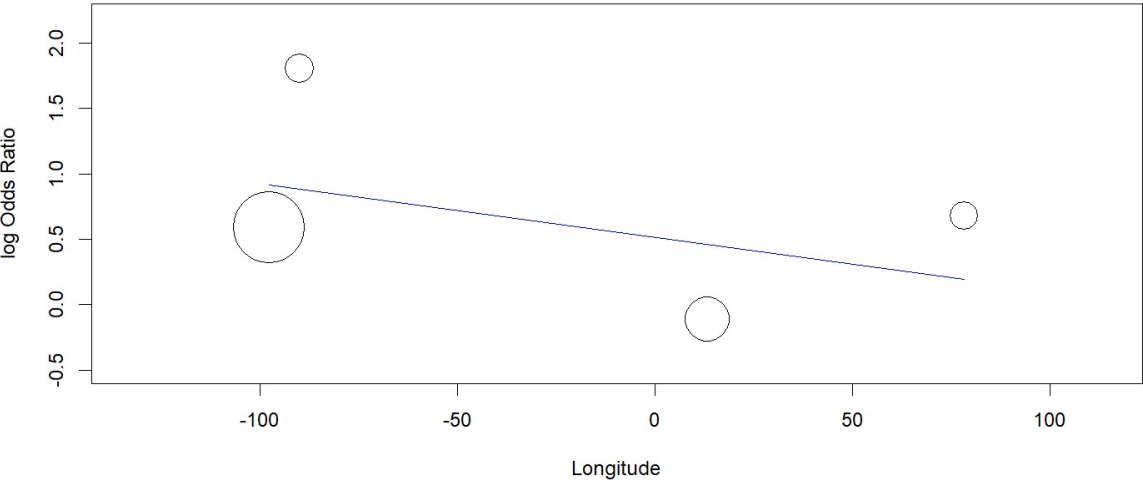


Figure A2 - Log OR meta-regression for the *IL-1B* +3954 polymorphism as a function of the longitude covariate (*p-value* = 0.295, Coefficient = -0.004).

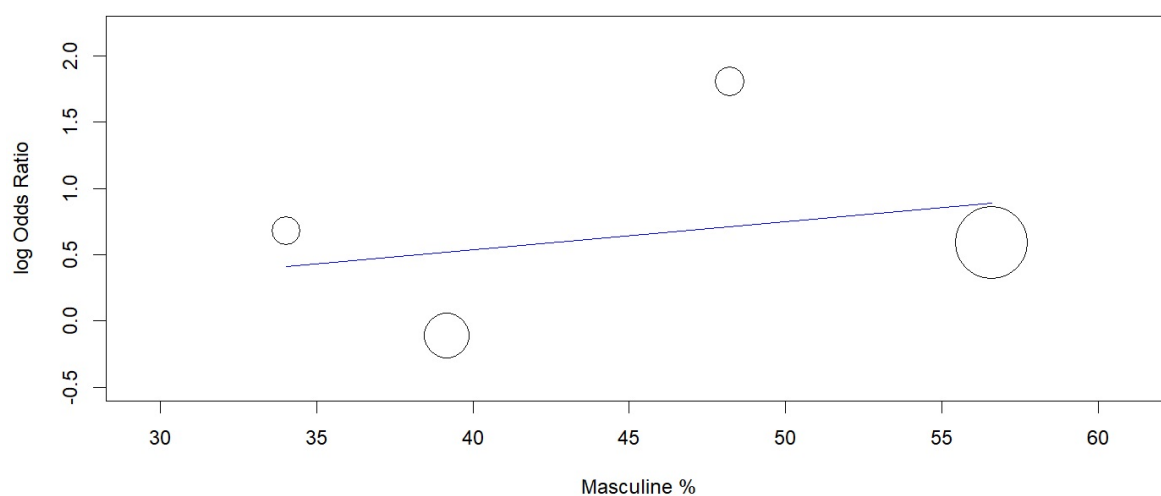


Figure A3 - Log OR meta-regression for *IL-1B* +3954 polymorphism as a function of the covariate percentage of male subjects (p -value = 0.531, Coefficient = 0.021).

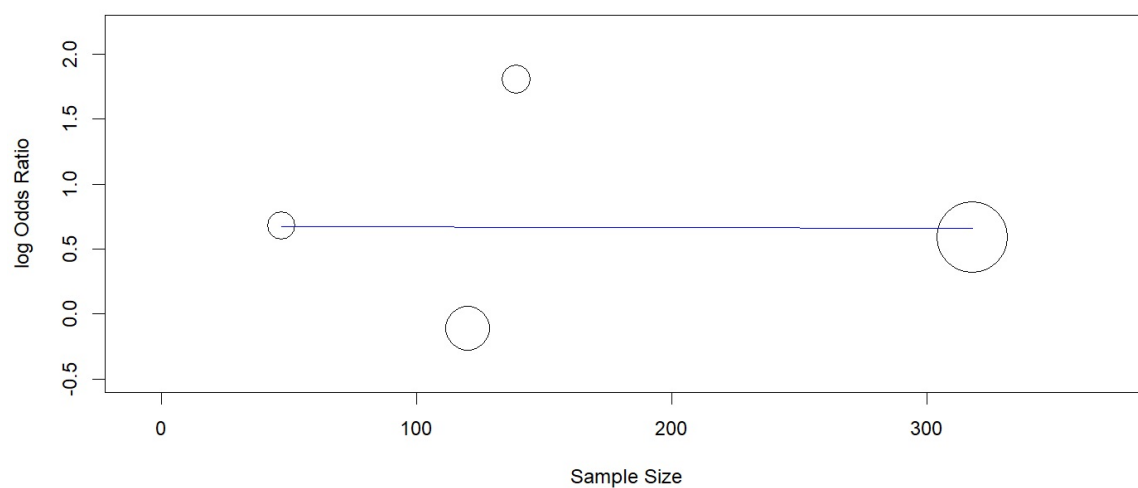


Figure A4 - Log OR meta-regression for the *IL-1B* +3954 polymorphism as a function of sample size (p -value = 0.989, Coefficient = -0.000).

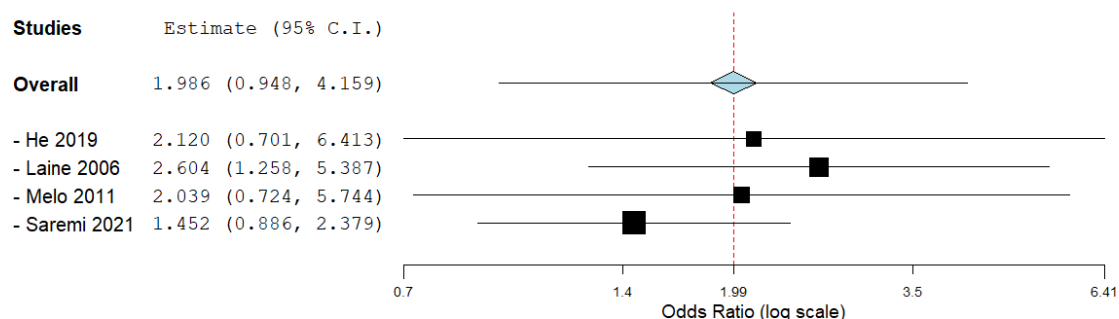


Figure A5 - Sensitivity Analysis (CI – confidence interval).

Table A1 – Systematic review table of the sample characteristics of the different articles.

Year & Author	Latitude; longitude (degrees)	Sample size	M/F (%)	Average age of the sample	Mean % of mutated allele
Garcia-Delaney, 2015	32.363890; -86.298190	Total: 54 PIG: 27 Control: 27	37/63	53	ND
Hamdy, 2011	-35.052770; 147.349560	Total: 50 PIG: 25 Control: 25	76/24	41	ND
He, 2019	29.894920; -97.677800	Total: 318 PIG: 144 Control: 174	57/43	45	<i>IL-1B</i> +3954: 25.65
Lachmann, 2007	-13.848923; -171.751145	Total: 29 PIG: 11 Control: 18	34/66	66	ND
Laine, 2006	55.702888; 13.194710	Total: 120 PIG: 71 Control: 49	39/61	67	<i>IL-1B</i> +3954: 29.45
Melo, 2011	17.433660; 78.339010	Total: 47 PIG: 16 Control: 31	34/66	46	<i>IL-1B</i> +3954: 33.22
Petkovic-Curcin, 2017	40.772770; -111.839100	Total: 98 PIG: 34 Control: 64	71/29	58*	ND
Saremi, 2021	32.331050; -90.170660	Total: 139 PIG: 50 Control: 89	49/51	41	<i>IL-1B</i> +3954: 12

ND – non-defined, PIG – peri-implantitis group, * In this article, the median age of the sample is exceptionally represented.

2.1.6 References

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3. AIMS

The primary objective of this study was to evaluate *IL-1* and *IL-1RN* gene polymorphisms in patients with peri-implantitis and to compare them with patients with peri-implant health, considering confounding variables such as smoking status and history of periodontitis. Our hypothesis was that individuals carrying the T allele in the *IL-1A* -889 and *IL-1B* +3954 genes, or the less frequent alleles in the *IL-1RN* gene (VNTR), were more susceptible to develop peri-implantitis in comparison with individuals who did not have these polymorphisms.

The aim of this investigation was also to investigate the possible relationships between *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* (VNTR) gene polymorphisms and the concentrations of the PICF mediators IL-1 β , TNF- α and PGE2. The second hypothesis was that, in both healthy individuals and patients with peri-implantitis, an altered genotype of *IL-1A*, *IL-1B* or *IL-1RN* would result in higher levels of pro-inflammatory mediators (IL-1 β , PGE2 and TNF- α) compared to patients without the altered genotype, and that this effect would be particularly pronounced in the peri-implantitis group.

To date, no consensus exists on whether genetic alterations contribute to an increased susceptibility to peri-implantitis. Furthermore, the number of studies in the literature evaluating this association is limited and some of these does not refer to confounding variables such as periodontal condition, ethnicity and smoking status. The inequality of these studies' design requires the conduction of further studies using proper methodologies and from different ethnic groups. Increased knowledge about this relationship can bring benefits such as identifying patients who are more likely to develop peri-implantitis, allowing better treatment and follow-up of certain patients with dental implants.

Next, a pilot study (Chapter 4) will be presented, which verified the feasibility of the study and allowed for sample calculation. Chapter 5 presents the study that evaluates the relationship between the *IL-1* and *IL-1RN* polymorphisms and the presence of peri-implantitis in the final sample. Chapter 6 includes the study relating genetic alterations to biochemical alterations in the peri-implant crevicular fluid. The possible association between these genetic polymorphisms and peri-

implantitis must be based on a change in the individual's immune-inflammatory response.

4. ASSOCIATION BETWEEN *IL-1A* AND *IL-1B* GENE POLYMORPHISMS AND PERI-IMPLANTITIS IN A PORTUGUESE POPULATION - A PILOT STUDY

4.1 The importance of pilot studies

Pilot studies or feasibility studies are preliminary, small-scale investigations that aim to assess whether the crucial components of the main study will be feasible [1]. Furthermore, a pilot study can also be a development, pre-testing and/or “trying out” of a particular research instrument [2]. They may be useful to predict issues, appropriate research protocols and facilitate the use of assessment tools. Consequently, if necessary, suitable, realistic and feasible methods can be added or removed to enrich and improve the various aspects before the main survey is carried out.

This chapter was based on the published work:

Paper II - Cardoso JM, Ribeiro AC, Palos C, Proença L, Noronha S, Alves RC. Association between *IL-1A* and *IL-1B* gene polymorphisms with peri-implantitis in a Portuguese population-a pilot study. PeerJ. 2022 Jul 14;10:e13729. doi: 10.7717/peerj.13729. PMID: 35855430; PMCID: PMC9288821.

4.2 Association between *IL-1A* and *IL-1B* gene polymorphisms with peri-implantitis in a Portuguese population – A pilot study

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Abstract:

Background. Scientific evidence indicates that biological complications in dental implants tend to be concentrated in a subset of individuals, which seems to imply that the host response may play a determining role in implant success. Over the last few decades, several polymorphisms have been studied. Polymorphisms in the interleukin (IL) 1 gene cluster have been associated with periodontitis. There are some similar features in the sequence of immunopathological events in peri-implant and periodontal infections. We aimed to investigate if individuals carrying the genetic single nucleotide polymorphism (SNP) in the *IL-1A* (rs1800587) and *IL-1B* (rs1143634) genes are more susceptible to develop peri-implantitis.

Methods. A cross-sectional analytic pilot study was conducted in 20 Caucasian Portuguese subjects divided into two groups: 10 subjects with peri-implantitis and 10 subjects with peri-implant health (control group). Samples containing cells from the buccal mucosa were stored at -20°C and later submitted to the DNA extraction process. Genetic analysis was performed using the PCR (polymerase chain reaction) and RFLP (restriction fragment length polymorphism) analysis. Data were analyzed by using descriptive and inferential statistical methodologies.

Results. For the *IL-1A* -889 gene polymorphism, it was observed that the mutated allele was present in a higher percentage in the peri-implantitis group compared to the control group (30% vs 15% respectively, Fisher's exact test, $p = 0.225$). For the *IL-1B* +3954 gene polymorphism, it was also observed that the altered allele was present in a higher percentage in the disease group compared to the control group (35% vs 10% respectively, Fisher's exact test, $p = 0.370$). The positive genotype (at least one allele with nucleotide sequence changed in both genes) was detected in 6 patients, 5 belonging to the disease group and 1 to the health group.

Conclusions. Regarding *IL-1* gene polymorphisms, there was no statistically significant difference between the health and disease group, however a trend should be highlighted, showing a potential link between the *IL-1* genotype and peri-implantitis. More studies are needed to clarify the role of genetic polymorphisms in the development of peri-implantitis.

Keywords: genetics, peri-implantitis, interleukin-1, single-nucleotide polymorphism, interleukin1-genotype, genetic polymorphisms, peri-implant disease

4.2.1 Introduction

Peri-implantitis is a pathological condition occurring in tissues around dental implants, characterized by inflammation in the peri-implant mucosa and progressive loss of supporting bone [3,4]. In 2013, Atieh et al., published a meta-analysis and concluded that the prevalence of peri-implantitis was 9.6% when assessed at the implant level and 18.8% when calculated per patient. In smokers, a high-risk group according to current evidence, the prevalence rates obtained for peri-implantitis increased to 36.3% [5]. More recently, Lee et al. (2017) conducted a systematic review and meta-analysis that included forty-seven articles, and concluded that the mean prevalence of peri-implantitis, at implant and subject level was 9.25% and 19.83%, respectively [6].

Scientific evidence indicates that biological complications in dental implants tend to be concentrated in a subset of individuals, which seems to imply that the host response might be a key determinant in the development of peri-implantitis [7,8]. The host immune system reacts to bacterial invasion by producing inflammatory mediators. The pro-inflammatory cytokine interleukin-1 (IL-1) is considered a major mediator of chronic inflammatory diseases, playing a crucial role in the process of destruction of the extracellular matrix of connective tissue and bone resorption [9]. It is also a primary activator of chemotactic cytokines and increases the expression of adhesion molecules that facilitate the migration of leukocytes. Additionally, IL-1 stimulates the production of other pro-inflammatory mediators such as tumor necrosis factor alpha (TNF- α) and prostaglandin E2 (PGE2) [10]. Several studies have shown high levels of IL-1 in peri-implant crevicular fluid in patients with peri-implant infections [11,12].

The *IL-1A* (rs1800587) and *IL-1B* (rs1143634) genes have been described as being able to regulate the production of IL-1 α and IL-1 β , respectively [13]. They are located close to each other, on the long arm of chromosome 2, specifically at position 2q13, and are considered potential candidates for genetic

markers in peri-implantitis, since the cytokines they encode play a crucial role in the development of the inflammatory response [14].

In 1997, Kornman et al. showed for the first time that the combined presence of allele 2 (allele with nucleotide sequence changed) of the *IL-1A* gene at nucleotide position – 889 (*IL-1A* -889T) and the allele 2 of *IL-1B* gene at nucleotide position + 3954 (*IL-1B* +3954T) was associated with an increased risk for developing severe periodontitis in nonsmoking Caucasian patients [15]. The replacement of the cytosine base by a thymine occurred in the promoter region of the *IL-1A* gene and in exon 5 of the *IL-1B* gene. The presence of at least one allele 2 at the *IL-1A* -889 locus and at least one allele 2 at the *IL-1B* +3954 locus is designated IL-1 positive genotype. These polymorphisms of *IL-1A* -889T and *IL-1B* +3954T are single nucleotide polymorphisms (SNP's).

The sequence of immunopathological events in peri-implant infections has some similarities to periodontal infections [16]. However, when compared to experimentally induced periodontitis, lesions associated with experimental peri-implantitis demonstrate larger inflammatory cell infiltrates and more rapid and pronounced bone loss. Similar to periodontitis, the lesions at peri-implantitis sites were also dominated by plasma cells and lymphocytes, but characterized by larger proportions of polymorphonuclear leukocytes and macrophages [17].

There are some scientific research studies that have analyzed the role of the polymorphisms *IL-1A* -889T and *IL-1B* +3954T in host response in the development of biological complications, in dental implants [14,18–25]. Some of these studies observed that there is no relationship between the prevalence of these polymorphisms and peri-implantitis [18–22]. However, in a subgroup analysis it is observed that the IL-1 positive genotype in heavy smokers corresponded to a higher risk for the development of more severe inflammatory complications and increased peri-implant bone loss [18,20].

Lachmann et al. (2007), evaluated the association between peri-implant crevicular fluid volume, crevicular inflammatory mediators' concentrations and the IL-1 positive genotype and the presence of peri-implantitis. In patients with peri-implantitis, no significant influence of altered genotype was observed [22].

In a study by He et al. (2020), with a sample of 318 Chinese patients, the authors observed that subjects carrying the T allele of *IL-1A* -889 and *IL-1B* +3954 had a significant 2.27-2.47-fold and 1.9-1.99-fold increased risk of peri-implantitis, respectively, when using the CC genotype as reference [25].

In 2019 Formousis and Vlachos performed a review in which concluded that the identification of genetic biomarkers associated with peri-implantitis risk could be valuable in daily clinical practice, however, no robust conclusions could be drawn from the current literature [26].

The number of studies in the literature evaluating this association is limited and some of these does not refer to confounding variables such as periodontal condition, ethnicity and smoking status. The definition of peri-implantitis also varies between studies. The inequality of these studies' design necessitates the conduction of further studies using proper methodologies and from different ethnic groups [26].

A clarification of the genetic basis associated with peri-implant pathology could be used to predict peri-implantitis occurrence and to improve treatment and monitoring of patients with dental implants [21].

According to the available evidence it is not possible to reach robust conclusions about the influence of IL-1 polymorphisms on the development of peri-implantitis. Furthermore, there are few studies that evaluate this possible relationship in certain ethnic groups, namely in Caucasians from southern Europe.

In the present study, we aimed to investigate the possible association of *IL-1A* -889T and *IL-1B* +3954T polymorphisms with the peri-implantitis in a Portuguese cohort. We hypothesize that individuals carrying the T allele in the *IL-1A* -889 and *IL-1B* +3954 genes are more susceptible to develop peri-implantitis in comparison with individuals that don't have these polymorphisms.

4.2.2 Materials and Methods

Study design and population

The study was conducted according to the principles of the Helsinki Declaration (reviewed in 2013). Appropriate measures were taken for the protection of patient data. The study was approved by the Ethics Committee of Egas Moniz School of Health and Science on July 24th, 2019 (process number 790).

The study included two groups, the disease group consisting of 10 patients with peri-implantitis (bleeding and/ or suppuration on probing, probing depth equal or greater than 6mm and bone loss equal to or greater than 3mm) and the control group formed by 10 patients with peri-implant health (peri implant mucosa without inflammatory signs and absence of peri-implant bone loss).

The definitions of peri-implantitis, peri-implant health, periodontal health and periodontitis case were in accordance with the new classification of periodontal and peri-implant diseases (American Academy of Periodontology - AAP and European Federation of Periodontology - EFP 2018) [27–29].

The convenience sample was obtained from patients referred to periodontal treatment at the Periodontology Department of Egas Moniz Dental Clinic (EMDC), between July of 2020 and February of 2021. All possible candidates received a screening questionnaire and if the patient's medical history was in accordance with study inclusion criteria, and if they agreed to participate, informed written consent was obtained.

The inclusion criteria were: - patients with Caucasian parents and grandparents, - patients with dental implants in function for at least 1 year, - patients with peri-implantitis diagnosis, - patients who agree to participate in the study and sign informed consent. The exclusion criteria were: - patients with any systemic condition that affects the immune system, - patients who have taken antibiotics or anti-inflammatory drugs on a chronic basis in the last 6 months - pregnant women, - patients who have undergone peri-implant treatment in the area to be evaluated.

Socio-demographic and clinical variables

Data were gathered on age, sex, smoking habit (yes or not) and history of periodontitis.

For patients with suspected periodontitis a full periodontal examination (six locations per tooth with a CP12 graduated periodontal probe) was performed recording probing pocket depth, bleeding on probing, gingival margin and tooth mobility.

A peri-implant probing examination (six locations with a CP12 graduated periodontal probe) was performed as usual at periodontology appointments. The presence of bleeding on probing or pus were also evaluated. An implant control radiograph was taken in cases where there was a probing depth more than 6mm (paralleling technique).

All clinical data was collected by the same examiner (J.M.C.). Prior to the study, a training and calibration exercise was performed for probing pocket depth (PPD) measurements using volunteer patients not included in the study. The calibration exercise demonstrated 95% concordance within ± 1 mm for measurements of PPD.

In the case of a peri-implant health situation, plaque control measures were taken, while in cases of peri-implantitis the patient was referred to receive further treatment, in addition to the reinforcement of plaque control measures. In situations of patients with more than one implant affected by peri-implantitis, the one with the greatest bone loss was chosen for the study.

Genetic analysis

The following genetic polymorphisms were analyzed: *IL-1A* -889 and *IL-1B* +3954 from patients with peri-implantitis and peri-implant health. At position +3954 of exon 5 of the *IL-1B* gene as well as at position -889 of promoter region of the *IL-1A* gene there were described single nucleotide polymorphisms (SNPs), which are characterized by replacing a cytosine base (C) with a thymine (T), resulting in two possible alleles: C allele and T allele. For the investigation of

genetic polymorphisms, a sample of cells from the jugal mucosa was collected with the aid of a sterile swab (Omniswab Whatman® FTA®). Samples were placed in a 1.5-ml test tube (Eppendorf tubes®) and kept at -20°C until DNA extraction. The genetic analysis was done at Egas Moniz Molecular Biology Laboratory with the DNA extraction technique followed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). The DNA extraction process was performed based on the "Protocol: Isolation of Total DNA from Surface and Buccal Swabs" taken from the QIAamp® DNA Investigator Handbook.

The DNA obtained was amplified using the PCR technique, and specific primers were used for each of the genes intended to be studied. Table 4.1 exhibits the sequences of the sense and antisense primers, that is, of the forward and reverse primers, which were described by Kornman et al. (1997) [15].

Table 4.1 – Sequence of primers chosen for each of the genetic polymorphisms studied.

Primers	Sequence
<i>IL-1A</i> -889 (Primer Forward)	5'-AAGCTTGTTCTACCACCTGAACTAGGC-3'
<i>IL-1A</i> -889 (Primer Reverse)	5'-TTACATATGAGCCTTCCATG-3'
<i>IL-1B</i> +3954 (Primer Forward)	5'-CTCAGGTGTCCTCGAAGAAATCAAA-3'
<i>IL-1B</i> +3954 (Primer Reverse)	5'-GCTTTTTTGCTGTGAGTCCCG -3'

PCR techniques were performed as described by Kornman et al. (1997) [15]. The PCR amplification was performed according to the protocol of the manufacturer of the commercial kit NZYTaQ II 2x Green Master Mix®. This commercial kit relates to a premixed solution, containing the Taq DNA polymerase enzyme NZYTaQ II (0.2 U/μL), 5 pmol of each primer, 10x PCR reaction buffer, contains 0,5 mM deoxyribonucleotides (dNTPs) and 2.5 mM of MgCl₂. PCR reaction occurred with a final volume of 25 μL, containing approximately 50 ng of genomic DNA.

The amplification reactions were carried out in a thermocycler (MJ MINITM Personal Thermal Cycler). For the *IL-1A* gene polymorphism at position -889, a cycle of 96°C for 2 minutes was performed, followed by 45 cycles of 94°C for 1 min, 50°C for 2 min and 72°C for 1 minute. Finally, a cycle of 72°C was carried out for 5 minutes. For the *IL-1B* gene polymorphism at position +3954, a cycle of 94°C for 3 min was performed in a first step, followed by 35 cycles of 94°C 30 seconds, 54°C 35 seconds and 72°C 30 seconds. Afterwards, a cycle of 72°C was carried out for 5 minutes.

After PCR amplification, PCR products were restricted with endonuclease NcoI (20,000 U/mL) (BioLabs). The restriction endonuclease was added to PCR products in a 30 µl reaction volume and consisting of 20 µl PCR products, 3 µl 10x NCoI buffer (BioLabs), 1 µl NCoI enzyme, 6 µl of nuclease-free water and were incubated at 37°C for 16 hours. The restriction fragments were visualized with ultraviolet light in the transilluminator (UltraLum). The restriction endonucleases and expected fragments are presented in Table 4.2.

Table 4.2 – Relation of restriction endonucleases, temperature and expected fragments.

Gene	Restriction endonucleases	Restriction profile	Temperature (°C)	Fragments
<i>IL-1A</i> -889	NcoI	5'-C/CATGG-3'	37°C	83bp + 16bp
		3'-GGTAC/C-5'		(allele 1)
				99bp (allele 2)
<i>IL-1B</i> +3954	TaqI	5'-T/CGA-3'	65°C	12bp + 85bp+
		3'-AGC/T-5'		97bp (allele 1)
				12bp + 182bp (allele 2)

The resulting fragments were separated by size in 5% MetaPhor agarose gel electrophoresis. After enzymatic digestion, 5 µl of DNA loading dye (Bromophenol blue 0.05%) were added to the samples. 15 µl of marker and DNA were placed in the wells. We used the molecular weight marker NZYDNA ladder VI and pUC19 / MspI (HpaII). The electrical current at 100 mA was established for performing the electrophoresis. For visualization and documentation of the agarose gel, an ultraviolet light transilluminator and a polaroid camera were used.

This procedure was repeated for the *IL-1B* +3954 polymorphism, with the difference that in this case, the restriction enzyme TaqI and the CutSmart® Buffer were used and the samples were stored at 65°C overnight.

Statistical analysis

Data were submitted to descriptive and inferential analysis methodologies. A 5% significance level ($p \leq 0.05$) was established in the latter case. The prevalence of *IL-1A* and *IL-1B* polymorphisms in patients with peri-implantitis and healthy ones was compared by using appropriate inferential statistics methodologies (bivariable analysis/association tests, Chi-square (χ^2) and Fisher's exact test), taking into account several sociodemographic and clinical variables: sex, smoking status and history of periodontitis. Median age was compared between groups by using Mann-Whitney test. Statistical analyses were performed using the software IBM SPSS Statistics v.27.

4.2.3 Results

Demographic and clinical characteristics of the study group

The study included 20 healthy subjects (9 men and 11 women) ages 32 to 79 years (median: 57 years) who had at least one dental implant inserted. All participants had the same ethnical origin (Caucasian). Factors examined for prognostic significance included cigarette smoking and history of periodontitis. The characteristics of the sample are presented in Table 4.3.

Table 4.3 – Baseline characteristics of patients with dental implants.

	Peri-implant Health (n=10)	Peri-implantitis (n=10)	P [#]
Age*	54.0 (22) 32-79	62.0 (14) 36-74	0.47 ^{##}
Sex, n (%)			
Male	6 (60.0)	3 (30.0)	0.37
Female	4 (40.0)	7 (70.0)	
Smoking, n (%)			
No	10 (100.0)	7 (70.0)	—
Yes	0 (0)	3 (30.0)	
History of Periodontitis, n (%)			
No	5 (50.0)	2 (20.0)	0.35
Yes	5 (50.0)	8 (80.0)	

Notes: *presented as medium (IQR), min–max, [#]Fisher's exact test, ^{##}Mann–Whitney test ($U = 40.5$, $p = 0.47$).

The percentage of smokers was 30% in the peri-implantitis group, while none of the patients in the peri-implant healthy group was classified as smoker. In addition, positive history of periodontitis was more frequent in the peri-implantitis group than in patients with peri-implant health, albeit the difference in proportion was not significant (80% vs 50%; Fisher's exact test, $p = 0.35$).

Allelic frequencies

Regarding the *IL-1A* gene polymorphism, in the health group the allele frequency for the C allele was 85% and for the T allele it was 15%. In the disease group, the allelic frequencies were 70% and 30%, for the C and T allele, respectively. Considering the total sample of alleles ($n=40$) present in this polymorphism, for the 20 patients, there were 31 (77.5%) C alleles and 9 (22.5%) T alleles. No statistically significant differences in proportions (Fisher's exact test, $p = 0.45$) were observed between the health and disease groups, when

considering the presence of the mutated allele. The allelic frequencies are presented in Table 4.4

Table 4.4 – Allelic frequencies of polymorphisms of *IL-1A* -889 and *IL-1B* +3954 in the peri-implant health group and in the peri-implantitis group.

Alleles, n (%)	Peri-implant health (n=20)	Peri-implantitis (n=20)	Total (n=40)	<i>p</i>
<i>IL-1A</i> -889				
C	17 (85)	14 (70)	31 (77.5)	0.45
T	3 (15)	6 (30)	9 (22.5)	
<i>IL-1B</i> +3954				
C	18 (90)	13 (65)	31 (77.5)	0.13
T	2 (10)	7 (35)	9 (22.5)	

Regarding the *IL-1B* gene polymorphism, in the health group the allele frequency for the C allele was 90% and for the T allele it was 10%. In the disease group, allelic frequencies were 65% and 35% for the C and T allele, respectively. Considering the total number of alleles present in this polymorphism in the 20 patients (n=40), the same number of C and T alleles as those registered for the *IL-1A* -889 polymorphism were observed. As already seen for the *IL-1A* gene polymorphism, no statistically significant differences were found in proportions of the *IL-1B* gene polymorphism, between the health and disease groups, when considering the presence of the mutated allele (Fisher's exact test, $p = 0.13$).

Genotypic frequencies

The genotypic frequencies obtained for the genes *IL-1A* -889 and *IL-1B* +3954, in the peri-implant health group, in the peri-implantitis group and for the total sample are presented in Table 4.5.

Table 4.5 – Genotypic frequencies in polymorphisms of *IL-1A* -889 and *IL-1B* +3954, in the peri-implant health group and in the peri-implantitis group.

Genotype, n (%)	Peri-implant health (n=10)	Peri-implantitis (n=10)	Total (n=20)	p
<i>IL-1A</i> -889				
CC	7 (70)	4 (40)	11 (55)	0.37
CT	3 (30)	6 (60)	9 (45)	
<i>IL-1B</i> +3954				
CC	8 (80)	4 (40)	12 (60)	—
CT	2 (20)	5 (50)	7 (35)	
TT	0 (0)	1 (10)	1 (5)	

Regarding the *IL-1A* -889 polymorphism, of the 10 patients in the healthy group, 7 (70%) had CC genotype, while the remaining 3 (30%) showed the presence of CT genotype. In the peri-implantitis group, of the 10 patients who are part of it, 4 (40%) had a CC genotype and 6 (60%) a CT genotype. The TT genotype was not observed in any of the groups. Analyzing the data obtained for the total sample, in the 20 patients involved in the study, approximately 55.0% had a CC genotype and 45.0% had a CT genotype. No statistically significant differences were found between the health and disease groups, when considering the proportion of the altered genotype (Fisher's exact test, $p=0.37$).

Regarding the *IL-1B* +3954 polymorphism, in the healthy group, the genotypic frequencies were 80.0% for the CC genotype, 20.0% for the CT genotype and 0% for the TT genotype. For the group with diagnosed peri-implantitis, a frequency of 40.0% for the CC genotype, 50.0% for the CT genotype and 10% for the TT genotype was obtained. Considering all individuals involved in the study, the observed genotypic frequencies were 60.0% for the CC genotype, 35.0% for the CT genotype and 5.00% for the TT genotype. For this polymorphism, it was not possible to perform the inferential analysis.

Characterization of the sample according to the presence of the positive genotype

Of the 10 patients in the healthy group, only 1 (10%) was genotype positive, while the remaining 9 (90%) didn't have this type of genotype. In the peri-implantitis group, of the 10 patients that constitute it, 5 (50%) were genotype positive and the remaining 5 (50%) were genotype negative. Of the 6 patients who were genotype positive, 5 belonged to the disease group. Analyzing the data for the total sample (n=20), 6 (30%) of the patients were genotype positive, while the remaining 14 (70%) were genotype negative. The table 4.6 shows the percentage of subjects who were genotype positive (*IL-1A* -889 allele 2 plus *IL-1B* +3954 allele 2).

Albeit the previous highlighted differences, no association (Fisher's exact test, $p = 0.14$) was found between the presence of the positive genotype and peri-implantitis.

Table 4.6 – Percentage of subjects who were genotype positive (*IL-1A* -889 allele 2 plus *IL-1B* +3954 allele 2).

		Peri-implant				Total	
		health (n=10)		Peri-implantitis (n=10)			
		n	%	n	%	n	%
Genotype	yes	1	10	5	50	6	30
	no	9	90	5	50	14	70

4.2.4 Discussion

The two most widely studied genetic variations, with respect to a possible relationship with peri-implantitis, are *IL-1A* -889T and *IL-1B* +3954T, which both have shown to be associated with changes in gene expression and protein secretion [30].

The *IL-1A* -889 polymorphism is located in the *IL-1A* promoter region, and the presence of the T allele has been associated with an increase in the transcriptional activity of the gene. The CC genotype has been associated with a decrease in transcriptional activity and, consequently, with lower levels of interleukin-1 α , when compared to the TT genotype. *IL-1B* +3954 is located in exon 5 of the *IL-1B* gene, and the replacement of the C allele by the T allele will cause an alteration similar to that described above [25].

Montes et al. (2009), in their study, performed a comparison of several studies that analyze the allelic frequencies of *IL-1B* +3954 polymorphisms in different populations, namely Chinese, Japanese, Italian, Swedish and Brazilian [31]. Analyzing the data obtained for this polymorphism in our study, the frequency of the mutated allele (T allele) in the *IL-1B* +3954 polymorphism was 22.5% in the total sample, which is close to the frequencies found in these populations. Comparing data from all populations, although with some discrepancies between them, all countries showed a higher prevalence of C allele in the *IL-1B* +3954 polymorphism, verifying the same trend appears at the global level and not only at the European level [31].

According to the study of Kornman et al. (1997), which was the first study to find an association of these polymorphisms with periodontitis, there is a reference that the frequency of the positive genotype is 29.1% in Caucasian individuals from northern Europe [15]. This observed data is in line with what was verified in the present study, in which it was found that 30% of all participants had at least one altered allele in the two genes *IL-1A* and *IL-1B*, at positions -889 and +3954, respectively. According to Kornman et al. (1997), patients with this genotype were

reported to be more likely to present an increase in periodontal tissue destruction [15].

In a study conducted by He et al. (2020), with 144 patients with peri-implantitis and 174 controls, aimed to analyze whether there was a relationship between polymorphisms of the *TNF- α* , *IL-1A* and *IL-1B* genes and peri-implantitis in a non-smoking Chinese population [25]. Regarding *IL-1A* -889 gene polymorphism, in the disease group, the frequency of the C allele was 70.1% and the frequency of the T allele (altered allele) was 29.9%. In the control group, the frequencies were 84.2% and 15.8% for the C and T allele, respectively. Thus, the allelic frequencies observed in that study are in agreement with the allelic frequencies obtained in the present study, in which a frequency of 70% for the C allele and 30% for the T allele in the peri-implantitis group and a frequency of 85% and 15% respectively, in the control group were observed.

With regard to the *IL-1B* +3954 polymorphism, He et al. (2020) observed that, in the peri-implantitis group, the allelic frequency for the C allele was 68.8% and for the T allele was 31.2%, while in the health group the allelic frequency was 79.9 % for the C allele and 20.1% for the T allele [25]. Thus, it appears that these frequencies are similar to those observed in the present study in the disease group, in which a frequency of 65% for the C allele and 35% was observed for the T allele. Comparing both studies, for both *IL-1A* -889 and *IL-1B* +3954 polymorphisms, there is a higher percentage of T alleles in the peri-implantitis group and a higher percentage of C alleles in the health group. He et al. (2020) concluded that both the *IL-1A* -889T polymorphism and the *IL-1B* +3954T polymorphism are associated with an increased risk of developing peri-implantitis [25]. In the present study, there was no statistically significant difference between the health group and the disease group, so that there was no association between these polymorphisms and peri-implantitis. However, although there is no association, it appears that there is a possible tendency for individuals with these genetic polymorphisms to be more susceptible to developing peri-implantitis, as patients with this disease have a greater number of mutated alleles (T allele) and of CT and TT genotypes, compared to individuals with peri-implant health.

A more recent study by Saremi et al. (2021), that included 50 patients with peri-implantitis and 89 patients with peri-implant health, in an Iranian population,

concluded that there is an association between the *IL-1B* +3954 polymorphism and peri-implantitis [32].

In contrast, Melo et al. (2012), in a study carried out with a total of 47 patients, concluded that there was no association of the *IL-1B* +3954 polymorphism with peri-implantitis [33].

Liao et al. (2014), carried out a meta-analysis with the aim of studying the relationship between IL-1 polymorphisms (*IL-1A* and *IL-1B*) and the risk of failure/complications at the level of dental implants [30]. In a total of 13 studies included, only four of these presented sufficient data regarding the relationship between positive genotype of *IL-1A* -889 and *IL-1B* +3954 and peri-implantitis. Thus, with the completion of the meta-analysis, it is observed that, in Caucasian European descendants, there is a significant association between the positive genotype of *IL-1A* -889 and *IL-1B* +3954 and peri-implantitis. A recent meta-analysis found that carriers of positive genotype of *IL-1A* -889 and *IL-1B* +3954 had 1.95-fold risk of peri-implant disease (which included implant failure/loss, marginal bone loss and peri-implantitis). The authors concluded that the positive genotype of IL-1 can be used as predictive marker for peri-implant disease [34].

Despite some agreement between the results obtained in the present study and the studies mentioned above, especially with regard to the allelic frequencies verified for the alleles with and without change between the health and disease groups, there were some differences regarding the influence of these polymorphisms in the development of peri-implantitis. These divergences may be related to differences regarding the ethnicity of the groups included. In the present study, Caucasian individuals from southern Europe were included, whereas in the study by He et al. (2020) [25], patients of Asian origin were included and in the study by Saremi et al. (2021) [32], only Iranian individuals participated. The influence of ethnic and racial variations in the frequency of gene polymorphisms in terms of the genetic susceptibility to a specific disease has been reported [15,35]. It has been demonstrated that there is low prevalence of the periodontitis-associated *IL-1A* +4845 and *IL-1B* +3954 gene polymorphisms in Chinese (2.3%) [35] compared with that reported for Caucasians (36%) [15]. Thus, it is of great importance to determine the occurrence of the genotypes in specific ethnic groups and not to extrapolate the information derived from one ethnic group to

another. To avoid including patients with different ethnic origins in the same sample, in our study, only patients with Caucasian parents and grandparents were included.

There were also differences in terms of smoking habits between the published studies. In the present study, smokers were included, whereas in the study by He et al. (2020) smoking patients were not selected [25]. According to some studies there is a synergism between smoking (heavy smokers) and IL-1 polymorphisms in the development of peri-implantitis [18,20]. Furthermore, there are studies that observed an association between smoking and peri-implantitis [36–38]. Therefore, this habit must be evaluated, its possible effect on the disease must be taken into account and the groups should be balanced regarding the existence of the smoking habit. However, in some studies there were no data on the smoking habits of the patients [22,32] and in the studies where this habit was evaluated there are differences in categorization of smokers and non-smokers which can lead to different results. Furthermore, all of the identified studies relied solely on patient-reported information for the assessment of smoking status.

On the other hand, our study is a pilot study with 20 patients, consisting of a convenience sample to test the feasibility of the study, which may not represent the population of Portuguese individuals with peri-implantitis. The sample size estimate can be determined by statistical estimation based on the two results of this pilot study: the proportion of altered genotypes (CT for *IL-1A* and CT/TT for *IL-1B*), which indicated that the sample size should be $n=84$, i.e., $n=42$ for each group (peri-implantitis and peri-implant health), based on an average effect size ($w=0.3$), with a power of 80% and an alpha level of 5%. Analyzing and comparing with the sample size of the remaining studies, in the one by He et al. (2020) [25] a total of 318 patients were included and a total of 139 subjects participated in the study by Saremi et al. (2021) [32]. Most studies that assess risk factors for peri-implant diseases are based on convenience samples. When we evaluate the samples of the studies that address the possible relationship between these polymorphisms and peri-implantitis, we observe that there are studies with samples from 29 [22] to 318 patients [39]. Such sampling methods are sensitive to selection bias, particularly in studies of cross-sectional and case-control

design. Subjects should ideally be included based on random selection, preferably having being treated in different environments.

Probably with a larger sample, we could observe statistically significant differences, regarding the presence of these polymorphisms, between the health and disease groups. In fact, there was a difference, although not statistically significant, in the frequencies of altered alleles in the *IL-1A* and *IL-1B* genes, at positions -889 and +3954 respectively, between the health and disease group.

In our study, positive history of periodontitis was more frequent in the peri-implantitis group than in patients with peri-implant health, albeit the difference in proportion was not significant. In studies where possible risk factors/indicators for peri-implantitis are investigated, it is important to evaluate the existence of a history of periodontitis. Previous studies have demonstrated that peri-implantitis-related biofilm was similar to that of periodontitis, comprising high levels of periodontal pathogens [40]. And it has been reported that the microflora present in the oral cavity before implantation determines the composition of the newly establishing microflora on implants [41], suggesting that patients with a history of periodontitis might be at high risk for peri-implantitis. Although there are several indices for the assessment of periodontal status, some of which particularly suitable for epidemiological studies, in our study the guidelines of the new classification of periodontal and peri-implant diseases (AAP /EFP 2018) were followed. According to the new classification case definitions of periodontitis and periodontal health may be applied in different contexts: patient care, epidemiological surveys and research studies.

The results described in the current scientific evidence are promising regarding the role of genetic factors, such as the studied polymorphisms, in the development of peri-implantitis. However, the number of studies in the literature regarding this association is quite limited, and some of them do not refer to variables that may impact/influence the results obtained (confounding variables), such as the periodontal status of individuals in the sample, their ethnicity and the presence or not of smoking habits. Thus, and due to the inequalities found between studies already published in the literature, in order to have a better understanding of this possible association, it is necessary to create uniform criteria between future studies. Furthermore, studies with larger samples in

different ethnic groups are needed to determine the real effect of genetic polymorphisms on this disease. In the future, it will be necessary to carry out more studies in this area, with larger samples, so that it is possible to identify and obtain results with greater accuracy and evidence regarding the relationship of polymorphisms of *IL-1A* -889 and *IL-1B* +3954 with the development of peri-implantitis.

4.2.5 Conclusions

According to the results of the present study, there was no statistically significant difference in proportions of IL-1 gene polymorphisms between the health and disease groups. So, within the limitations of the present study we can conclude, there seems to be no association between *IL-1A* genetic polymorphisms (*IL-1A* -889) and *IL-1B* (*IL-1B* +3954) and the development of peri-implantitis. However, although no statistic association was found, it appears that there is a possible tendency for individuals with these genetic polymorphisms to be more susceptible to developing peri-implantitis, as patients with peri-implantitis have a greater frequency of mutated allele (T allele), compared to individuals with peri-implant health. Regarding the allelic and genotype frequencies found, these are similar to those found in European populations.

Further prospective studies with a larger group of patients will confirm whether the studied polymorphisms can be used for screening patients prior to implant treatment and may possibly help in increasing the implant success rates, through the development of new point of care tests.

4.2.6 References

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5. ANALYSIS OF THE ASSOCIATION OF *IL-1A*, *IL-1B* AND *IL-1RN* POLYMORPHISMS WITH PERI-IMPLANTITIS IN A PORTUGUESE POPULATION

This chapter was based on the submitted work to the International Journal of Oral and Maxillofacial Implants:

Paper III – Cardoso JM, Ribeiro AC, Proença L, Noronha S, Alves RC. Analysis of the association of *IL-1A*, *IL-1B* and *IL-1RN* polymorphisms with peri-implantitis in a Portuguese population.

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Abstract

Purpose: The aim of this study was to investigate whether genetic variations in cytokine genes involved in the pathogenesis of peri-implantitis, could be associated with its occurrence, an issue that remains controversial and may vary according to the population evaluated.

Material and Methods: A cross-sectional analytical study was carried out on 102 Portuguese Caucasian individuals divided into two groups: 43 individuals with peri-implantitis and 59 individuals with peri-implant health. Samples from the buccal mucosa were obtained and genetic analysis was performed using the Real-time polymerase chain reaction (PCR) technique for *IL-1A* and *IL-1B* and using PCR analysis for *IL-1RN*.

Results: The *IL-1A* -889 C/T polymorphism showed a higher prevalence of the less common allele (T allele) in cases of peri-implantitis than in healthy cases (27.9% vs 16.9%, respectively), but without statistical significance ($p = 0.060$). For the *IL-1B* +3954 C/T and *IL-1RN* (variable number of tandem repeats) polymorphisms, the analysis revealed that the allele and genotype frequencies did not differ significantly between groups. There was a significant association between a history of periodontitis and peri-implantitis ($p = 0.020$).

Conclusions: The genetic polymorphisms evaluated had no influence on the occurrence of peri-implantitis in the population studied. Further research into genetic variations in different populations is needed to elucidate the role of genetic factors in the onset and progression of peri-implant disease.

Keywords: dental implants, peri-implantitis, interleukin-1 receptor antagonist protein, interleukin-1, genetic polymorphisms.

5.1 Introduction

Peri-implantitis is a pathological condition associated with bacterial plaque that occurs in the tissues around dental implants, characterized by inflammation of the peri-implant mucosa and progressive loss of supporting bone [1,2]. The onset as well as the severity of the disease also depend on concomitant factors specific for the individual [3,4]. Besides oral hygiene habits, a key factor for the progression of peri-implant tissue breakdown seems to be the local immunological reaction at the site of infection [3,5]. Several studies have investigated the impact of numerous mediators and cytokines on periodontal and peri-implant diseases, with interleukin (IL)-1 being one of the most studied [6–10].

As in periodontitis and other infections, pathogens and their virulence factors stimulate the release of various immunoinflammatory biomarkers by peri-implant cells [11]. Among the numerous biomarkers, the pro-inflammatory cytokines tumor necrosis factor (TNF)- α , interferon- γ , IL-1 β , IL-6, IL-12, IL-17 and receptor activator of nuclear factor kappa-beta ligand (RANKL); anti-inflammatory cytokines IL-4, IL-10 and IL-1 receptor antagonist (IL-1Ra); and chemokines IL-

8, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 alpha (MIP-1 α) have been suggested as important mediators of inflammation and immunity in the pathogenesis of peri-implantitis [6,7,11,12]. According to the review by Alassy et al., the assessment of the levels of pro-inflammatory cytokines IL-1 β , TNF- α and matrix metalloproteinase-8 in peri-implant crevicular fluid may be relevant in the diagnosis of peri-implantitis and peri-implant mucositis [6]. However, more controlled longitudinal studies are needed to understand the role of these cytokines in predicting peri-implantitis progression [6,11,13]. The results of a recent systematic review showed that the levels of IL-1 β , IL-6, IL-10 and RANKL/osteoprotegerin are not balanced in peri-implant disease, suggesting that these mediators are involved in the osteo-immuno-inflammatory response of the host related to peri-implantitis [7].

IL-1 is the pivotal mediator of the immunoinflammatory response that acts both in response to bacterial infection and in bone metabolism [14–16]. IL-1 is strongly induced by lipopolysaccharides from the cell walls of Gram-negative bacteria and acts directly or indirectly to initiate and amplify inflammatory responses by inducing the expression of a substrate of effectors, including cytokines/chemokines and matrix metalloproteinases [14]. There are two genetically and biochemically distinct IL-1 molecules, IL-1 α and IL-1 β , which have similar biologic functions [15]. IL-1Ra plays an anti-inflammatory role by preventing the transmission of pro-inflammatory signals and preventing the immune response by using the same receptor as pro-inflammatory IL-1 [17,18].

In recent years, a hypothesis has emerged suggesting that an individual's genetic background can influence the severity and progression of oral inflammations by affecting the expression of cytokines and their regulators [19–23]. The IL-1 family includes 11 cytokines located on the long arm of chromosome 2q [15]. The two most extensively studied members are the *IL-1A*, *IL-1B* genes, which encode IL-1 α and IL-1 β , respectively [24].

The *IL-1A* -889 polymorphism is situated in the promoter region of the *IL-1A* gene and the presence of the T allele has been linked to an increase in gene transcriptional activity [25]. *IL-1B* +3954 polymorphism is located in exon 5 of the *IL-1B* gene, and the replacement of the C allele by the T allele is associated with an increase in IL-1 β production [16,26]. Several studies have investigated these

single nucleotide polymorphisms (SNPs) in the IL-1 genes, with the aim of identifying possible links between these genetic variations and increased susceptibility to biological complications related to dental implants. However, these studies have achieved contradictory results [3,16,27–32].

The *IL-1RN* gene, which encodes IL-1Ra [17], contains a genetic polymorphism in intron 2, characterized by a variable number of tandem repeats (VNTR) of 86 base pairs [33]. The presence of IL-1RN allele 2 has been linked to a reduction in the production of IL-1Ra protein [34]. Additionally, *in vitro* studies have demonstrated that mononuclear cells from carriers of *IL-1RN* allele 2 produce higher levels of IL-1 β [35]. It is possible that *IL-1RN* allele 2 mainly increases the levels of IL-1 β and therefore results in an imbalance in the IL-1 β /IL-1Ra ratio leading to an increased susceptibility or more severe outcome of inflammatory diseases [18,36]. The association between the VNTR polymorphism in this gene and peri-implantitis has only been analyzed in two studies, with conflicting results [18,37].

To date, there is no consensus on whether these genetic alterations contribute substantially to a greater susceptibility to peri-implantitis [38,39]. No robust conclusions could be drawn from the current literature about the possible link between genetic variations and the occurrence of peri-implantitis. [19,24,38,40]. The inequality of the design of these studies requires more studies to be carried out using larger population samples and from different ethnic groups [24,38,39]. Studies have reported that the frequency of gene polymorphisms associated with genetic susceptibility to a particular disease can be influenced by ethnic and racial variations [41,42].

The aim of this study was therefore to investigate whether the three genetic variants (*IL-1A* - 889C/T, *IL-1B* + 3954C/T and *IL-1RN* (VNTR)) were associated with the occurrence of peri-implantitis in a Portuguese Caucasian population.

5.2 Materials and Methods

Study design and population

The study was conducted in accordance with the principles of the Declaration of Helsinki (revised in 2013). Appropriate measures were taken to

protect patient data. The study was approved by the Ethics Committee of Egas Moniz School of Health and Science on 24 July 2019 (process number 790).

A total of 43 patients diagnosed with peri-implantitis (bleeding and/ or suppuration on probing, probing depth (PD) equal or greater than 6 mm and bone loss equal to or greater than 3 mm) and 59 individuals with peri-implant health (peri implant mucosa without inflammatory signs and absence of peri-implant bone loss) were recruited between July 2021 and July 2022. The case definitions of peri-implantitis, peri-implant health, periodontal health and periodontitis were in accordance with the new classification of periodontal and peri-implant diseases [2,43,44].

The sample was obtained from patients referred for periodontal treatment or supportive care at the Periodontology Department of Egas Moniz School of Health and Science. The sample size and the selection of two groups were determined by statistical estimation based on the two results of a pilot study: proportion of altered genotypes (CT for *IL-1A* and CT/TT for *IL-1B*), which indicated that the sample size for this study should be $n=84$, i.e., $n=42$ for each group (peri-implantitis and peri-implant health), based on an average effect size ($w=0.3$), with a power of 80% and an alpha level of 5% [45]. All potential candidates received a screening questionnaire and, if the patient's medical history met the criteria for inclusion in the study and the patient agreed to participate, written informed consent was obtained.

The inclusion criteria were: - unrelated patients who born in Portugal, - patients with Caucasian parents and grandparents, - patients with at least one dental implant in function for at least one year, - patients with peri-implantitis or peri-implant health diagnosis, - patients who agree to participate in the study and sign an informed consent form. The exclusion criteria were: - patients with any systemic condition that affected the immune system, - patients who have taken antibiotics or anti-inflammatory drugs chronically in the last six months - pregnant women, - patients who have undergone peri-implant treatment in the area to be evaluated, - patients with non-osseointegrated implants, - patients with mucositis diagnosis.

Socio-demographic and clinical variables

Data were collected regarding the following variables: age, gender, smoking status (yes or no), history of periodontitis, stage III or IV of periodontitis, and full mouth plaque score (FMPS).

Patients with suspected periodontitis underwent a complete periodontal examination (six sites per tooth with a CP12 graduated periodontal probe) to record PD, bleeding on probing, gingival margin and tooth mobility.

Peri-implant probing (six sites with a CP12 graduated periodontal probe) was carried out as usual during periodontal appointments. FMPS was recorded as the percentage of total surfaces (4 aspects per tooth) that revealed the presence of bacterial plaque (adapted from O'Leary, Drake, Naylor, 1972 [46]). The presence of bleeding on probing or pus was also evaluated.

All clinical data was collected by the same examiner (J.M.C.). Prior to the study, a training and calibration exercise for PD measurements was carried out using volunteer patients not included in the study. The calibration exercise demonstrated 95% agreement with a margin of ± 1 mm for PD measurements.

Plaque control measures were implemented for cases of peri-implant health, while patients with peri-implantitis were referred for additional treatment in conjunction with reinforcement of plaque control measures. If a patient had multiple implants affected by peri-implantitis, the implant with the greatest degree of bone loss was selected for analysis in the study.

Genetic analysis

For the investigation of genetic polymorphisms, a sample of cells from the jugal mucosa was collected with the aid of a sterile swab (Omniswab Whatman® FTA®). Samples were placed in a 1.5-ml test tube (Eppendorf tubes®) and kept at -20 °C until DNA extraction. The DNA extraction process was performed based on the "Protocol: Isolation of Total DNA from Surface and Buccal Swabs" taken from the QIAamp® DNA Investigator Handbook.

The polymorphism of the *IL-1RN* gene (rs2234663) analyzed consists of a genetic variation of a repetitive sequence of 86 bp, consisting of a VNTR polymorphism. This sequence can be repeated two, three, four, five or six consecutive times, providing five possible alleles: 1, 2, 3, 4 and 5. Thus, its genotyping using the basic methodology of molecular biology techniques becomes the most viable technique. The DNA obtained through the DNA extraction technique was amplified by specific sequences of oligonucleotides for this polymorphism (primers). The primers used were 5'-CTC AGC AAC ACT CCT AT-3' and 5'-TCC TGG TCT GCA GGT AA-3'. For the *IL-RN* gene polymorphism, the primers were aliquoted at a concentration of 10 µM and the Master Mix was prepared. The Master Mix solution included NZYtaq II 2x Green Master Mix - NZYtech (consisting of the Taq DNA Polymerase enzyme, dNTP's, Buffer and MgCl₂), forward and reverse primers and RNase/ Dnase-free Water. The samples obtained with a final volume of 25 µl were inserted into the MJ MINI™ Personal Thermal Cycler for carrying out the amplification reactions. The initial step involved subjecting the samples to a heating cycle at 96°C for 1 minute. Subsequently, 35 cycles were performed, consisting of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, extension at 70°C for 2 minutes, and final extension at 70°C for 5 minutes. For revealing the genotyping of the polymorphic regions associated with the *IL-1RN* gene (intron 2, VNTR), the technique of electrophoresis in 3% agarose gel was used.

For the analysis of the *IL-1A* -889 (rs1800587) and *IL-1B* +3954 (rs1143634) polymorphisms, the Real-time polymerase chain reaction (PCR) technique was performed, using the commercial kit *AppliedBiosystems*™ (Thermo Fisher SCIENTIFIC), using TaqMan™ SNP Genotyping Assay solution, which contains the forward and reverse primers, as well as two probes with different fluorescent reporters. This solution is specifically defined for each polymorphism and is available commercially. Then, a Master Mix solution was prepared that contained the TaqMan™ Genotyping Master Mix solutions and TaqMan™ SNP Genotyping Assay. The reference values were 10 µl for TaqMan™ Genotyping Master Mix and 1 µl for TaqMan™ SNP Genotyping Assay, which were multiplied by the 102 samples. Subsequently, 11 µl of the previously prepared solution and 9 µl of each DNA sample were added to

“eppendorf” minitubes. The samples were inserted into the Corbett Research RG 3000 thermocycler to start the amplification reactions in Real-time PCR.

Some results obtained by the TaqMan assay were confirmed by DNA sequencing. Patients of each genotype type were selected for analysis. The results obtained by Sanger sequencing and the TaqMan assay were identical.

Statistical analysis

A set of descriptive and inferential statistics was performed. After confirming the non-normality of the data using the Kolmogorov-Smirnov test, the chi-square test and Fisher's exact test compared categorical variables, while the Mann-Whitney test was used to compare continuous variables between the groups. The dependent variable was the peri-implant status, and the unit of analysis was the participant. The significance was set at p equal to or less than 0.05 for the inferential analyses. Data analyses were performed using the software IBM SPSS Statistics v.28.

5.3 Results

Demographic and clinical characteristics of the study group

The study included 102 healthy individuals (45 men and 57 women) aged between 24 and 90 who had at least one dental implant in function. All the participants had the same ethnic origin (Caucasian) and were born in Portugal. The factors examined for prognostic significance included smoking habits, a history of periodontitis and full-mouth plaque score. The characteristics of the sample are presented in Table 5.1.

There were no statistical differences between the groups in terms of gender, age and FMPS. The percentage of smokers was higher in the peri-implantitis group than in patients with peri-implant health, although the difference in proportion was not significant (46.5.2% vs 28.8%, $p = 0.066$). In addition, a positive history of periodontitis was more frequent in the group with peri-

implantitis than in patients with peri-implant health, and the difference was statistically significant (76.7% vs 54.2%, $p = 0.020$). In addition, the most severe stages of periodontitis were more frequent in the group with peri-implantitis.

Table 5.1 – Demographic and clinical data of patients with dental implants.

	Peri-implant Health (n=59)	Peri-implantitis (n=43)	<i>P</i>
Age, Mean (SD) (years)	59.6 (11.7)	58.8 (11.6)	0.645
Range (years)	24-85	38-90	
Sex, n (%)			
Male	25 (42.4)	20 (46.5)	0.678
Female	34 (57.6)	23 (53.5)	
Smoking, n (%)			
No	42 (71.2)	23 (53.5)	0.066
Yes	17 (28.8)	20 (46.5)	
History of Periodontitis, n (%)			
No	27 (45.8)	10 (23.3)	0.020
Yes	32 (54.2)	33 (76.7)	
More severe Stages of Periodontitis Stage III			
Stage IV			
No	40 (67.8)	18 (41.9)	0.009
Yes	19 (32.2)	25 (58.1)	
FMPS			
≤ 15%	30 (50.8)	14 (32.6)	0.066
> 15 %	29 (49.2)	29 (67.4)	

Abbreviations: standard deviation (SD), full-mouth plaque score (FMPS), statistically significant differences ($p < 0.05$) are denoted in bold.

Allelic frequencies

With regard to the *IL-1A* gene polymorphism, in the healthy group, the allele frequency for the C allele was 83.1% and for the T allele 16.9%. In the group with peri-implantitis, the allele frequencies were 72.1% and 27.9% for the C and T alleles, respectively. There were no statistically significant differences in the proportions between the health and disease groups when the presence of the less common allele (allele T) was taken into account ($p = 0.060$). The allelic frequencies are shown in Table 5.2

Table 5.2 – Allelic frequencies of *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* (VNTR) polymorphisms, in the peri-implant health group, in the peri-implantitis group and in the total sample.

Alleles, n (%)	Peri-implant health (n=118)	Peri-implantitis (n=86)	Total (n=204)	<i>p</i>
<i>IL-1A</i> -889				
C	98 (83.1)	62 (72.1)	160 (78.4)	0.060
T	20 (16.9)	24 (27.9)	44 (21.6)	
<i>IL-1B</i> +3954				
C	103 (87.3)	71 (82.6)	174 (85.3)	0.346
T	15 (12.7)	15 (17.4)	30 (14.7)	
<i>IL-1RN</i> (VNTR)				
1	102 (86.4)	76 (88.4)	178 (87,3%)	0.683
2/3/5	16 (13.6)	10 (11.6)	26 (12.7%)	

Abbreviations: variable number of tandem repeats (VNTR).

In relation to the *IL-1B* gene polymorphism, in the healthy group the allele frequency for the C allele was 87.3% and for the T allele it was 12.7%. In the group with the disease, the allele frequencies were 82.6% and 17.4% for the C and T alleles, respectively. As already observed for the *IL-1A* gene polymorphism, no statistically significant differences were found in the proportions of the altered allele between the groups ($p = 0.346$).

With respect to the *IL-1RN* polymorphism (VNTR), in the healthy group, the allele frequencies were 86.4% for allele 1 and 13.6% for the less common alleles. In the group with the disease, the allele frequencies were 88.4% and

11.6% for allele 1 and the altered alleles, respectively. Among the altered alleles, allele 2 is the most common. The allele frequencies did not vary significantly between the groups for this polymorphism ($p = 0.683$).

Genotype frequencies

The genotype frequencies obtained for the genes *IL-1A* -889, *IL-1B* +3954 and *IL1-RN* (VNTR), in the peri-implant health group, in the peri-implantitis group and for the total sample are presented in Table 5.3. Table 5.3 also shows the percentage of carriers of positive composite genotype (at least one allele with nucleotide sequence changed in both genes) of *IL-1A* -889 C/T and *IL-1B* +3954 C/T.

With regard to the *IL-1A* -889 polymorphism, 67.8% of the subjects had the CC genotype, while 32.2% showed the presence of the CT or TT genotypes. In the peri-implantitis group, 55.8% had the CC genotype and 44.2% had CT or TT genotypes. No statistically significant differences were found between the health and disease groups, when considering the proportion of the altered genotype ($p = 0.216$).

With respect to the *IL-1B* +3954 polymorphism, in the healthy group, the genotype frequencies were 74.6% for the CC genotype and 25.4% for the CT and TT genotypes. For the group with diagnosed peri-implantitis, frequencies of 69.8% were obtained for the CC genotype and 30.2% for the CT and TT genotypes. No statistically significant differences were found in the proportions of the *IL-1B* gene polymorphism between the health and disease groups when the presence of the modified genotype was taken into account ($p = 0.591$). The genotype frequencies obtained for *IL-1A* -889 and *IL-1B* +3954 did not deviate from the Hardy-Weinberg expectation in the control group.

Table 5.3 – Genotype frequencies of *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* (VNTR) polymorphisms, in the peri-implant health group, in the peri-implantitis group and in the total sample.

Genotype, n (%)	Peri-implant health (n=59)	Peri-implantitis (n=43)	Total (n=102)	<i>p</i>
<i>IL-1A</i> -889				
CC	40 (67.8)	24 (55.8)	64 (62.7)	0.216
CT/ TT	19 (32.2)	19 (44.2)	38 (37.3)	
<i>IL-1B</i> +3954				
CC	44 (74.6)	30 (69.8)	74 (72.5%)	0.591
CT/ TT	15 (25.4)	13 (30.2)	28 (27.5%)	
<i>IL-1RN</i> (VNTR)				
1/1	50 (84.7)	38 (88.4)	88 (86.3)	0.599
1/3, 1/5, 2/2	9 (15.3)	5 (11.6)	14 (13.7)	
IL-1 composite genotype				
Yes	9 (15.3)	10 (23.3)	19 (18.6)	0.305
No	50 (84.7)	33 (76.7)	83 (81.4)	

Abbreviations: variable number of tandem repeats (VNTR).

In relation to the *IL-1RN* polymorphism (VNTR), in the healthy group, the genotype frequencies were 84.7% for the 1/1 genotype and 15.3% for the altered genotypes (1/3, 1/5 and 2/2). For the group with diagnosed peri-implantitis, the frequencies were 88.4% for the 1/1 genotype and 11.6% for less common genotypes. The distribution of the different genotypes for this polymorphism showed no differences between the groups in the proportions found ($p = 0.599$).

The percentage of carriers of IL-1 positive composite genotype, although higher in the disease group, did not differ significantly between groups ($p = 0.305$).

Analysis of clinical variables according to genotype observed in each polymorphism

Clinical variables such as FMPS, PD and maximum PD were also analyzed according to the presence or absence of the polymorphism, as shown in table 5.4.

Table 5.4 – Comparison of clinical variables according to the observed genotype in each polymorphism.

Genotype, n (%)	FMPS (mean ± SD)	<i>p</i>	PD (mean ± SD)	<i>p</i>	Maximum PD (mean ± SD)	<i>p</i>
<i>IL-1A</i> -889						
CC	20.6 ±14.7	0.158	3.7 ±1.9	0.151	5.0±2.2	0.166
CT/ TT	23.5 ± 14.1		4.4 ± 2.3		5.6±2.5	
<i>IL-1B</i> +3954						
CC	20.5 ±13.6	0.154	4.0 ±2.3	0.769	5.3±2.6	0.606
CT/ TT	24.8 ±16.4		3.9 ±1.6		5.0±1.6	
<i>IL-1RN</i> (VNTR)						
1/1	21.8 ±14.6	0.957	4.1 ±2.2	0.143	5.3±2.4	0.424
1/3, 1/5, 2/2	20.6 ±14.2		3.3 ±1.4		4.7±1.9	
<i>IL-1</i> composite genotype						
Yes	26.3 ± 13.1	0.024	4.3 ± 1.6	0.161	5.3±1.6	0.252
No	20.6 ± 14.6		3.9 ±2.2		5.2±2.5	

Abbreviations: standard deviation (SD), full mouth plaque score (FMPS), probing depth (PD), statistically significant differences ($p < 0.05$) are denoted in bold.

The aim of this analysis was to verify whether there was any relationship between the presence of a particular polymorphism and the clinical variables analyzed. In this evaluation, the groups were divided into the presence or absence of the polymorphism for each gene, regardless of whether they had peri-implant health or peri-implantitis. It was observed that the clinical variables did

not differ significantly between the groups with and without the polymorphism. The only exception was the percentage of individuals with FMPS above 15%, which was more frequent in individuals with *IL-1* positive composite genotype compared to patients without this genotype ($p = 0.024$).

5.4 Discussion

Peri-implantitis is among the most common complications of implant-supported dental restorations [47–49]. However, the etiology and pathogenesis of peri-implantitis are currently not well understood, and more research is needed to clarify them [38]. The accumulation of microbial plaque around dental implants can trigger a host inflammatory response (in the peri-implant tissues), but its progression and severity can vary between individuals [50]. The question of the influence of genetic alterations on the development of peri-implantitis is a relevant topic because, if this association is confirmed, it is possible to identify patients who have a particular polymorphism and the physician's approach can be adjusted in order to prevent the onset of this disease [38]. Genetic polymorphisms are constant and can be measured before the onset of the disease, so they can be very useful for treatment planning and prognosis at an early stage [38,51].

Trevilatto, et al., in their study, compared various studies analyzing the genotype frequencies of the *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* (VNTR) polymorphisms in different populations [52]. Analyzing the data obtained for the *IL-1A* -889 polymorphism in our study, the frequency of the altered genotype (CT/TT) is 37.3.% in the total sample, which is lower than in studies carried out in Brazil [52,53] and in a study carried out in northern Europe [18], where a genotype frequency of 64% of this polymorphism was found. In relation to the data obtained for the *IL-1B* +3954 polymorphism in our study, the frequency of the altered genotype (CT/TT) is 27.5% in the total sample, which is slightly lower than other studies carried out in southern [54] and northern Europe [18] with values of approximately 40 and 50% respectively, although higher than studies carried out in Asia [55]. As for the *IL-1RN* polymorphism (VNTR), the frequency

recorded for carriers of less common genotypes (13.7%) is similar to that found in other studies carried out in southern Europe [54,56].

IL-1A -889 and *IL-1B* +3954 are two of the most studied genetic variants in the field of implant failure and peri-implant disease [24]. Several studies have investigated their association with the risk of peri-implantitis, with some obtaining positive results [16,30–32] and others negative [3,18,57,58]. In the present study, there were no statistically significant differences between the health and disease groups in relation to the polymorphisms assessed, so there was no association between the polymorphisms studied and peri-implantitis. Published data on the association between the *IL-1RN* (VNTR) polymorphism and the risk of peri-implantitis is scarce in the current literature [18,37].

Liao et al. carried out a meta-analysis to examine the potential correlation between IL-1 polymorphisms (specifically, *IL-1A* -889 and *IL-1B* +3954) and the incidence of dental implant failures/complications [24]. Of the 13 studies analyzed, only four provided sufficient data to explore the possible link between these polymorphisms and peri-implantitis. Upon completion of the meta-analysis, it was observed that there is a borderline significant association among Caucasian Europeans between the positive composite genotype of *IL-1A* -889 and *IL-1B* +3954 and peri-implantitis. A more recent meta-analysis found that individuals carrying the positive composite genotype of *IL-1A* -889 and *IL-1B* +3954 had 1.95 times greater risk of peri-implant disease, which encompasses implant failure/loss, marginal bone loss and peri-implantitis. The authors concluded that the IL-1 positive composite genotype could serve as a predictive marker for peri-implant disease [20].

The differences between the results of the studies may be due to differences in the ethnicity of the groups included. In the present study, Caucasian individuals from southern Europe were included. There is evidence that ethnic and racial differences can have an impact on the prevalence of genetic polymorphisms linked to susceptibility to a specific disease [41,42]. To ensure the homogeneity of our study population, we exclusively recruited patients born in Portugal, with Caucasian ancestry, spanning three generations, and excluded those with different ethnic origins. Vaz et al. observed in a Portuguese population that alleles 1 and 2 (allele with altered nucleotide sequence) of the *IL-1A* gene

and alleles 1 and 2 of the *IL-1B* gene were statistically associated with the success or no success of dental implants, respectively [31]. However, the inclusion criteria vary from our study. In another study carried out in the Spanish population, it was observed that IL-1 genotypes (*IL-1A* -889 and *IL-1B* +3954 polymorphisms) do not appear to be good predictors of peri-implantitis in the vast majority of patients who smoke [57].

On the other hand, our study consisted of 102 patients, a number that is in line with the sample calculation carried out in a pilot study [45]. However, many of the studies investigating risk factors for peri-implant disease were based on convenience sampling, which is prone to selection bias, especially in cross-sectional and case-control studies [59].

In our study, the guidelines of the new classification of periodontal and peri-implant diseases were followed [2,43,44]. There is a wide range of different definitions of peri-implant mucositis and peri-implantitis that have been used in different studies. This misdiagnosis of peri-implantitis and the inclusion of cases of peri-implant mucositis can affect the results of studies [6,40,48,60].

Peri-implantitis is a common, complex and multifactorial disease [51,61]. Among its established risk factors are history of periodontal disease, lack of maintenance and smoking [39,62]. Local risk factors include inadequate plaque control [39], mucositis [63], poor implant positioning and poorly designed prosthesis [64] or the presence of excess cement [39]. Furthermore, despite the lack of consensus among the studies and the fact that they were not identified by longitudinal studies, certain diseases, characteristics of the implant or the area where the implant was placed have been pointed out by some authors as possible risk indicators for peri-implantitis [65,66]. It is therefore understandable that in diseases with a multifactorial etiology, and especially in studies with a cross-sectional design, it is difficult to control all the risk factors/indicators and understand the real effect of a particular factor (in this case, genetics) on the development of the disease.

Our study included patients who smoked, while other studies on this subject did not include this type of patients. The smoking status of the participants should be assessed and balanced between the groups [40]. However, some

studies did not present data on smoking habits [3,32] and, in the studies in which smoking status was assessed, there were variations in the categorization of smokers and non-smokers, which could lead to divergent results [18,29,31,37,57].

Data on the periodontal status of the patients were not reported or clarified in some studies that evaluate the possible relationship between these polymorphisms and peri-implantitis [3,18,30]. There is strong evidence from both longitudinal and cross-sectional studies that a history of periodontitis is a risk factor/indicator for peri-implantitis [39]. In the present study, our results also demonstrated that there was a significant association between the history of periodontitis and peri-implantitis.

Some limitations of our study should be addressed. A longitudinal design would have allowed more adequate control of the various risk factors/indicators. A greater number of local and systemic risk factors/indicators should have been assessed. The results of the present study would have been more authentic if quantitative detection of cytokine levels would have been done in peri-implant crevicular fluid samples of patients with and without the genetic polymorphism. To our knowledge there is only one study that relate genetic alterations with changes at the biochemical level in the peri-implant crevicular fluid [3]. In that study, with 29 participants, the IL-1 polymorphism investigated had minimal effect in the peri-implant crevicular immune response. There is evidence that biomarkers are not necessarily present at a single moment of peri-implant crevicular fluid collection due to various systemic or local factors [6]. As a consequence of the cyclical progression of peri-implant diseases, biomarkers of immunoinflammatory events responsible for tissue degradation may not always be active in cross-sectional studies with a single moment of fluid collection [13]. In addition, the effects of these polymorphisms could have been evaluated on the transcriptional activity of the genes. The presence of the IL-1 positive composite genotype was assessed, but future research should analyze the presence of other haplotypes and relate them to the presence of peri-implantitis.

The number of studies investigating the potential involvement of genetic factors, including the polymorphisms studied, in the development of peri-implantitis is relatively limited, and some studies do not take into account

confounding variables that may impact or influence the results obtained. Given the disparities observed between previously published studies, it is imperative to establish standardized criteria for future research in order to obtain a comprehensive understanding of the potential association between genetic factors, including the polymorphisms studied, and peri-implantitis. Furthermore, in order to verify the true impact of genetic polymorphisms on peri-implantitis, it is essential to carry out studies with larger samples, covering different ethnic groups.

5.5 Conclusions

Based on the results obtained, it can be concluded that there was no correlation between the *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* polymorphisms and peri-implantitis in the population studied. However, more studies on genetic variations in different populations are needed to elucidate the role of genetic factors in the onset and progression of peri-implant disease.

5.6 References

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6. THE INFLUENCE OF GENETIC POLYMORPHISMS ON THE EXPRESSION OF INTERLEUKIN-1BETA, PROSTAGLANDIN E2 AND TUMOR NECROSIS FACTOR ALPHA IN PERI-IMPLANT CREVICULAR FLUID: A CROSS-SECTIONAL STUDY

This chapter was based from the published work:

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Abstract:

The aim of this study was to evaluate the possible relationships between polymorphisms in the interleukin-1 (*IL-1*) *A*, *IL-1B*, and *IL-1RN* genes and concentrations of the inflammatory mediators IL-1 β , tumor necrosis factor-alpha (TNF- α) and prostaglandin E2 (PGE2) in peri-implant crevicular fluid (PICF). A cross-sectional analytical study was conducted on 51 patients with dental implants. Samples from the buccal mucosa were obtained and genetic analysis was performed using the real-time polymerase chain reaction (PCR) technique for *IL-1A* and *IL-1B*, and PCR for *IL-1RN*. For the biochemical analysis, the concentrations of IL-1 β and TNF- α were analyzed using multiplexed fluorescent sphere immunoassays, and PGE2 by enzyme-linked immunosorbent assay. In

patients with detected *IL-1RN* polymorphism, there was an increase in the concentration of the three mediators with statistically significant differences in the mean values of TNF- α and PGE2, regardless of peri-implant health status ($p = 0.002$ and $p = 0.049$, respectively). The concentrations of all three mediators were positively and significantly correlated (IL-1 β vs. TNF- α Rho = 0.480, $p < 0.001$; IL-1 β vs. PGE2 Rho = 0.382, $p = 0.006$; and TNF- α vs. PGE2 Rho = 0.528, $p < 0.001$). We can conclude that the *IL-1RN* polymorphism exerts an influence on the PICF immune response, which may explain the influence of this genetic polymorphism on the occurrence of peri-implantitis.

Keywords: peri-implantitis; interleukin-1 beta; interleukin-1 receptor antagonist protein; genetic polymorphisms; prostaglandin E2; tumor necrosis factor-alpha

6.1 Introduction

Although endosseous implants demonstrate significant success and survival rates, it is important to recognize that complete prevention of biological complications is not achievable [1–3]. One of the biological complications around dental implants is peri-implantitis, which is a plaque-associated pathological condition that occurs in the tissues around dental implants and is characterized by inflammation of the peri-implant mucosa and progressive loss of the supporting bone [4,5]. According to the new classification of periodontal and peri-implant diseases, in the absence of previous examination data, the diagnosis of peri-implantitis should be based on the combination of the presence of bleeding and/or suppuration on gentle probing, probing depths of ≥ 6 mm and bone levels ≥ 3 mm apical to the most coronal portion of the intraosseous part of the implant [4]. Although specific bacteria, dental plaque and environmental factors are associated with peri-implant disease, there is currently a lack of reliable indicators to predict the occurrence and severity of peri-implantitis [6]. Important risk factors/indicators have been identified, including a history of periodontitis, poor plaque control and no regular supportive peri-implant care following implant therapy [7,8]. Less conclusive evidence was found for smoking and diabetes, or local factors such as the presence of submucosal cement following prosthetic

restoration of the implant, or positioning of implants limiting access to oral hygiene and maintenance [7,8].

There is controversy regarding significant differences in the incidence of peri-implantitis and implant loss between smokers and non-smokers [7,8]. According to a review study on the influence of smoking on implant therapy, there is insufficient evidence on the effect of smoking on peri-implantitis, as well as on long-term implant loss [9]. On the other hand, in a study evaluating the phenomenon of corticalization of the peri-implant jawbone in tobacco smokers, a correlation was observed between smoking and changes in bone structure in radio textures near the implants [10]. Other studies have shown that smoking may affect the outcome of peri-implant disease treatment [11,12].

According to a recent systematic review of changes in molecular signatures associated with dental implant material in peri-implantitis, differences in transcriptomic signatures between peri-implant lesions and periodontal tissues may be related to titanium particles arising from dental implants [13]. In a comparative analysis of the transcriptome of periodontitis and peri-implantitis in humans, it was observed that although periodontitis and peri-implantitis shared a common genetic expression that was clearly distinct from healthy conditions, there were also unique genetic patterns that were differentially expressed only in peri-implantitis [14].

Interleukin-1 (IL-1) is a multifunctional cytokine that plays a key role in the pathogenesis of inflammatory diseases, with a variety of diverse activities and functions in the immune response, inflammation, tissue breakdown and tissue homeostasis, as shown in Figure 6.1 [15,16].

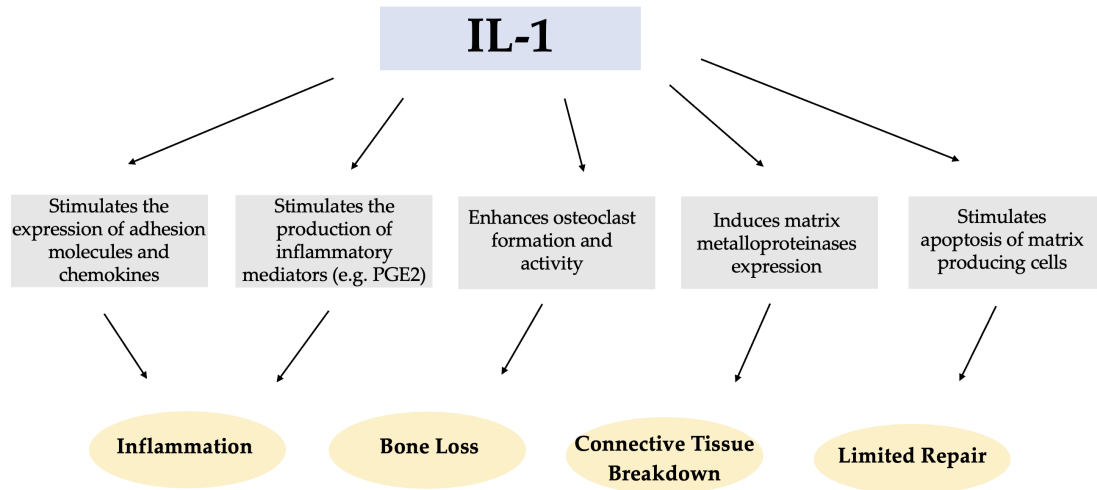


Figure 6.1 - Potential impact of Interleukin-1 (IL-1) in the pathogenesis of inflammatory diseases.

The existence of two genetically and biochemically distinct IL-1 molecules, IL-1 alpha (IL-1 α) and IL-1 beta (IL-1 β), is well-established. These molecules exhibit comparable biological functions but share only 27% homology at the amino acid level [17]. Both forms of interleukin (IL1 α and IL-1 β) bind to a common receptor that is present at varying densities in numerous cell types. Within the IL-1 family of proteins, there is also the IL-1 receptor antagonist (IL-1Ra) [18]. IL-1Ra plays an anti-inflammatory role by preventing the transmission of pro-inflammatory signals and the immune response by using the same receptor as pro-inflammatory IL-1 [19].

Several studies have reported higher levels of IL-1 β and tumor necrosis factor-alpha (TNF- α) in the peri-implant crevicular fluid (PICF) of implants with peri-implantitis than in healthy implants [20–24]. IL-1 β , a powerful pro-inflammatory protein, plays a crucial role in the inflammatory process that occurs in the oral cavity. It acts as a key mediator in the production of prostaglandins, leukotrienes and platelet-activating factors in various cell types. In addition, IL-1 β stimulates osteoclast formation and contributes to bone resorption [25,26]. IL-1 β is one of the candidate biomarkers that has been identified as being strongly associated with an increase in immuno-infiltrating cells in soft tissue samples obtained from peri-implantitis sites [27]. Another pro-inflammatory cytokine, TNF-

α , shares several functions with IL-1 β [28,29]. TNF- α also stimulates bone resorption and fibroblast apoptosis, resulting in limited repair of the periodontium, and increasing local secretion and activity of metalloproteinases that degrade connective tissue [30,31].

Prostaglandin E2 (PGE2) is a vasodilator that increases vascular permeability at sites of inflammation and also plays a role in bone resorption. Some studies agree that higher levels of PGE2 are found in the PICF of patients with peri-implant disease [20,32,33].

Genetic polymorphisms can influence gene expression, protein synthesis and cytokine secretion [34]. Numerous observational studies have investigated the potential association between different gene polymorphisms and the development of peri-implantitis, with a significant emphasis on IL-1 [16,19,35–39]. Among them, the *IL-1A*, *IL-1B*, and *IL-1RN* genes have been studied. These genes encode IL-1 α , IL-1 β and IL-1Ra, respectively [18,40]. The *IL-1A* (rs1800587) and *IL-1B* (rs1143634) genes are known to regulate the production of IL-1 α and IL-1 β , respectively [40,41]. They are located close to each other on the long arm of chromosome 2, specifically at position 2q13, and are considered potential candidates for genetic markers in peri-implantitis since the cytokines they encode play a crucial role in the development of the inflammatory response [19]. The presence of the *IL-1RN* allele 2 has been linked to a reduction in the production of IL-1Ra protein [18,42]. Furthermore, mononuclear cells obtained from individuals carrying the *IL-1RN* allele 2 exhibited an elevated production of IL-1 β in vitro [43]. These findings indicate that the presence of the *IL-1RN* allele 2 can potentially disrupt the balance between IL-1 β and IL-1Ra, leading to greater vulnerability or more pronounced symptoms of inflammatory diseases. Although there is no consensus in the literature regarding the role of genetic polymorphisms in the development of peri-implantitis, most studies have focused on *IL-1A* -889, *IL-1B* +3954, and *IL-1RN* variable number of tandem repeats (VNTR) polymorphisms. Some studies have found an association between the presence of these polymorphisms and peri-implantitis [16,19,36,37,44], while others have not [35,39,45,46].

Moreover, over the past 25 years, numerous single nucleotide polymorphisms have been associated with an increased incidence or severity of

periodontal inflammation. The most studied polymorphisms were *IL-1A* -889 and *IL-1B* +3954. Meta-analyses seem to show an association between these polymorphisms and periodontal disease [47–49]. There is evidence that the presence of the positive composite genotype (i.e., the presence of at least one altered allele in the *IL-1A* -889 C/T and *IL-1B* +3954 C/T genes) increases susceptibility to periodontitis [47,50]. The sequence of immunopathological events in peri-implant infections has some similarities to periodontal infections [51].

Although there are studies that try to relate the existence of certain genetic polymorphisms and the presence of peri-implantitis, to our knowledge, there is only one study that relates genetic alterations with changes at the biochemical level in the PICF [35]. In this study, with 29 participants, the *IL-1* polymorphism investigated had a minimal effect on the peri-implant crevicular immune response. A study with a larger sample size and better-defined criteria for cases of peri-implant health and peri-implantitis may help to evaluate the effect of these polymorphisms at the level of inflammatory mediators. The possible association between genetic polymorphisms and peri-implantitis must be based on an alteration in the individual's immune-inflammatory response. Therefore, it is important that studies evaluating genetic polymorphisms in patients with peri-implantitis investigate the functional consequences of these polymorphisms. This can be obtained by analyzing the protein levels in PICF. Table 6.1 describes the various genetic polymorphisms that have been tried to be associated with peri-implantitis.

Table 6.1 – Genetic polymorphisms have been studied in cases of peri-implantitis.

Genetic Polymorphisms Evaluated in the Studies
<i>IL-1A</i> -889
<i>IL-1A</i> +4845
<i>IL-1B</i> -511
<i>IL-1B</i> +3954
<i>IL-1RN</i> +2018
<i>IL-1RN</i> (VNTR)
<i>IL-6</i> -174
<i>IL-17</i> (rs10484879)
<i>IL-17R</i> (rs879576)
<i>TNF-α</i> -308
<i>OPG</i> T950C
<i>OPG</i> G1181C
<i>RANKL</i> (rs9533156)
<i>RANKL</i> (rs2277438)
<i>CD-14</i> -159

Abbreviations: variable number of tandem repeats (VNTR), tumor necrosis factor-alpha (TNF-α), osteoprotegerin (OPG), receptor activator of nuclear factor-Kappa B ligand (RANKL), cluster of differentiation 14 (CD14).

In the present study, we aimed to investigate the possible relationships between *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* (VNTR) gene polymorphisms and the concentrations of the PICF mediators IL-1β, TNF-α and PGE2. The main hypothesis of the study was that, in both healthy individuals and patients with peri-implantitis, an altered genotype of *IL-1A*, *IL-1B* or *IL-1RN* would result in higher levels of pro-inflammatory mediators compared to patients without the altered genotype, and that this effect would be particularly pronounced in the peri-implantitis group. A second hypothesis was that the concentrations of the three mediators would be correlated in health and disease since they all orchestrate the same biological process.

6.2 Materials and Methods

Study design and population

The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki, last revised in 2013. Stringent measures were

implemented to ensure the general data protection regulations in place. Approval for this research was granted by the Institutional Review Board (Egas Moniz School of Health and Science, Almada, Portugal) on 24 July 2019 (process number 790).

A total of 27 patients diagnosed with peri-implantitis (bleeding and/or suppuration on probing, probing depth (PD) equal to or greater than 6 mm and bone loss equal to or greater than 3 mm) and 24 individuals with peri-implant health (peri-implant mucosa without inflammatory signs and absence of peri-implant bone loss) were recruited. The diagnostic criteria were in accordance with the updated classification of periodontal and peri-implant diseases [4,52,53].

The convenience sample was obtained from patients referred for periodontal treatment at the Periodontology Department of the Egas Moniz Dental Clinic (EMDC) between July 2021 and July 2022. A screening questionnaire was provided to all potential candidates, and written informed consent was obtained from those whose medical history met the study eligibility criteria and accepted to be enrolled.

Patients were considered eligible if they were born in Portugal, of Caucasian ancestry (i.e., first and second line), unrelated, had a dental implant in function for at least twelve months, were diagnosed with either peri-implant health or peri-implantitis, agreed to participate and provided informed consent. Patients were excluded if they were pregnant, had an immune disorder with systemic involvement, had recently taken antibiotics (6 months or less), had been on chronic anti-inflammatory therapy (for 6 months or less), had received treatment for the peri-implant condition in the oral region being evaluated, had nonosseointegrated implants or had a diagnosis of mucositis.

Socio-demographic and clinical variables

Data were collected regarding the following variables: age, sex, smoking status (yes or no), history of periodontitis and full-mouth plaque score (FMPS).

Patients suspected of having periodontitis underwent a comprehensive periodontal examination. Peri-implant probing was performed using a CP12 graduated periodontal probe, at six sites around each dental implant, as well as the evaluation of the presence of bleeding or suppuration on probing. Bone loss was assessed using a retroalveolar radiograph (paralleling technique). FMPS was determined by calculating the percentage of total tooth surfaces (four aspects per tooth) that exhibited the presence of plaque. This scoring method was adapted from O'Leary, Drake and Naylor in 1972 [54].

A single examiner collected all the clinical data in the study (J.M.C.). To ensure consistency and accuracy, a training and calibration exercise was conducted before the study, specifically for PD measurements. The results were statistically analyzed using the intra-class correlation coefficient (intra-examiner reproducibility). The calibration exercise resulted in a high level of agreement, with 95% concordance observed within a range of ± 1 mm for the PD measurements.

Plaque control measures were implemented for peri-implant healthy cases, while patients with peri-implantitis were referred for additional treatment in conjunction with reinforcing plaque control measures. If a patient presented multiple implants affected, we included data from the implant with the highest bone loss.

Genetic analysis

To study genetic polymorphisms, cells were collected from the buccal mucosa, inside of the cheek, using a sterile swab (specifically, Omniswab Whatman® FTA®, South Miami, FL, USA, was utilized). They were then transferred to 1.5-mL test tubes (Eppendorf tubes®, Hamburg, Germany) and stored at -20°C for later analysis. The DNA extraction process followed the manufacturer's protocol as detailed elsewhere (QIAamp® DNA Investigator Handbook, Venlo, The Netherlands).

The polymorphism of the *IL-1RN* gene (rs2234663) analyzed consists of a genetic variation of a repetitive sequence of 86 bp, consisting of a VNTR

polymorphism. This sequence can be repeated two, three, four, five or six consecutive times, providing five possible alleles: 1, 2, 3, 4 and 5. Thus, genotyping using the basic methodology of molecular biology techniques has become the most viable technique. The DNA obtained through the DNA extraction technique was amplified using oligonucleotide sequences specific for this polymorphism (primers). The primers used were: 5'-CTC AGC AAC ACT CCT AT-3' and 5'-TCC TGG TCT GCA GGT AA-3'. For the *IL-RN* gene polymorphism, the primers were aliquoted at a concentration of 10 µM and the Master Mix was prepared. The Master Mix solution included NZYtaq II 2x Green Master Mix—NZYtech, Lisbon, Portugal (consisting of the Taq DNA Polymerase enzyme, dNTP's, Buffer and MgCl₂), forward and reverse primers and RNase/Dnase-free water. The samples obtained with a final volume of 25 µL were inserted into the MJ MINITM Personal Thermal Cycler (Hercules, CA, USA) to carry out the amplification reactions. The initial step involved subjecting the samples to a heating cycle at 96 °C for 1 min. Subsequently, 35 cycles were performed, consisting of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 70 °C for 2 min and final extension at 70 °C for 5 min. The technique of electrophoresis in 3% agarose gel was used to reveal the genotypes of the polymorphic regions associated with the *IL-1RN* gene (intron 2, VNTR).

For the analysis of the *IL-1A* -889 (rs1800587) and *IL-1B* +3954 (rs1143634) polymorphisms, the real-time polymerase chain reaction (PCR) technique was performed using the commercial kit AppliedBiosystems™ (Thermo Fisher SCIENTIFIC, Waltham, MA, USA) and using the TaqMan™ SNP Genotyping Assay solution (Thermo Fisher SCIENTIFIC, Waltham, MA, USA), which contained the forward and reverse primers and two probes with different fluorescent reporters. This solution is specifically defined for each polymorphism and is commercially available. A Master Mix solution was then prepared containing the TaqMan™ Genotyping Master Mix solution (Thermo Fisher SCIENTIFIC, Waltham, MA, USA) and the TaqMan™ SNP Genotyping Assay. The reference values were 10 µL for TaqMan™ Genotyping Master Mix and 1 µL for Taq-Man™ SNP Genotyping Assay, which were multiplied by the 51 samples. Subsequently, 11 µL of the previously prepared solution and 9 µL of each DNA sample were added to Eppendorf minitubes. The samples were

inserted into the Corbett Research RG 3000 thermocycler (Venlo, The Netherlands) to initiate the amplification reactions in real-time PCR. The results obtained by the TaqMan assay were confirmed by DNA sequencing. Patients with each genotype type were selected for analysis. The results obtained by Sanger sequencing and the TaqMan assay were identical.

Biochemical analysis

For biochemical analysis of the inflammatory mediators IL-1 β , TNF- α and PGE₂, PICF was collected using paper strips (Periopaper®, New York, NY, USA). In cases of peri-implantitis, the paper strips were inserted into the pocket at the most affected site, while in cases of peri-implant health, the collection was performed from the mesio-buccal location. Two collections were made, with an interval of 5 min between them. The analysis was performed one week after the clinical examination. Prior to collection, plaque deposits were removed with a curette, and the collection site was dried and isolated to prevent contamination of the sample with saliva. The procedure involved inserting paper strips into the peri-implant sulcus or pocket until resistance was felt, followed by a 30-s waiting period before removal. Samples that were visually contaminated with blood or saliva were excluded from the analysis. The collected paper strips were then placed in a Periotron® 8000 device (Periotron Ide-Interstate, New York, NY, USA) to measure the volume of PICF collected. Subsequently, the paper strips were carefully transferred to Eppendorf tubes with a filter and stored at -80 °C until they were sent to the Complutense University of Madrid. At the university, the concentrations of the inflammatory mediators IL-1 β and TNF- α were analyzed by multiplexed fluorescent sphere immunoassays using the Luminex® 100/200™ system (Austin, TX, USA) and PGE₂ by enzyme-linked immunosorbent assay (ELISA). For the analysis of IL-1 β and TNF- α , a high-sensitivity kit was used: the MILLIPLEX MAP Human High Sensitivity T-Cell Panel - Immunology Multiplex Assay from Merck Millipore® (Darmstadt, Germany). For PGE₂ analysis, a PGE₂ ELISA kit from Alpha Diagnostic International® (San Antonio, TX, USA) was used. In the laboratory, samples were processed and analyzed according to the

standardized protocols provided by the manufacturers of each of the commercially available kits.

Statistical analysis

The data were submitted for descriptive and inferential analysis. The association between categorical variables was checked using appropriate inferential statistical methodologies (bivariable analysis/association tests, chi-square (χ^2), and Fisher's exact test). Due to the non-normality characteristics of the considered continuous variables, the Mann–Whitney test was used for inferential comparison between groups. For *IL-1A* -889 and *IL-1B* +3954 polymorphisms, the odds ratio (OR) and 95% confidence interval (95%CI) were calculated to assess its association strength with risk of peri-implant disease in two comparison models: dominant (homozygous + heterozygous variant vs. homozygous wildtype) and recessive (homozygous variant vs. homozygous + heterozygous wildtype). The correlation between inflammatory marker concentrations was assessed using Spearman's rank correlation coefficient. A 5% significance level ($p \leq 0.05$) was established in the inferential analyses. Data analyses were performed using IBM SPSS Statistics v.28.

6.3 Results

Demographic and clinical characteristics of the study group

A total of 51 healthy individuals, consisting of 26 men and 25 women, with ages ranging from 38 to 90 years, were included in the study. The mean age in the peri-implant health group was 58.74 years and in the peri-implantitis group 59.17 years. The median values for age are 60 and 57.5 for the health and peri-implantitis groups, respectively. All the participants shared the same ethnic background (Caucasian) and were born in Portugal. Each participant had at least one dental implant in function. Cigarette smoking, history of periodontitis and full-mouth plaque score were among the factors investigated for their prognostic significance. The details of the sample's characteristics are shown in Table 6.2. Although there was a higher proportion of smokers in the disease group com-

pared to patients with peri-implant health (54.2% vs. 33.3%, $p = 0.134$), the difference was not statistically significant. The peri-implantitis group demonstrated a higher prevalence of a positive history of periodontitis compared to patients with peri-implant health, albeit the differences were not significant (66.7% vs. 59.3%, $p = 0.585$). There was no statistical difference between the groups in terms of the full-mouth plaque score (FMPS). The implants were in function in the health group for 8.6 years and in the peri-implantitis group for 8.7 years. The mean time in function of the implants was not significantly different between the groups.

Table 6.2 - Demographic and clinical data of patients with dental implants.

	Peri-Implant Health (n = 27)	Peri- Implantitis (n = 24)	p
Age, Mean (SD) (years)	58.74 (8.72)	59.17 (12.40)	0.865
Median (years)	60	57.5	
Range (years)	41–74	38–90	
Sex, n (%)			0.121
Male	11 (40.7)	15 (62.5)	
Female	16 (59.3)	9 (37.5)	
Smoking, n (%)			0.134
No	18 (66.7)	11 (45.8)	
Yes	9 (33.3)	13 (54.2)	
History of Periodontitis, n (%)			0.585
No	11 (40.7)	8 (33.3)	
Yes	16 (59.3)	16 (66.7)	
FMPS (%)			0.234
≤15%	11 (40.7)	6 (25)	
>15%	16 (59.3)	18 (75.0)	
Implant function time			0.960
Mean (SD) (years)	8.6 (4.8)	8.7 (5.8)	
Median (years)	9	6.5	
Range (years)	1–20	1–20	

Abbreviations: Standard deviation (SD), full-mouth plaque score (FMPS).

Genotype Frequencies and Genetic Models

The genotype frequencies obtained for the genes *IL-1A* -889, *IL-1B* +3954, and *IL1-RN* (VNTR), in the peri-implant health group, in the peri-implantitis group and for the total sample are presented in Table 6.3.

Table 6.3 - Genotype frequencies of *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* (VNTR) polymorphisms, in the peri-implant health group, in the peri-implantitis group and in the total sample.

Genotype, n (%)	Peri-Implant Health (n = 27)	Peri-Implantitis (n = 24)	Total (n = 51)	p
<i>IL-1A</i> -889				
CC	16 (59.3)	13 (54.2)	29 (56.9)	0.714
CT/TT	11 (40.7)	11 (45.8)	22 (43.1)	
<i>IL-1A</i> -889				
CC	16 (59.3)	13 (54.2)	29 (56.9)	—
CT	10 (37.0)	6 (25.0)	16 (31.4)	
TT	1 (3.7)	5 (20.8)	6 (11.7)	
<i>IL-1B</i> +3954				
CC	18 (66.7)	17 (70.8)	35 (68.6)	0.749
CT/TT	9 (33.3)	7 (29.2)	16 (31.4)	
<i>IL-1B</i> +3954				
CC	18 (66.7)	17 (70.8)	35 (68.6)	—
CT	9 (33.3)	6 (25.0)	15 (29.4)	
TT	0 (0.0)	1 (4.2)	1 (2.0)	
<i>IL-1RN</i> (VNTR)				
1/1	23 (85.2)	21 (87.5)	44 (86.3)	0.811
1/5, 2/2	4 (14.8)	3 (12.5)	7 (13.7)	
<i>IL-1RN</i> (VNTR)				
1/1	23 (85.2)	21 (87.5)	44 (86.3)	—
1/5	1 (3.7)	0 (0.0)	1 (1.9)	
2/2	3 (11.1)	3 (12.5)	6 (11.8)	

Abbreviations: variable number of tandem repeats (VNTR).

With regard to the *IL-1A* -889 polymorphism, 59.3% of the subjects had the CC genotype, while 40.7% showed the CT or TT genotypes. In the peri-implantitis group, 54.2% had the CC genotype and 45.8% had the CT or TT genotypes. No statistically significant differences were found between the health and disease groups ($p = 0.714$).

With respect to the *IL-1B* +3954 polymorphism, in the healthy group, the genotype frequencies were 66.7% for the CC genotype and 33.3% for the CT and TT genotypes. For the group with diagnosed peri-implantitis, frequencies of 70.8% were obtained for the CC genotype and 29.2% for the CT and TT genotypes. No statistically significant differences were found in the proportions of the *IL-1B* gene polymorphism between the health and disease groups ($p = 0.749$).

In relation to the *IL-1RN* polymorphism (VNTR), in the healthy group, the genotype frequencies were 85.2% for the 1/1 genotype and 14.8% for the altered genotypes (1/5 and 2/2). For the group with diagnosed peri-implantitis, the frequencies were 87.5% for the 1/1 genotype and 12.5% for less common genotypes. The distribution of the different genotypes for this polymorphism showed no differences between the groups in the proportions found ($p = 0.811$).

When the genotypes (homozygous/heterozygous variant) were analyzed separately for the *IL-1A* -889 and *IL-1B* +3954 polymorphisms it was not possible to perform the inferential analysis. When the genotypes were analyzed separately for the *IL-1RN* polymorphism (1/1, 1/5 and 2/2), inferential analysis was also not possible.

The odds ratio and corresponding 95% confidence interval are shown for the *IL-1A* -889 and *IL-1B* +3954 polymorphisms in the dominant and recessive genetic models in Tables 6.4 and 6.5, respectively.

Table 6.4 - Analysis of the association between *IL-1A* -889 polymorphism and the risk of peri-implantitis in dominant and recessive genetic models.

Genetic Model <i>IL-1A</i> -889	Peri-Implant Health (n = 27)		Peri-Implantitis (n = 24)		OR (95% CI) *	p
	Events	Total	Events	Total		
Dominant TT + CT vs. CC	11	27	11	24	0.81 (0.27–2.47)	0.934
Recessive TT vs. CC + CT	1	27	5	24	0.15 (0.02–1.36)	0.148

Abbreviations: * odds ratio (OR) and correspondent 95% confidence intervals (95% CI), Mantel-Haenszel.

Table 6.5 - Analysis of the association between *IL-1B* +3954 polymorphism and the risk of peri-implantitis in dominant and recessive genetic models.

Genetic Model <i>IL-1B</i> +3954	Peri-Implant Health (n = 27)		Peri-Implantitis (n = 24)		OR (95% CI) *	p
Dominant TT + CT vs. CC	Events 9	Total 27	Events 7	Total 24	1.21 (0.37–3.99)	0.986
Recessive TT vs. CC + CT	0	27	1	24	—	—

Abbreviations: * odds ratio (OR) and correspondent 95% confidence intervals (95% CI), Mantel-Haenszel.

As shown in Tables 6.4 and 6.5, the *IL-1A* -889 and *IL-1B* +3954 polymorphisms were not associated with the risk of peri-implantitis in the dominant and recessive models.

Influence of *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* (VNTR) polymorphisms on PICF volume and selected biochemical markers of the peri-implant immune response

The analysis of the influence of *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* (VNTR) polymorphisms on the mean concentrations of IL-1 β , TNF- α and PGE2 (pg/ μ L) when the healthy and diseased groups were grouped, is described in Table 6.6.

Table 6.6 - Immunological findings of the total sample in relation to *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* (VNTR) polymorphisms, calculated as relative concentrations (pg/μL of peri-implant crevicular fluid).

Genotype	Number of Individuals	IL-1β (pg/μL) Mean (SD)	TNF-α (pg/μL) Mean (SD)	PGE2 (pg/μL) Mean (SD)
<i>IL-1A</i> -889				
CC	29	19.3 (21.9)	1.2 (1.1)	258.5 (332.3)
CT/TT	22	29.0 (59.0)	1.6 (3.5)	177.7 (221.9)
<i>p</i>		0.518	0.313	0.231
<i>IL-1B</i> +3954				
CC	35	26.1 (48.7)	1.2 (1.3)	262.0 (339.6)
CT/TT	16	18.0 (20.4)	1.6 (3.9)	139.7 (91.3)
<i>p</i>		0.776	0.490	0.543
<i>IL-1RN</i> (VNTR)				
1/1	44	23.4 (44.2)	0.9 (1.2)	193.8 (265.5)
1/5, 2/2	7	24.1 (24.7)	3.9 (5.5)	411.0 (384.8)
<i>p</i>		0.339	0.002	0.049
IL-1 positive composite Genotype				
Yes	13	18.5 (22.1)	1.8 (4.3)	145.0 (96.9)
No	38	25.2 (46.9)	1.2 (1.3)	250.5 (328.2)
<i>p</i>		0.713	0.364	0.634

Abbreviations: interleukin-1 beta (IL-1β), prostaglandin E2 (PGE2), standard deviation (SD), tumor necrosis factor-alpha (TNFα), variable number of tandem repeats (VNTR), statistically significant differences ($p < 0.05$) are denoted in bold.

For the *IL-1A* -889 polymorphism, it was observed that patients with the altered genotype exhibited higher mean concentrations of IL-1β ($p = 0.518$) and TNF-α ($p = 0.313$) and a lower mean concentration of PGE2 ($p = 0.231$) than patients without the presence of this polymorphism. However, these changes were not statistically significant.

For the *IL-1B* +3954 polymorphism, it was observed that patients with the altered genotype had a higher mean concentration of TNF-α ($p = 0.490$) and lower mean concentrations of IL-1β ($p = 0.776$) and PGE2 ($p = 0.543$) than patients without genotype changes. However, these changes were not statistically significant.

For the *IL-1RN* gene, it was noted that patients who had the polymorphism had higher mean concentrations of IL-1 β ($p = 0.339$), TNF- α ($p = 0.002$), and PGE2 ($p = 0.049$) than patients who did not have this polymorphism. These differences were statistically significant ($p < 0.05$) for TNF- α and PGE2 levels.

In patients with the IL-1 positive composite genotype, there was an increase in the mean TNF- α concentration ($p = 0.364$) and a decrease in the mean IL-1 β and PGE2 concentrations ($p = 0.713$ and $p = 0.634$, respectively) compared to patients with the IL-1 negative composite genotype. However, these differences were not statistically significant ($p > 0.05$).

We also evaluated whether the influence of the genetic component at the biochemical level was more pronounced in individuals with peri-implantitis. For this purpose, only individuals with the IL-1 positive composite genotype or polymorphism in the *IL-1RN* (VNTR) were evaluated, comparing patients with peri-implant health and patients with peri-implantitis (Table 6.7).

Table 6.7 - Immunological findings of patients with peri-implant health and patients with peri-implantitis in relation to the presence of the IL-1 positive composite genotype and *IL-1RN* (VNTR) polymorphism, calculated as relative concentrations (pg/ μ L of peri-implant crevicular fluid) and relative to peri-implant crevicular fluid volume.

Patients	PICF Mean Volume (μ L) (SD)	IL-1 β (pg/ μ L) Mean (SD)	TNF- α (pg/ μ L) Mean (SD)	PGE2 (pg/ μ L) Mean (SD)
IL-1 positive composite genotype patients				
Healthy patients ($n = 8$)	0.51 (0.28)	15.31 (18.31)	2.59 (5.53)	119.06 (65.74)
Patients with peri-implantitis ($n = 5$)	0.66 (0.44)	23.65 (28.81)	0.60 (0.50)	186.54 (130.81)
p	0.042	0.612	0.499	0.043
Patients with <i>IL-1RN</i> (VNTR) polymorphism				
Healthy patients ($n = 4$)	0.41 (0.56)	31.28 (29.31)	5.56 (7.20)	365.81 (346.89)
Patients with peri-implantitis ($n = 3$)	0.48 (0.29)	14.60 (17.30)	1.68 (0.15)	471.32 (504.10)
p	0.660	0.464	0.558	0.464

Abbreviations: peri-implant crevicular fluid (PICF), statistically significant differences ($p < 0.05$) are denoted in bold.

It was observed that among patients with IL-1 positive composite genotype, patients with peri-implantitis had a higher mean volume of PICF (healthy 0.51 μ L vs. peri-implantitis 0.66 μ L, $p = 0.042$) and higher mean concentrations of IL-1 β (healthy 15.31 pg/ μ L vs. peri-implantitis 23.65 pg/ μ L, $p = 0.612$) and PGE2 (healthy 119.06 pg/ μ L vs. peri-implantitis 186.54 pg/ μ L, $p = 0.043$) than healthy patients. These differences were statistically significant for PICF volume and mean PGE2 concentration.

Regarding patients with the VNTR polymorphism, the mean volume of PICF and the mean concentration of PGE2 were higher in the disease group than in the healthy group. However, the differences in PICF volume and PGE2 concentration were not statistically significant ($p = 0.660$ and $p = 0.464$, respectively).

Correlation between Concentrations of Biochemical Markers (IL-1 β , TNF- α , and PGE2)

IL-1 β levels were positively and significantly correlated with TNF- α ($\rho = 0.480$, $p < 0.001$) and PGE2 levels ($\rho = 0.382$, $p = 0.006$). TNF- α levels were also positively related to PGE2 levels ($\rho = 0.528$, $p < 0.001$) (Table 6.8). All three mediator concentrations were moderately positively correlated.

Table 6.8 - Correlations between concentrations of biochemical markers (IL-1 β , TNF- α , and PGE2) (pg/ μ L).

	Correlation (Rho, Spearman)	
	Rho	p
IL-1 β vs. TNF- α	0.480	<0.001
IL-1 β vs. PGE2	0.382	0.006
TNF- α vs. PGE2	0.528	<0.001

6.4 Discussion

The primary etiological factor for the onset and progression of peri-implantitis is the accumulation of a peri-implant plaque biofilm [7,8]. The aim of treatment is to stop the inflammatory processes in the peri-implant tissues and to control local and systemic risk factors that may sustain it [8]. Disruption of the locally accumulating microbial biofilms is a key target [8]. The aim of prevention is to obtain and preserve peri-implant tissues free of clinical inflammation. This is achieved by enabling appropriate self-performed and professionally delivered oral hygiene measures that need to be customized according to the design of implant-supported restorations [8].

Our research group carried out a pilot study in which we observed that although there were no statistically significant differences in the *IL-1A* -889 and *IL-1B* +3954 polymorphisms between the healthy and peri-implantitis groups, there was a trend towards a higher prevalence of genetic polymorphisms in the disease group [55]. The *IL-1RN* (VNTR) polymorphism was not assessed in that study. In the present study, there was no association between the various polymorphisms analyzed (*IL-A* -889, *IL-1B* +3954, and *IL-1RN* (VNTR)) and peri-implantitis. However, the possible association between these genetic polymorphisms and peri-implantitis may be based on alterations in the individual's immune-inflammatory response. Therefore, the functional impact of these polymorphisms is the appropriate way to study their role in peri-implant disease. Studies that have attempted to associate these polymorphisms only with the occurrence of peri-implantitis are dependent on the multifactorial etiology of the disease [49]. For this reason, especially in cross-sectional studies, it can be difficult to control all the risk factors/indicators and understand the real effect of a particular factor (in this case, genetics) on the development of the disease.

There are studies that try to relate the existence of certain genetic polymorphisms and the occurrence of peri-implantitis, with contradictory results, and there are also studies that try to assess whether patients with peri-implantitis have higher concentrations of certain inflammatory mediators in the PICF than patients with peri-implant health. However, very few studies have identified genetic determinants, such as polymorphisms, that might be responsible for the

overproduction of biochemical mediators (hyperactive phenotype) and, therefore, could be important in determining the risk profile of a patient for peri-implantitis. To the best of our knowledge, only one study has related the presence of polymorphisms in the *IL-1A* and *IL-1B* genes with changes in the levels of the inflammatory mediators IL-1 β , plasminogen activator inhibitor type 2 (PAI-2) and PGE2 [35]. In the aforementioned study, when the healthy and peri-implantitis patient groups were combined, subjects who tested negative for IL-1 exhibited higher levels of IL-1 β and PGE2 in the PICF than those who tested positive for IL-1 ($p = 0.02$ for IL-1 β and $p = 0.04$ for PGE2). In addition, there was a trend towards increased PAI-2 concentrations in IL-1-negative individuals, although the difference was not statistically significant ($p = 0.052$). In our study, we observed that patients with the IL-1 negative composite genotype also had higher concentrations of IL-1 β and PGE2, although the difference was not statistically significant ($p = 0.713$ and $p = 0.634$, respectively). For the *IL-1RN* polymorphism (VNTR), it was found that individuals with this polymorphism had higher concentrations of TNF- α , IL-1 β and PGE2 in PICF than individuals with the most common genotype, with statistical significance for TNF- α and PGE2 ($p = 0.002$ and $p = 0.049$, respectively). This may indicate that individuals with the *IL-1RN* polymorphism (VNTR) may have a more vigorous immune-inflammatory response and that although they have not yet developed the disease, they are more likely to have peri-implantitis. This polymorphism was not evaluated in the study by Lachamann et al. [35].

In our study, we also observed that among the IL-1 positive composite genotype individuals, those who had peri-implantitis showed a greater volume of PICF ($p = 0.042$) and a greater concentration of PGE2 ($p = 0.043$). However, in this analysis, the groups had few individuals, and the data must be interpreted with caution. Based on the available evidence, it can be concluded that although some studies do not find a relationship between IL-1 β , TNF- α and PGE2 levels in PICF and peri-implantitis, most of them, as evidenced in systematic reviews and clinical studies, demonstrate a positive association between elevated concentrations of these biomarkers in the PICF of diseased versus healthy sites [20,22,23,32,33,56,57]. The divergent results observed in the aforementioned studies can be attributed to variations in the study design, materials and methods

employed, including differences in sample collection, processing techniques and assay sensitivity.

Based on our study, there was a correlation between the concentrations of the three mediators evaluated (IL-1 β , TNF- α and PGE2), a fact that was also observed in the study by Lachman et al. for IL-1 β , PGE2 and PAI-2 [35]. This fact makes sense, as these inflammatory mediators orchestrate the same biological process. Despite some similarities between studies, differences in some results may be due to the collection methods and biochemical tests used, which differed between studies. The definition of peri-implantitis also varied between studies.

Some limitations of our study should be addressed. The sample size of 51 patients, although a total of 306 analyses were performed, 153 at the biochemical level (IL-1 β , TNF- α and PGE2) and 153 at the genetic level (*IL-1A*, *IL-1B* and *IL-1RN*), is one of the limitations of the study. However, the only published study that evaluated the relationship between genetic and biochemical aspects in cases of peri-implantitis and peri-implant health included a sample of 29 patients [35]. On the other hand, studies investigating the effect of genetic alterations on the periodontal crevicular immune response have smaller sample sizes [58,59]. Longitudinal studies should be preferred because, although the genetic profile does not vary, biomarkers may not be consistently present during a single instance of PICF collection due to various systemic or local factors [57]. Furthermore, since peri-implantitis has a multifactorial etiology, a study with a longitudinal design would allow better control of the various factors that can contribute to the development of the disease. One of these factors is the potential lack of regular supportive peri-implant care following implant therapy. Follow-up from the planning stage of implant placement would allow better control of the various local risk factors/indicators related not only to the characteristics of the implant, but also to the rehabilitation (the material of the abutment or prosthesis, shape of the abutment), and the surgical site (the amount of keratinized gingiva, the amount of tissue above the neck of the implant). The implants were not all placed at our university, which may make it difficult to analyze various factors that may interfere with the development of peri-implant pathology. Another limitation was the assessment of bone loss using a retroalveolar radiograph (paralleling technique). Due to the 2D nature of this imaging technique, there is an una-

avoidable overlap of anatomical structures and a lack of 3D information. In addition, it is important to standardize the sampling process for PICF because of the atypical morphology of implant prostheses. The use of different paper strips, cones, membranes or collection devices can be technique sensitive and potentially lead to inaccurate or misleading results.

The etiology and pathogenesis of periimplantitis are not currently well understood, and more research is still needed to clarify them. Microbial plaque accumulation around dental implants can trigger inflammatory host response in peri-implant tissues but its progression and severity can vary between individuals. The number of studies that evaluate the role of genetic factors, such as the studied polymorphisms, in the development of peri-implantitis, is quite limited, and some of them do not refer to variables that may impact/influence the results obtained. It would also be important to carry out more studies that try to relate genetic alterations to the consequences at the biochemical level in patients with dental implants. Since peri-implantitis is usually latent in the early stages, the analysis of cytokine levels in the PICF of susceptible individuals can be a valuable analytical tool. Understanding the active components and mechanisms that contribute to this destructive process is crucial in this context. This knowledge has the potential to guide the development of new diagnostic strategies and to identify potential disease markers for peri-implant conditions.

According to the observed results, it is reasonable to suppose that increased levels of TNF- α and PGE2 in the peri-implant tissues would result in more aggressive destruction of both bone and connective tissue in response to the bacterial challenge.

6.5 Conclusions

Individuals with the *IL-1RN* gene polymorphism, regardless of peri-implant health status, presented higher levels of TNF- α and PGE2 in PICF compared to patients without the polymorphism, which is believed to place them at a higher risk for peri-implant inflammatory complications.

There are individuals who have not yet developed the disease but may be at greater risk for its development. Since peri-implantitis is a multifactorial disease, it is understood that a single factor may not be sufficient for its development.

If the association between this polymorphism and the incidence of peri-implantitis is confirmed, carrying out a genetic test will allow the identification of patients susceptible to the disease and the application of rigorous measures to control their risk factors, especially those related to the reduction in inflammation. When there is a history of periodontitis, a similar approach should be implemented.

However, it should be emphasized that in all patients with dental implants, even if they have no risk factors/indicators for the disease, strict plaque control measures are essential for the prevention of peri-implantitis.

6.6 References

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7. EVALUATION OF LABORATORY RESULTS OF GENETIC ANALYSIS, EVALUATION OF HARDY-WEINBERG EQUILIBRIUM AND EVALUATION OF THE PRESENCE OF POLYMORPHISMS IN SMOKERS AND NON-SMOKERS SEPARATELY

In the first 20 patients, the analysis of genetic polymorphisms of the *IL-1A* and *IL-1 B* genes was performed using the PCR-RFLP analysis. In the pilot study, basic molecular biology techniques were used, and the results were later confirmed through the real time PCR (TaqMan assay). In the remaining 82 patients, the polymorphisms analysis was performed using Real-time PCR analysis.

The polymorphism of the *IL-1RN* gene consists of a VNTR polymorphism of 86 bp. Thus, its genotyping using PCR analysis becomes the most viable technique.

The results obtained by the TaqMan assay were confirmed by DNA sequencing. Patients with each genotype type were selected for analysis. The results obtained after the laboratory genetic analysis will be described in more detail below.

7.1 Analysis of laboratory results of *IL-1A* -889 and *IL-1B* +3954 polymorphisms

7.1.1 PCR-RFLP analysis of *IL-1A* -889 and *IL-1B* +3954 polymorphisms

In the figures 7.1 and 7.2 we can observe the electrophoretic profile (MetaPhor™ Agarose gel 5%, Lonza Bioscience) of the *IL-1A* -889 gene polymorphism. This SNP is characterized by the replacement of a cytosine (C) base by a thymine (T), originating two possible alleles: C allele and the T allele. Two bands were observed, one of 83bp corresponding to allele 1 (83bp+16bp) and one of 99bp corresponding to allele 2. Allele 2 corresponds to the allele with nucleotide sequence changed while allele 1 corresponds to the wild type allele. The molecular weight markers NZYDNA ladder VI, pUC19 DNA/MspI (HpaII) and

GeneRuler™ Low Range DNA Ladder were used. Each patient was assigned a number, which is represented in the images of the agarose gel electrophoresis.

According to the figures 7.1 and 7.2 we can conclude that individuals 1, 6, 11, 16, 20, 21, 24, 26 present the *IL-1A* -889 polymorphism. They are heterozygous, presenting a genotype 1/2, because the corresponding samples present a fragment of 99pb and another fragment of 83pb. According to the figure 7.2, patient 2 also has genotype 1/2.

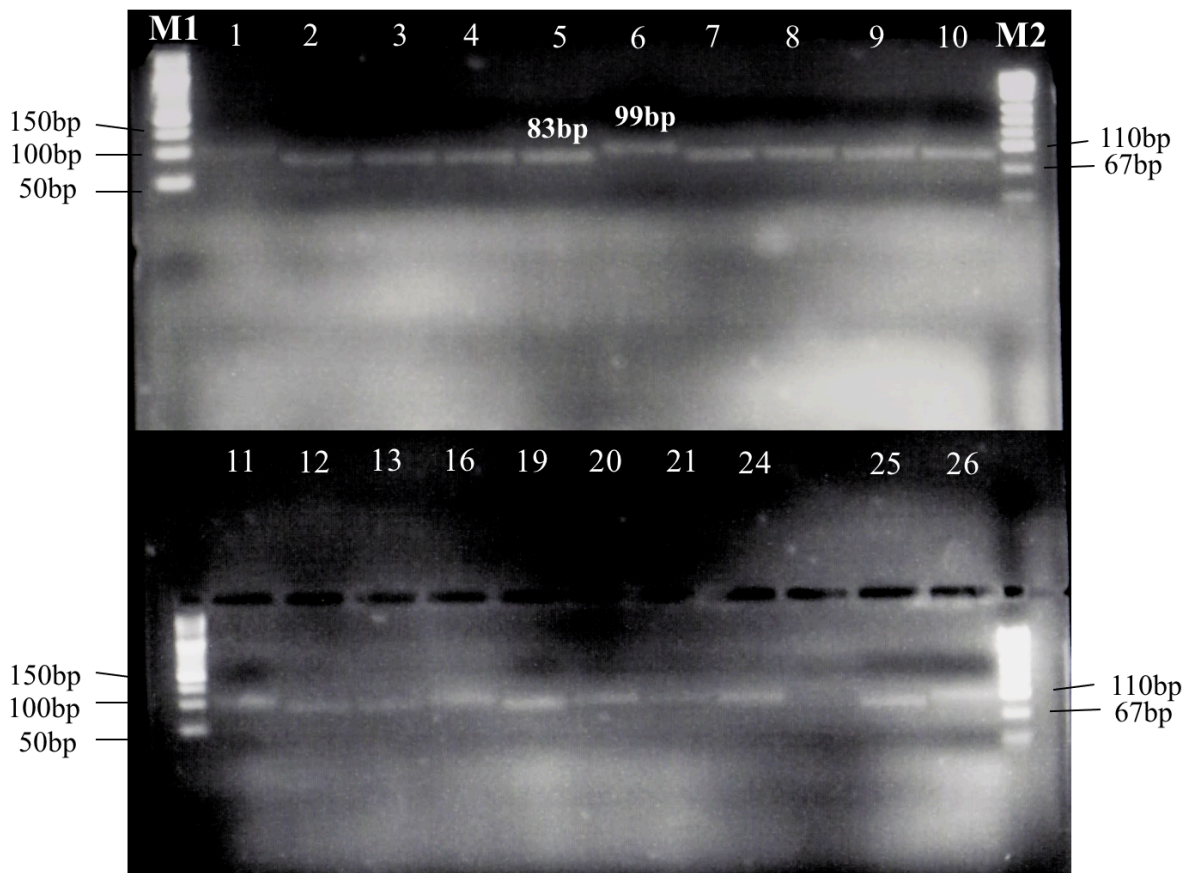


Figure 7.1 - Agarose gel electrophoresis of restriction profile of *IL-1A* -889 gene. M1- Molecular weight marker, NZYDNA ladder VI. M2- Molecular Weight Marker, pUC19/Mspl (HpaII).

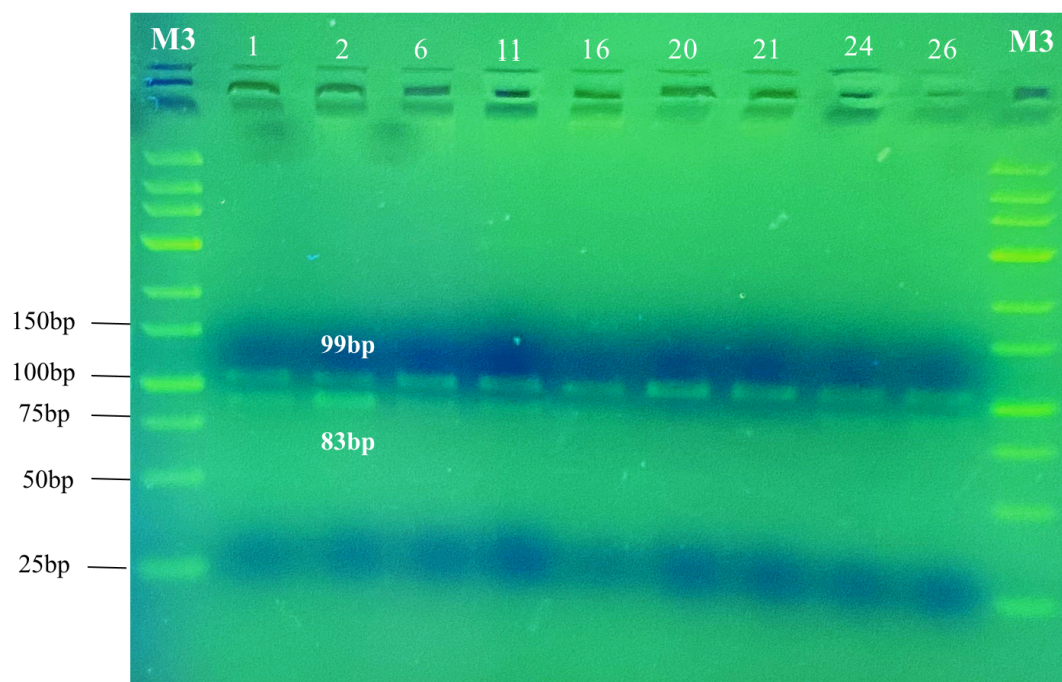


Figure 7.2 - Agarose gel electrophoresis of restriction profile of *IL-1A* -899 gene. M3- Molecular weight marker, GeneRuler™ Low Range DNA Ladder. Confirmation of some samples.

In figures 7.3 and 7.4 we can observe the electrophoretic profile (MetaPhor™ Agarose gel 5%, Lonza Bioscience) of the *IL-1B* +3954 gene polymorphism. This SNP is characterized by the replacement of a cytosine (C) base by a thymine (T), originating two possible alleles: C allele and the T allele. Three bands were observed, one of 97bp and 85bp corresponding to allele 1 (97bp+85bp) and one of 182bp corresponding to allele 2. Allele 2 corresponds to the allele with nucleotide sequence changed while allele 1 corresponds to the wild type allele. The molecular weight marker GeneRuler™ Low Range DNA Ladder was used. Each patient was assigned a number, which is represented in the images of agarose gel electrophoresis.

According to the figures 7.3 and 7.4 we can conclude that individuals 6, 13, 16, 19, 20, 21, 24 and 26 present the polymorphism and are all heterozygous (genotype 1/2), except for 24 which is homozygous for the altered allele (genotype 2/2).

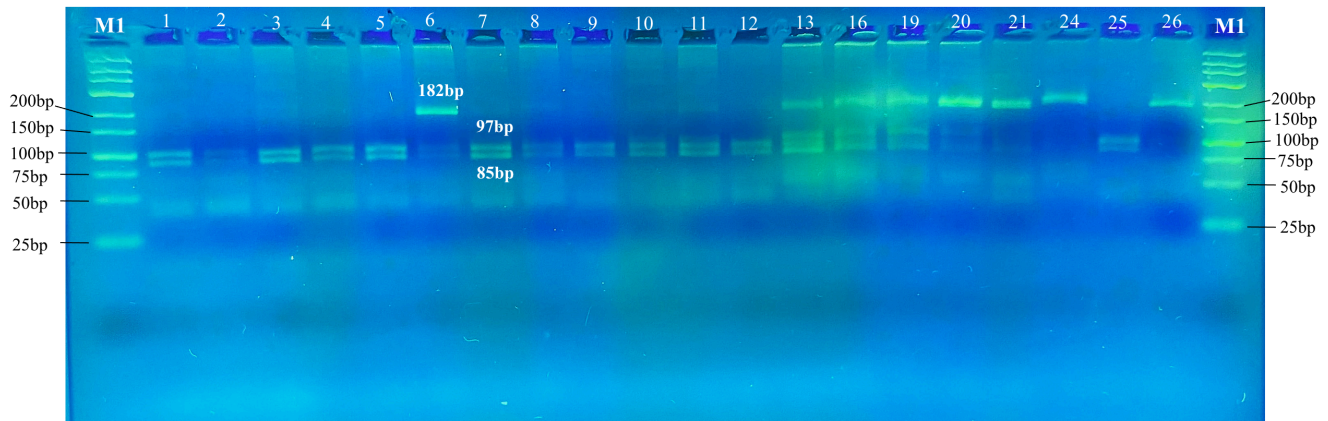


Figure 7.3 - Agarose gel electrophoresis of restriction profile of *IL-1B* +3954 gene. M1- Molecular weight marker, GeneRuler™ Low Range DNA Ladder.

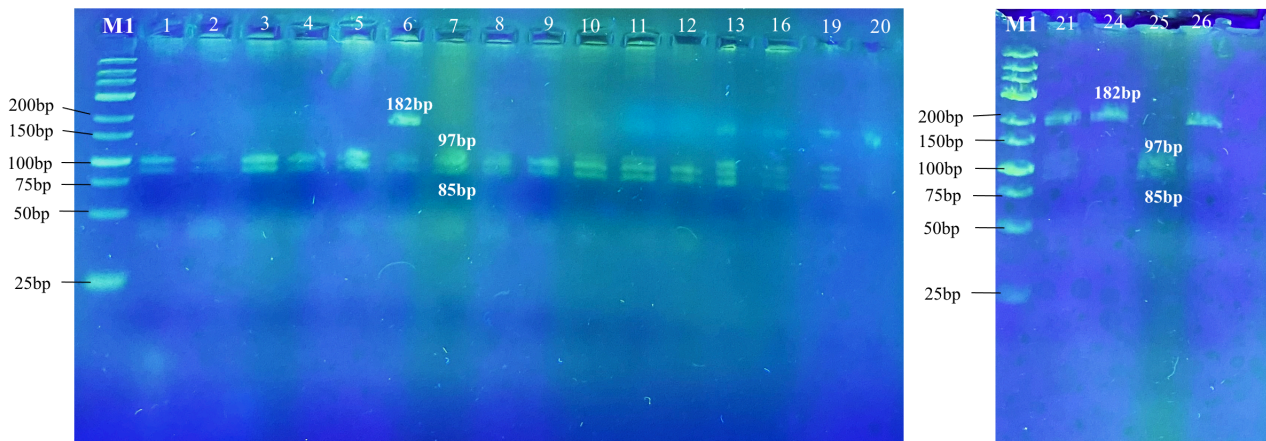


Figure 7.4 - Agarose gel electrophoresis of restriction profile of *IL-1B* +3954 gene. M1- Molecular weight marker, GeneRuler™ Low Range DNA Ladder. Confirmation of the results.

7.1.2 RT-PCR analysis (TaqMan assay) of *IL-1A* -889 and *IL-1B* +3954 polymorphisms

Polymorphisms of the *IL-1A* -889 and *IL-1B* +3954 genes were analyzed in 82 patients using Applied Biosystems™ TaqMan real-time PCR assay. The specific probes of the TaqMan assay used for the detection of these SNP's were labeled with fluorophores (FAM, JOE or VIC), with specific emission wavelengths, allowing the detection of the C and T alleles and, thus, conclude on which the genotype of each sample. In addition to the oligonucleotide probes added, the forward and reverse primers also act in the recognition and delimitation of the SNP's to be analyzed. In this sense, each sample corresponds to an amplification

curve which, depending on the configuration shown (circles or plain) and the number of curves, determines the genotype of that sample. The FAM probe is associated with allele 2 (allele with nucleotide sequence changed, allele T), showing a smooth curve, and the VIC or JOE probes associated with allele 1 (wild allele, allele C), representing a curve with circles.

For the *IL-1A* -889 polymorphism 53 patients had the genotype 1/1, being homozygous for allele 1, 23 patients showed a genotype 1/2, being heterozygous and 6 patients had the genotype 2/2, being homozygous for the altered allele. Figure 7.5 shows the amplification curves of 3 patients, one corresponding to a homozygous patient for the wild allele, another corresponding to a heterozygous patient and another to a homozygous for the mutated allele.

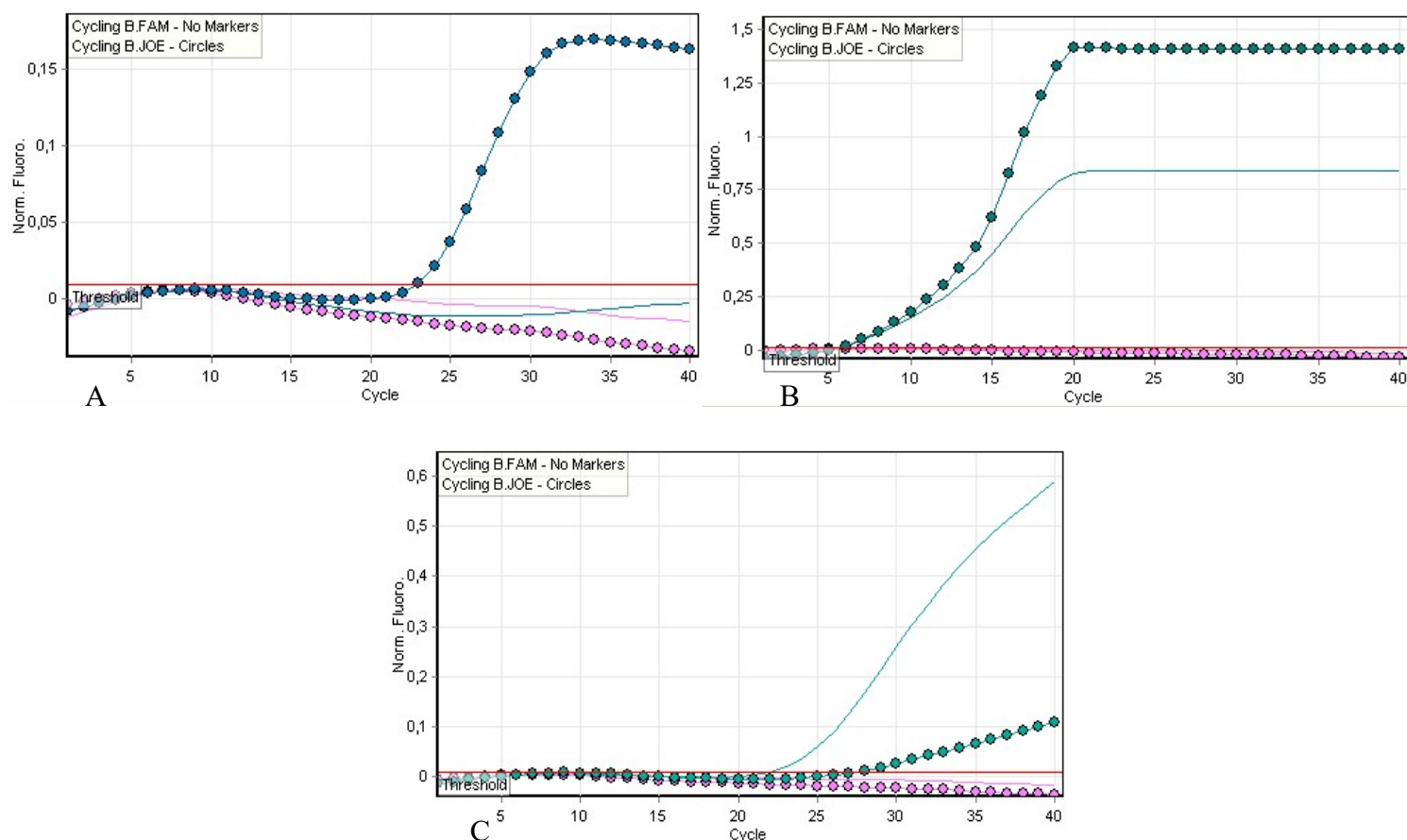


Figure 7.5 - A) Exemplary amplification curve of a patient carrying the homozygous genotype type 1/1, relative to the *IL-1A* gene (position -889). Blue curve: detected by the JOE probe (allele 1); Pink curve: negative control (white); Red line: Threshold. B) Exemplary amplification curve of a patient carrying the type 1/2 heterozygous genotype, relative to the *IL-1A* gene (position -889). Green curve with circles: detected by the JOE probe (allele 1); Flat green curve: detected by the FAM probe (allele 2); Pink curve: negative control (white); Red line: Threshold. C) Exemplary amplification curve of a patient carrying the type 2/2 homozygous genotype, relative to the *IL-1A* gene (position -889). Flat green curve: detected by the FAM probe (allele 2); Pink curve: negative control (white); Red line: Threshold.

For the *IL-1B* +3954 polymorphism 62 patients had the genotype 1/1, being homozygous for allele 1, 19 patients showed a genotype 1/2, being heterozygous and 1 patient had the genotype 2/2, being homozygous for the altered allele. Figure 7.6 shows the amplification curves of 3 patients, one corresponding to a homozygous patient for the wild allele, another corresponding to a heterozygous patient and another to a homozygous for the altered allele.

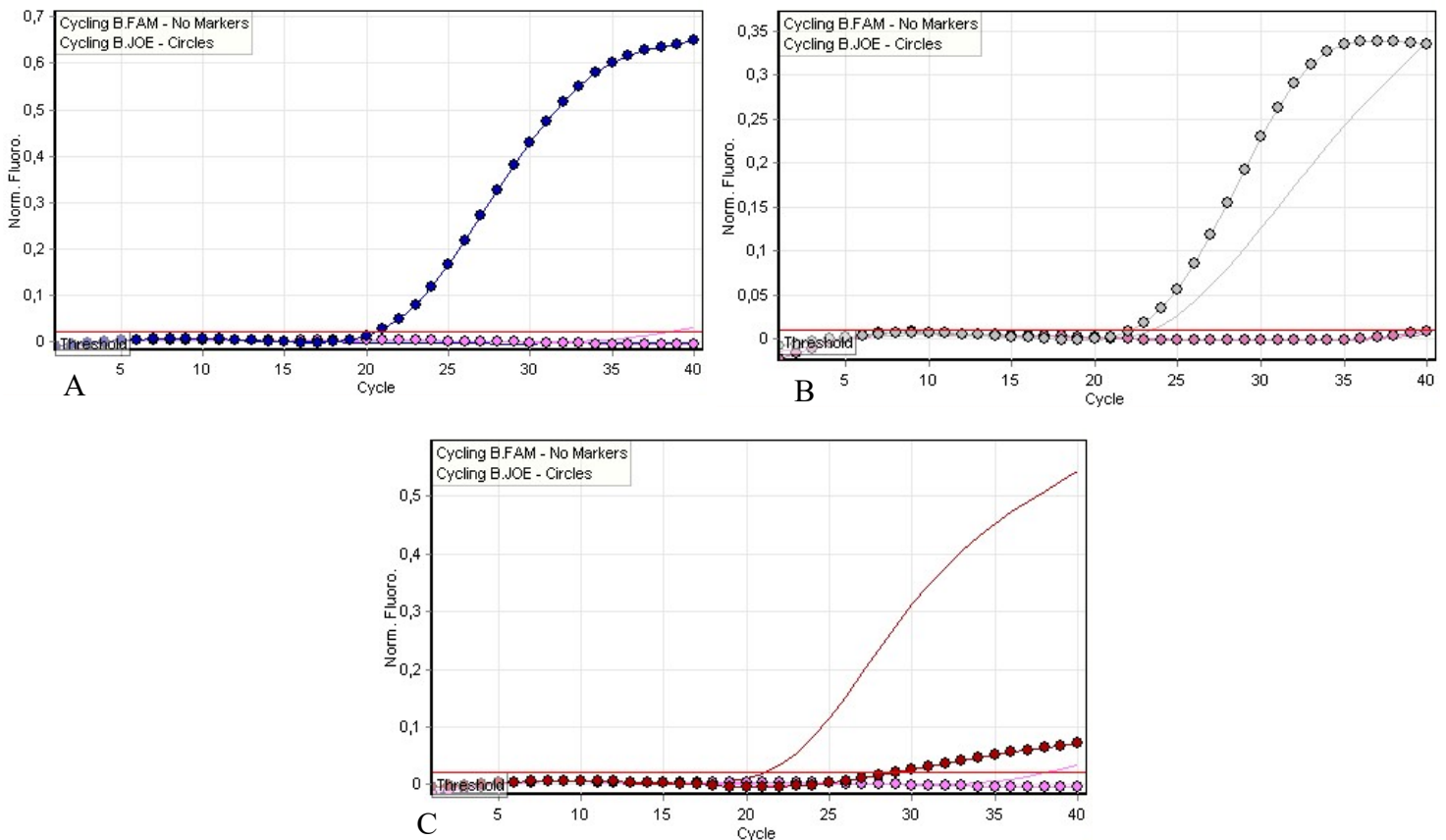


Figure 7.6 - A) Exemplary amplification curve of a patient carrying the homozygous genotype type 1/1, relative to the *IL-1B* gene (position +3954). Blue curve: detected by the JOE probe (allele 1); Pink curve: negative control (white); Red line: Threshold. B) Exemplary amplification curve of a patient carrying the type 1/2 heterozygous genotype, relative to the *IL-1B* gene (position +3954). Gray curve with circles: detected by the JOE probe (allele 1); Flat gray curve: detected by the FAM probe (allele 2); Pink curve: negative control (white); Red line: Threshold. C) Exemplary amplification curve of a patient carrying the type 2/2 homozygous genotype, relative to the *IL-1B* gene (position +3954). Flat dark red curve: detected by the FAM probe (allele 2); Pink curve: negative control (white); Red line: Threshold.

7.1.3 DNA sequencing polymorphism analysis

Some results obtained by the TaqMan assay were confirmed by DNA sequencing. The results obtained by Sanger sequencing and TaqMan assay were identical. In figure 7.7 we can observe a sequencing chromatogram of a patient homozygous for the wild allele (allele C) for the polymorphism *IL-1A* -889. This patient corresponds to a genotype 1/1. Figure 7.8 shows a sequencing chromatogram of a patient homozygous for the mutated allele (allele T) for the polymorphism *IL-1A* -889. This patient corresponds to a genotype 2/2.

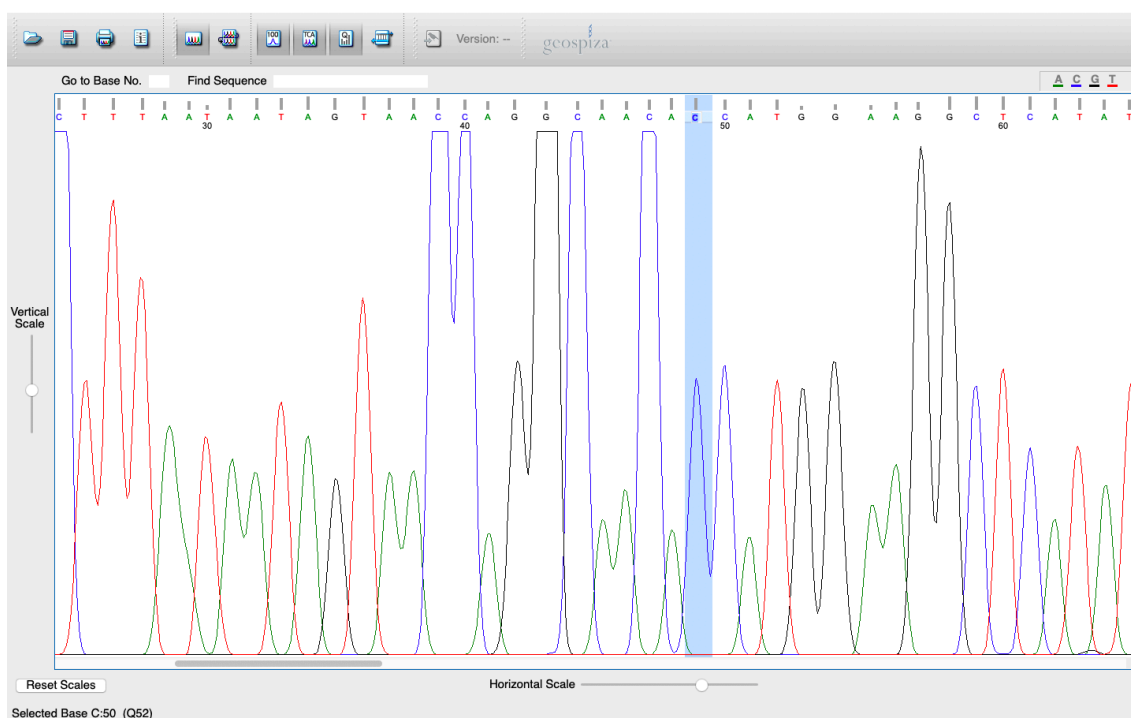


Figure 7.7 - Sequencing chromatogram of a patient homozygous for the wild allele (allele C) for the polymorphism *IL-1A* -889. The shaded area corresponds to the position where the *IL-1A* -889 polymorphism may exist.

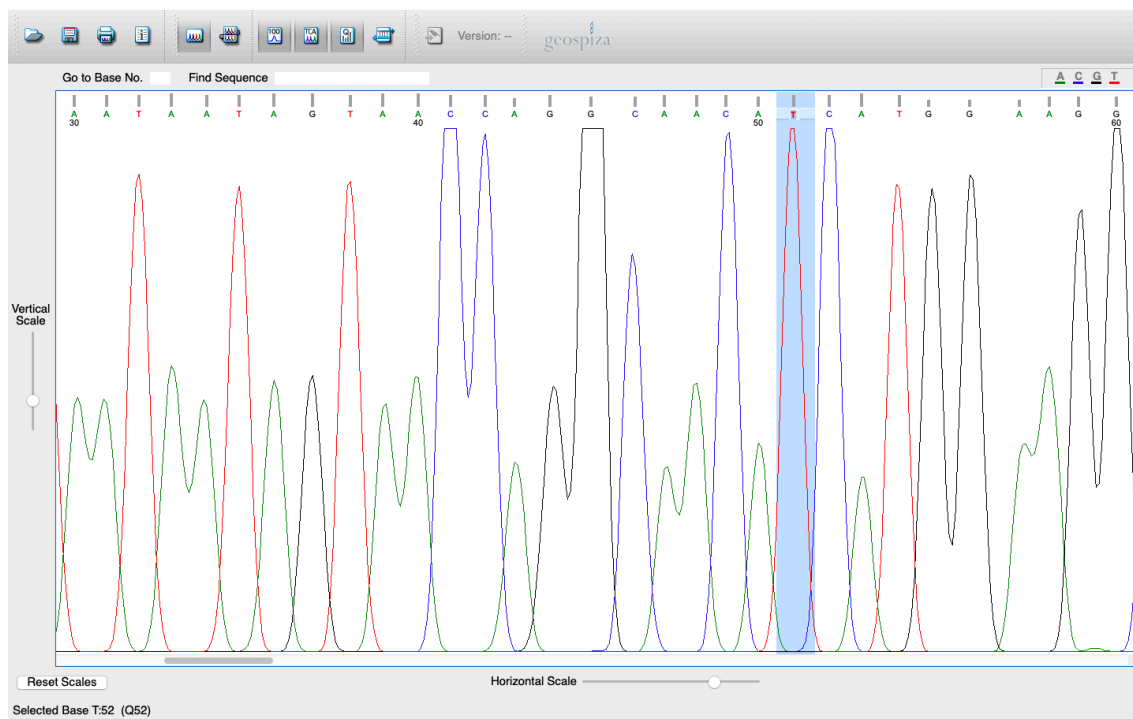


Figure 7.8 - Sequencing chromatogram of a patient homozygous for the altered allele (allele T) for the polymorphism *IL-1A* -889. The shaded area corresponds to the position where the *IL-1A* -889 polymorphism is present.

Regarding the *IL-1B* +3954 polymorphism in figure 7.9 we can observe a chromatogram of a patient with genotype 1/1, being homozygous for the C allele (wild allele). Figure 7.10 concerns a patient with genotype 1/2, heterozygous, presenting a C and T allele (altered allele) at position +3954. Figure 7.11 shows a chromatogram of a patient homozygous for the altered allele (T allele), presenting the genotype 2/2.

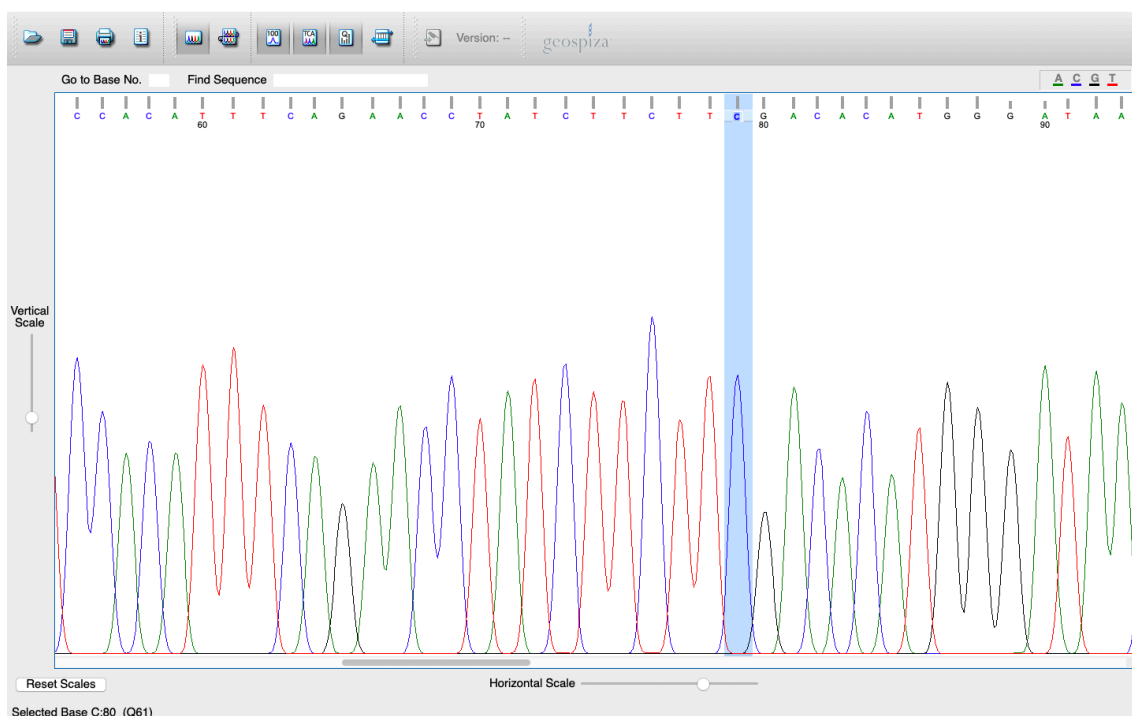


Figure 7.9 - Sequencing chromatogram of a patient homozygous for the wild allele (allele C) for the polymorphism *IL-1B* +3954. The shaded area corresponds to the position where the *IL-1B* +3954 polymorphism may exist.

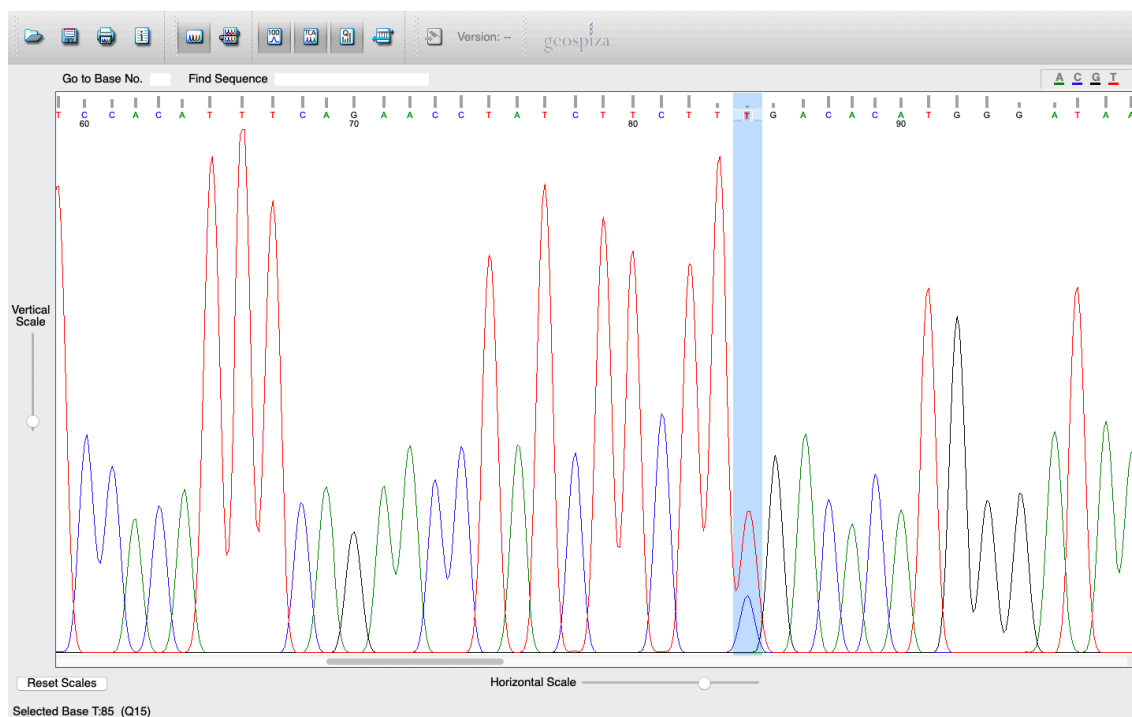


Figure 7.10 - Sequencing chromatogram of a patient heterozygous, presenting a C and T allele (altered allele) for the polymorphism *IL-1B* +3954. The shaded area corresponds to the position where the *IL-1B* +3954 polymorphism is present.

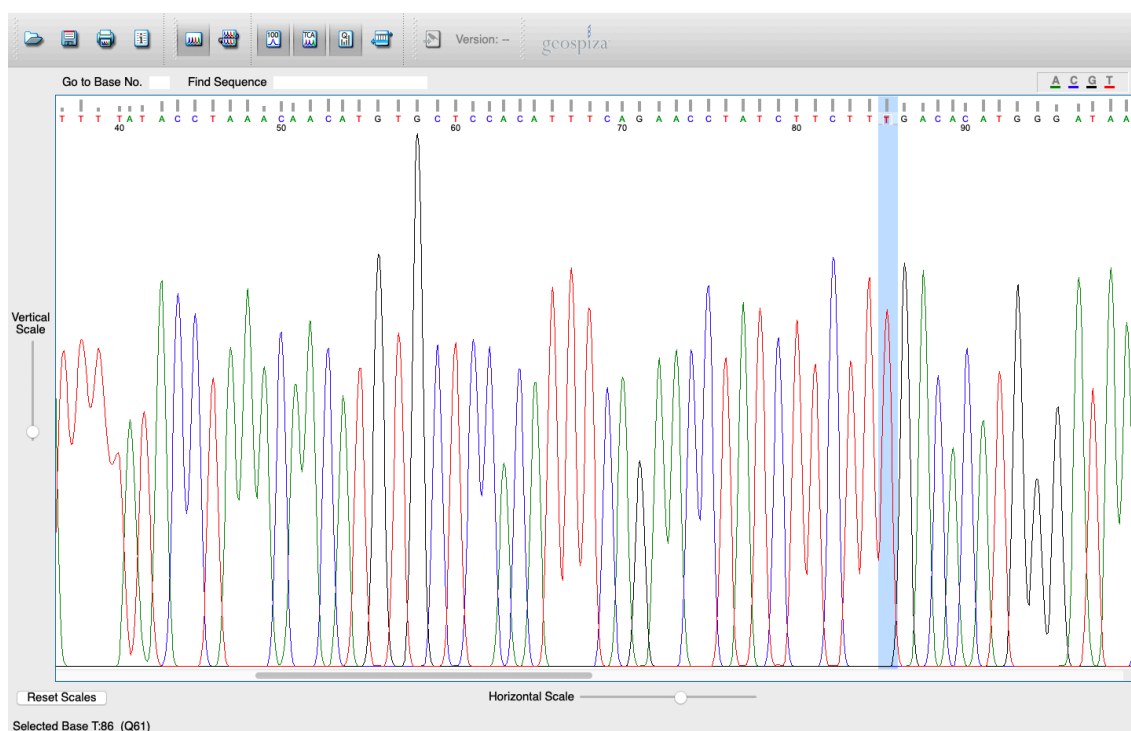
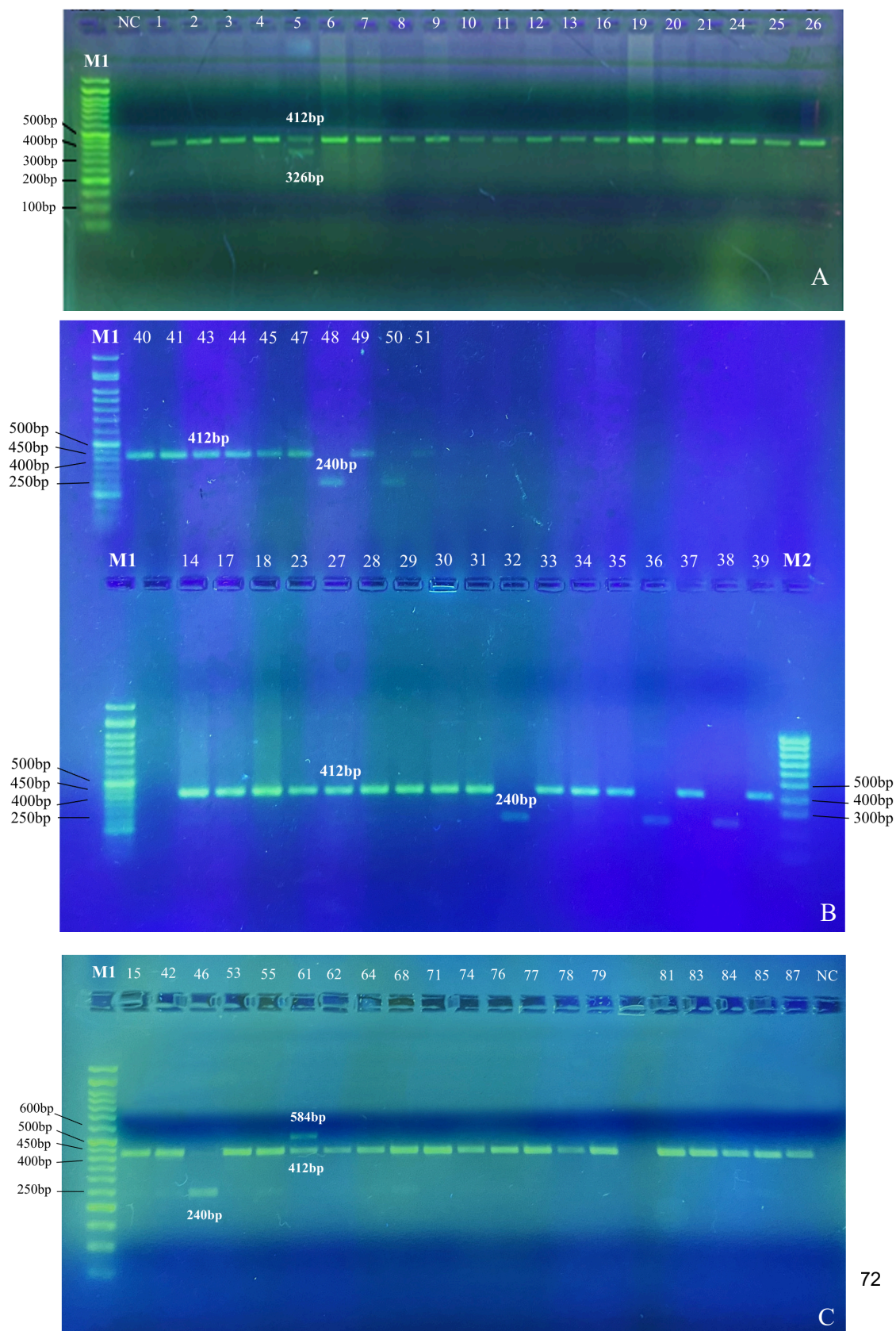


Figure 7.11 - Sequencing chromatogram of a patient homozygous for the altered allele (allele T) for the polymorphism *IL-1B* +3954. The shaded area corresponds to the position where the *IL-1B* +3954 polymorphism is present.

7.2 Analysis of laboratory results of *IL-1RN* polymorphism (VNTR)

The polymorphism in intron 2 of the *IL-1RN* gene (VNTR), associated with five possible alleles, according to the number of times they are repeated in tandem along an 86 bp sequence, was analyzed using the conventional PCR technique. Subsequently, the PCR products were analyzed in a 3% agarose gel (SeaKem® LE Agarose, Lonza Bioscience) by electrophoresis, allowing the visualization of numerous bands, corresponding to the different possible genotypes. The molecular weight markers NZYDNA Ladder VI (M1) and HyperLadder™ 100bp (M2) were used. Of the 102 individuals evaluated, the majority was homozygous for allele 1, corresponding to the 412 bp band (Figure 7.12). Patient 5 was heterozygous, presenting allele 1 and allele 3, corresponding to bands 412bp and 326, respectively (Figure 7.12 - A, F). According to Figure 7.12 – B, patients 32, 36, 38, 48 and 50 were homozygous for allele 2, showing a single 240bp band. As shown in Figure 7.12 – C, patient 46 had the 2/2 genotype, while patient 61 had the 1/5 genotype, the latter having a band of 412bp and another of 584, corresponding to alleles 1 and 5, respectively. In

figures 7.12 - D, E we can observe that patients 69, 70, 73, 93, 96 and 97 had genotype 2/2, showing only a band of 412 bp. In conclusion, of the 102 individuals evaluated, 14 had the less common genotypes, 12 of which were homozygous for allele 2 (genotype 2/2) and another 2 were heterozygous (genotype 1/3 and genotype 1/5).



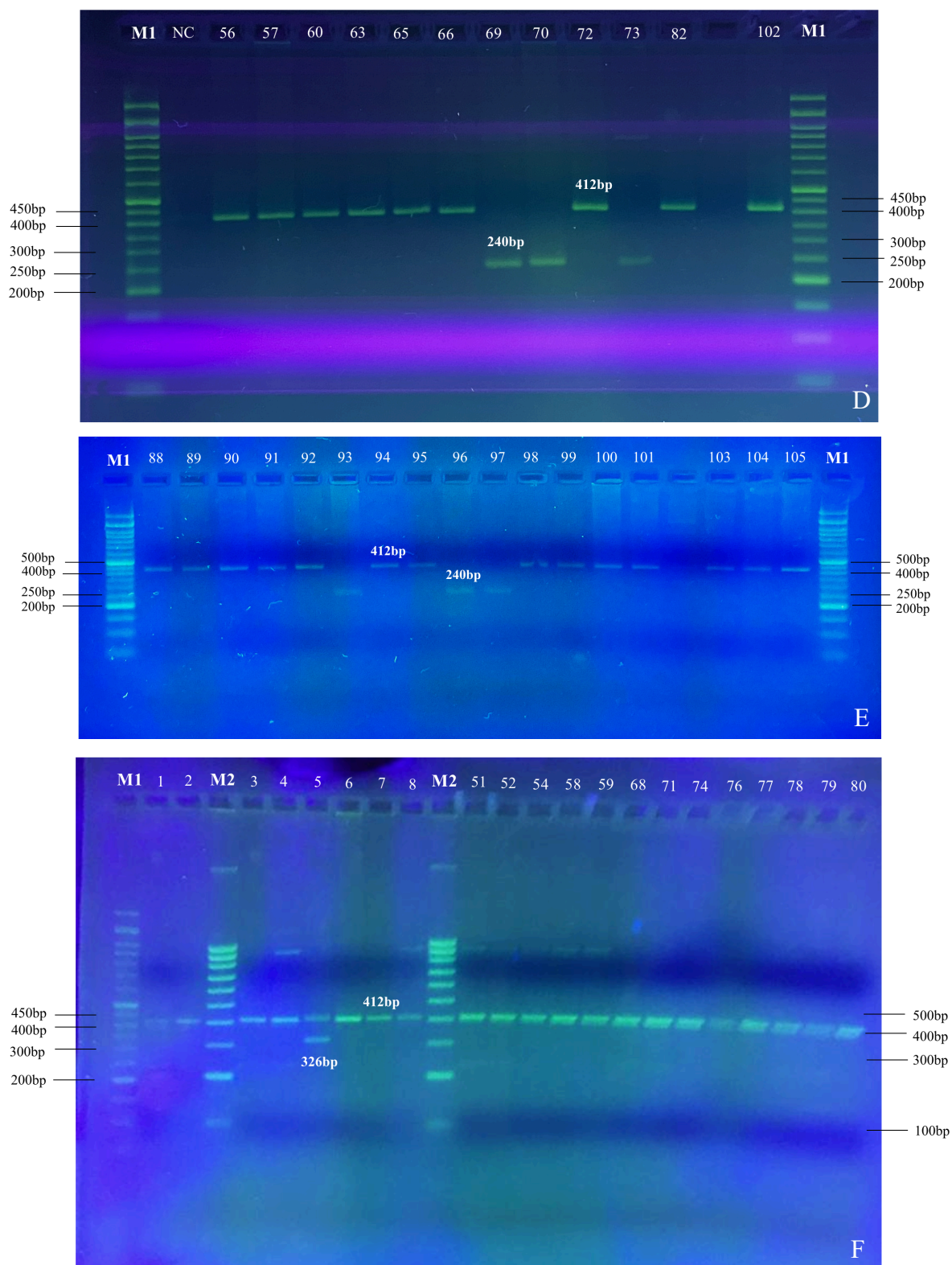


Figure 7.12 - Agarose gel electrophoresis with results for the *IL-1RN* gene (intron2 – VNTR) for the 102 patients analyzed. The 102 patients analyzed are distributed by figures from A to F. The genetic analysis of some patients was confirmed by more than one analysis. M1- Molecular weight marker, NZYDNA Ladder VI, M2 - Molecular weight marker, HyperLadder™ 100bp.

7.3 Evaluation of Hardy-Weinberg equilibrium

Tables 7.1 and 7.2 show the genotype and allelic frequencies, respectively, for each of the groups, peri-implant health and peri-implantitis, and for the total sample.

Table 7.1 – Genotype frequencies of *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* (VNTR) polymorphisms, in the peri-implant health group, in the peri-implantitis group and in the total sample.

Genotype, n (%)	Peri-implant health (n=59)	Peri-implantitis (n=43)	Total (n=102)
<i>IL-1A</i> -889			
CC	40 (67.8%)	24 (55.8%)	64 (62.7%)
CT	18 (30.5%)	14 (32.6%)	32 (31.4%)
TT	1 (1.70%)	5 (11.6%)	6 (5.9%)
<i>IL-1B</i> +3954			
CC	44 (74.6%)	30 (69.8%)	74 (72.5%)
CT	15 (25.4%)	11 (25.6%)	26 (25.5%)
TT	0 (0%)	2 (4.6%)	2 (2%)
<i>IL-1RN</i> (VNTR)			
1/1	50 (84.7%)	38 (88.4%)	88 (86.3%)
1/3	1 (1.7%)	0	1 (0.98%)
2/2	7 (11.9%)	5 (11.6%)	12 (11.8%)
1/5	1 (1.7%)	0	1 (0.98%)

Abbreviations: variable number of tandem repeats (VNTR).

To assess if the population (control group) was in Hardy-Weinberg equilibrium for the *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* genotypes and alleles, a Hardy-Weinberg-Allele Calculator (<https://www.had2know.org/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html>) was used. To use the calculator, we entered the observed frequencies of the 3 different genotypes. The calculator computed the frequency of each allele and the Hardy-Weinberg equilibrium expected frequencies of each genotype. It also calculated the chi-square value

so we could decide whether to reject or accept the null hypothesis that the population was in Hardy-Weinberg equilibrium.

For *IL1-A* -889 and *IL-1B* +3954 the obtained genotype frequencies did not deviate from the expected ones according to the Hardy-Weinberg equilibrium ($p = 0.520$, $p = 0.263$, respectively).

For *IL-1RN*, since many of the possible genotypes had very low frequencies or even zero, only alleles 1 and 2 were considered for analysis. The obtained genotype frequencies deviated from the expected ones according to the Hardy-Weinberg equilibrium ($p < 0.05$).

Table 7.2 – Allelic frequencies of *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* (VNTR) polymorphisms, in the peri-implant health group, in the peri-implantitis group and in the total sample.

Alleles, n (%)	Peri-implant health (n=118)	Peri-implantitis (n=86)	Total (n=204)
<i>IL-1A</i> -889			
C	98 (83,1%)	62 (72,1%)	160 (78,4%)
T	20 (16,9%)	24 (27,9%)	44 (21,6%)
<i>IL-1B</i> +3954			
C	103 (87,3%)	71 (82,6%)	174 (85,3%)
T	15 (12,7%)	15 (17,4%)	30 (14,7%)
<i>IL-1RN</i> (VNTR)	Allele 1 – 102 (86,4%) Allele 2 – 14 (11,9%) Allele 3 – 1 (0,85%) Allele 5 – 1 (0,85%)	Allele 1 – 76 (88,4%) Allele 2 – 10 (11,6%) Allele 3 – 0 Allele 5 – 0	Allele 1 – 178 (87,3%) Allele 2 – 24 (11,8%) Allele 3 – 1 (0,5%) Allele 5 – 1 (0,5%)

Abbreviations: variable number of tandem repeats (VNTR).

7.4 Evaluation of the presence of polymorphisms in smokers and non-smokers separately

Tables 7.3 and 7.4 analyze the presence of the *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* (VNTR) polymorphisms in the peri-implant health and peri-implantitis groups, analyzing only non-smoking and smoking patients, respectively.

Table 7.3 – Genotype frequencies of *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* (VNTR) polymorphisms, in the peri-implant health group and in the peri-implantitis group in non-smoking patients.

Genotype, n (%)	Peri-implant health (n=59)	Peri-implantitis (n=43)	Total (n=102)	<i>p</i>
<i>IL-1A</i> -889				
CC	31 (75.6)	15 (62.5)	46 (70.8)	0.262
CT/ TT	10 (24.4)	9 (37.5)	19 (29.2)	
<i>IL-1B</i> +3954				
CC	30 (73.2)	17 (70.8)	47 (72.3)	0.839
CT/ TT	11 (26.8)	7 (29.2)	18 (27.7)	
<i>IL-1RN</i> (VNTR)				
1/1	37 (90.2)	20 (83.3)	57 (87.7)	0.454
1/3, 1/5, 2/2	4 (9.8)	4 (16.7)	8 (12.3)	
IL-1 composite genotype				
Yes	5 (12.2)	5 (20.8)	10 (15.4)	0.479
No	36 (87.8)	19 (79.2)	55 (84.6)	

Abbreviations: variable number of tandem repeats (VNTR).

Table 7.4 – Genotype frequencies of *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* (VNTR) polymorphisms, in the peri-implant health group and in the peri-implantitis group in smoking patients.

Genotype, n (%)	Peri-implant health (n=59)	Peri-implantitis (n=43)	Total (n=102)	<i>p</i>
<i>IL-1A</i> -889				
CC	9 (50.0)	9 (47.4)	18 (48.6)	0.873
CT/ TT	9 (50.0)	10 (52.6)	19 (51.4)	
<i>IL-1B</i> +3954				
CC	14 (77.8)	13 (68.4)	27 (73.0)	0.714
CT/ TT	4 (22.2)	6 (31.6)	10 (27.0)	
<i>IL-1RN</i> (VNTR)				
1/1	14 (77.8)	17 (89.5)	31 (83.8)	0.405
1/3, 1/5, 2/2	4 (22.2)	2 (10.5)	6 (16.2)	
IL-1 composite genotype				
Yes	4 (22.2)	5 (26.3)	9 (24.3)	1.000
No	14 (77.8)	14 (73.7)	28 (75.7)	

Abbreviations: variable number of tandem repeats (VNTR).

When analyzed separately for non-smoking and smoking patients, the genotype frequencies showed no statistically significant differences ($p > 0.05$) between the peri-implant health and peri-implantitis groups for the different polymorphisms evaluated.

8. GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

8.1 General discussion

Peri-implantitis is among the most common complications of implant-supported dental restorations [1,2]. However, the etiology and pathogenesis of periimplantitis are not currently well understood, and more research is still needed to clarify them [3]. Microbial plaque accumulation around dental implants can trigger inflammatory host response in peri-implant tissues but its progression and severity can vary between individuals [4]. Since peri-implantitis represents a public health issue, it is essential to identify the various relevant risk factors to the onset and progression of the disease. This study aimed to verify if there is an association between the polymorphisms of the *IL-1A*, *IL-1B* and *IL-1RN* and peri-implantitis. It is a relevant topic since, if this association is confirmed, it is possible to identify patients who have this polymorphism and the physician's approach can be adjusted in order to prevent the onset of the disease. The identification of susceptible patients will help in the development of individualized maintenance programs, eventually contributing to the primary prevention of the disease. In addition, in this type of patient, an even more rigid attitude must be implemented in the control of the various risk factors for the disease. Genetic polymorphisms are constant and can be measured before disease onset, thus it could be of great benefit for treatment planning and prognosis in an early stage. Although the etiology and pathology of peri-implantitis are complex, the identification of genetic biomarkers associated with the risk of peri-implantitis can serve as a valuable tool in daily clinical practice. These biomarkers can aid in the early identification of individuals who are predisposed to an elevated risk of peri-implantitis. Consequently, this information would assist practitioners in personalizing treatment plans after estimating the prognosis of dental implants.

The future of disease diagnosis should encompass the identification of not only more susceptible patients but also disease-associated biomarkers. Biomarkers are biological indicators that possess significant prognostic and

predictive value, as they can be linked to the onset or progression of a pathology. Frequently, they serve as predictors for the presence or progression of a disease.

8.1.1 Genotype and allelic frequencies in the total sample

Trevilatto et al. (2011), performed a comparison of several studies that analyze the genotype frequencies of *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* (VNTR) polymorphisms in different populations, namely Chinese, Japanese, German, Italian, Swedish and Brazilian [5]. Analyzing the data obtained for *IL-1A* -889 polymorphism in our study, the frequency of altered genotype (CT/TT) was 37.3% in the total sample, a value that is lower than studies carried out in Brazil [5,6] and in a study carried out in northern Europe [7], where a genotype frequency of 64% of this polymorphism was verified. As far as allele frequencies are concerned, in our study T allele was observed in 21.6% of patients. This value is below the values observed in studies carried out in northern [7] and central Europe [8] or in Brazil [6], where values are around 30%.

Analyzing the data obtained for *IL-1B* +3954 polymorphism in our study, the frequency of altered genotype (CT/TT) was 27.5% in the total sample, value that is lower than other studies carried out in southern [9] and northern Europe [7] with approximated values of 40 and 50%, respectively. This frequency is, however, higher than studies carried out in Asia [10,11], where values below 10% were observed. In relation to allele frequencies, an allele frequency of 14.7% was observed for the T allele. This value is below the value found in other studies carried out in Europe and Brazil [5,7,9], but above the values found in studies carried out in Asia [10,11].

As for the *IL-1RN* polymorphism (VNTR), it can be observed that the frequency registered for carriers of the 2/2 genotype (11.8%), which were all patients with genetic alteration with the exception of 2 patients who had the 1/5 and 1/3 genotypes, is similar to that of verified in other studies carried out in southern Europe [9,12]. However, in these studies, genotype frequencies for genotype 1/2 of around 30% were observed, while in our study, no individual was observed with this genotype. Even in studies carried out in Asia, values between

10 and 20% were observed for this genotype [10,11]. In relation to allele frequencies, a percentage of 11.8% were observed for allele 2 in our study. This value is similar to that found in studies carried out in Asia [10,11]. However, in Europe and Brazil values between 20 and 30% were observed [5,8,9,12].

According to the study of Kornman et al. (1997), which was the first study to find an association of *IL-1A* -889 and *IL-1B* +3954 polymorphisms with periodontitis, the frequency of the IL-1 positive composite genotype was 29.1% in Caucasian individuals from northern Europe [13]. Vaz et al. (2011) observed, in a Portuguese population, that the frequency of patients with positive composite genotype was 33.5% [14]. This is not in line with the results found in the present study, in which it was observed that 18,6% of all participants had at least one altered allele in the two genes *IL-A* and *IL-1B*, at positions -889 and +3954, respectively. The discrepancies observed between both studies may be related with differences in inclusion criteria and in the technique of genetic analysis performed.

In general, it was observed, according to the aforementioned studies, that the genotypic frequencies (CT/TT) and allelic frequencies (T allele) in *IL-1A* -889 and *IL-1B* +3954 in our study are lower than those observed in studies carried out in Europe, but higher than those verified in Asian countries. For these genes, the population was in Hardy-Weinberg equilibrium.

We also consulted the genome browser (Ensembl.org), which contains allele frequencies in different populations according to scientific publications, to compare with the allele frequencies observed in our study. For *IL-1A* -889, an allelic frequency for the mutated allele (allele T) of 29% was observed in Europe, this value being similar to that observed in America (28%), lower than Africa (40%) but higher than East Asia (7%). At a subpopulation level, there is no information for the Portuguese subpopulation, but among the Finnish (28%), English (36%), Italian (29%) and Spanish (26%) subpopulations, the latter is where there is a lower allelic frequency for the altered allele. In our study, an allele frequency of 21.6% was found for the altered allele (allele T), a value that among all populations evaluated is close to that observed in Spain.

For *IL-1B* +3954, an allelic frequency for the altered allele (allele T) of 25% was observed in Europe, value that is higher than that found in Africa (12%), America (13%) and East Asia (2%). At a subpopulation level, there is no information for the Portuguese subpopulation, but among the Finnish (24%), English (31%), Italian (27%) and Spanish (20%) subpopulations, the latter is where there is a lower allele frequency for the altered allele. In our study, an allele frequency of 14.7% was found for the altered allele (allele T), a value that is lower than that observed in Spain.

For *IL-1 RN* it was observed that, as in other studies, within the altered alleles, allele 2 is the most common. However, while in other studies the second most common genotype was 1/2, in ours it was 2/2. For *IL-1RN* the population was not in Hardy-Weinberg equilibrium. HWE may be violated because of genotyping error, chance, inbreeding, non-random mating, differential survival of marker carriers, genetic drifting, population stratification, or combinations of these reasons. Some of these effects may also occasionally act in different directions, canceling each other. Unfortunately, most gene-disease association studies report very limited information to provide any detailed insight into these potential problems. Thus, also at a meta-analysis level, typically there is no way to decipher for which of the above reasons the violation has occurred, except perhaps for population stratification under special conditions [15].

Genetic polymorphisms are variations in DNA that occur naturally in the human population. The relationship between genetic polymorphisms and ethnicity is complex and multifaceted. Different human populations show distinct genetic variations, reflecting evolutionary history and migrations that have occurred over time [16]. These variations can be found at different frequencies in specific ethnic groups, which may be related to factors such as natural selection, genetic drift and migration. Genetic studies have sought to identify and characterize these genetic variations in different ethnic groups. These polymorphisms may be associated with physical characteristics, susceptibility to disease, drug metabolism, response to therapies and other aspects related to health and well-being. In addition, ethnicity is a complex social and cultural construction, which is not only based on genetic differences, but also on historical, cultural, linguistic and geographic aspects. The relationship between

genetic polymorphisms and ethnicity is influenced by these factors and cannot be simplified into a single direct connection [16].

In summary, genetic polymorphisms are related to the genetic diversity observed among individuals and ethnic groups. However, the relationship between genetic polymorphisms and ethnicity is complex and influenced by a variety of genetic, historical, cultural and environmental factors. It is important to avoid simplistic generalizations and recognize the importance of individuality and diversity within each ethnic group.

8.1.2 Association between *IL-1A*, *IL-1B* and *IL-1RN* polymorphisms and peri-implantitis

The *IL-1A* -889 and *IL-1B* +3954 polymorphisms are two of the most widely studied genetic variants in the issue of implant failure and peri-implant disease [17]. There are some studies that have investigated its associations with the risk of peri-implantitis, in which some obtained positive results [18,19,19,20] and others yielded negative results [7,21–24]. In the present study, there were no statistically significant differences between the health and the disease groups with regard to the polymorphisms evaluated, so that there was no association between these polymorphisms and peri-implantitis. Published data on the association of *IL-1RN* polymorphism (VNTR) and peri-implantitis risk are very scarce in the current literature. Previous studies have reported that allele 2 carriers were linked with a threefold increased peri-implantitis risk in the Swedish population [7] and implant loss in the Brazilian population [25].

In a systematic review published by our study group (chapter 2), which included eight studies, it was observed in four articles that individuals with *IL-1* gene polymorphisms (*IL-1A* -889, *IL-1B* +3954, *IL-1B* -511 or *IL-1RN* (VNTR)) showed a higher risk for the development of peri-implantitis [26]. Within the limits of that systematic review, it might be concluded that there is no obvious association between specific genetic polymorphism of *IL-1* and *IL-1RN* and periimplantitis, although a tendency should be underlined showing the potential

link between *IL-1* genotype and peri-implantitis. For the meta-analysis, it was only possible to include four articles [7,18,20,23], and it was observed that individuals who had the polymorphism in the *IL-1B* +3954 had an almost twice higher risk of developing peri-implantitis (OR 1.986).

Evaluating the published studies about the possible relationship between the polymorphisms evaluated in our study and peri-implantitis, it appears that there is no consensus regarding the role of these genetic variations in the onset and progression of the disease.

The reasons for the contradictory results between the studies may be related to certain variables that may impact/influence the results obtained (confounding variables) such as ethnicity, smoking habits and periodontal status. The size of the samples used in the different studies, as well as the definition of peri-implantitis used, may also explain the differences found between the studies.

Ethnicity

The differences between the results of the studies may be due to differences regarding the ethnicity of the groups included. In the present study, Caucasian individuals from southern Europe were included, whereas in the study by He et al. (2020) [18] and by Saremi et al. (2021) [20] patients of Asian origin were included. On the other hand, some studies do not refer to specific information about the population included [19,21]. Studies have reported that the frequency of gene polymorphisms associated with genetic susceptibility to a particular disease can be influenced by ethnic and racial variations [13,27]. For instance, research has shown that the prevalence of *IL-1A* +4845 and *IL-1B* +3954 gene polymorphisms linked to periodontitis is low among the Chinese population (2.3%) [27], in contrast to the higher prevalence reported for Caucasians [13]. It is crucial to identify the prevalence of these genotypes in particular ethnic groups and not generalize the findings from one group to another. To ensure homogeneity in our study population, we exclusively recruited patients born in Portugal, with Caucasian ancestry spanning three generations, and excluded those with diverse ethnic backgrounds. Vaz et al. (2011) [14] observed, in a Portuguese population, that the alleles 1 and 2 (allele with nucleotide sequence

changed) of *IL-1A* gene and the alleles 1 and 2 of *IL-1B* gene were statistically associated with the success or no success of the dental implants, respectively. However, the inclusion criteria varied from our study. The implants were considered unsuccessful if they failed (implant loss after osseointegration) or developed any of these biological complications: mobility; pain on palpation, percussion or function; recurrent infection (fistula or suppuration); peri-mucositis, periimplantitis, and vestibular metal exposing during or after the abutment connection. In our study, only patients with peri-implantitis or peri-implant health were included, following the guidelines of the new classification of periodontal and peri-implant diseases (AAP and EFP 2018) [28–30]. In another study carried out in the Spanish population, it was observed that *IL-1* genotypes (*IL-1A* -889 and *IL-1B* +3954 polymorphisms) do not seem to be good predictors of peri-implantitis in the great majority of smoking patients [22].

Furthermore, population stratification is another cause of possible false-positive findings in some studies. It occurs when cases and controls have different allele frequencies attributable to diversity in the population background, unrelated to health status. This is due to non-random mating between ethnic groups, mainly caused by geographical separation which is then followed by genetic drift of allele frequencies. In studies that try to relate certain genetic polymorphisms and the occurrence of a specific disease, population stratification has been often addressed selecting cases and controls from the same country, same ethnicity, and in some sparse case asking for ancestry. However, these methods have proven to be inconsistent, especially in areas cohabited by different ethnicities [15].

In our study, the genotypic frequencies found for the altered genotypes in the total sample were lower than those found in other European countries. These data may indicate that the Portuguese population has ethnic characteristics that justify these differences. This situation may explain the absence of differences between groups regarding the role of these polymorphisms in the occurrence of peri-implantitis, in the evaluated population. This situation emphasizes how important it is to develop this type of studies in different ethnic groups. This is the only way to understand the real role of these polymorphisms in the onset and progression of peri-implantitis.

Smoking habits

There were also differences in terms of smoking habits between the published studies. In the present study, smokers were included, whereas in the studies by Hamdy et al. (2011) [19], He et al. (2020) [18] and Melo et al. (2012) [23] smoking patients were not selected. According to some studies there is a synergism between smoking (heavy smokers) and *IL-1* polymorphisms in the development of peri-implantitis [31,32]. For this reason, we carried out an analysis of these polymorphisms (*IL-1A* -889, *IL-1B* +3954 and *ILRN* (VNTR)) in patients with peri-implant health and with peri-implantitis, only in patients who smoked. There were no statistically significant differences in genotype frequencies between the groups when only smoking patients were assessed.

On the other hand, there is evidence that the genetic association with chronic periodontitis was more evident when smokers were excluded from the study groups, confirming the importance of this risk factor and suggesting that its effect is strong even in individuals who are not genetically susceptible to the disease [13]. These data suggest that smoking-related risk can often obscure polymorphism-related risk, as described by Kornman et al. [13]. For this reason, polymorphism analysis was also carried out only on non-smoking patients, and there were no statistically significant differences in genotype frequencies between the peri-implant health and peri-implantitis groups.

Thus, smoking status may be a confounding factor for the genetic associations. There are studies that observed an association between smoking and peri-implantitis [33–35]. Therefore, this habit must be evaluated, its possible effect on the disease must be considered and the groups should be balanced regarding the existence of the smoking habit. However, in some studies there were no data on smoking habits of the patients [20,21] and in the studies where this habit was evaluated there were differences in categorization of smokers and non-smokers which can lead to different results. Furthermore, all the identified studies relied solely on patient-reported information for the assessment of smoking status.

Sample

Our study comprised 102 patients, in accordance with the sample calculation carried out in a pilot study [36]. When we evaluated the samples of the studies that addressed the possible relationship between these polymorphisms and peri-implantitis, we found that there were studies with samples ranging from 29 [21] to 318 patients [18]. Most studies that investigated risk factors for peri-implant diseases relied on convenience sampling, which is prone to selection bias, especially in cross-sectional and case-control studies. There are studies that have a longitudinal character [21,31,32] while others are cross-sectional studies [18,20]. A longitudinal assessment allows for better control of local and systemic risk factors/ indicators that may influence the onset and progression of the disease.

It has been stated that sample size affects the precision of the estimated parameters and that consecutive sampling with clearly defined inclusion and exclusion criteria must be used [37]. Furthermore, large populations are the prerequisite to overcome any bias due to heterogeneity [38].

Periodontal Status

Data on the periodontal status of the patients were not reported or clarified in some studies that evaluate the possible relationship between these polymorphisms and peri-implantitis [7,19,21]. When investigating potential risk factors or indicators for peri-implantitis, it is crucial to assess whether patients have a history of periodontitis. There is strong evidence from both longitudinal and cross-sectional studies that a history of periodontitis is a risk factor/indicator for peri-implantitis [39]. The concept that microorganisms are essential for the development of infections around dental implants is well supported in the literature. It has been suggested that periodontal pockets of teeth may act as a reservoir for microorganisms to colonize the newly inserted implants [40]. Although bacteria may be considered the initiating factor of periodontal disease, other aspects like genetic factors may be extremely important. In fact, most authors agree that the host response to bacterial challenge is a critical factor,

especially in the progression of the disease [41]. These 2 factors (similar bacterial profile and genetic predisposition) might explain why patients with periodontitis are at great risk of developing peri-implant diseases [42]. In the present study, our results also demonstrated that there was a significant association between the history of periodontitis and peri-implantitis ($p = 0.020$).

Peri-implantitis definition

There is a wide range of different definitions regarding peri-implant mucositis and peri-implantitis that were employed in different studies, situation that may lead to different results between studies and difficulties in comparing them. Many studies, including published systematic reviews, assessed the relationship of these polymorphisms with the peri-implant disease, pooling in the same group patients with bone loss, implant loss and peri-implantitis [37,43,44]. On the other hand, some studies [25,45] did not differentiate between early and late implant failure, situations that have different causes. It is noteworthy, that in two studies [19,21] peri-implantitis was defined as PD >4 mm accompanied with clinical signs of peri-implant tissue inflammation and radiographic evidence of bone loss around implant whereas in another study [7] it was defined as bone loss >3 threads and evidence of bleeding and/or pus on probing. In the study by He et al. (2020) [18] peri-implantitis was defined as PD $\geq$ 4 mm accompanied with clinical signs of peri-implant tissue inflammation and recorded radiographic signs of pathologic bone loss involving $\geq$ 2 threads compared with the radiograph taken at the time of prosthetic placement. In the studies by Gruica et al. (2004) [31] and Feloutzis et al. (2003) [32], the assessment of peri-implantitis was based on peri-implant bone loss. However, there is evidence that peri-implant tissue health can exist around implants with variable levels of bone support [28].

In our study the guidelines of the new classification of periodontal and peri-implant diseases (AAP and EFP 2018) [28–30] were followed. The existence of a classification with objective diagnostic criteria will help to standardize the diagnosis of peri-implant diseases.

Multifactorial etiology of peri-implantitis

Peri-implantitis is a common, complex and multifactorial disease [46,47]. Important risk factors have been identified, including a history of periodontitis, poor plaque control and no regular supportive peri-implant care following implant therapy [39,48]. Less conclusive evidence was found for smoking and diabetes, or local factors such as the presence of submucosal cement following prosthetic restoration of the implant, or positioning of implants limiting access to oral hygiene and maintenance [39,48,49]. Furthermore, despite the lack of consensus among the studies and the fact that they were not identified by longitudinal studies, certain diseases, characteristics of the implant or the area where the implant was placed have been pointed out by some authors as possible risk indicators for peri-implantitis [35,52]. It is therefore understandable that in diseases with a multifactorial etiology, and especially in studies with a cross-sectional design, it is difficult to control and assess all the risk factors/indicators and understand the real effect of a particular factor (in this case, genetics) on the development of the disease.

8.1.3 The influence of genetic polymorphisms on expression of interleukin-1beta, prostaglandin E2 and tumor necrosis factor alpha in peri-implant crevicular fluid

There are studies that try to relate the existence of certain genetic polymorphisms and the occurrence of peri-implantitis, and there are also studies that try to assess whether patients with peri-implantitis have higher concentrations of certain inflammatory mediators in the PICF, compared to patients with peri-implant health. However, to our knowledge, there is only one study that related the existence of polymorphisms in the *IL-1A* and *IL-1B* genes with changes in the levels of inflammatory mediators IL-1 β , plasminogen activator inhibitor type 2 (PAI-2) and PGE2 in dental implants [21].

When we refer to periodontitis the situation is different. Kornman & di Giovine (1998) described a twofold increase of sulcular IL-1 β production in periodontitis

patients with positive composite genotype [53]. In another study where the influence of IL-1 gene polymorphisms on the expression of IL-1 β and TNF- α in periodontal tissue and GCF was evaluated, the authors stated that patients with the positive composite genotype may demonstrate phenotypic differences as indicated by elevated levels of IL-1 β in the GCF [54]. In another study of Pani et al. (2021) whose aim was to compare IL-1 β levels in GCF from healthy and periodontitis sites of *IL-1B* +3954 positive and *IL-1B* +3954 negative periodontitis subjects in association with their bacterial profiles, the results indicated an independent association of both *IL-1B* +3954 and red complex bacterial species with increased IL-1 β levels in GCF of periodontitis sites [55].

The possible association between these genetic polymorphisms and peri-implantitis must be based on a change in the individual's immune-inflammatory response. One of the ways to assess this effect is to analyze the volume and content of the PICF. In the study of Lachmann et al. (2007), when healthy and the peri-implantitis patient groups were pooled together, carriers of IL-1 negative composite genotype showed higher IL-1 β and PGE2 PICF levels than their IL-1-positive counterparts ($p=0.02$ for IL-1 β and $p=0.04$ for PGE2) and a tendency of increased PAI-2 concentrations that did not, however, reach statistical significance ($p=0.052$) [21]. In our study, we observed that carriers of IL-1 negative composite genotype also had higher concentrations of IL-1 β and PGE2, although statistical significance was not obtained ($p=0.713$ and $p=0.634$, respectively). For the *IL-1RN* polymorphism (VNTR), it was found that individuals with this polymorphism had higher concentrations of TNF- α , IL-1 β and PGE2 in the PICF, compared to individuals with the most common genotype, with statistical significance for TNF- α and PGE2 ($p=0.002$ and $p=0.049$, respectively). In this group, 53% of patients had peri-implant health while 47% had peri-implantitis. When a separate analysis was performed within patients with polymorphism in the *IL-1RN* gene between healthy and peri-implantitis patients, no significant differences were found. Thus, the presence of peri-implantitis did not appear to be a factor influencing the results. The observed data regarding the biochemical mediators TNF- α and PGE2 may indicate that individuals with the *IL-1RN* polymorphism (VNTR) may have a more vigorous immune-inflammatory response and that, although they have not yet developed the disease, they are

more likely to have peri-implantitis. Cullinan et al. (2001) concluded from the results of their longitudinal study on patients undergoing periodontal therapy that the positive composite genotype effect is outplayed in sites of deep periodontal pockets by other factors of greater impact on periodontal health [56]. The results of that study have shown an interaction of the IL-1 positive genotype with age, smoking and *Porphyromonas gingivalis* which suggests that IL-1 genotype is a contributory but non-essential risk factor for periodontal disease progression in that population.

In our study, we also observed that among the IL-1 positive composite genotype individuals, those who had peri-implantitis showed a greater volume of PICF ($p=0.042$) and a greater concentration of PGE2 ($p=0.043$). However, in this analysis the groups had few individuals, and the data have to be interpreted with caution. According to the available evidence, it can be concluded that although certain studies have not found a relationship between IL-1 β , TNF- α , PGE2 and peri-implantitis, most of them, as evidenced in systematic reviews and clinical studies, demonstrated a positive association between elevated concentrations of these biomarkers in the PICF of diseased versus healthy sites [57–63]. The conflicting results of the above studies may be due to differences in study design, material and methods utilized, such as sample collection, processing and assay sensitivity.

Based on our study, there was a correlation between concentrations of the three evaluated mediators (IL-1 β , TNF- α and PGE2), a fact that was also observed in the study by Lachman et al. for IL-1 β , PGE2 and PAI-2 [21]. This fact makes sense, as these inflammatory mediators orchestrate the same biological process. Despite some similarities between both studies, differences in some results may be due to the collection method and biochemical tests used, which were different between studies. The definition of peri-implantitis also varied between the two studies.

8.2 Limitations of the present study

Some limitations of our study should be addressed. Ideally, the study should be longitudinal and not cross-sectional. Longitudinal studies should be preferred because, although the genetic profile does not vary, biomarkers may not be consistently present during a single instance of PICF collection due to various systemic or local factors. Studies with a longitudinal design will also allow better control of the various factors that can influence the onset or progression of a disease with a multifactorial etiology such as peri-implantitis. One of these factors is the potential lack of regular supportive peri-implant care following implant therapy. Follow-up from the planning stage of implant placement would allow better control of the various local risk factors/indicators related not only to the characteristics of the implant, but also to the rehabilitation and the surgical site. The implants were not all placed at our university, which may make it difficult to analyze various factors that may interfere with the development of peri-implant pathology.

Another limitation of the study was a sample of 51 patients in the part that evaluates the influence of genetic alterations on the peri-implant immune response. It was a convenience sample. However, the sample of 51 patients involved a total of 306 analyses, 153 biochemical (IL-1 β , TNF- α and PGE2) and 153 genetic (*IL-1A*, *IL-1B* and *IL-1RN*). The only published study that evaluated the relationship between the genetic and the biochemical aspects in cases of peri-implantitis and peri-implant health contained a sample of 29 patients [21]. On the other hand, the studies that addressed the impact of genetic alterations on the periodontal crevicular immune response had smaller samples than our study. The study by Engebretson et al. (1999) that evaluated the Influence of *IL-1* gene polymorphisms on expression of IL-1 β and TNF- α in periodontal tissue and GCF, included a sample of 22 patients [54]. In the study of Pani et al. (2021) whose aim was to compare IL-1 β levels in GCF from healthy and periodontitis sites of *IL-1B* +3954 positive and *IL-1B* +3954 negative periodontitis subjects in association with their bacterial profiles, a sample of 32 patients was included [55].

Furthermore, it was observed that for the *IL-1RN* polymorphism (VNTR) the population was not in Hardy-Weinberg equilibrium. Therefore, this polymorphism should be tested in a similar population to understand the possible cause of this finding.

8.3 Conclusions

Based on the results obtained in this study, it can be concluded that there was no correlation between *IL-1A* -889, *IL-1B* +3954 and *IL-1RN* polymorphisms and peri-implantitis, on the studied population. Therefore, our hypothesis that individuals carrying the T allele in the *IL-1A* -889 and *IL-1B* +3954 genes, or the less frequent alleles in the *IL-1RN* gene (VNTR), were more susceptible to developing peri-implantitis in comparison with individuals who did not present these polymorphisms, it was not observed. On the other hand, it was observed that the prevalence of these polymorphisms in the population evaluated was lower than in other European countries. This situation may hypothetically explain the absence of differences between groups regarding the role of these polymorphisms in the occurrence of peri-implantitis. This situation emphasizes how important it is to develop this type of studies in different ethnic groups.

It was observed that individuals with *IL-1RN* gene polymorphism, regardless of peri-implant health status, presented higher levels of TNF- α and PGE2 in PICF, as compared with patients without the polymorphism, which is believed to place them at a higher risk for peri-implant inflammatory complications. Thus, our second hypothesis that in both healthy individuals and patients with peri-implantitis, an altered genotype of *IL-1A*, *IL-1B* or *IL-1RN* would result in higher levels of pro-inflammatory mediators (IL-1 β , PGE2 and TNF- α) compared to patients without the altered genotype, was verified for the *IL-1RN* gene. It is reasonable to suppose that increased levels of TNF- α and PGE2 in the peri-implant tissues would result in a more aggressive destruction of both bone and connective tissue in response to the bacterial challenge. These individuals have not yet developed the disease but may be at greater risk for its development. Since peri-implantitis is a multifactorial disease, it is understood that a single factor may not be sufficient for its development. Among the patients

with the positive composite genotype, it was observed that those with peri-implantitis had a greater volume of PICF and a higher concentration of PGE2.

8.4 Future Directions

The number of studies that evaluated the role of genetic factors, such as the studied polymorphisms, in the development of peri-implantitis is quite limited, and some of them do not refer to variables that may impact/influence the results obtained (confounding variables), such as the periodontal status of individuals in the sample, their ethnicity and the presence or not of smoking habits. In this way, an analysis of the variables that may impact/influence the results is essential. There should also be uniformity regarding the definition of peri-implantitis used in the studies. The Hardy-Weinberg equilibrium must be calculated, which often does not happen in studies. Thus, and due to the inequalities found between studies already published in the literature, in order to have a better understanding of this possible association, it is necessary to create uniform criteria between future studies. Optimal sample size and statistical power should be calculated. Studies with larger samples in different ethnic groups are needed to determine the real effect of genetic polymorphisms on this disease. Studies with larger samples, mainly those that relate the genetic part with the peri-implant crevicular immune response are necessary. Longitudinal studies should be preferred because, although the genetic profile does not vary, biomarkers are not necessarily present in a single moment of PICF collection, due to several systemic or local factors. In addition, longitudinal studies allow for better control of risk factors for the disease. On the other hand, standardization of PICF sampling is relevant due to the atypical morphology of the implant prosthesis. The insertion of different paper strips, cones, membranes or other devices would be technique sensitive and may offer misleading results.

It would also be important to carry out more studies that try to relate genetic alterations with the consequences at the biochemical level in patients with dental implants. Since peri-implantitis is usually latent in early stages, cytokine analysis in the PICF of susceptible individuals may serve as a valuable analytical tool. Active components and mechanisms involved in the destructive process may

thus be important perspectives within this field. Such knowledge may potentially lead to new diagnostic strategies and candidate disease markers for peri-implant conditions.

8.5 References

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9. ANNEXES

Annex 1. Ethical clearance from Egas Moniz School of Health and Science Committee

Comissão de Ética EGAS MONIZ



Proc. Interno nº 790
ADENDA

Ex.mo Senhor
José Maria C. G. Neves Cardoso

Monte de Caparica, 24 de julho de 2019.

Ex.mo Senhor,

Em resposta ao Pedido de Parecer que submeteu à apreciação da Comissão de Ética da Egas Moniz, com o tema denominado **“Associação dos Polimorfismos dos genes da IL-1 e do IL-1RA com a peri-implantite”**, foi aprovado.

Com os melhores cumprimentos,

A Presidente da Comissão de Ética da Egas Moniz

Profª. Doutora Maria Fernanda de Mesquita

Annex 2. Permission to use the diagram in figure 1.1

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Title	Associação dos polimorfismos da IL-1 e do IL-1RA com a peri-implantite	Portions	Figure 1
Institution name	Egas Moniz School of Health and Science		
Expected presentation date	Nov 2023		

Annex 3. Copyright Permission granted for “Association between *IL-1A*, *IL-1B* and *IL-1RN* Polymorphisms and Peri-Implantitis: A Systematic Review and Meta-Analysis” and for “The Influence of Genetic Polymorphisms on the Expression of Interleukin-1beta, Prostaglandin E2 and Tumor Necrosis Factor Alpha in Peri-Implant Crevicular Fluid: A Cross-Sectional Study”.

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