SINGLE NUCLEOTIDE POLYMORPHISM OF PROSTAGLANDIN-ENDOPEROXIDE SYNTHASE (*PTGS2*) AND DEFENSIN BETA 1 (*DEFB1*) GENES IN MALAY CHRONIC PERIODONTITIS SUBJECTS.

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JASMIN KAUR A/P JAGENDER SINGH

FACULTY OF DENTISTRY
UNIVERSITY OF MALAYA
KUALA LUMPUR

2020

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JASMIN KAUR A/P JAGENDER SINGH

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FACULTY OF DENTISTRY
UNIVERSITY OF MALAYA
KUALA LUMPUR

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Field of Study: Genetics & Molecular Biology

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SINGLE NUCLEOTIDE POLYMORPHISM OF PROSTAGLANDIN-ENDOPEROXIDE SYNTHASE (*PTGS2*) AND DEFENSIN BETA 1 (*DEFB1)* GENES IN MALAY CHRONIC PERIODONTITIS SUBJECTS

ABSTRACT

The association of prostaglandin-endoperoxide synthase 2 (*PTGS2*) and defensin beta 1 (*DEFB1*) single nucleotide polymorphisms (SNP) with chronic periodontitis (CP) have been widely studied amongst different ethnic groups such as the Chinese and European population. Thus far, no study has been done on the Malay ethnic group. Hence, this study assessed the allele and genotype frequencies of *PTGS2* and *DEFB1* variants in subjects with CP and periodontally healthy individuals in Malaysian Malays. Samples of 78 Malay CP subjects and 62 periodontally healthy controls were obtained from Malaysian Periodontal Database and Biobanking system (MPDBS) for this study. DNA samples were genotyped for 3 candidate SNPs from *PTGS2* (rs5275, rs20417, rs689466) and 1 from *DEFB1* (rs1047031) using TaqMan SNP genotyping assays. The rs20417 and rs689466 are SNPs in the promoter region while rs5275 and rs1047031 are located in the 3′ untranslated region of the transcript. The allelic and genotypic associations between the SNPs and CP were investigated. We did not find any significant associations between these SNPs and CP risk ($p>0.05$) in the Malay ethnic group. No association was found in this study and this might be due to the small sample size and hence further study using larger sample size is required to confirm our findings.

Keywords: *PTGS2, DEFB1*, cytokines, Defensin, single nucleotide polymorphism, chronic periodontitis, allele, gene, genotyping

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POLIMORFISME NUKLEOTIDA TUNGGAL PROSTAGLANDIN-ENDOPEROXIDE SYNTHASE (*PTGS2*) DAN DEFENSIN BETA 1 (*DEFB1*) GEN DALAM SUBJEK PERIODONTITIS KRONIK MELAYU

ABSTRAK

Hubungan Prostaglandin-endoperoxide Synthase *(PTGS2*) dan Defensin Beta 1 *(DEFB1*) polimorfisme nukleotida tunggal (SNP) dengan peridontitis kronik (CP) telah dikaji secara meluas di kalangan etnik yang berbeza seperti penduduk China dan Eropah. Sehingga kini, tiada kajian telah dilakukan terhadap kumpulan etnik Melayu. Oleh itu, kajian ini menilai frekuensi alel dan genotip dari varian *PTGS2* dan *DEFB1* dalam subjek peridontitis kronik Melayu dengan subjek peridontik Melayu sihat sebagai kawalan. Subjek CP Melayu dan kawalan periodontik sihat diperolehi dari Pangkalan Data dan biobank Periodontal Malaysia (MPDBS) untuk kajian kawalan kes ini. Sampel DNA digenotip untuk 3 kandidat SNP, rs689466, rs5275, rs20417 *(PTGS2)* dan rs1047031 (*DEFB1*). Genotaiping dilakukan menggunakan kaedah genotaiping TaqMan di Malaysia. Hubungan antara SNP dan kumpulan kajian dinilai dengan menggunakan analisis regresi logistik.Sampel DNA dari 140 individu, 78 kes CP dan 62 kawalan sihat the digenotip. Kandidat SNP rs20417 dan rs689466 berada di kawasan promoter sementara rs5275 dan rs1047031 terletak di kawasan transkrip 3' yang tidak diterjemahkan. Perkaitan alelik dan genotip antara SNP dan CP disiasat. Tiada hubungan yang signifikan antara risiko SNP dan CP dalam kumpulan etnik Melayu ditemui ($p > 0.05$). Tidak hubungan yang signifikan antara risiko SNP dan CP yang dijumpai dalam kajian ini dan ini mungkin disebabkan oleh sampel yang kecil dan oleh itu kajian lebih lanjut menggunakan sampel yang lebih besar diperlukan untuk mengesahkan penemuan kami.

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Kata kunci: *PTGS2, DEFB1*, Cytokines, Defensin, polimorfisme nukleotida tunggal, periodontitis kronik, alel, gen, penapisan

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TABLE OF CONTENTS

Abstract....................iii
Abstrak....................iv
Acknowledgements....................vi
Table of Contents....................vii
List of Figures....................x
List of Tables....................xi
List of Symbols and Abbreviations....................xii
List of Appendices....................xv

CHAPTER 1: INTRODUCTION....................1
1.1 Background of Study....................1
1.2 Aim of Study....................3
1.3 Objective of Study....................3
1.4 Hypothesis....................4
1.4.1 Null Hypothesis....................4
1.4.2 Alternative Hypothesis....................4

CHAPTER 2: LITERATURE REVIEW....................5
2.1 Periodontal Disease....................5
2.1.1 Classification of Periodontal Disease....................6
2.1.2 Case Definition....................10
2.1.3 Chronic Periodontitis....................10
2.1.3.1 Case definition for CP by American Academy of Periodontology (AAP, 1999)....................10
2.1.3.2 Case Definition for CP by Eke....................10
2.1.4 Aggressive Periodontitis....................12
2.2 Epidemiology of Periodontal Disease....................13
2.3 Aetiology of Periodontal Disease....................14
2.4 Pathogenesis of Periodontitis....................14
2.4.1 Innate Immune response....................15
2.4.2 Adaptive Immune Response....................16
2.5 Risk Factors for Periodontitis....................16
2.5.1 Modifiable Risk Factors....................17
2.5.1.1 Smoking....................17
2.5.1.2 Diabetes....................17
2.5.1.3 Stress....................18
2.5.1.4 Obesity....................18
2.5.2 Non-modifiable Risk Factor....................19
2.5.2.1 Genetics....................19
2.6 Single Nucleotide Polymorphisms (SNPs)....................19
2.7 Genetic Studies in Periodontitis....................20
2.7.1 Familial Aggregation Studies....................20
2.7.2 Twin Studies....................20
2.7.3 Candidate Gene Association Studies....................21
2.7.4 Genome Wide Association Studies and Next Generation Sequencing....................23
2.7.5 Validated Candidate Genes for Periodontitis in a Very Large Sample Size....................25

Universiti Malaya

2.8 Candidate Genes for Periodontal Disease 28
2.8.1 Prostaglandin-endoperoxide Synthase *(PTGS2)* 28
2.8.1.1 Role of PTGS2 in Pathogenesis of Periodontitis 28
2.8.1.2 Selection of *PTGS2* SNPs Associated with CP 29
2.8.2 Beta-Defensin 30
2.8.2.1 Role of *DEFB1* in Pathogenesis of Periodontitis 32
2.8.2.2 Selection of *DEFB1* SNPs Associated with CP 33

CHAPTER 3: MATERIALS AND METHOD 35
3.1 Study Subjects and Clinical Characteristics 35
3.2 Data Collection Procedure 37
3.2.1 Sociodemographic Data 37
3.2.2 Clinical Periodontal Parameters 38
3.2.2.1 Visible Plaque Index (VPI). (Ainamo & Bay, 1975) 38
3.2.2.2 Gingival Bleeding Index (GBI). (Ainamo & Bay, 1975). 38
3.2.2.3 Probing Pocket Depth (PPD). 39
3.2.2.4 Clinical Attachment Level (CAL) 39
3.3 Blood Sample Collection 39
3.4 Selection of SNPs 40
3.5 Laboratory Work 43
3.5.1 Genomic DNA Extraction and Quantification 43
3.5.2 TaqMan® SNP Genotyping Method 45
3.6 Data and Statistical Analysis 48
3.7 Allele frequency, Genotype Frequency and Model Association Test Analysis 48
3.8 Experiment Summary 50

CHAPTER 4: RESULTS 51
4.1 Demographic Characteristics and Clinical Data 51
4.2 Comparisons in Periodontal Parameters Between Case and Control Groups 53
4.3 Genomic DNA Extraction 54
4.4 Interpretation of TaqMan SNP Genotyping Result 55
4.5 Quality Control for Results 57
4.6 Single Nucleotide Polymorphism 58
4.6.1 Genotype Frequency Analysis for SNPs 58
4.6.2 Allele Frequency Analysis for SNPs 59
4.7 Association Study Between the *PTGS2* and *DEFB1 SNPs* and CP 59

CHAPTER 5: DISCUSSION 61
5.1 Discussion of Materials and Methods 61
5.1.1 Study Subjects 61
5.1.2 Case Definition 62
5.1.3 Selection of *PTGS2* and *DEFB1* SNPs 63
5.1.4 TaqMan SNP Genotyping Method 63
5.2 Discussion of Results 64
5.2.1 Characteristics of Study Population 64
5.2.2 Periodontal Parameters 65
5.3 Single Nucleotide Polymorphism (SNP) 65
5.3.1 Genotypes and Allele Frequencies for *PTGS2* gene 66
5.3.2 Genotypes and Allele Frequencies for *DEFB1* Gene 68
5.4 Strengths and Limitations of Study 69
5.4.1 Strengths 69

Universiti Malaya

5.4.2 Limitations 69

CHAPTER 6: CONCLUSION AND FUTURE RECOMMENDATIONS 72

References 73

Appendix 88

Universiti Malaya

LIST OF FIGURES

Figure 2.1: Healthy gums vs diseased gums (periodontitis) 6

Figure 2.2: Distribution of *DEFB1* in periodontal tissue 33

Figure 3.1: DNA extraction procedure flow chart 44

Figure 3.2: TaqMan SNP genotyping assay procedure.. 47

Figure 3.3: Flow chart for experiment 50

Figure 4.1: A representive graphical output of a SNP (rs1047031) detected by applied biosystem 7500 real-time PCR system. 56

Universiti Malaya

LIST OF TABLES

Table 2.1: Classification system for periodontal disease by AAP from 1977–1989 8

Table 2.2: Abbreviated version of the 1999 classification of periodontal diseases and conditions. (Adapted from Armitage, 1999) 9

Table 2.3: Case definition for severe, moderate and mild CP (Eke *et al.,* 2012)............ 12

Table 2.4: Percentage of Chonic Periodontitis status amongst Malaysians based on age group (Mohd-Dom *et al*., 2013) 13

Table 2.5: Validated candidate genes for periodontitis in a very large sample size 27

Table 3.1: Summary of SNPs examined in this study.. 41

Table 3.2: *PTGS2* and *DEFB1* SNP rs number, TaqMan probes and context sequences of the SNPs …………………………………………………………………………..42

Table 3.3: Calculation table for working solution for genotyping................................ 46

Table 4.1: Demographic characteristics and social habits of the study group 52

Table 4.2: Comparison of VPI, GBI, PPD and CAL among control and CP groups 54

Table 4.3: Genotyping call rates and HWE for case and control group 57

Table 4.4: Genotype distribution and frequency analysis of SNPs rs5275, rs20417, rs689466 and *rs1047031* in CP and control group...................................... 58

Table 4.5: Allele frequencies of rs5275, rs20417, rs689466 and rs1047031 and association analysis with chronic periodontitis 60

Table 4.6: Association study of SNPs in *PTGS2* and *DEFB1* genes and CP 60

Universiti Malaya

LIST OF SYMBOLS AND ABBREVIATIONS

Symbol		Description
α	:	Alpha
β	:	Beta
>	:	Greater than
<	:	Lesser than
g	:	Gram
µL	:	Microliter
ng	:	Nanogram
%	:	Percentage
ºC	:	Degree Celsius
ml	:	Milliliter
mm	:	Millimeter
µg	:	Microgram
N	:	Number of sample
p	:	p-value
rpm	:	Rotation per minute
3’	:	3-prime
5’	:	5-prime
A	:	Adenine
AgP	:	Aggressive Periodontotitis
bp	:	Basepair
C	:	Cysteine
CAL	:	Clinical attachment loss
CP	:	Chronic periodontitis
DEFB	:	β-defensins

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DNA	:	Deoxyribonucleic acid
EDTA	:	Ethylenediaminetetraacetic acid
G	:	Guanine
GBI	:	Gingival bleeding index
GWAS	:	Genome-wide association studies
hBDs	:	Human β-defensins
HWE	:	Hardy-Weinberg equilibrium
IL	:	Interleukin
IL1	:	interleukin-1
IL6	:	interleukin-6
MAF	:	Minor allele frequency
MPDBS	:	Malaysian Periodontal Biobank and Database System
OR	:	Odds ratio
PCR	:	Polymerase chain reaction
PGs	:	Prostaglandins
PPD	:	Probing pocket depth
PTGS	:	Prostaglandin endoperoxide synthetase
SD	:	Standard deviation
SNP	:	Single nucleotide polymorphism
T	:	Thymine
TNF-α	:	Tumor necrosis factor-α
VBI	:	Visible plaque index
WHO	:	World Health Organisation
3'UTR 3'	:	Untranslated region
95% CI	:	95% confidence interval

Universiti Malaya

Egr-1	:	Human early growth response factor-1
PMNs	:	Polymorphonuclear neutrophils

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LIST OF APPENDICES

Appendix A. Ethics Approval from UM 88

Appendix B. Ethics Approval from NMRR 89

Appendix C. Questionnaire form 90

Universiti Malaya

CHAPTER 1: INTRODUCTION

1.1 Background of Study

Periodontitis results in the loss of connective tissue and alveolar bone support of the teeth. Severe chronic periodontitis (CP) has been reported to have a prevalence of about 8% of the world population, with about 40% suffering from mild to moderate forms of periodontitis (Albandar *et al.,* 1999; Eke *et al.*, 2012). According to World Health Organization (WHO) (2007), human populations worldwide are affected at prevalence rates up to 30-35% for the more severe forms of periodontal disease. Moreover, it is the major cause of tooth loss in adults above 40 years (Schaefer *et al.,* 2010a).

Most of the previous studies (epidemiological & microbiological) have suggested that a subgingival biofilm also known dental plaque is necessary but not sufficient to cause periodontal disease and it is the host response that drives the disease process (Armitage, 1999; Laine *et al.,* 2012; Offenbacher *et al.,* 2008; Bartold *et al.*, 2013). The hyper and hypo-responsiveness of the immune system towards the microbial challenge CP have been said to be the main reason for the progression of CP. Immune response will be triggered upon attack of periodontopathic bacteria on the host, thus activating the inflammatory process that may slowly cause damage to the host (alveolar bone support and connective tissue). However, the periodontal destruction may not be caused by the interaction between microorganisms and host responses alone. Therefore, every individual carry an individual dose-dependent response towards the microbial challenge, which will then determine their susceptibility to periodontitis. Thus, host susceptibility is essentially the summation of genetic and lifestyle factors, which in turn, determines disease severity (Kinane *et al*, 2007; Laine *et al*., 2010).

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Prostaglandin endoperoxide synthetase 2 (PTGS2) plays an important role as the main enzyme in converting arachidonic acid to prostaglandins (PGs) which involved in inflammation progression (Noguchi & Ishikawa, 2007). Defensins can be defined as proteins produced by the host defense system, which assists in maintaining the balance between healthy and/or disease state in the oral cavity. Defensins have antimicrobial activity against pathogenic bacteria (Schaefer *et al.,* 2010b).

Genetic variations, such as single nucleotide polymorphisms (SNPs) have been suggested to be related with increased periodontal disease severity or/and also susceptibility (Loos *et al*., 2008; Laine *et al*., 2010). SNPs can be defined as DNA sequence variations that is caused by the alteration of a single nucleotide which occur in appreciable frequency (>1%) in the population that may alter gene function or gene expression, therefore altering individuals' susceptibility to disease including CP. It is important in identifying susceptibility genes to CP in order to improve the understanding of the biological and etiological mechanism involved in the progression of CP. Notably, there is a definite difference between the allelic and genotype frequency of CP associated SNPs, between different population such as Caucasians and Asians. Genetic and environmental heterogeneity may cause the genotype to vary between different ethnicities and population (Schaefer *et al.,* 2010a; Xie *et al.,* 2009; Ho *et al.,* 2008). This may lead to inconclusive results from published papers. Thus, it is necessary to investigate the association between SNPs in candidate genes with CP susceptibility in different ethnicities and population.

Schaefer *et al*. (2013) carried out a large-scale replication study of 23 genes that have been reported from previous studies. They then concluded that *GLT6D1, ANRIL, IL-10*, *PTGS2* and *DEFB1* genes were considered to carry validated susceptibility variants for periodontitis.

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Among these susceptibility variants for periodontitis, 2 genes have been reported to show clear evidence of association with CP susceptibility which are *PTGS2* (Schaefer *et al.,* 2010a; Xie *et al.,* 2009; Ho *et al.,* 2008) and *DEFB1* (Schaefer *et al.,* 2010b). Among these 2 genes, *PTGS2* gene polymorphism which is involved in immune response and inflammation regulation was reported to be the only gene reported to show association in an Asian CP population (Xie *et al.,* 2009) but has not been examined in the Malay population. Meanwhile, *DEFB1* gene polymorphism which is involved in antimicrobial and immunomodulatory activity has been reported to show association in a population of European CP subjects (Schaefer *et al.*, 2010b), but no studies have assessed in any Asian CP population.

Thus, this study aims to use the candidate gene approach to examine *PTGS2* SNPs rs5275 (8473T>C), rs20417 (-765G>C), rs689466 (-1195G>A) and *DEFB1* SNPs rs1047031 (c*5G>A) and rs2293958 (61 + 19T>A) for their associations with CP susceptibility in the Malaysian Malay ethnic group.

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1.2 Aim of Study

The aim of this study is to investigate the presence of *PTGS2* and *DEFB1* SNPs in Malaysian Malay CP subjects and to assess its association with CP susceptibility in these subjects.

1.3 Objective of Study

1. To determine the allele and genotype frequencies of *PTGS2* SNPs rs689466, rs5275, rs20417 and *DEFB1* SNPs rs1047031 and rs2293958 in case group with CP as compared to healthy controls.

2. To investigate the association between *PTGS2* SNPs rs689466, rs5275, rs20417 and *DEFB1* SNPs rs1047031 and rs2293958 in Malaysian Malay subjects with CP.

1.4 Hypothesis

1.4.1 Null Hypothesis

There is NO association between *PTGS2* and *DEFB1* and its genetic variants with periodontal disease in Malaysian Malay subjects with CP.

1.4.2 Alternative Hypothesis

There is association between *PTGS2* and *DEFB1* and its genetic variants with periodontal disease in Malaysian Malay subjects with CP.

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CHAPTER 2: LITERATURE REVIEW

2.1 Periodontal Disease

Periodontal disease is defined as a chronic inflammatory disease of the periodontal structures which comprises the gingiva, cementum, alveolar bone and periodontal ligament (Figure 2.1). It ranges from gingivitis to chronic and aggressive forms of periodontitis (Armitage, 1999). If left untreated, the disease results in irreversible attachment loss and bone destruction that finally leads to tooth loss (Philstrom *et al.*, 2005). The two major forms of periodontal disease are gingivitis when it involves only the gingiva, and periodontitis when the destruction progresses into the alveolar bone, periodontal ligament and cementum (Armitage, 1999).

CP is a multifactorial chronic inflammation disease of the tissues supporting the tooth and the alveolar bone (Daing *et al.,* 2012). CP is a slow progressive disease. CP can happen in most age groups but is most prevalent among older generation (>35 years old) worldwide (Page & Eke, 2007). WHO previously reported that 10–15% of the world populations have severe form periodontitis (Peterson & Ogawa, 2005).

The main aetiology of periodontal disease is the dental biofilm (plaque) (Löe *et al.*, 1965). The bacterial plaque (biofilm) contains complex communities of bacteria and which are commonly found in the human body (Gurenlian, 2007). Biofilms form on almost all surfaces immersed in the oral cavity (Chandki *et al.*, 2011). The biofilm will boost the pathogenic bacteria's resistance towards the hosts immune system and antimicrobials. Thus, if the dental biofilm is not regularly removed, it will become more mature and form a complex pathogenic

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bacterial microflora. This will contribute to the aetiology in gingivitis, periodontitis and dental caries (Gurenlian, 2007).

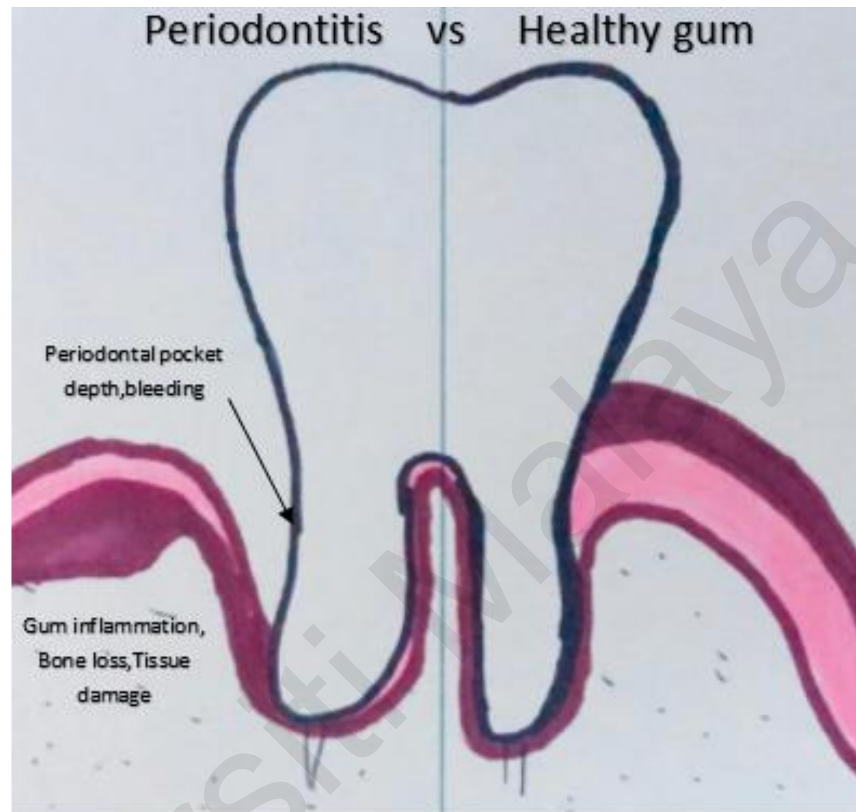

Figure 2.1: Healthy gums vs diseased gums (periodontitis)

2.1.1 Classification of Periodontal Disease

In the past few decades, there have been numerous classification systems for periodontal disease. The American Academy of Periodontology (AAP) classification system reported 2 main periodontal disease categories in the year 1977 which were juvenile periodontitis and chronic marginal periodontitis (The American Academy of Periodontology, 1989). In 1986, this was then modified to four periodontal disease categories which were juvenile periodontitis, adult periodontitis, necrotizing ulcerative periodontitis and refractory

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periodontitis (The American Academy of Periodontology, 1989). In the year 1989, the AAP classification system went from 2 main categories in 1977 to 5 categories which included early onset periodontitis, adult periodontitis, necrotizing ulcerative periodontitis, refractory periodontitis and periodontitis associated with systemic disease (Table 2.1) (The American Academy of Periodontology, 1989). The changes in the classification system from 1977 to 1989 were due to some unclear classification criteria and also difficulty in fitting certain patients into any of the existing categories (International Workshop for a Classification of Periodontal Diseases and Condition, 1999).

Compared to previous classifications, the 1989 periodontal disease classification was a major improvement. In this classification, a new category was introduced on the effect of systemic disease on periodontal health was added. In addition, more criteria for early-onset diseases were also added in this classification.

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Table 2.1: Classification system for periodontal disease by AAP from 1977–1989

1977	1986	1989
I. Juvenile periodontitis Chronic marginal periodontitis	I. Juvenile periodontitis A. Prepubertal B. Localized juvenile periodontitis C. Generalized juvenile periodontitis	I. Early-onset periodontitis A. Prepubertal periodontitis 1. Localized 2. Generalized B. Juvenile periodontitis 1. Localized 2. Generalized C. Rapidly progressive periodontitis
	II. Adult periodontitis	II. Adult periodontitis
	III. Necrotizing ulcerative gingivo-periodontitis	III. Necrotizing ulcerative periodontitis
	IV. Refractory periodontitis	IV. Refractory periodontitis
		V. Periodontitis associated with systemic disease

In 1999, there was a new periodontal disease classification system introduced by the 1999 International Workshop for a Classification of Periodontal Disease and Conditions (Table 2.2) which was also accepted by the AAP. This classification had 8 main subcategories including gingival diseases and various forms of periodontitis. However, the most common forms of periodontitis are CP and aggressive periodontitis (AgP) (American Academy of Periodontology, 2015).

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Table 2.2: Abbreviated version of the 1999 classification of periodontal diseases and conditions. (Adapted from Armitage, 1999)

A. Gingival Diseases i. Dental plaque-induced gingival diseases ii. Non-plaque-induced gingival lesions
B. Destructive Periodontal Disease i. Chronic Periodontitis ii. Aggressive Periodontitis iii. Periodontitis as a Manifestation of Systemic Diseases iv. Necrotizing Periodontal Diseases a. Necrotizing ulcerative gingivitis b. Necrotizing ulcerative periodontitis v. Abscesses of the Periodontium vi. Periodontitis Associated with Endodontic Lesions vii. Developmental or Acquired Deformities and Conditions

Recently, a new classification of Periodontal and Peri-implant Diseases and Conditions have been adopted by the American Academy of Periodontology (AAP) and European Federation of Periodontology (EFP) (Caton *et al.,* 2018). Based on to this classification, the two forms of periodontitis which were known as CP and AgP are now under the same category, which is “periodontitis”. Besides that, periodontitis can be further categorised and characterised based on staging and grading system according to this 2017 classification (Caton *et al.,* 2018). Classification of periodontitis based on stages are defined by the disease’s severity and complexity of disease management as well as its extent and distribution ranging from stage I to IV**,** which represent an increased severity and complexity. The grading system is used to reflect the features of disease progression, which includes risk of progression, treatment response and its effects on systemic health. In the grading system, each stage of extent and distribution of periodontitis can be classified based on the number of diseased sites involved. It is considered as localized periodontitis if it affects <30% of total sites and generalized if >30% of sites are involved (Caton *et al.,* 2018).

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2.1.2 Case Definition

In population-based surveillance of the disease, a standard case definition of a disease is an essential requirement. Various definitions for periodontitis have been used previously in the literature for population-based studies, however there is no specific accepted standard. Although the recent classification for periodontal diseases (Caton *et al.,* 2018) was launched in 2018, however in this review we will be covering case definitions of the AAP 1999 classification as all subjects were sampled using this case definition.

2.1.3 Chronic Periodontitis (CP)

The most common case definition for CP are the ones proposed by Armitage (1999) and Eke *et al.* (2012).

2.1.3.1 Case definition for CP by American Academy of Periodontology (AAP, 1999)

According to AAP (1999), the case definition for CP is characterized by extent and severity. Extent of CP can be defined as the percentage or number of sites affected (Brown & Loe, 1993). The extent of CP is classified as localized or generalized depending on whether less than 30% or more than 30% of sites are involved (International Workshop for a Classification of Periodontal Diseases and Conditions, 1999).

2.1.3.2 Case Definition for CP by Eke

In 2003, the Division of Oral Health at the Centre for Disease Control and Prevention (CDC) and AAP have cooperated in inspecting the practicability of, and to identify valid nonclinical measures for, population-based surveillance of periodontitis in developing the standardized

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clinical case definitions for population-based studies of periodontal disease (Page & Eke, 2007). They initially proposed case definitions for moderate and severe CP (Page & Eke, 2007) and subsequently proposed the case definition for mild CP (Eke *et al.*, 2012) (Table 2.4).

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Table 2.3: Case definition for severe, moderate and mild CP (Eke *et al.*, 2012).

Stage of CP	Age	Case definition
Mild/Early	≥35 years	• ≥ 2 interproximal sites with CAL ≥ 3 mm **OR** • ≥ 2 interproximal sites with PPD ≥ 4 mm (not on same tooth) **OR** • 1 site with PPD ≥ 5mm
Moderate	≥35 years	• ≥ 2 interproximal sites with CAL ≥ 4 mm (not on same tooth), **OR** • ≥ 2 interproximal sites with PPD ≥ 5mm (not on same tooth)
Severe	≥35 years	• ≥2 interproximal sites with CAL ≥6 mm (not on same tooth), **OR** • ≥1 interproximal site with PPD ≥ 5mm
CAL: clinical attachment loss; PPD: probing pocket depth		

2.1.4 Aggressive Periodontitis (AgP)

AgP is defined as a periodontal disease which affects systemically healthy young individuals (<30 years old). AgP is characterized by rapid loss of alveolar bone support surrounding the tooth. The rate of alveolar bone loss is not proportionate with the quantity of microbial deposits (Albandar, 2014).

In general, AgP affects systemically healthy individuals less than 30 years of age but patients may be older. AgP causes rapid destruction of the periodontal attachment apparatus and the supporting alveolar bone and is usually characterized in two forms which are localized or generalized. CP and AgP have a number of significant clinical differences including the age of onset, the rate of progression, composition of the subgingival microflora, alteration in the host immune response, familial aggregation of diseases in individuals, and a strong racial influence. Although its prevalence has been reported to be much lower than that of CP, it can result in early tooth loss in the affected individuals if not diagnosed in the early stages and

Universiti Malaya

treated appropriately (Prakasam *et al*., 2012; Susin & Albandar, 2005; Albandar & Rams, 2002).

2.2 Epidemiology of Periodontal Disease

Periodontal disease is one of the two major dental diseases that affects human populations at high prevalence rates in the world, the other being dental caries (Frencken *et al.,* 2017). WHO reported that 10–15% of the world population suffers from severe CP (Petersen & Ogawa., 2005). Other studies have shown a wide range in the prevalence of periodontitis with severe CP found in 8% of the total population, while 40% suffer from mild to moderate forms of CP (Albandar *et al*., 1999; Eke *et al*., 2012).

An epidemiological study conducted by Corbet and Leung (2011) comparing Asians and Caucasians concluded that Asians are particularly susceptible to periodontitis. In Malaysia, about 18% of the Malaysian population suffer from severe forms of periodontal disease (Oral Health Division, Ministry of Health Malaysia, 2012). Prevalence of CP in the age group of 35–44 have observed an increase from 1990 (31.9%), 2000 (35.7%) and 2010 (59.5%). For age group 65–74, prevalence declined between 1990 (40.6%) to 2000 (36.3%) but a rise was observed in the 2010 survey (54.8%) as seen in table 2.5 (Mohd-Dom *et al*., 2013).

Table 2.4: Percentage of Chonic Periodontitis status amongst Malaysians based on age group (Mohd-Dom *et al*., 2013)

Age Group	1990(%)	2000(%)	2010(%)
35-44	31.9	35.7	59.5
65-74	40.6	36.3	54.8

Universiti Malaya

2.3 Aetiology of Periodontal Disease

The search for aetiological agents of periodontal diseases has been conducted by many investigators as early as 1880–1920 during the golden era of medical bacteriology (Feres *et al.*, 2004). Periodontal Disease is a complex disease as there are various factors simultaneously play a role in the onset and disease progression (Laine *et al.*, 2012). However, the dental biofilm is the main aetiology for periodontal disease (Löe *et al.,* 1965).

Dental biofilm (plaque) is a structured, yellowish-greyish biofilm that accumulates on the hard surfaces of the oral cavity including teeth. With the gingival margin serving as an anatomical reference, dental plaque is located supra- or subgingivally. Subgingival plaque is a complex ecosystem containing vast numbers of micro-organisms and periodonto-pathogens. The accumulation of bacterial plaque on the tooth surface is enhanced by predisposing factors such as dental calculus (Mandel & Gaffar, 1986), overhanging restoration (Lang *et al.,* 1983; Pack *et al.,* 1990; Jansson *et al.*, 1994; Matthews & Tabesh, 2004), subgingival restorative margin (De Waal & Castellucci, 1994; Schatzle *et al.*, 2001), prescription of partial dentures (Behr *et al.,* 2012; Zlataric *et al.,* 2002; Ogunrinde *et al.*, 2014) as well as crowded teeth, bulbous crowns and grooves on teeth (Jensen & Solow, 1989). The interaction between plaque bacteria and host defense mechanisms plays an important role in the severity of tissue damage. The host response to bacterial plaque are altered by systemic or local modifying factors (Szkaradkiewicz & Karpiński, 2013; Kebschull & Papapanou, 2011).

2.4 Pathogenesis of Periodontitis

To maintain periodontal health, the response of the immune system to bacteria in the dental biofilm (plaque) is an essential process. The response begins with pathogen recognition,

Universiti Malaya

followed by activation of innate and adaptive immunity and concludes with healing process (Löe *et al.*, 1965; Ishikawa, 2007).

Page and Schroeder (1976) have described periodontitis in stages which are, the initial lesion; the established lesion; and the advanced lesion. The initial lesion shows the response of local leukocytes and endothelial cells to the bacterial plaque. No signs of clinical inflammation is observed at this stage. The subsequent stage is the established lesion. This stage shows the transition of the innate immune response to the acquired immune response. In this stage, macrophages, plasma cells, and T and B lymphocytes increases, together with IgG1 and IgG3 subclasses of B lymphocytes, leading to the formation of the advanced lesion. Thus, the alveolar bone loss can be observed clinically and histologically, and this loss cannot be reversed. If left untreated, the inflammatory lesion will spread deeper into the alveolar bone causing tooth loss (Fiorellini *et al*., 2005; Page & Schroeder, 1976).

2.4.1 Innate Immune response

The innate immune response involves a homeostatic system, which is the first line of defence against foreign bodies. The barrier effect of an intact epithelium is able to recognize attacking microorganisms as non-self, thus triggering immune responses to eliminate them (Ishikawa, 2007). The saliva protects the oral mucosa from pathogenic bacteria which contains a number of protective factors. Pattern recognition receptors (PRRs) distinguishes between the host and the bacteria (Rietschel & Brade, 1992) by identifying lipopolysaccharide (LPS) from gram-negative bacteria and peptidoglycan from gram-positive bacteria (Silva *et al.,* 2015). Cytokines will be produced upon activation of junctional epithelium cells and produce neuropeptides by neuron stimulation, resulting in vasodilatation of local blood vessels.

Universiti Malaya

Finally, in response to chemokines, the neutrophils will travel towards the site of inflammation (Ishikawa, 2007).

2.4.2 Adaptive Immune Response

If the innate immune response fails to eliminate the stimuli, the adaptive immune system will be triggered and releases macrophages and dendritic cells (Ishikawa, 2007). The association between the innate and adaptive immune response is coordinated by cytokines like interleukin (IL-12, IL-18 and Interferon gamma (INF-γ) (Gemmell & Seymour, 2004; Tew *et al.,* 2011). As the disease progresses, the tissue experiences continuous stress from the pro-inflammatory molecules produced by the immune cells leading to a chronic inflammatory state if the condition continues to persist. Thus, IL-8 (Ekhlassi *et al.,* 2008; Ara *et al.,* 2009) and monocyte chemotactic protein 1 (MCP-1) (Gupta *et al.,* 2013) will attract more neutrophils and macrophages to the inflamed site. Like the other immune cells, neutrophils secrete inflammatory mediators such as IL-1β, tumour necrosis factor alpha (TNF-α), defensins, prostaglandins and matrix metalloproteinases (MMPs) (Zhang *et al.,* 2002).

2.5 Risk Factors for Periodontitis

As CP is multifactorial disease, it is important to have a clear understanding on associated risk factors. It is well well-known that periodontal disease is caused by pathogenic bacteria located in dental biofilm (dental plaque). However, the interaction of multiple different gene loci, as well as behavioral and environmental factors plays an important role in CP progression (Chai *et al.,* 2010; Kinane *et al.,* 2007). Risk factors can be divided into two categories which are modifiable or non-modifiable. Modifiable risk factors are usually environmental or lifestyle factors that can be altered while non-modifiable risk factors are

Universiti Malaya

typically intrinsic to the particular individual and thus may not easily be changed (Van Dyke & Sheilesh, 2005).

2.5.1 Modifiable Risk Factors

2.5.1.1 Smoking

Smoking is recognized as a major risk factor towards the progression of periodontal disease (Bergström & Preber, 1994; Grossi *et al*., 1994; Al-Bayaty *et al.,* 2008). Grossi *et al*. (1997) reported that smokers exhibited greater clinical attachment loss (CAL) and radiographic bone loss than non-smokers. Smoking affects the pathogenesis of periodontitis by changing the host response, changing the periodontitis patterns that will indirectly affect periodontal healing and treatment outcomes (Barbour *et al.,* 1997; Palmer *et al.,* 2005) the toxic substances emitted from the tobacco smoke such as nicotine is able to coat the root surfaces of diseased teeth, resulting in interference with post-surgical healing (Arffin *et al.,* 2012).

2.5.1.2 Diabetes

Diabetes is categorized as type 1 and type 2 diabetes mellitus (DM). Type 1 DM develops due to impaired production of insulin, while type 2 DM is caused by deficient utilization of insulin (Mealey & Ocampo, 2006). Oral symptoms of diabetes includes burning mouth, xerostomia, altered taste sensation, candidiasis and periodontitis (Mealey & Ocampo, 2007).

A study conducted on the Pima Indian population, which has reported to have exceptionally high prevalence of Type 2 DM, showed higher prevalence of either periodontal attachment loss or radiographic bone loss. This indicates that diabetes is a risk factor for periodontal disease (Emrich *et al.,* 1991; Schlossman *et al*., 1990). Diabetes is reported to affects all these

Universiti Malaya

periodontal parameters which includes gingival bleeding, probing depth, and attachment loss (Nassar *et al.,* 2007). Previous studies have also reported that there is a two-way relationship between diabetes mellitus and periodontal disease (Grossi & Genco, 1998; Lalla *et al.,* 2000; Lalla *et al*., 2001; Mealey & Ocampo, 2007).

2.5.1.3 Stress

The association of psychological stress is related with poor oral hygiene, increased glucocorticoid secretion that depresses the immune function, increased insulin resistance, and potentially increased risk of periodontitis. Patients with increased stress are at higher risk to get severe periodontal disease (Genco *et al.,* 1998; Kinane *et al*., 2006; Hugoson *et al*., 2002). Studies have demonstrated that some periodontal disease indicators such as tooth loss and gingival bleeding are associated with work stress and financial strains (Genco *et al.,* 1998). Hugoson *et al*. (2002) suggested that emotional stress due to bereavement may increase the risk of CP.

2.5.1.4 Obesity

Obesity is a condition whereby there is excessive deposition of fat in the adipose tissue. Obesity does not only result in adverse metabolic effects on one's health, but it can also increase oxidative stress, leading to endothelial dysfunction and increase in pro-inflammatory cytokines, which is related to periodontitis. Thus, obesity appears to be one of the main risk factors of periodontitis. In inflammatory processes, the adipose tissue secretes a variety of cytokines and hormones. This shows similar pathways involved in pathogenesis of periodontitis, however, the causal relationship between obesity and periodontitis is yet to be established (Jagannathachary & Kamaraj, 2010).

Universiti Malaya

2.5.2 Non-modifiable Risk Factor

2.5.2.1 Genetics

Researchers have proposed that genetic risk factors plays a role in progression of periodontitis (Kinane *et al.,* 2007; Loos *et al.,* 2008; Laine *et al.,* 2010). Michalowicz *et al.* (1991) conducted classic twin studies which suggests that genetic factors might explain variance in periodontal disease progression in approximately 50% of the population (Michalowicz, 1994; Michalowicz *et al.,* 1991). Recently, many studies have been carried out on the identification of genetic polymorphisms as a risk factor of periodontal disease (Laine *et al.,* 2012; Zhang *et al.,* 2011; Schaefer *et al.,* 2010a; Schaefer *et al.,* 2010b; Xie *et al.,* 2009). These studies have focused on identifying specific single nucleotide polymorphisms (SNPs) as a risk factor for AgP and CP. Genetic diseases have been categorized into two groups which are simple mendelian diseases and complex diseases (Kinane & Hart, 2003).

2.6 Single Nucleotide Polymorphisms (SNPs)

SNPs are DNA sequence variations that occurs as a result from the alteration of a single nucleotide. Intronic SNPs does not result in amino-acid substitution but may alter gene function or gene expression in contrast with some exonic SNPs (Suzuki *et al.*, 2004). More than 10 million single-nucleotide polymorphisms (SNPs) have been identified in the human genome (Taba *et al.*, 2012).

A single SNP variation may contribute to a moderate or limited role in disease progression, however, it may also work together with other genetic variations or environmental factors to

Universiti Malaya

cause this disease progression (Chai *et al.*, 2010). Previous genetic research in oral disease have been concentrated on SNPs that are involved in the immune response or metabolic mechanism.

In certain situations, genetic polymorphisms can result in changes in the protein (amino acid) or its expression, that may result in alterations in innate and adaptive immunity that may lead to disease progression. Besides that, genetic polymorphisms may also play a role as a protector or a destructive factor in disease progression (Vaz *et al.*, 2012).

Researchers have suggested that SNP analysis will assist in detecting multiple genes that are associated with periodontitis, acting as a genomic marker for investigating the risk factors of periodontal disease (Suzuki *et al.*, 2004).

2.7 Genetic Studies in Periodontitis

2.7.1 Familial Aggregation Studies

Genetic aetiology is associated to familial aggregation of a trait or disease. Familial aggregation is caused by various factors such as environmental and socio-economic risk factors and most importantly, shared genes. Previous studies have reported familial aggregation for AgP, however, it was hard to compare the reports because of the differences in disease terminology, disease characterization and lack of standardized method for clinical examination (Marazita *et al.,* 1994).

2.7.2 Twin Studies

Commonly, monozygous (MZ) twins share 100% and dizygous (DZ) twins share an average of 50% of their genes. For MZ twins, environmental factors may be the cause of a disease

Universiti Malaya

while for DZ twins, environmental and genetic factors may play a role in the causation of disease. Twin studies are beneficial in detecting the different variations in disease which is caused by environmental or genetic factors. Michalowicz *et al.* (1991) conducted a study on 110 twin pairs (63 monozygotic and 33 dizygotic twin pairs) with CP. The results of this study reported that higher levels of alveolar bone loss were observed in monozygotic twins as compared to dizygotic twins. The heritability of 38% was estimated for alveolar bone loss among reared-apart monozygotic twins. However, twin studies are not reliable because of its limited scope and small sample size (Michalowicz *et al.,* 1991).

2.7.3 Candidate Gene Association Studies

Candidate gene association studies are hypothesis driven studies where a targeted candidate gene which is related to the mechanism of the disease trait which is being studied is analysed (Patnala *et al.*, 2013). SNPs of the candidate gene are assessed and selected if they have a role in the candidate gene regulation or protein product function (Kwon & Goate, 2000; Collins *et al.*, 1997). Verification of gene variants for trait (disease) association is done by observing the occurrence of the gene variant in diseased (cases) or healthy (control) groups. Thus, the candidate gene can be a potential biomarker which may be used as a diagnostic tool in the future treatment of genetic related diseases (Peters *et al.*, 2010).

Most of the current candidate gene studies for CP have been conducted on cytokines, chemokines, antigen recognition and cell-surface receptors (Vaz *et al.,* 2012). The most common studied candidate genes are interleukin (IL)-1, IL-6, Fc gamma receptor (FCGR2A), TNF-α, human vitamin D receptor (VDR), cluster of differentiation (CD)-14, matrix metalloproteinase-1, toll-like receptor (TLR), *PTGS2*, Defensins and C-reactive protein

Universiti Malaya

genes (Laine *et al.,* 2012; Zhang *et al.,* 2011). However, most of these candidate gene association studies have been conducted in recent years in periodontitis subjects with reports of contradictory results (Laine *et al.,* 2012; Zhang *et al.,* 2011).

The main limitation of candidate gene association studies is that they use a small sample size, resulting in lack of power and replication (Schaefer *et al.,* 2011a). Besides that, the complete genetic information of a specific region of interest is not able to be obtained when using candidate gene studies approach. This is because only a single or a few SNPs were genotyped.

Moreover, there are limited studies on periodontal genetics due to the insufficient phenotype classification for periodontal disease. As periodontitis is a multicausal disease, the clinical phenotype may be expressed in various severity and biological pathways. CP is known as a complex disease, thus having mild, moderate to severe variable phenotypes (Albandar *et al.,* 1999; Eke *et al.,*2012). Currently, there are different phenotype classification for CP (disease and control subjects), such as classifications defined by Armitage (1999), the Centers for Disease Control and Prevention and the American Academy of Periodontology (Eke *et al.*, 2012), the European Workshop in Periodontology (Tonetti, 2005) and the latest classification was introduced at the World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions (Caton *et al.,* 2018).

There is a definite difference between the allelic and genotype frequency between different population such as Caucasians and Asians. Different ethnic populations may show different genetic variants; however, it is said that many of the associated variants may also be shared between different ethnicities. The effects of these genetic variants may be large and of clinical significance, while the effects of others are probably minor and not clinically significant (Kwon & Goate, 2000; Collins *et al.,* 1997).

Universiti Malaya

In complex diseases, it is believed that the risk variant has a smaller contribution for the disease progression (McCarthy *et al.,* 2008). If the genetic variants are strong enough to produce clinical significance, it is useful in predicting the risk of the disease with combination with other risk factors (environmental or lifestyle). On the other hand, if the effects of the genetic variant are minor, it can still be used to determine a disease-associated gene or even genetic pathway (Need & Goldstein, 2010).

2.7.4 Genome Wide Association Studies and Next Generation Sequencing

In the past years, genome-wide association studies (GWAS) have been conducted in genetic studies. GWAS is a powerful technique in screening hundreds of thousands or even millions of genetic polymorphisms simultaneously and identify any locus that is associated with a certain disease phenotype. In contrast to candidate gene studies, GWAS approach is hypothesis free thus it is unbiased with respect to genomic structure for identifying disease-associated genetic variants (Laine *et al.,* 2014; Vaithilingam *et al*., 2014). Currently, there are very few GWAS that have been performed in association with CP and AgP (Schaefer *et al*., 2010c; Divaris *et al.,* 2013; Teumer *et al.,* 2013).

In 2009, the first GWAS for periodontitis was conducted on AgP. This study was conducted in Germany with 283 AgP subjects (cases) and 972 healthy controls and the Netherlands with 164 AgP subjects (cases) and 368 healthy controls. In this GWAS on 1758 subjects, rs1537415 reached a genome-wide significance level of P= 5.51 x 10(-9), OR = 1.59 (95% CI 1.36-1.86) (Schaefer *et al.,* 2010c). This was followed by a GWAS conducted in 2013 in USA on Americans of European ancestry, however they did not detect any specific gene or locus to be associated with CP (Divaris *et al.,* 2013). Subsequently, a GWAS on a German

Universiti Malaya

population also did not detect any gene to be associated with CP, however they confirmed that genetic factors play a role in the pathobiology of CP (Teumer *et al.,* 2013).

Most of the current published GWAS studies have small sample sizes with different disease phenotypes on periodontitis. Thus, there is very limited data in identifying potential low frequency variants in order to produce large genetic effects (Vaithilingam *et al.,* 2014). Besides that, environmental and lifestyle risk factors such as smoking, oral health, age play a huge role in determining CP expression or/and progression. These risk factors (non-genetic) may interact with genetic variants (gene–environment and gene–lifestyle interactions) while genetic variants may also react with each other (gene–gene interaction). These interrelated factors may be able to explain the difference between disease phenotypes among individuals and populations (Vaithilingam *et al.,* 2014).

Currently, limited success has been achieved by GWAS in finding genetic markers in periodontitis. This may be caused by the expression of a mild disease phenotype of CP, whereby there is a lack of statistical power to detect significant genetic variants which may be too low due to the stringent significance value threshold of ($P<5\times10^{-8}$), and also small sample size. Due to these limitations, GWAS is not able to detect weaker genetic variant associations, which may have true association to disease susceptibility (Laine *et al.,* 2014). Besides that, GWAS is unable to identify the full genetic component for complex diseases as the data only represents minor proportions of the genetic determinant. Researchers face a big challenge in this 'missing heritability' in GWAS studies, with varying proposed explanations. GWAS has limitations in identifying low-frequency genetic variants which may have larger effect sizes compared to common variants that normally have low to modest effect sizes (Manolio *et al.,* 2009). In addition, there are also limitations due to environmental

Universiti Malaya

risk factors (smoking, oral health, age) that contribute to disease progression (Laine *et al.,* 2014). These risk factors may act simultaneously and be linked with each other (Laine & Loos, 2012).

Recently, a new technique called Next generation sequencing (NGS) was introduced for genetic testing. NGS has the potential to find causal mutations, including de novo, novel and familial mutations, which are associated with a disease. NGS has the ability to sequence large numbers of genes, the whole exome (protein-coding regions) or entire genome at once (Dunn *et al.,* 2018). Current studies using NGS technique on periodontal disease are more focused on microbiota sequencing (Camelo-Castillo *et al*., 2015; Zheng *et al*., 2018; Carda-Diéguez *et al*., 2020).

2.7.5 Validated Candidate Genes for Periodontitis in a Very Large Sample Size

After performing his GWAS study, Schaefer *et al.* (2013) conducted a large-scale replication study of 23 genes that have been reported from previous studies. This replication study was performed on a European population consisting of AgP subjects from Germany and the Netherlands (755 cases and 3042 controls). The observed association was then tested in another study group with CP from Germany (1437 cases and 1125 controls). They then concluded that *GLT6D1, ANRIL, IL-10, PTGS2* & *DEFB1* genes were considered to carry validated susceptibility variants for periodontitis. Their findings are shown in Table 2.5.

Among these susceptibility variants for periodontitis, 2 genes have been reported to show clear evidence of association with CP susceptibility which are *PTGS2* (Schaefer *et al.,* 2010a; Xie *et al.,* 2009; Ho *et al.,* 2008), and *DEFB1* (Schaefer *et al.*, 2010b), while the other variants were associated with AgP subjects (Table 2.5). Among the 2 validated genes for CP, only

PTGS2 has previously been associated with an Asian CP population while *DEFB1* has been found in both Dutch and German CP population. Thus, the current study will be assessing these 2 candidate genes.

Universiti Malaya

Table 2.5: Validated candidate genes for periodontitis in a very large sample size

Gene/SNP	Population	Cases	Control	P-value	OR (95% CI)	MAF (%)		Publication
						Cases	Control	
GLT6D1 (rs1537415)	German generalized AgP	141	500	1.8 x 10^{-4}	1.67 (1.27–2.18	50.0	37.5	Schaefer *et al.,* 2010c
	German localized AgP	142	472	3.1 x 10^{-4}	1.65 (1.26–2.17)	49.3	37.1	
	Dutch AgP	164	368	5.7 x 10^{-3}	1.47 (1.12–1.93)	49.0	39.6	
ANRIL (rs3217992)	Dutch AgP	159	421	7.2 x 10^{-3}	2.53 (1.3–5.1)	34.0	37.1	Schaefer *et al.,* 2010c; Schaefer *et al.,* 2011b
	German AgP	301	962	4.4 x 10^{-4}	1.48 (1.2–1.9)	43.3	35.0	
	Dutch CP	154	421	0.06	1.82 (1.0–3.4)	38.9	37.1	
IL-10 (rs61815643)	German AgP	600	1448	0.009	0.78 (0.65–0.94)	14.7	18.1	Schaefer *et al.,* 2013
	Dutch AgP	164	1045	0.0326	1.46 (1.03–2.07)	18.9	14.7	
(rs6667202)	German AgP	703	1921	0.003	0.81 (0.71–0.93	38.7	43.8	
PTGS2 (rs6681231)	Dutch/German AgP	520	1043	7.7 x 10^{-3}	1.05 (0.42–2.62)	17.4	13.0	Schaefer *et al.,* 2010a
(rs20417)	Taiwanese AgP	85	153	1 x 10^{-4}	0.071 (0.02–0.22)	3.0	22.0	Ho *et al.,* 2008
(rs689466)	Chinese CP	146	148	5 x 10^{-3}	2.49 (1.33–4.69	39.4	48.6	Xie *et al.,* 2009
DEFB1 (rs1047031)	Dutch/German AgP	532	1472	0.0342	1.5 (0.8–2.7)	21.2	17.5	Schaefer *et al.,* 2010b
	Dutch/German chronic periodontitis	805	1415	0.0253	0.4 (0.2–2.4)	20.0	16.7	

Adapted from Vaithilingam *et al.,* 2014

Universiti Malaya

2.8 Candidate Genes for Periodontal Disease

2.8.1 Prostaglandin-endoperoxide Synthase *(PTGS2)*

Prostaglandins (PGs) consist of a group of hormones found in tissues which are potent biochemical mediators of the immune response to infection. PTGS2 is a key enzyme to convert arachidonic acid to PG, which plays an important role in the process of inflammation and carcinogenesis (Xie *et al.,* 2009; Schaefer *et al.,* 2010a; Ho *et al.,* 2007; Noguchi *et al.*, 2007).

There are 2 main isoforms of PTGS which are PTGS1 and PTGS2 that catalyses the same reaction even though they are encoded in distinct genes. PTGS1 is known as a housekeeping enzyme that is constitutively expressed in many tissues while PTGS2 is involved in the inflammation process of periodontitis (Dubois *et al.*, 1998). When there is inflammation in the periodontal tissue, production of PTGS2- mediated prostaglandin E2 will be induced by the release of cytokines such as interleukin [IL]-1, IL-6, tumor necrosis factor-α, growth factors and lipopolysaccharides (Offenbacher & Salvi, 1999; Xu, 2000; Miyauchi *et al.,* 2004).

2.8.1.1 Role of PTGS2 in Pathogenesis of Periodontitis

Periodontopathic bacteria such as *Tannerella forsythia, Porphyromonas gingivalis,* and *Treponema denticola* (Holt *et al.,* 2005) are considered as primary pathogens that are involved in pathogenesis of periodontal disease and are present in dental plaque. The host pathogen releases lipopolysaccharides, antigens and other virulence factors that will trigger the inflammatory and immune response that will induce cytokines and prostanoids which are inflammatory molecules. This further activates host defence cells including PMNs and

Universiti Malaya

triggers an antibody response directed towards eliminating the microbial challenge. However, a chronic inflammatory response will be triggered. When host immune and inflammatory responses are not enough to eliminate the microbial invasion. Thus, resulting in periodontal inflammation (such as redness, swelling and bleeding) and periodontal damage (clinical attachment loss) and if left untreated can contribute to tissue destruction and bone resorption (Paquette & Wiliams, 2000; Yucel-Lindberg *et al.,* 2013).

PTGS2 expression is reported to be increased in inflamed gingival tissues and plays a role in producing prostaglandinE2 in the cells stimulated with pro-inflammatory molecules, thus suggesting that PTGS2 plays a pivotal role in CP progression (Noguchi & Ishikawa, 2007). Many studies have indicated that prostaglandin E2 is involved in the pathogenesis of periodontal disease. Increased prostaglandin E2 levels have been reported to be present in gingiva, gingival cervicular fluid and human gingival biopsies of patients with periodontal diseases (Goodson *et al.,* 1974; El Attar, 1976; Ohm *et al*., 1984; Offenbacher *et al.,* 1984; Offenbacher *et al.,* 1986; Zhang *et al.,* 2003).

2.8.1.2 Selection of *PTGS2* SNPs Associated with CP

Studies have revealed that the levels of PTGS2 was up regulated and have been detected in inflammatory conditions like periodontitis (Morton & Dongari-Bagtzoglou, 2001; Zhang *et al*., 2003). Researchers have suggested the genetic association of *PTGS2* to periodontitis (Kornman *et al.*, 1997; Soga *et al.*, 2003; Trevilatto *et al.*, 2003; Loos *et al.*, 2005).

In addition, some studies have suggested that *PTGS2* gene as a biomarker in CP, because PTGS2 inhibitors such as celecoxib, loxoprofen and meloxicam have been shown to alleviate periodontal conditions by reducing alveolar bone resorption (Gurgel *et al.,* 2004; Nassar *et al.,* 2005; Pinho *et al.*, 2008; Yen *et al.,* 2008).

Universiti Malaya

In the Taiwanese population (343 cases vs 153 controls), Ho *et al.* (2008) reported the association of *PTGS2* SNP rs6681231, a haplotype tagging SNP (htSNP) for rs20417 (near-perfect linkage disequilibrium, r2 > 0.95). Later, two studies were conducted on the Chinese population by Loo *et al.* (2011) and Li *et al.* (2012) and also reported associations between *PTGS2* SNP rs20417 and Chinese CP patients (280 cases vs 250 controls, 122 cases and 532 controls, respectively). Another study on the Chinese population (146 cases vs 148 healthy control) for *PTGS2* SNP rs689466 was identified to be associated with CP group in the Chinese population carried out by Xie *et al.* (2009). Schaefer *et al.* (2010a) carried out a study on association of *PTGS2* gene polymorphism on European AgP population (disease group, n=520; control group, n=1043) and found *PTGS2* SNP of rs6681231 to be associated with AgP.

Most previous studies have focused more on Caucasians and only few studies reported the association of *PTGS2* SNPs in Asian population. Associations of the *PTGS2* gene with periodontitis have been reported and positively validated in the Chinese and Taiwanese populations (Ho *et al.,* 2008; Xie *et al.,* 2009). By using candidate gene study approach, the association of 3 *PTGS2* SNPs (rs5272, rs20417 & rs689466) with risk of CP in Malay population was examined in this study. These SNPs were selected based on their role as functional SNP (Ho *et al.,* 2008; Loo *et al.,* 2011; Li *et al.,* 2012; Xie *et al.,* 2009; Prakash *et al.,* 2015).

2.8.2 Beta-Defensin

Defensins which consist of cysteine-rich antimicrobial peptides of the epithelium are recognized to play a role in the innate host response system, which plays a role in maintaining the balance between healthy state and diseased condition of the mouth (Dale & Fredericks,

Universiti Malaya

2005). They have broad-spectrum antimicrobial activity against bacteria, fungi and some viruses (Huttner & Bevins, 1999).

Generally, defensins can be divided into 2 subfamilies, α and β -defensins. In humans, polymorphonuclear leukocytes and intestinal Paneth cells produce α-defensins, while on the other hand, epithelial cells from the epithelium of the airway, gingiva, kidney, and skin express human β -defensins (Ouhara *et al.,* 2006; Devine, 2003).

According to genomic targeting, more than twenty β -defensins have been suggested, however, currently, only four human β-defensins (DEFB1-4) have been discovered and characterized functionally (Gursoy & Könönen, 2012; Vardar-Sengul, 2007). Currently, DEFB 1-3 have been reported to be expressed in the human oral cavity (Dale, 2002; Vardar-Sengul, 2007; Gomes & Frenandez, 2010).

DEFB1 can be found in epithelial tissues, salivary glands and also in genitourinary system (kidneys, ureters, bladder, and urethra) and possess high susceptibility against gram-negative bacteria (Islam *et al.*, 2001; Goldman *et al.*, 1997; Krisanaprakornkit *et al.*, 1998). Human β-defensin-2 (*DEFB-2*) shows a strong antibacterial effect on gram-negative bacteria and antifungal properties. Harder *et al.* (2001) reported the presence of *DEFB2* as identified in keratinocyte cell culture, and it was up-regulated together with *TNF-α* and *Pseudomonas aeruginosa*. Human b-defensin-3 (*DEFB3*) is expressed in various oral tissues, keratinocytes and lung epithelium (Dunsche *et al.,* 2002; Harder *et al.,* 2001).

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2.8.2.1 Role of *DEFB1* in Pathogenesis of Periodontitis

DEFB1 has been reported to be expressed in both healthy and infected periodontal tissues and is said to be induced by *Fusobacterium nucleatum* (Dale & Fredericks, 2005). *Fusobacterium nucleatum* is present at different levels in both healthy and diseased sites and also has been found to play a pivotal role in the formation of biofilm (Lovegrove, 2004). Figure 2.1 shows the location of *DEFB1* in oral epithelium and sulcular epithelium of the gingival tissue.

In a recent research study, researchers have tried to link the presence of *DEFB1* with periodontal diseases. The mechanisms involved includes the colonization of bacteria which results in production of *DEFB1*, which plays a role as an inhibitor. However, a few pathogenic periodontal bacteria (*A. actinomycetemcomitans, P. gingivalis* and *T. forsythia*) are able to invade the gingival tissue (Amano *et al*., 2014). Dendritic cells produce chemokines such as interleukin-8 which is stimulated by *DEFB1,* and becomes chemo-attractants to attract phagocytes and lymphocytes to the site of infection.

Another possible mechanism of defensins during infection was described by Wang *et al.* (2014). First, recruitment of immature dendritic cells will be stimulated by defensins and brought to the site of infection resulting in antigen uptake by producing a 'Defensin-Ag' complex.

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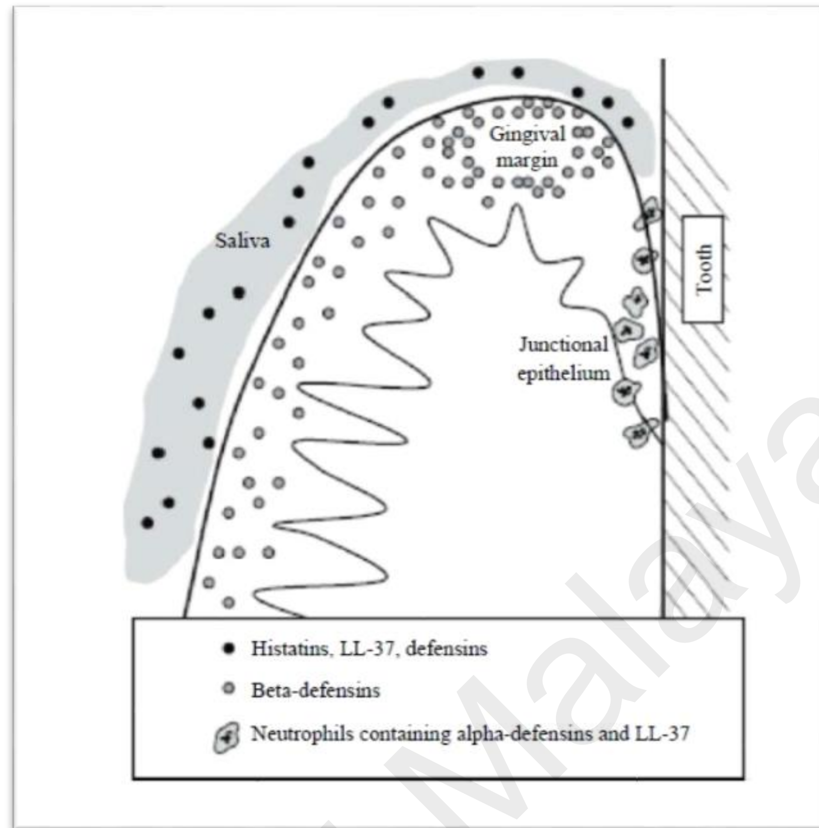

Figure 2.2: Distribution of DEFB1 in periodontal tissue (Taken from Sulijaya *et al*., 2017)

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2.8.2.2 Selection of *DEFB1* SNPs Associated with CP

Studies have tried to link clinical association of *DEFB1* level with the severity of periodontitis (Sulijaya *et al*., 2016; Ertugrul *et al.,* 2013; Vardar-Sengul *et al.,*2007; Schaefer *et al*., 2010a). Dale (2002) came up with the hypothesis that different *DEFB1* play a pivotal role in the junctional epithelium region. In association with severity of disease, Ertugrul *et al*. (2013) carried out a study with CP, CP with diabetes type-2, and gingivitis subjects and discovered a significantly higher protein level of DEFB1 in CP and diabetes type-2 CP groups compared to gingivitis groups ($p<0.05$). According to Vardar-Sengul *et al.* (2007), they found an up-regulation of mRNA gene expression of *DEFB1* in CP but showed down-

regulated expression in AgP and gingivitis. A study conducted by Sulijaya *et al.* (2016) reported that *DEFB1* protein level was higher in mild CP than severe CP group (p=0.087).

DEFB1 is known to affect peptide expression (Zupin *et al*., 2017). Dale and Krisanaprakornkit (2001) stated that *DEFB1* peptides were detected in all gingival samples conducted in their study. Schaefer *et al.* (2010b), carried out a study on the Dutch and German CP and AgP population looking into associations with genetic polymorphisms of *DEFB1* rs1047031 SNP. Both AgP (p-value = 0.0342) and CP (p-value= 0.0253) subjects reported an association with *DEFB1* SNPs rs1047031. rs1047031 is located in microRNA binding sites that are potentially able to alter *DEFB1* expression (Prado-Montes, 2010). Another study reported that *DEFB1* mRNA expression was up-regulated in CP (Vardar-Sengul *et al.,* 2007). Schaefer *et al.* (2010) also reported the association of *DEFB1* intronic SNP rs2293958 with CP group, but lost the significance after covariate adjustment.

Most previous *DEFB1* SNP studies have yielded inconsistent association results with CP susceptibility between Caucasians and Asians such as Chinese & Japanese population. Moreover, most studies have focused more on Caucasians and only few studies reported the association of *DEFB1* SNPs in Asians (Ikuta *et al.,* 2013; Shao *et al.,* 2019). These SNPs were selected based on their role as functional SNP located in 3'UTR that was previously reported to be associated with CP (Ho *et al.,* 2008; Loo *et al.,* 2011; Li *et al.,* 2012; Xie *et al.*, 2009; Prakash *et al.,* 2015). Thus, in this study we will investigate the association between *DEFB1* rs1047031 and rs2293958 SNPs with CP risk in Malaysian Malay population.

Universiti Malaya

CHAPTER 3: MATERIALS AND METHOD

3.1 Study Subjects and Clinical Characteristics

This study is a retrospective comparative cross-sectional study looking at CP subjects (case group) and periodontally healthy controls (control group). All data and blood samples used in this study was obtained from the Malaysian Periodontal Database and Biobank System (MPDBS) situated in the Faculty of Dentistry University Malaya (Vaithilingam *et al.,* 2015). The candidate for the present study was not involved in collecting patient data. All data and samples used for this study was obtained from MPDBS.

Patient data and blood samples used in this study were collected from eligible subjects who fulfilled the inclusion and exclusion criteria from the Faculties of Dentistry in University of Malaya, Universiti Sains Islam Malaysia and Universiti Teknologi Mara and also from Ministry of Health periodontal specialist clinics in the states of Penang, Johor, Negeri Sembilan and Kuala Lumpur. All sociodemographic data, clinical examinations and sample collection were performed by dedicated periodontists in these institutions. All examiners were calibrated at the beginning of the study prior to subject recruitment with Kappa scores of 75–92% and 73–91%, respectively PPD & CAL, whereby Kappa values of 0.61–0.8 are considered as substantial agreement whilst Kappa values >0.8 are almost perfect agreement (World Health Organization, 2013). All collected data and blood samples were then stored in the Malaysian Periodontal Database and Biobank System (MPDBS) situated in the Faculty of Dentistry University Malaya (Vaithilingam *et al.,* 2015).

Written informed consent was obtained from all subjects prior to participating in this study. Ethical approval to conduct this study was obtained from Medical ethics committee, Faculty

Universiti Malaya

of Dentistry, University of Malaya (DF PE1103/0037(L)) (Appendix A) and the National Medical Research Registry (NMRR-12-814012063) (Appendix B).

Sample size for this study was calculated using genetic power calculator to achieve 50% statistical power. The sample size required for control group and case group for CP was 350 each. However, due to time constraints and our strict inclusion criteria we only managed to obtain a total of 78 Malay CP and 62 healthy control subjects. We were not able to collect the desired number of samples as our inclusion criteria was limited to the Malay ethnic group and the patients from the MPDBS were from the various ethnic groups in Malaysia. This small sample size is a limitation in this study. Moreover, genetic heterogeneity occurs if different ethnic populations have been mixed that can affect the SNP detection (Vaithilingam *et al*., 2014). In order to ensure that the study subjects were only Malay and not from mixed background, we have collected info regarding paternal and maternal grandparents ethnicity.

This study commenced in 2015 and data analysis was completed in 2018. Since this study commenced prior to the new classification for periodontitis being introduced (Caton *et al.*, 2018), therefore the subjects were diagnosed following the criteria defined by Eke *et al.* (2012) for CP. This classification categorized the disease severity based on the severity of clinical attachment loss (CAL) and extent of disease. For this study, we selected subjects with mild, moderate and severe CP disease severities. All data and samples obtained from the MPDBS used the following inclusion and exclusion criteria:

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The inclusion criteria for the subjects were;

1. Subjects from the Malay ethnic group
2. Patients should have at least 12 teeth present
3. CP subjects
    i. Mild, Moderate & Severe CP (Eke *et al.,* 2012)
    ii. Aged 35 years and above
4. Healthy controls
    i. Pocket depths less than 4mm, alveolar bone loss < 15%
    ii. Age 35 years and above

The exclusion criterion was non-Malay subjects.

3.2 Data Collection Procedure

3.2.1 Sociodemographic Data

The sociodemographic data of subjects were obtained via a questionnaire form (Appendix C). During subject recruitment, they filled up a questionnaire which involved collection of data on demographic & socioeconomic status (age, gender, ethnicity, education level), social habits such as smoking (current or former smoker, number of years of smoking), alcohol intake, and oral health (mouth rinse, brushing, interdental cleaning) and medical history (diabetes, cardiovascular disease, arthritis and pulmonary disease) (Vaithilingam *et al.,* 2015).

During sample selection, we did not exclude smokers. We acknowledge that smoking is also a confounder for CP that contributes to an increase in prevalence and severity of CP (Papapanou, 1996; Susin *et al.,* 2004). However, it was difficult to exclude smokers from the

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study. This is because of the high prevalence of smokers reported in Malaysia whereby 22.8% of Malaysian adults have been reported to be tobacco smokers (Lim *et al.,* 2018) Besides that, we were also concerned that the exclusion of smokers may hinder the desired sample size within the sampling time frame. Thus, it was decided not to exclude smokers during recruitment and to use statistics to control for this confounder.

3.2.2 Clinical Periodontal Parameters

The clinical periodontal data obtained from the MPDBS comprised full mouth periodontal examination excluding the third molars.

The following parameters were obtained:

3.2.2.1 Visible Plaque Index (VPI). (Ainamo & Bay, 1975)

The VPI was recorded at four sites per tooth which was the mesiobuccal, midbuccal, distobuccal and lingual/palatal surfaces. The absence or presence of plaque was recorded as a dichotomous recording. Those surfaces, which had soft accumulations at the dentogingival junction, were coded as 'Yes'. In the absence of plaque at these sites, they were coded as 'No'.

3.2.2.2 Gingival Bleeding Index (GBI). (Ainamo & Bay, 1975).

The GBI was recorded as a dichotomous recording at four sites per tooth which were the mesiobuccal, midbuccal, distobuccal and lingual/palatal surfaces. If bleeding occurred within 10 seconds following probing, a positive finding was recorded. Similarly, if there was no bleeding a negative finding was recorded.

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3.2.2.3 Probing Pocket Depth (PPD).

PPD was measured with a Williams' probe to the nearest 1mm at 6 sites per tooth. These measurements were done at the mesiobuccal, midbuccal, distobuccal, mesiopalatal/lingual, mid-palatal/lingual and disto palatal/lingual sites. The measurement was made from the gingival margin to the base of the pocket or sulcus using a force of 20g.

3.2.2.4 Clinical Attachment Level (CAL)

CAL was measured with a Williams' probe to the nearest 1mm at 6 sites per tooth which were the mesiobuccal, mid buccal, distobuccal, mesiopalatal/lingual, mid palatal/lingual and disto palatal/lingual sites. The measurement was done from the cemento-enamel junction to the base of the sulcus or periodontal pocket.

3.3 Blood Sample Collection

All blood samples for this study were obtained from MPDBS. From each patient, 10 ml of blood was collected by venipuncture in the ante-cubital fossa was taken and collected in sodium EDTA vacutainers and stored at -20°C. Buffy coat was prepared by centrifuging whole blood at 2500 × g for 10 minutes at room temperature (15–25°C). After centrifugation, three different fractions were distinguishable: the upper clear layer was plasma; the intermediate layer was the buffy coat containing concentrated leukocytes; and the bottom layer contained concentrated erythrocytes. After the upper plasma layer was removed, the buffy coat was extracted and stored at -80°C.

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3.4 Selection of SNPs

Genomic sequence information were attained from NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP). For *PTGS2*, three SNPs, rs689466 (Xie *et al.,* 2009; Ho *et al.,* 2008; Prakash *et al.,* 2015), rs5275 (Xie *et al.,* 2009; Daing *et al.,*2012; Prakash *et al.,* 2015) and rs20417 (Xie *et al.,* 2009; Prakash *et al.,* 2015) were examined. Meanwhile, for *DEFB1,* SNPs, rs1047031 (Schaefer *et al*., 2010b) and rs2293958 (Schaefer *et al*., 2010b) were examined (Table 3.1). All the sequences were referred to the human *PTGS2* (GenBank accession no. D28235.1) and *DEFB1* (GenBank accession no NM_005218.4) gene sequence. The TaqMan probes were purchased from Applied Biosystems, Foster City, CA. The SNP rs ID and the context sequence were shown in Table 3.2. These SNPs were based on previous reported findings or functional prediction from the dbSNP web database (https://www.ncbi.nlm.nih.gov/snp). Most SNPs to date has yielded inconsistent results with CP association between Caucasians and Chinese population (Schaefer *et al.*, 2010a; Schaefer *et al.*, 2010b; Xie *et al.,* 2009; Ho *et al.,* 2008). In addition, there are only few studies reported the association of these SNPs in Asian population (Daing *et al.,* 2012; Ho *et al.*, 2008; Xie *et al.,* Schaefer *et al*., 2010a). Hence, we would like to investigate the association between these SNPs with CP in the Malay study group.

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Table 3.1: Summary of SNPs examined in this study

Gene	Chromosome	SNP rs no	Alleles
PTGS2	GRCh38.p12	rs5275	C>T
	GRCh38.p12	rs20417	C>G
	GRCh38.p12	rs689466	A>G
DEFB1	GRCh38.p12	rs1047031	G>A
	GRCh38.p12	rs2293958	T>C
			Taken from dbSNP (https://www.ncbi.nlm.nih.gov/snp)

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Table 3.2: *PTGS2* and *DEFB1* SNP rs number, TaqMan probes and context sequences of the SNPs

Gene	SNP rs no	Reporter 1 Dye/Quencher	Reporter 2 Dye/Quencher	Context sequence
PTGS2	rs5275 (G>A)	VIC/NFQ	FAM/NFQ	TGTTTTTGTTTGATGACAGAAAAAT[A/G]ACCAAAAGTACTTTAAAATTTCAAA
	rs20417 (G>C)	VIC/NFQ	FAM/NFQ	CCTCCTTGTTTCTTGGAAAGAGAGG[C/G]GGGAAAGGTAAATTCTCCTCATAAT
	rs689466 (T>C)	VIC/NFQ	FAM/NFQ	TTAGATGGAAGGGAGATTTTGACAG[C/T]TGGAATTTCATCTTTGCTTTTGTTT
DEFB1	rs1047031 (T>C)	VIC/NFQ	FAM/NFQ	TCTGCGTCATTTCTTCTGGTCACTC[C/T]CAGCTCACTTGCAGCACTTGGCCTT
	rs2293958 (T>A)	VIC/NFQ	FAM/NFQ	CCCAGCCCTGGGGATGGGAAACTCT[A/T]GCAGGTACCAGAGCTTACCTGAGGC
				Taken from TaqMan Assays (https://www.thermofisher.com/order/genome-database)

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3.5 Laboratory Work

3.5.1 Genomic DNA Extraction and Quantification

Genomic DNA was extracted from blood (QIAamp DNA Blood Mini Kit, Qiagen, Germany) following the manufacturer's spin protocol. The spin protocol was used for purification of total genomic DNA obtained from Buffy coat using a microcentrifuge (Figure 3.1).

Samples were equilibrated to room temperature (15–25°C). Twenty µL of Qiagen Protease is pipetted into the bottom of a 1.5ml microcentrifuge tube. Then, 200 µL of sample (buffy coat) was added to the microcentrifuge tube. For samples with volume less than 200 µL, appropriate volume of PBS was added. After that, 200 µL of Buffer AL (264 ml lysis buffer) was added to the sample followed by pulse-vortexing for 15 seconds to mix the sample. The samples were then incubated at 56°C on a heating block for 10 minutes. After incubation, the samples were briefly centrifuged to remove drops from the inside of the lid. Next, 200 µL of ethanol (100%) was added to the sample, and mixed again by pulse-vortexing for 15 seconds. After mixing, the samples were briefly centrifuged to remove drops from inside the lid. The mixture was transferred to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim followed by centrifuging at 6000 x g (8000 rpm) for 1 minute. The QIAamp spin column was placed in a clean 2ml collection tube, by discarding the tube containing the filtrate. Next, QIAamp spin column was carefully opened and 500 µL of buffer AW1 was added followed by centrifuging at 8000rpm for 1 minute. Then, 500 µL of buffer AW2 was added followed by centrifuging at full speed 13,200 rpm for 6 minutes. After that, the QIAamp spin column was placed in a clean 1.5 microcentrifuge tube, and the tube containing the filtrate was discarded. Finally, 200 µL of Buffer AE was added into the spin column

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which was incubated at room temperatute (15–25°C) for 5 minutes, and then centrifuged at 6000 × g (8000 rpm) for 1 minute (Figure 3.1).

The quality and quantity of extracted DNA were determined by UV spectrophotometry using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). All the extracted DNA that showed $A_{260/280}$ ratio of ~ 1.70–1.90 were considered relatively pure. The extracted DNA was then stored at -20°C till further analysis.

Figure 3.1: DNA extraction procedure flow chart
(Taken from QIAamp DNA Mini and Blood Mini Handbook, 2016)

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3.5.2 TaqMan® SNP Genotyping Method

In previous studies, target DNA sequence will be amplified and followed by detection using fluorescence gel electrophoresis to detect the presence of the SNPs (Daing *et al.,* 2012; Loo *et al.,* 2012). However, genotyping technology has been developing from low-throughput technologies to high-throughput technology. This includes microarrays and can genotype millions of SNPs in large populations, producing accurate results and the technique is fairly cost-effectively (Kim & Misra, 2007).

Genotyping was performed using TaqMan® SNP Genotyping method (Applied Biosystems) according to the manufacturer's instructions. By using TaqMan® SNP Genotyping Assays, proven TaqMan® probes, allelic discrimination will be detected by incorporating minor groove binder (MGB) technology at the 3' end. The MGB molecule will bind to the minor groove of the DNA helix. The allelic discrimination is detected by proven 5' nuclease chemistry by means of exonuclease cleavage of an allele-specific 5' dye label (VIC® dye and FAM™ dye) that generates the assay signal.

The TaqMan probes and TaqMan Universal PCR Master Mix were designed and purchased from Applied Biosystems, Foster City, CA. The reaction mixture was prepared as shown in Table 3.3.

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Table 3.3: Calculation table for working solution for genotyping

Component	1 working reaction (uL)
TaqMan Universal PCR Master Mix (2✕),	5.0
20✕ working stock of SNP Genotyping Assay	0.5
2 ng/µl of gDNA	2.0
DNase-free Water	2.5
Total Volume per Well	10.0

In every cycle, at least one non-template controls using DNAase-free water. Working reaction in the final volume of 10µl in each well was prepared in the MicroAmp Optical 96-well reaction plate. The PCR amplification was performed using Real Time thermocycler AB 7500 (Applied Biosystems, Foster City, CA.).

The qPCR conditions were as follows:

Hold step for 10 min at 95°C was done to allow AmpliTaq Gold Enzyme (Applied Biosystems, USA) activation followed by 40 cycles of initial denaturation step for 15 seconds at 92°C and annealing and extension step of 1 min at 60°C. The samples were run on 96-well plates. After completion of PCR amplification, the fluorescent signal intensities and the ratio of FAM/ROX and VIC/ROX were generated. Based on the signals generated from each well, specific alleles were determined using ABI PRISM Sequence Detection System (SDS) software. SNPs showing genotype call rates >95% were retained for statistical analysis in order to evaluate the concordance of genotyping calls, 5% of the samples for each SNP were randomly selected for re-genotyping.

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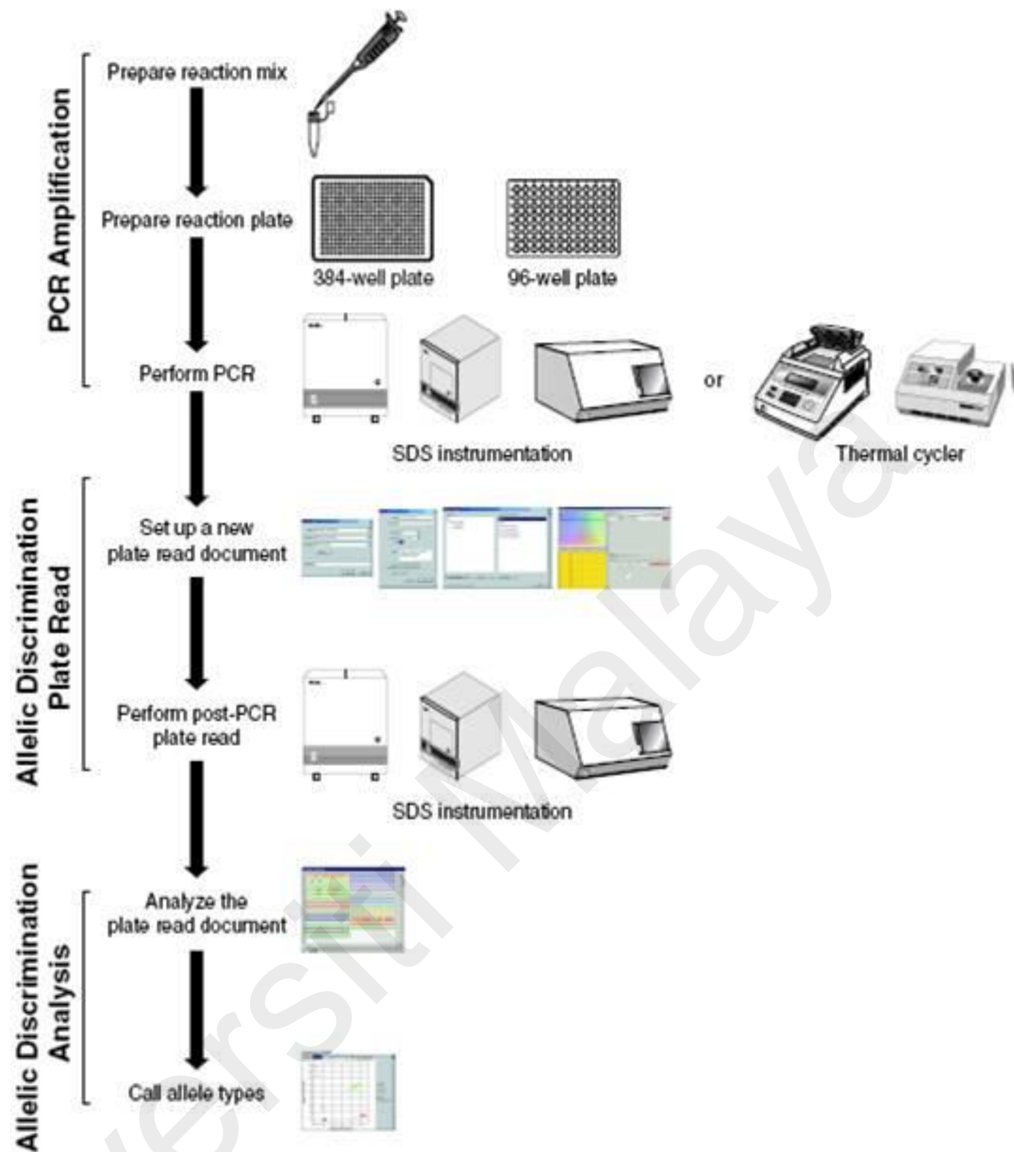

Figure 3.2: TaqMan SNP genotyping assay procedure. (taken from TaqMan® SNP Genotyping Assays Protocol, Applied Biosystems, 2010)

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3.6 Data and Statistical Analysis

Statistical analysis was performed using Statistical Analysis System Software (V.22.0: SAS Institute, SPSS Inc., CH, USA) and PLINK 1.9 software (Purcell *et al.,* 2007). PLINK is a free, open-source whole genome association analysis toolset, designed to perform a range of basic, large-scale analyses in a computationally efficient manner. Normality of the quantitative data was assessed using Kolomogorov-Smirnov test. Continuous data were presented as mean ± standard deviation and categorical data were presented as percentages (%). Independent t-test was conducted to compare the clinical parameters (periodontal pocket depth and clinical attachment loss) between case and control groups. When appropriate, Mann-Whitney U test was done in order to compare differences between mean of clinical parameters (Visible Plaque Index and Gingival Blood Index) in case and control groups, where the data was not normally distributed. Fisher's exact test was used to test each SNP for Hardy-Weinberg equilibrium (HWE) in controls and in combined case-control samples. Chi-square tests were used to examine the allelic and genotypic association of all the SNPs with CP respectively. The genotypic association were analysed according to additive (A1A1 vs A1A2 vs A2A2), dominant (A1A1 and A1A2 vs A2A2) and recessive (A1A1 vs A1A2 and A2A2) models, where A1 is the variant and effect allele using model association test analysis. The strength of association was expressed as odds ratio (OR) with a 95% confidence interval (CI). All statistics were considered statistically significant when $p<0.05$.

3.7 Allele frequency, Genotype Frequency and Model Association Test Analysis

Allele & genotype frequency can be defined as relative frequency of an allele & genotype of candidate gene variants such as SNPs at a genetic locus in a population (Rezaei & Hedayat, 2013). Allele & genotype frequency is determined in order to identify genetic association

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between CP with *PTGS2* and *DEFB1* SNPs. Allele and genotype frequencies are the basic genetic analysis for association studies between SNPs and disease whereby the alleles & genotypes frequency is quantified between cases and control group, and is measured as odds ratio (Frazer *et al.,* 2009; Scott *et al.,* 2007). Allele/Genotype frequency is calculated by counting how many times the allele/genotype appears in the case or control group then dividing by the total number of copies of the gene. Hardy Weinberg equilibrium (HWE) is used to test deviation in controls in order to check genotyping quality (Anderson *et al.,* 2010). A good genotyping quality is ensured when control genotypes are in HWE, and the cases may be further tested (Lewis, 2002).

Genetic association analysis is conducted to explain the genetic factors that makes an individual more susceptible to a disease. Model association test is conducted to identify the genotypic association of candidate gene variants with the risk of disease susceptibility. For example, if single SNP carries alleles A1 and A2, where A1 is the risk allele (the allele that causing an effect) which are usually tested to generate data which involves genotypes (A1A1, A1A2 and A2A2) in cases and controls study group. Generally, model association test includes three genotypic test models which are dominant, recessive and additive models (Zhao *et al.,* 2016). In dominant model, association is tested by having at least one minor allele A1 (either A1A1+A1A2) versus not having it at all (A2A2). While in recessive model, association is tested by having minor allele A1 as both alleles (A1A1) versus having at least one major allele A2 (A1A2+A2A2). For additive model, the association is based on minor and major allele (A1 vs A2). This model association is determined by 2-by-3 table of disease-by-genotype (Zhao *et al.,* 2016).

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3.8 Experiment Summary

The following figure 3.3 demonstrates the flow chart summarizing the whole experiment from obtaining the blood samples from MPDBS, conducting laboratory work (DNA extraction, DNA quantification, and SNP genotyping) and followed by analysis of results.

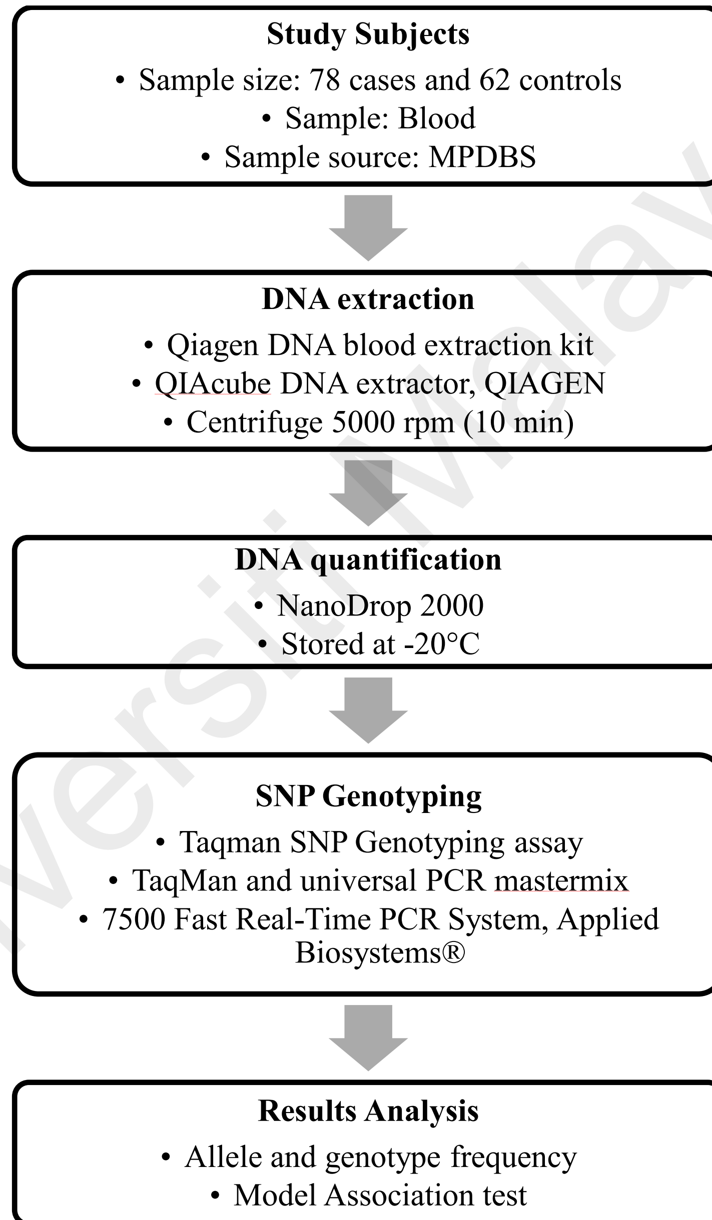

Figure 3.3: Flow chart for experiment

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CHAPTER 4: RESULTS

4.1 Demographic Characteristics and Clinical Data

The demographic characteristics and habits of CP and control groups are described in Table 4.1. The differences between the two groups with regard to age groups reflects the nature of the selection of the cases and controls. This is because CP is a chronic inflammatory disease and it is found more in older age group as shown in our data. The demographic characteristics and habits of CP and control groups are described in Table 4.1.

A total of 78 Malay CP and 62 healthy control subjects were included in this study. Females were dominant in this study population as compared to males in both control (61.3%) and case (55.1%) groups ($p>0.05$). The cases and controls were classified into four age group categories. Most controls were between 35 and 45 years old (61.3%). Meanwhile, subjects in the case group were between 35 to 45 years (43.6%) and 46 to 55 years (39.7%). The age distribution between cases and controls was significantly different ($p=0.015$). Most subjects in the control (50.0%) and case (53.8%) groups had received secondary education. Smokers comprised 24.2% of subjects in the control group and 16.7% subjects in the case group. There were no differences in relation to gender, education level and smoking habits between the two groups ($p>0.05$).

Smoking in a known risk factor for periodontitis (Albandar & Rams, 2002; AlJehani, 2014). However, due to the high prevalence of smokers among the Malaysian population we included smokers in our study with the intention of controlling for the bias using regression analysis if there was a positive association. The smokers and non-smokers among the case and control groups were found to be equally distributed. Smokers comprised 24.2% of

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subjects in the control group and 16.7% subjects in the case group. There were no differences in relation to gender, education level and smoking habits between the two groups (p>0.05).

Table 4.1: Demographic characteristics and social habits of the study group

Characteristics	Groups (N=140)		*p*–value
	Control (N=62)	Case (N=78)	
	N (%)	N (%)	
Gender			
Male	24 (38.7)	35 (44.9)	0.468[a]
Female	38 (61.3)	43 (55.1)	
Age (years)			
35 – 45	38 (61.3)	34 (43.6)	0.028[a*]
46 – 55	11 (17.7)	31 (39.7)	
56 – 65	13 (21.0)	12 (15.4)	
66 – 75	0 (0.0)	1 (1.3)	
Education level			
Tertiary	28 (45.2)	24 (30.8)	0.129[a]
Secondary	31 (50.0)	42 (53.8)	
Primary	2 (3.2)	9 (11.5)	
Others	1 (1.6)	3 (3.8)	
Smoking habit			
Smoking	15 (24.2)	13 (16.7)	0.269[a]
Non–smoker	47 (75.8)	65 (83.3)	

[a]Chi-Square test; p value<0.05

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4.2 Comparisons in Periodontal Parameters Between Case and Control Groups

Table 4.2 shows the comparison of means and standard deviations (SDs) for VPI, GBI, PPD and CAL for the control and case groups. As expected, the mean percentage of both VPI and GBI were significantly higher in the case group [50.67% (± 31.23) and 45.00% (± 28.36) respectively] compared to control group [23.12% (± 24.88) and 15.35% (± 26.32) respectively].

Similar observations were noted for mean PPD and CAL. In the case group, the means for PPD and CAL [2.72 (± 0.94) mm and 3.39 (± 1.41) mm respectively] were significantly higher compared to the means for PPD and CAL in the control group [1.24 (± 0.54) mm and 1.41 (± 0.54) mm respectively] ($p<0.001$).

Means for PPD and CAL were further analyzed based on the severity of the disease (Table 4.2). In both parameters, severity was indicated by the pocket depth (PPD < 4 mm, PPD 4–6 mm and PPD > 6 mm) and attachment loss (CAL < 4 mm, CAL 4–6 mm and CAL > 6 mm). In the case group, only 3.70% subjects had PPD > 6 mm while 9.52% subjects had CAL > 6 mm. This indicates that majority of the patients had mild to moderate CP while subjects with severe CP had very localised disease. CP is considered localized when less than 30% of the sites assessed in the mouth showed loss of attachment (Armitage, 1999).

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Table 4.2: Comparison of VPI, GBI, PPD and CAL among control and CP groups

Clinical parameters	Groups (N=140)		*p* – value
	Control (N=62)	Case (N=78)	
	Mean (±SD)	**Mean (±SD)**	
Mean VPI (%)	23.12 (24.88)	50.67 (31.23)	< 0.001[b*]
Mean GBI (%)	15.35 (26.32)	45.00 (28.36)	< 0.001[b*]
Mean PPD (mm)	1.24 (0.54)	2.72 (0.94)	< 0.001[a*]
% sites with PPD > 6 mm	0.00 (0.00)	3.70 (6.77)	< 0.001[b*]
% sites with PPD 4-6 mm	0.00 (0.00)	19.03 (14.08)	< 0.001[b*]
% sites with PPD < 4mm	100.00 (0.00)	77.27 (17.40)	< 0.001[b*]
Mean CAL (mm)	1.41 (0.54)	3.39 (1.41)	< 0.001[a*]
% sites with CAL > 6 mm	0.00 (0.00)	9.52 (13.74)	< 0.001[b*]
% sites with CAL 4-6 mm	0.00 (0.00)	28.64 (17.98)	< 0.001[a*]
% sites with CAL < 4 mm	100.00 (0.00)	61.84 (24.92)	< 0.001[b*]

SD: Standard deviation; [a] Independent T test; [b] Mann-Whitney U Test; **VPI**: Visible Plaque Index, **GBI**: Gingival Bleeding Index, **PPD**: Probing Pocket Depth; **CAL**: Clinical Attachment Loss; [*] $p < 0.001$

4.3 Genomic DNA Extraction

Genomic DNA was successfully extracted from the buffy coat of blood samples (2–5 ml), which obtained approximately 10–50 µg DNA. These DNA samples were then diluted to a concentration of 2 ng/µl for subsequent experiment. NanoDrop ND-1000 spectrophotometer was used to measure the purity of the DNA samples, and all the extracted DNA samples were relatively pure (A_{260}/A_{280} ratio ~ 1.70–1.90).

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4.4 Interpretation of TaqMan SNP Genotyping Result

The genotyping results presented in Figure 4.1 shows the graphical output of a SNP allele 1 and allele 2 as detected by the software of Applied Biosystems 7500 Real-Time PCR System. The genotyping results were attained by detecting the fluorescent signal intensities of the TaqMan assay and display as a scatter plot of Allele X Rn versus Allele Y Rn (Figure 4.1).

As shown in Figure 4.1, the allelic discrimination plot contains three distinct clusters which represents three different genotypes along the horizontal axis (Allele X), the vertical axis (Allele Y) or diagonal axis (Alelle X/Alelle Y). The cluster of red spot signifies a strong VIC/ROX signal and represents those individuals homozygous for allele 1. The cluster of green spot represents heterozygous individuals (having both allele 1 and 2), which caused an increase in fluorescence signal from both reporter dyes (VIC and FAM). The cluster of blue spot indicated strong FAM/ROX signals representing individuals homozygous for allele 2.

In order to ensure the reproducibility of the results and to confirm the allelic discrimination, normalized reported signal (Rn) values were generated. Rn for allele 1(VIC/ROX) were represented by Allele X Rn while Rn for allele 2 was represented by allele Y Rn.

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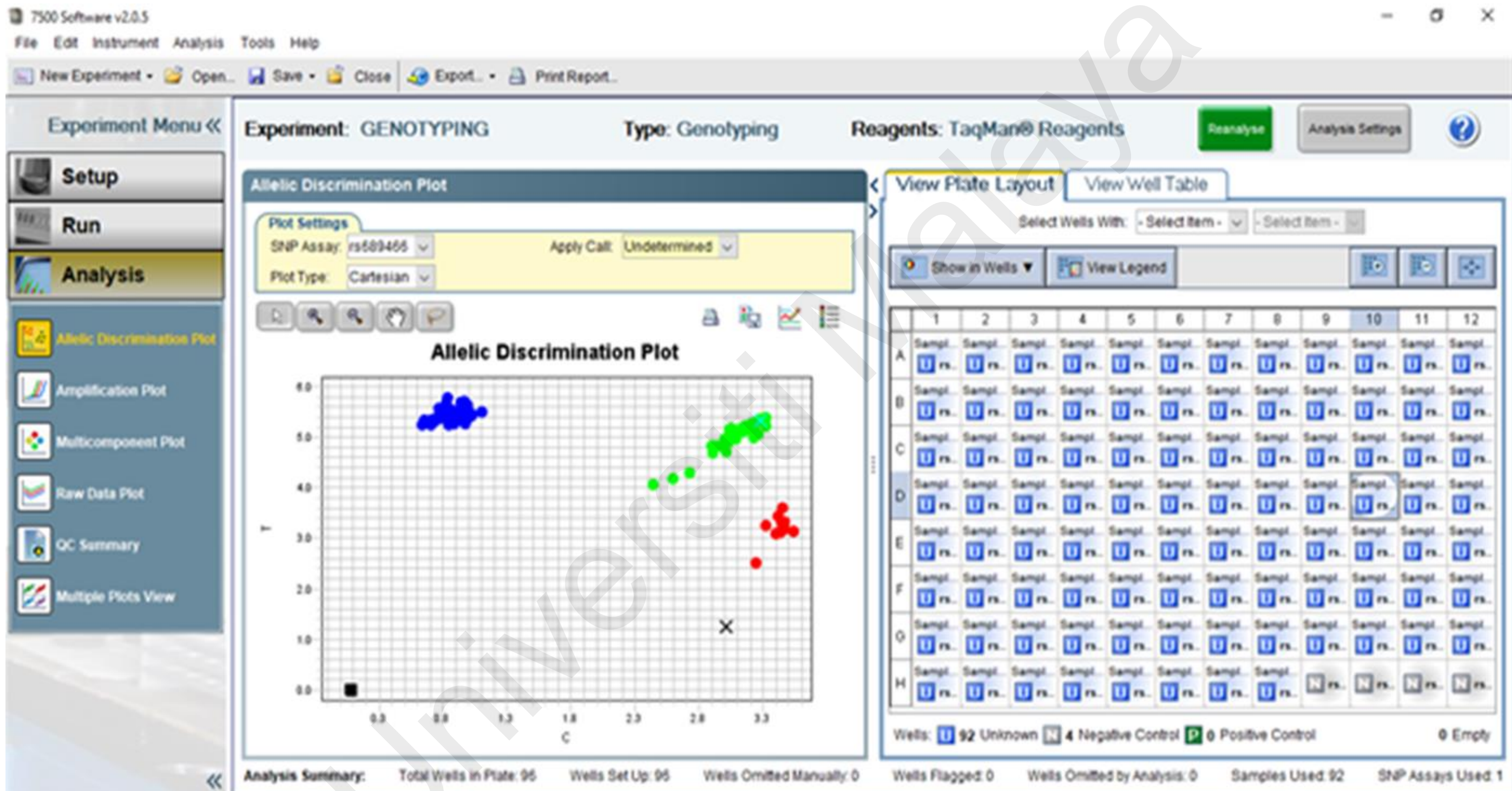

Figure 4.1: A representive graphical output of a SNP (rs1047031) detected by applied biosystem 7500 real-time PCR system.

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4.5 Quality Control for Results

The CP cases (n=78) and controls (n=62) were compared with respect to their minor allele frequencies (MAF). All the SNPs except rs2293958, showed 100% genotyping call rates. SNP rs2293958 was excluded from further analysis as it was undetermined in more than 40% of samples. Five percent of the samples were randomly selected to be re-genotyped for each SNP and the genotype concordance rates were 100% (Table 4.3). All 4 SNPs were in HWE (P>0.05) in control and case group (Table 4.3). We acknowledge that Yates correction is recommended to reduce bias results as our expected cell frequency is below than 5. However, we did not use it for correction as it may be too strict (Camilli & Hopkins, 1979; Larntz, 1978; Thompson, 1988). The alleles (A1/A2) for *PTGS2* and *DEFB1* SNPs were transcribed from reverse strand (Table 4.3).

Table 4.3: Genotyping call rates and HWE for case and control group

	Genotyping call rate (%)	Allele (A1/A2)	p-value	HWE P-value	
				case	control
PTGS2					
rs5275	100	C/T	0.329	0.598	0.562
rs20417	100	C/G	0.641	1.000	0.200
rs689466	100	G/A	0.849	0.467	0.757
DEFB1					
rs1047031	100	A/G	0.729	0.819	0.436

Abbreviations: SNP: Single nucleotide polymorphism; HWE: Hardy-Weinberg equilibrium, A1-minor allele, A2-major allele. *chi square, $p < 0.05$ considered significant; HWE, $P > 0.05$

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4.6 Single Nucleotide Polymorphism

4.6.1 Genotype Frequency Analysis for SNPs

Genotype distributions were tested and all four SNPs were in HWE. However, none of these SNPs was found to be significantly associated ($P>0.05$) with the risk of CP. (Table 4.4)

Table 4.4: Genotype distribution and frequency analysis of SNPs rs5275, rs20417, rs689466 and rs1047031 in CP and control group.

Genotype	Groups (N=140)		*p – value
	Control (N=62)	Case (N=78)	
	N (%)	N (%)	
PTGS2			
rs5275			
CC	6 (7.70)	5 (8.10)	0.329
TC	36 (46.15)	30 (48.40)	
TT	36(46.15)	27 (43.50)	
rs20417			
CC	0 (0)	2 (3.23)	0.641
CG	14 (17.90)	11(17.74)	
GG	64 (82.10)	49 (79.03)	
rs689466			
GG	8 (10.30)	6 (9.68)	0.849
AG	39 (50.00)	24 (38.71)	
AA	31 (39.70)	32 (51.61)	
DEFB1			
rs1047031			
AA	14 (17.95)	9 (14.52)	0.729
GA	37 (47.44)	34 (54.84)	
GG	27 (34.61)	19 (30.64)	

**chi square, p<0.05 considered significant, SNP: Single nucleotide polymorphism*

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4.6.2 Allele Frequency Analysis for SNPs

The distribution of allele frequencies within groups is shown in Table 4.5. The allele frequencies of each SNP were compared between the CP and control groups. Although the allelic frequency for the rs689466-G allele was higher in CP group at 35.2% versus control group at 29.0%, while A allele was lower in CP group at 64.7% for the CP group versus 71.0% of A allele for the control group, the differences were not statistically significant. Our results did not show any significant differences in the allelic frequencies of all the SNPs between the CP and control groups (Table 4.5).

4.7 Association Study Between the *PTGS2* and *DEFB1 SNPs* and CP

The association between the *PTGS2* and *DEFB1* SNPs with CP susceptibility was further analysed using three different genetic models namely additive, dominant and recessive models. No significant associations ($p>0.05$) were observed for all the SNPs (Table 4.6).

Universiti Malaya

Table 4.5: Allele frequencies of rs5275, rs20417, rs689466 and rs1047031 and association analysis with CP.

			Cases n (%)		Controls n (%)			
			A1	A2	A1	A2		
PTGS2								
rs5275	C	T	48 (30.8)	108 (69.2)	40 (32.3)	84 (67.7)	0.780	0.927 (0.544-1.579)
rs20417	C	G	14 (9.0)	142 (91.0)	15 (12.1)	109 (87.9)	0.406	0.725 (0.339-1.548)
rs689466	G	A	55 (35.2)	101 (64.7)	36 (29.0)	88 (71.0)	0.265	1.343 (0.800-2.255)
DEFB1								
rs1047031	A	G	65 (41.7)	91 (58.3)	52 (41.9)	72 (58.1)	0.963	0.989 (0.607-1.611)

Differences between groups was analyzed by Fisher's exact test and p<0.05 was considered significant. SNP: Single nucleotide polymorphism, OR: Odds ratio, CI: confidence interval, A1-minor allele, A2-major allele n (%): number (percentage), SNP: Single nucleotide polymorphism, OR: Odds ratio, CI: confidence interval, A1-minor allele, A2-major allele

Table 4. 6: Association study of SNPs in *PTGS2* and *DEFB1* genes and CP

	Additive model (A1 *vs.* A2)		Dominant model (A1A1, A1A2 *vs.* A2A2)		Recessive model (A1A1 *vs.* A1A2, A2A2)	
	OR (95% CI)	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**
PTGS2						
rs5275	0.927 (0.544-1.579)	0.780	0.900 (0.460-1.761)	0.758	0.950 (0.276-3.272)	0.935
rs20417	0.725 (0.339-1.548)	0.406	0.825 (0.355-1.913)	0.653	NA	
rs689466	1.343 (0.800-2.255)	0.265	1.617 (0.825-3.172)	0.162	1.067 (0.350-3.254)	0.910
DEFB1						
rs1047031	0.989 (0.607-1.611)	0.963	0.835 (0.409-1.704)	0.620	1.288 (0.517-3.210)	0.587

**Abbreviations: OR: Odds ratio; CI: Confidence interval, CP: Chronic periodontitis; A1-minor allele, A2-major allele, NA: not applicable*
Logistic regression analysis, P-value that showed association (p<0.05)

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CHAPTER 5: DISCUSSION

5.1 Discussion of Materials and Methods

5.1.1 Study Subjects

This study was a retrospective comparative cross sectional study, which was conducted to investigate the association between *PTGS2* and *DEFB1* single nucleotide polymorphisms with CP in Malaysian Malay subjects.

Recently, different study designs have been used in studies that have looked at genetic polymorphisms in CP patients ranging from candidate gene studies (Jiang *et al.,* 2014; Daing *et al*., 2012; Schaefer *et al*., 2010a) to genome-wide association studies (GWAS) (Vaithilingam *et al*., 2014). As GWAS was not feasible due to prohibitive costs in conducting large-scale genotyping, we chose a candidate gene study design approach instead to determine genetic variations that are involved in CP progression. According to Risch and Merikangas (1996), candidate gene association studies were suggested to have more statistical power to detect modest effects of genetic loci in complex diseases. Till date, the results of candidate gene association studies are often criticized because of their small sample sizes and low statistical power (Burgner *et al.*, 2006).

Sample size for this study was calculated using genetic power calculator to achieve 50% statistical power. The sample size required for control group and case group for CP was 350 each. However, due to time constraints and our strict inclusion criteria we only managed to obtain a total of 78 Malay CP and 62 healthy control subjects. We were not able to collect the desired number of samples as our inclusion criteria was limited to the Malay ethnic group and the patients from the MPDBS were from the various ethnic groups in Malaysia. This small sample size is a limitation in this study. Moreover, genetic heterogeneity occurs if different ethnic populations have been mixed that can affect the

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SNP detection (Vaithilingam *et al*., 2014). In order to ensure that the study subjects were only Malay and not from mixed background, we have collected info regarding paternal and maternal grandparents ethnicity.

During sample selection, we did not exclude smokers. We acknowledge that smoking is also a confounder for CP that contributes to an increase in prevalence and severity of CP (Papapanou, 1996; Susin *et al.*, 2004). However, it was difficult to exclude smokers from the study. This is because of the high prevalence of smokers reported in Malaysia whereby 22.8% of Malaysian adults have been reported to be tobacco smokers (Lim *et al.,* 2018). Besides that, we were also concerned that the exclusion of smokers may hinder the desired sample size within the sampling time frame. Thus, it was decided not to exclude smokers during recruitment and to use statistics to control for this confounder.

5.1.2 Case Definition

In this study, the case definition by Eke was used to define cases of periodontitis (Eke *et al*., 2012). This classification categorized the disease severity based on the severity of clinical attachment loss (CAL) and extent of disease. For this study, we selected subjects with mild, moderate and severe CP disease severities. We were not able to use the case definition given in the new classification for periodontitis (Caton *et al.,* 2018) as the study was designed and initiated before this classification was introduced. According to the new classification, healthy periodontium defined as <10% bleeding sites, with pocket depths less than 3 mm while healthy periodontium as defined by Eke *et al*. (2012), is pocket depths less than 4mm with alveolar bone loss <15%. If the case definition for healthy periodontium by Caton *et al.* (2018) were to be used in this study, the sample size for control group will be reduced as the pocket depth is reduced from less than 4mm to less than 3mm, which will affect the overall number of samples in the control group. If the patient selection was more strict following the new classification, the chances of

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significant results may be higher. Previous studies investigating association of SNPs and CP had also used other classifications such as the classification from the World Workshop for Periodontics and The American Academy of Periodontology (Armitage, 1999) to assign and define disease severity for their CP participants which has added to the heterogeneity among different studies (Ho *et al*., 2008; Daing *et al.,* 2012).

5.1.3 Selection of *PTGS2* and *DEFB1* SNPs

The selection of *PTGS2* rs5275 (Daing *et al.,* 2012) and *DEFB1* rs2293958 (Schaefer *et al.,* 2010a) SNPs for this study were done as these SNPs had been previously associated with the occurrence of periodontal disease or with changes in the subgingival biofilm. These SNPs *PTGS2 rs689466* (Ho *et al.,* 2008), *rs20417* (Xie *et al.,* 2009), and *DEFB1* rs1047031 have been replicated in a large analysis population. They have provided clear evidence of association or strong suggestive evidence of association with CP (Schaefer *et al.*, 2010a; Vaithilingam *et al.,* 2014).

5.1.4 TaqMan SNP Genotyping Method

Currently, SNP genotyping technologies such as SNaPShot, TaqMan, SNP-IT, and Mass Array have been developed, that can genotype a candidate gene polymorphisms/SNP on a single sample at a time. This is well suited for low to medium throughput genotyping applications as it is limited to genotyping one SNP per assay and thus suitable for our small study design (Kim & Misra, 2007). Ding *et al.* (2015) used the TaqMan® assay for investigating genes associated with periodontitis in Han Chinese population. Another study used TaqMan® assay to successfully determine single nucleotide polymorphisms (SNPs) of the *DEFB1* in Crohn's disease (Kocsis *et al.,* 2008). TaqMan® SNP Genotyping Assays does not require optimizing of probe and primer and also temperature. This is because all assays use universal reagent concentrations and thermal cycling

Universiti Malaya

conditions. Moreover, no transfers, washes, or additional reagents are required to be conducted post thermal cycling. As the plate are sealed, the software will read the plate and analyze the genotypes (TaqMan® SNP Genotyping Assays Product Bulletin, Applied Biosystems, 2010). This reduces the chance of contamination, sample mix-up, and sample loss. Therefore, we have chosen TaqMan SNP Genotyping assay (Applied Biosystems, CA) in this study.

5.2 Discussion of Results

5.2.1 Characteristics of Study Population

In 2010, the Malaysian National Oral Health Survey reported that 94% of adults in Malaysia had some form of periodontal disease, in which, the number was similar to the findings held 20 years ago; whereby, a total of 92% of adults possessed periodontal diseases (NOHSA, 2010; Mohd-Dom, *et al.*, 2013). In the current research, the mean age for both control and study population were 45.32 and 47.19, respectively. Noteworthy, the incidence of severe periodontitis increases the most among the age group of 35–44 and 65–74 (Mohd-Dom *et al.,* 2013).

Periodontal diseases have become a major global oral health burden (Tonetti *et al.,* 2017) which can be prevented by healthy life style and adequate oral hygiene. Smoking, diabetes, hypertension, obesity, and oral hygiene are factors that increases the risk of periodontal disease (Almerich-Silla *et al.,* 2017). In this study, the smokers and non-smokers among the control and study populations were identified and we found there was no significant difference between the numbers of smokers in both groups. Thus smoking habit would not have caused a bias in the outcome of the study. However, since smoking is a well-known risk factor for CP, we have controlled the possibility of this bias being introduced in the outcome of our results by performing logistic regression analysis.

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In this study, there was also no significant difference between the case and control groups of gender and education level (p>0.05) and therefore, no bias was introduced from these parameters into the study. In terms of participants' age, however, the control group comprised of significantly younger participants as compared to the case group. However, this bias was controlled by logistic regression analysis.

5.2.2 Periodontal Parameters

According to Almerich-Silla *et al.* (2017), periodontal probing depth, bleeding on probing, and clinical attachment loss level are the vital clinical signs used to diagnose periodontal disease. The clinical examination to diagnose these subjects was performed by senior experienced periodontists. All examiners had undergone a periodontal standardisation exercise prior to subject examination and sampling (Vaithilingam *et al.,* 2015). The scores were between 75–92% and 73–91%, respectively for PPD & CAL, whereby Kappa values of 0.61–0.8 are considered as substantial agreement whilst Kappa values >0.8 are almost perfect agreement (World Health Organization, 2013). The candidate was not involved in the data collection and all data was obtained from the MPDBS database.

Clinical parameters that were considered in this study included VPI, GBI, PPD, and CAL. The observation made in the CP group, VPI and GBI was identified at 50.67% and 45.00%, respectively. Overall, subjects in the control group showed that all of them had PPD and CAL less than 4 mm. Meanwhile, the case group showed that 3.70% subjects had PPD > 6 mm while 9.52% subjects had CAL > 6 mm. The subjects in this study were mainly generalized mild to moderate CP subjects and localized severe CP subjects.

5.3 Single Nucleotide Polymorphism (SNP)

The first objective of this study was to determine the allele and genotype frequencies of *PTGS2* SNPs rs689466, rs5275, rs20417 and *DEFB1* SNPs rs1047031 in case group with

CP as compared to healthy controls. *DEFB1* rs2293958 was omitted from this study due to the presence of more than 40% undetermined genotype calls. This may be due to presence of other allelic variants (C or G) which could not be detected during genotype calling in this study. This may occur due to samples that only carrying one reported allele. These allele may or may not run together with those carrying two reported alleles, thus forming homozygote cluster splitting that could not be detected.

5.3.1 Genotypes and Allele Frequencies for *PTGS2* gene

The human *PTGS2* gene is located at chromosome 1q25.2-q25.3, consisting of 10 exons which is about 8.3 kbp in size (Kosaka *et al*., 1994). The genotype distribution and allele frequencies of SNPs rs5275, rs20417, and rs689466 in gene *PTGS2* were analysed. All SNPs in test and control group were not significantly associated with CP (p>0.05) (Table 4.4). Notably, the results of minor allele frequency analysis showed that the frequency of minor allele in CP group for rs5275, rs20417, rs689466 and rs1047031 was greater with values of 30.8%, 9.0%, 37.2% and 42.9.6% as compared to control group, respectively. Among all the SNPs that were studied, only rs689466 showed a significant difference (p=0.03) between CP group (66.2%) versus control group (70.5%).

Xie *et al.* (2009) reported that that the A allele of rs689466 was associated with severe CP. The A allele frequency was higher in CP group (60.62%) than in control group (51.35%), and there was a higher frequency for the AA/GA genotypes compared with the GG genotype of rs689466 (adjusted OR: 2.49, 95% CI: 1.33–4.69, p=0.005) in the Chinese population. We found that the A allele was more prevalent in Malay controls compared to CP cases and there was no association with CP. *PTGS2* rs689466 is situated in the 5' flanking region of the *PTGS2* gene. Papafili (2002) suggested that many possible transcription factor binding sites exist in the promoter region of the *PTGS2* gene. There is a possibility that the interference with the specific binding between the transcription

factors and the promoter sequences by variant alleles could alter the gene expression. Zhang *et al.* (2005) suggested that the A allele at rs689466 site will increase gene expression by creating a C-MYB binding site. Most of the reports indicate that the A allele will increase the gene expression or the enzymatic activity resulting in increased disease susceptibility (Guo *et al.,* 2007).

Our study shows that rs5275 polymorphism was not associated with CP, which is in agreement with previous studies (Xie *et al.,* 2009; Daing *et al.,* 2012; Prakash *et al.,* 2015). However, a meta-analysis of these three studies suggested a role of rs5275 polymorphisms in overall CP susceptibility (Prakash *et al.,* 2015). The possible explanation of no significant association of rs5275 SNP in CP progression may be due to rs5275 polymorphism being located within the functional region of 3'UTR that affects mRNA stability and translational efficiency (Dixon *et al.,* 2013; Cok & Morrison, 2001). This may modulate susceptibility to periodontitis through differential expression of *PTGS2*.

Most of the previous studies carried out for rs20417 polymorphism were on the Han Chinese population (Ho *et al.,* 2008; Loo *et al.,* 2011; Li *et al.,* 2012). In the meta-analysis conducted by Prakash *et al.* (2015), rs20417 showed a significant association in CP progression in the Chinese. The possible mechanism could be that the C variant allele of rs20417 may cause disruption in the stimulatory protein-1 binding site, leading to around 30% lower promoter activity (Papafili, 2002). In the current study however, we did not find any significant association between rs20417 polymorphism with the progression of CP, which is similar to the findings reported by Prakash *et al.* (2015) in a North Indian population (200 cases versus 200 controls). No CC genotype was found in case group, and the frequency of C allele was lower in Malays. A study conducted by Szczeklik *et al.* (2004) reported contradicting results, patients with rs20417 showed increased

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biosynthesis of PGs by C allele. They reported that peripheral monocytes that produced PGE2 and PGD2 was significantly higher in the CC homozygotes compared with the GG. PGs induces *EGR1* production which promotes *PTGS2* gene positively or negatively. This is because, gene expression can be activated or inhibited by *EGR1* (induced during inflammatory response) (Gashler *et al.,* 1993; Tan *et al.,* 2003). This may be the reason of contradicting results in studies conducted on *PTGS2* SNP rs20417.

5.3.2 Genotypes and Allele Frequencies for *DEFB1* Gene

Schaefer *et al*. (2010b) reported contrasting results, showing significant associations of the A allele of the *DEFB1* rs1047031 with periodontal diseases (OR: 2.2 ;95% CI: 1.2–4.3; P=0.021), prior and after adjustment for confounders (smoking and gender). Schaefer *et al*. (2010b) showed that, the 3' untranslated region SNP rs1047031 of *DEFB1* reported the most significant association for homozygous carriers of the rare A allele (*p*-value=0.002) with an increased genetic risk of 1.3 (95% confidence interval: 1.11–1.57) in European population. They also observed that both AgP (p-value = 0.0342) and CP (p-value= 0.0253) subjects reported an association with SNPs rs1047031.The association was consistent with the specific forms of CP with odds ratio of 2.2 (95% confidence interval: 1.16–4.35, *p*-value=0.02) (Schaefer *et al*., 2010b).

DEFB1 rs1047031 is located in microRNA binding sites that are potentially able to alter hBD-1 expression (Prado-Montes, 2010). The exact mechanism of *DEFB1* in CP is still unknown. There might be few possibilities on why rs1047031 was not significantly associated with CP in Malay study group. A study reported low levels of DEFB1 mRNA in CP patients' biopsies as compared to healthy subjects (Lu *et al.,* 2004). Another study reported that DEFB1 mRNA expression was up -regulated in CP respect to healthy subjects (Vardar-Sengul *et al.,* 2007). There was still debate by researchers on how the rs1047031 variant damages the function of the maintenance of the epithelial barrier and

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the actual role of *DEFB1* in CP progression (Schaefer *et al*., 2010b). Besides that, there was also a possibility of missing out on the true or additional causal variant which is located within *DEFB1* gene. The contrasting results of rs1047031 polymorphism in different studies may due different ethnicity population, individual variation in cytokine production.

5.4 Strengths and Limitations of Study

5.4.1 Strengths

This study is the first to report the *PTGS2* and *DEFB1* genetic polymorphism and their association with CP in Malay ethnic group, which is the main ethnic group in Malaysia. Genetic heterogeneity occurs if different ethnic populations have been mixed that can affect the SNP detection (Vaithilingam *et al.,* 2014). In order to ensure that the study subjects were only Malay and not from mixed background, we have collected info regarding paternal and maternal grandparents ethnicity. The controls were ethnically matched and were examined to have no periodontal disease. Data from the present study could assist researchers and practitioners in downstream studies not limited to the Malaysian population, but also to other populations in the Malay Archipelago.

5.4.2 Limitations

The main limitation in this study was the small sample size, as we were unable to retrieve large number of samples that fulfilled our inclusion and exclusion criteria. As CP is a complex disease, a very large sample size is required in order to be able to determine the risk variants involved (Vaithilingam *et al.,* 2014). We were not able to collect the desired number of samples as our inclusion criteria was limited to the Malay ethnic group and the patients from the MPDBS were from the various ethnic groups in Malaysia. Besides that, as CP is a complex (multifactorial and polygenic) disease, it may be highly influenced by

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environmental and lifestyle factors, which may contribute many underlying low-risk variants. These variants may have gene-gene interaction, gene–environment and gene–lifestyle interactions. Currently, candidate gene association studies that involves complex diseases easily encounter problems, because genomic markers often show very low and insignificant relative risks of lesser than OR: 1.3 when associated with the disease (Jostins *et al*., 2012). As the required case numbers increases exponentially with decreasing relative risk, thus it is impossible to achieve sufficient statistical power in these studies (Stüber *et al.*, 2005). As GWAS was not feasible due to prohibitive costs in conducting large-scale genotyping, thus we chose a candidate gene study design approach instead to determine genetic variations that are involved in CP progression. During sample selection, we did not exclude smokers. We acknowledge that smoking is also a confounder for CP that contributes to an increase in prevalence and severity of CP (Papapanou, 1996; Susin *et al.,* 2004). However, it was difficult to exclude smokers from the study.

Besides that, limitations in many studies of genetics in periodontitis may be due to other unidentified factors such as the use of antibiotics or drugs, underlying systemic diseases as well as in consideration of lifestyle factors such as smoking. For this study, we chose to exclude participants who had other autoimmune diseases and diabetes mellitus which are potential risk factors that might affect our study outcomes. Furthermore, the complete genetic information of a particular region of interest in most of the candidate gene studies in periodontitis have not been captured. According to Laine *et al*. (2014), only one or a few candidates of SNPs of the genetic locus of interest in most of the studies were genotyped instead of complete haplotypes. As discussed by Schaefer *et al.* (2011a), only one or a few candidates of SNPs of the genetic locus of interest in most of the studies were genotyped instead of complete haplotypes in candidate gene studies. In order to

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capture rare variants, ‘next-generation sequencing’, of the entire genomes can be conducted. Thus, a future genome-wide association study is advocated.

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CHAPTER 6: CONCLUSION AND FUTURE RECOMMENDATIONS

In conclusion, although the results from this study suggest no association between the *PTGS2* SNPs rs5272, rs20417, rs689466 and *DEFB1* SNP rs1047031 with CP in Malays, more studies with larger sample size are needed to validate the findings. Knowledge obtained from these investigations will further improve our understanding in the pathogenesis of CP. This can lead to prevention and improvement of periodontal disease diagnosis and treatment in the future.

In order to investigate the role of *PTGS2* and *DEFB1* gene in CP, further studies should be conducted in a larger sample size and a prospective study for validating the current findings. CP is a multifactorial and polygenic disease, which may be associated with combination of genes or environmental factors. Future studies may consider to conduct epistasis/gene-gene, gene-environment or gene-environment-CP interaction analysis to yield a larger effect size on the disease susceptibility (Yang *et al.,* 2009 Cordell, 2009), which will further increase our understanding of CP pathogenesis, leading to prevention and improvement of periodontal disease diagnosis and treatment in the future. Besides that, GWAS study should be carried out in the future as it is allows 'hypothesis-free' and unbiased analysis of the genome, which is the contrast of the candidate gene approach in identifying the genetic variants for CP.

Universiti Malaya

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