PERIODONTAL AND MICROBIAL RESPONSES OF OBESE INDIVIDUALS AFTER SHORT TERM NON-SURGICAL PERIODONTAL THERAPY

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DEPARTMENT OF RESTORATIVE DENTISTRY
FACULTY OF DENTISTRY
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF DENTAL SCIENCE

DEPARTMENT OF RESTORATIVE DENTISTRY
FACULTY OF DENTISTRY
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

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ABSTRACT

The association between obesity and chronic periodontitis has been well documented. However, the response of obesity on periodontal and microbial following non–surgical periodontal therapy (NSPT) remains unclear. Thus, this study is aimed to investigate the response of obesity on the clinical parameters and periodontal pathogens profile following short term of NSPT. The study also assessed the correlation between clinical parameters and periodontal pathogens profile. This prospective study involved 30 obese (test group) and 30 non–obese (control group). Obese is defined as individual who have BMI $\geq$ 30.0 kg/m^2. Ethical approval was obtained (DF PE1501/0085(L)). The clinical parameters (visible plaque index (VPI), gingival bleeding index (GBI), probing pocket depth (PPD) and clinical attachment loss (CAL)) were obtained from the Malaysian Periodontal Database and Biobank Database (MPDBS). The subgingival plaques samples were collected at baseline and at 12 weeks following NSPT by previous researchers. Quantitative real–time polymerase chain reaction (qPCR) was used to quantify the mean count of periodontal pathogens. The obese group showed greater reduction in mean differences of VPI and GBI (69% and 53%) at 12 weeks post NSPT compared to the non–obese group (15% and 15%) respectively. After adjusting the confounding factors, the obese group was found as a potential associated ($p < 0.0001$) factor in mean changes of VPI and GBI. Following NSPT, means PPD and CAL in both groups was significantly reduced after 12 weeks except for CAL in the non–obese groups. In terms of periodontal pathogens profile, the obese group showed significant reduction only on *P. intermedia* (up to 49%) compared with reduction means count of *P. gingivalis*, *T. forsythia* and *A. actinomycetemcomitans* (up to 100%) in the non–obese group. No correlation was found between the clinical parameters and periodontal pathogens profiles in the obese groups with chronic periodontitis following 12 weeks

NSPT. From the findings, it can be summarized that the obese group showed higher VPI and GBI but lower PPD and CAL compared to the non–obese group following 12 weeks NSPT. Furthermore, lower *P. gingivalis* and *T. forsythia* but higher *P. intermedia* were observed in the obese group at 12 weeks post NSPT. No correlation was found between clinical parameters and periodontal pathogens profile in the obese group.

ABSTRAK

Perkaitan di antara obesiti dan penyakit kronik periodontitis telah didokumentasikan dengan jelas. Walaubagaimanapun, respon obesiti terhadap periodontal dan mikrobial selepas rawatan periodontium tanpa pembedahan (NSPT) masih belum jelas. Oleh itu, penyelidikan ini bertujuan menyelidik respon obesiti terhadap parameter klinikal dan profil patogen periodontal dalam jangka masa pendek selepas NSPT. Kajian juga turut menilai sekiranya terdapat hubungkait di antara parameter klinikal dan profil patogen periodontal. Kajian prospektif ini melibatkan 30 peserta obes (kumpulan ujian) dan 30 peserta bukan obes (kumpulan kawalan). Definisi obesiti adalah individu yang mempunyai BMI $\geq$ 30.0 kg/m^2. Kelulusan etika diperolehi (DF PE1501/0085(L)). Parameter klinikal indek plak visibel (VPI), indek pendarahan gingiva (GBI), kedalaman poket periodontium (PPD) and kehilangan atakmen klinikal (CAL) diperolehi daripada pangkalan data Malaysian Periodontal Database dan Biobank Database (MPDBS). Sampel plak subgingiva telah diambil pada masa permulaan dan 12 minggu selepas NSPT oleh penyelidik terdahulu. Kuantitatif masa sebenar reaksi polimerase berantai (qPCR) digunakan untuk mengira purata bilangan patogen periodonal. Kumpulan obes menunjukkan pengurangan purata perbezaan lebih besar untuk VPI dan GBI (69% dan 53%) selepas 12 minggu NSPT berbanding dengan kumpulan bukan obes (15% dan 15%). Setelah faktor pembaur diselaraskan, kumpulan obes telah dikenal pasti sebagai salah satu faktor yang berpotensi untuk dikaitkan (p < 0.0001) dengan perubahan purata VPI dan GBI. Selepas NSPT, purata PPD dan CAL untuk kedua-dua kumpulan menunjukkan pengurangan yang signifikan kecuali CAL pada kumpulan bukan obes. Dari segi profil patogen periodontal, kumpulan obes menunjukkan pengurangan yang signifikan hanya terhadap *P. intermedia* (sehingga 49%) berbanding dengan purata bilangan *P. gingivalis*, *T. forsythia* dan *A. actinomycetemcomitans* (sehingga 100%) untuk kumpulan bukan obes. Tiada

hubungkait yang ditemui di antara klinikal parameter dan profil patogen periodontal dalam kumpulan obes selepas 12 minggu NSPT. Hasil daripada kajian penyelidikan ini, dapat dirumuskan bahawa kumpulan obes menunjukkan VPI dan GBI yang lebih tinggi tetapi PPD dan CAL yang lebih rendah berbanding kumpulan bukan obes selepas 12 minggu NSPT. Tambahan lagi, *P. gingivalis* dan *T. forsythia* yang lebih rendah tetapi *P. intermedia* yang lebih tinggi telah diperhatikan dalam kumpulan obes pada minggu ke 12 selepas NSPT. Tiada hubungkait ditemui di antara parameter klinikal dan profil patogen periodontal dalam kumpulan obes.

ACKNOWLEDGEMENTS

Alhamdulillah, in the name of Almighty God, ALLAH S. W. T, The Most Merciful, I would like to express my sincere gratitude for His blesses and guidance that had finally enabled me to complete this writing successfully on required time.

First and foremost, I would like to extend my greatest gratitude to my supervisor, Assoc. Prof. Dr. Nor Adinar Baharuddin from Department of Restorative Dentistry, Faculty of Dentistry, for her continual and tireless assistance and constructive suggestions during the planning, and development of this research work. Her willingness to spend her time so generously is very much appreciated.

I would like to express my very utmost appreciation to my co-supervisor Assoc. Prof. Dr. Aied Mohamed from Department of Oral Science and Craniofacial, Faculty of Dentistry for his guidance, constructive recommendations and encouragement throughout the span of this study.

A word of thanks also goes to Assoc. Prof. Dr. Roslan Saub as the statistician of this study for his endless assistance and supports. Besides that, a word of thanks also goes to postgraduate students; Dr Shahrukh Khan, Dr Zohaib Akram, Dr Samira Bashir, Dr Ainul Haliza and Dr Azzyytie for their efforts in samples selections and collections to ensure that this study flows smoothly.

Particularly, I would like to acknowledge Assoc. Prof. Dr. Rathna Devi and Nur Solehah, for valuable discussions that helped me understand my research area better.

From the bottom of my heart, special thanks go to all of the staffs of Postgraduate Clinic in University of Malaya and Craniofacial & Molecular Biology Research Laboratory (CMBRL).

Special thank also goes to my friend, Nurul Anis Ridzuan, who always supported and accompanied me while I was completing my lab work over the weekends and till midnight.

I would also like to express my sincere gratitude to my beloved parents; Mr. Md. Tahir and Mdm Asmah, my siblings especially my sister, Hanamasayu for their endless prays, never ending love, supports and encouragements to me in this journey. Not forgetting to extend my most sincere thanks and appreciation to my beloved husband, Shaiful Azhwat who helped me through the hurdles, and stays sane through all those difficult years of studying.

Finally, I appreciate the financial support from UMRG and IPPP that funded this dissertation.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
°C	Celsius
-	Minus
%	Percentage
C_T	Threshold Cycle
kb	Kilo base pair
kg	Kilogram
m	Meter
ml	Milliliter
mm	Millimeter
ng	Nanogram
rpm	Round Per Minute
µl	Microliter
AAP	American Academy of Periodontology
A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
ATCC	American Type Culture Collection
Av	*Actinomyces* sp.
BMI	Body Mass Index
BPE	Basic Periodontal Examination
CAL	Clinical Attachment Loss
Cg	*Capnocytophaga* sp.
CI	Confident Interval
CO_2	Carbon Dioxide
CNS	Central Nervous System

CPG	Clinical Practice Guideline
DNA	Deoxyribonucleic acid
FEME	Full Mouth Periodontal Examination
Fn	*Fusobacterium nucleatum*
GBI	Gingival Bleeding Index
H_2	Hydrogen
H_A	Alternative Hypotheses
H_O	Null Hypotheses
IDF	International Diabetic Federation
IL	Interleukin
MMPs	Matrix Metalloproteinase
MPDBS	Malaysian Periodontal Database and Biobank System
N_2	Nitrogen
NHANES	National Health and Nutrition Examination Survey
NHMS	National Health and Morbidity Survey
NOHSA	National Oral Health Survey
NSPT	Non–Surgical Periodontal Therapy
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
P. gingivalis/*Pg*	*Porphyromonas gingivalis*
P. intermedia/*Pi*	*Prevotella intermedia*
PPD	Periodontal Pocket Depth
PPP	Postgraduate Research Grant
qPCR	Quantitative Real–Time Polymerase Chain Reaction

R	Correlation Coefficient
RT–PCR	Real–Time Polymerase Chain Reaction
RTX	Repeats in Toxins
Ss	*Streptococcus sanguinis*
SD	Standard Deviation
SPSS	Statistical Package for the Social Sciences
T. denticola	*Treponema denticola*
T. forsythia	*Tannerella forsythia*
TNF–α	Tumor Necrosis Factor–α
UMRG	University Malaya Research Grant
USA	United State of America
VPI	Visible Plaque Index
WC	Waist Circumference
WHO	World Health Organization

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Obesity is an important, yet most neglected public health problem. Obesity has been shown to have an impact to the overall health issues in both developing and developed countries. It is characterized as excessive amount of body fat in proportion to lean body mass. Recently, some factors such as economic, social and lifestyle changes could be a burden and predispositions of obesity (Rampal *et* al., 2007; Khambalia & Seen, 2010).

Globally, obesity is an epidemic, with increasing prevalence in various age populations. The World Health Organization (WHO) European region reported that the prevalence of obesity has tripled since 1980s (Doak *et al*., 2012). Study in the United States of America (USA) showed the increment of the prevalence of obesity across both genders (Ogden *et al*., 2006). In Malaysia, 1.85 million Malaysians were obese (Rampal *et al*., 2007). The prevalence of obesity among the Malaysian population was reported higher as compared to other Southeast Asian countries (Kee *et al*., 2008).

Chronic periodontitis is a group of conditions that cause gingival inflammation and destruction of hard tissues, if left untreated it will lead to tooth loss (Strohm & Alt, 1998). Epidemiological and microbiological studies have accepted that dental plaque is the aetiology for chronic periodontitis (Loe *et al*., 1965).

The prevalence of chronic periodontitis among general population showed the increment of about 30 to 35% in worldwide (WHO, 2007) and about 48.5% in Malaysia (NOHSA, 2010). Interestingly, the prevalence of chronic periodontitis among obese population continues the increasing trend by 47 to 52% in worldwide (Eke *et al*., 2012;

Dye, 2012; Khader *et al*., 2009) and again Malaysia reported the highest number with 74% (Khan *et al*., 2015).

For chronic periodontitis treatment, periodontal therapy is necessary which is aimed to provide a dentition that function in health and comfort for the life of the patient (Zander *et al*., 1976). Non–surgical periodontal therapy (NSPT) focuses on elimination of bacterial plaque on the root surface by means of scaling and root planing. The effects of NSPT can be measured on clinical parameters and periodontal pathogens profile.

The oral cavity is dominated by hundreds of bacterial species including periodontal normal flora and pathogens. More than 700 species have been detected in several studies (Aas *et al*., 2005; Paster *et al*., 2006). Among all, *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia* (*T. forsythia)*, *Prevotella intermedia* (*P. intermedia*) and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) have been linked with chronic periodontitis in adult populations (Figuero-Ruiz & Martínez, 2005). The quantitative real–time polymerase chain reaction (qPCR) has been shown to be more reliable and sensitive method for detection and quantify of individual periodontal pathogens (Boutaga *et al*., 2007).

The response of NSPT can be seen through the changes of clinical parameters of chronic periodontitis includes visible plaque index (VPI), gingival bleeding index (GBI), periodontal pocket depth (PPD) and clinical attachment loss (CAL). Sites with initial higher PPD showed significantly greater pocket depth reduction post 12 months NSPT (Haffajee *et al*., 2006).

Furthermore, the response of NSPT also can be seen through the changes of microbial counts. The prevalence of *P. gingivalis, T. forsythia, P. intermedia* and *A. actinomycetemcomitans* prior to 12 weeks NSPT was reduced in 7% (Ivić-Kardum *et al*., 2001) and up to 45% (Predin *et al*., 2014) of chronic periodontitis subjects.

Obesity has been recognized as risk for complex diseases such as cardiovascular diseases, hypertension and Types 2 diabetes (Ritchie, 2007; Matsushita *et al*., 2014). Meanwhile, in dentistry, obesity has been suggested as potential risk factor for chronic periodontitis (Mathur *et al*., 2011). In Malaysia, the highest prevalence of these two conditions of obesity and chronic periodontitis has become worrisome.

To date, there is not so much research that examined on the clinical parameters and periodontal pathogens profile in obese Malaysian population with chronic periodontitis following NSPT. Thus, the aims of this study were to determine the response of obesity with chronic periodontitis in terms of clinical parameters (VPI, GBI, PPD and CAL) and periodontal pathogens (*P. gingivalis, T. forsythia, P. intermedia* and *A. actinomycetemcomitans*) profiles following 12 weeks NSPT.

The objectives for this study were:

1. To compare clinical parameters (VPI, GBI, PPD and CAL) and periodontal pathogens (*P. gingivalis, T. forsythia, P. intermedia* and *A. actinomycetemcomitans*) profiles in obese with chronic periodontitis following 12 weeks NSPT
2. To assess correlation between clinical parameters (VPI, GBI, PPD and CAL) and periodontal pathogens (*P. gingivalis, T. forsythia, P. intermedia* and *A. actinomycetemcomitans*) profiles in obese with chronic periodontitis following 12 weeks NSPT

The hypotheses for this study were:

1. To determine the clinical parameters and periodontal pathogens profiles between obese and non–obese with chronic periodontitis following 12 weeks NSPT.

   H_O : There is no difference in clinical parameters and periodontal pathogens profiles between obese and non–obese with chronic periodontitis following 12 weeks NSPT.

   H_A : There is a difference in clinical parameters and periodontal pathogens profiles between obese and non–obese with chronic periodontitis following 12 weeks NSPT.

2. To assess correlation in clinical parameters and periodontal pathogens profiles between obese and non–obese with chronic periodontitis following 12 weeks NSPT.

   H_O : There is no correlation in clinical parameters and periodontal pathogens profiles between obese and non–obese with chronic periodontitis following 12 weeks NSPT.

   H_A : There is a correlation in clinical parameters and periodontal pathogens profiles between obese and non–obese with chronic periodontitis following 12 weeks NSPT.

CHAPTER 2: LITERATURE REVIEW

2.1 Obesity

2.1.1 Definition of Obesity

Obesity is characterized as excessive accumulation of body–fat in proportion to lean body mass. The excess of adipose tissue or excess lipid deposition in obesity might cause co–morbidity conditions (Prentice & Jebb, 2001).

WHO classified obesity on the basis of body mass index (BMI) levels, which varies depending on populations. Currently, the international guidelines recommended waist circumference (WC) alongside with BMI by means of measurement of visceral abdominal fat i.e. for adiposity assessment (Ritchie, 2007).

Obesity is associated with economic, social, and lifestyle changes. In this modern world, people are more incline towards fast food which is rich in calories and adopting low physical activity lifestyle (Rampal *et al*., 2007; Khambalia & Seen, 2010). These changes in lifestyle could contribute to overweight and obesity across all age groups. Prolonged unhealthy lifestyle practices have been shown to increase the risk and prevalence of obesity and subsequently lead to chronic conditions. In addition, obesity has been recognized as an established risk for common metabolic disorder, such as, cardiovascular diseases, hypertension and Types 2 diabetes (Eckel *et al*., 2005).

2.1.2 Body Mass Index (BMI)

BMI is a measurement tool for classifying underweight, overweight and obesity. The BMI of a person can be calculated based on one's weight in kilograms (kg) divided with square of one's height in meter (m).

$$\text{BMI (kg/m}^2\text{)} = \frac{\text{Body weight (kg)}}{\text{Height (m}^2\text{)}}$$

Table 2.1: BMI for adults based on WHO and Asian classification (WHO, 2004; Ismail *et al*., 2004; WHO, 2012)

Classification	BMI (kg/m^2)		Risk of comorbidities	
	WHO	Asian	WHO	Asian
Underweight	< 18.5	< 18.5	Low*	Low*
Normal range	18.5 – 24.9	18.5 – 22.9	Average	Increase
Overweight	≥ 25.0	≥ 23.0		
Pre obese	25.0 – 29.9	23.0 – 27.4	Increased	Increased
Obese I	30.0 – 34.9	27.5 – 34.9	Moderate	High
Obese II	35.0 – 39.9	35.0 – 39.9	Severe	Very high
Obese III	≥ 40.0	≥ 40.0	Very severe	Extremely high

*but increased risk of other clinical problem

The cut–off point for obesity in the Asian population is lower compared to the Caucasian/European populations (WHO, 2004; Ismail *et al*., 2004). For the Caucasian/European population, obesity is defined as individuals having BMI of ≥ 30.0 kg/m^2 (WHO, 2012). On the other hand, for the Asian population the obesity is defined as individuals having BMI of ≥ 27.5 kg/m^2 (Ismail *et al*., 2004). Table 2.1 summarized the BMI for adults based on WHO and Asian classifications. However, the disadvantage of the BMI is solely weight measurements with muscle and does not include measurements of body fat mass. Thus, it is recommended that BMI must be used together with abdominal fat mass (WHO, 2004; Ismail *et al*., 2004).

The underweight and normal weight categories have the same BMI range for both classifications. The different in cut–off point between both classifications (WHO and Asian) can be seen from overweight until Obese I categories. The WHO classification has been used by many previous studies including those studies conducted in Malaysia (Rampal *et al*., 2007; Sidik & Rampal., 2009). Thus, this would allow easy comparison of outcomes. In contrast, the Asian cut–off point for normal BMI have a small range (18.5 to 22.9 kg/m^2). Together with high prevalence of obesity in Malaysia, this will restrict the number of subject recruitments for the control non–obese. Therefore, WHO classification was the classification of choice to ensure adequate sample size in the current study.

2.1.3 Prevalence of Obesity

(a) Prevalence of Obesity Worldwide

The WHO European region reported that the prevalence of obesity has tripled since 1980s (Doak *et al*., 2011). Scotland is the most obese population amongst the European

developed countries. There has been a steady upward trend in the prevalence of overweight and obesity for both genders, between ages of 16 to 64 since 1995. It has been reported that 15.9% of men (16 to 64 years age group) were obese in 1995 compared to 24.9% in 2008. The equivalent figures for women were 17.3% and 26.5% respectively. In both genders, age pattern for those who were overweight and obese were higher among those 50 to 64 years age group compared to the 25 to 49 years age group.

The information on prevalence of obesity in the United States of America (USA) from 1999 to 2004 was obtained from the National Health and Nutrition Examination Survey (NHANES). Test of trend using logistic regression for 6 single–year data showed a significant increase in the prevalence of obesity among men between 1999 to 2000 (27.5%) and between 2003 to 2004 (31.1%) (Ogden *et al*., 2006). Obese analysis based on age between 2003 to 2004 showed approximately 28.5% of adults aged between 20 to 39 years, 36.8% of adults aged between 40 to 59 years, and 31.0% of those aged 60 years or older were obese. Comparison studies among the USA ethnic populations reported that Mexican American and non–Hispanic black women were significantly more likely to be obese compared with Hispanic white women but inversely proportional to the men (Ogden *et al*., 2006).

Meanwhile, studies in the Eastern Saudi Arabia showed middle–aged (30 to 60 years age group) adults have extremely high prevalence rates of obesity which increases over time especially in Kuwait and Saudi Arabia (Ng *et al*., 2011). The trends of obesity have increased from 79% (1994) up to 100% (2006) in Kuwait while the pattern continues with 63% (1992) to 82% (2005) in Saudi Arabia. In both countries, the prevalence of obesity was reported higher among middle–aged compared to young adult (16 to 30 years) groups, however, the prevalence was not more than 55%. It was suggested that gender

disparity between men and women across all age groups with more prevalence among women could possibly due to marriage and childbirth. In middle–age group this disparity between both genders was around 10% to 24% differences.

The prevalence of overweight and obesity (adults above 20 years) in the Asia Pacific region showed a wide range from 5% in India (the lowest) to 60% in Australia (the highest) (Collaboration, 2007). The prevalence in overweight and obesity in China and Japan was reported to be 2 to 3 times less than those in Australia. However, their overall increment in prevalence over 20 years was up to 400% compared to only 20% in Australia. The increment could be associated with geographical areas. In the mainland of China, the urban area has been reported to have higher percentages of obese individuals than the rural area (Reynolds *et al*., 2007). The differences in obesity prevalence were also observed among Chinese in the Northern China compared to the Southern China region.

(b) Prevalence of Obesity in Malaysia

A population–based cross sectional study conducted in all states of Malaysia has identified approximately 1.85 million obese Malaysians, comprised of 0.76 million men and 1.09 million women. The prevalence of obesity was reported to increase with age, especially among men in the age 40 to 49 years age group (12.1%) and among women in the 50 to 59 years age group (27.2%) (Rampal *et al*., 2007)

In term of ethnicity, the Malay and Indian populations have been reported to be dominantly obese compared to other races with obesity prevalence 13.6% and 13.5% respectively. Interestingly, education level and smoking habit status also have an influence on the prevalence of obesity. Those with at least tertiary education and those who were

current smokers had lower prevalence of obesity with 8.8% and 8.3% respectively (Rampal *et al*., 2007).

The same cross sectional study also reported prevalence of obesity in one of the states in Malaysia, Selangor. There was a significant association between obesity and age (p = 0.013). Those respondents from the 50 to 59 years age group had higher prevalence of obesity (58.2%) compared to those of 20 to 49 years age group (45.6%) (Sidik & Rampal., 2009).

A study in Malaysia showed a correlation between prevalence of obesity and work. Desk work related lifestyle increases the tendency for overweight and obesity among working adults. However, no significant relationship was established between types of occupation, working days per week and duration of computer use in workplace with obesity (Cheong *et al*., 2010).

A study that investigated the sociodemographic and health-lifestyle in Malaysia has identified racial ethnic group, gender, education level, family history and smoking status as statistically significant determinant risk factors for obesity. In details, those from the Malay ethnic group, female gender, least year formal education, family history with systemic disease and non-smoker were more likely to be obese (Tan *et al*., 2011).

In recent decades, Malaysia has experienced rapid development, which in turn gives rise to urbanization that contributes to changes in lifestyle. This may exert a strong social impact on the people's diet and subsequently a raise in the prevalence of obesity in Malaysia. The National Health and Morbidity Survey III (NHMS III) conducted in 2006 reported a higher (47.3% in women and 30.1% in men) prevalence of obesity among the Malaysian population as compared to other Southeast Asian countries. The study used

waist circumference (WC) to determine abdominal obesity; with International Diabetic Federation cut–off points for Southeast Asian population (Malay, Indian and Chinese) i.e. > 90 cm for men and 80 cm for women. Interestingly, when the abdominal obesity was defined using the WHO cut off points (102 cm for men and 88 cm for woman) the prevalence was substantially lower with 17.4% for women and 7.2% for men (Collaboration, 2007).

The NHMS III data also reported the prevalence of obesity was 29.8% and abdominal obesity was 54.8% among the Malaysian elderly population (Suzana *et al*., 2012). Moreover, there was a decreasing trend of obesity with an increase in age. Those between 60 to 64 years age group has a higher obesity prevalence (12.8%) compared to those who are much older (> 80 years old) (3.9%). The findings were based on WHO standard cut off value for both BMI and WC.

2.1.4 Obesity Related Inflammatory Diseases/Conditions

Adipose tissue is an important endocrine organ. Besides fat cells, adipose tissues consist of blood vessels, nerve cells and act as storage and release energy when body is in need. Adipose tissue include adipocytes are responsible for synthesizing and secreting numerous hormones, such as, leptin and adiponectin.

Leptin signal carried through peripheral and central nervous system (CNS) pathway. The signals responsible to suppress appetite and increase energy consume through glucose uptake in muscle and adipose tissue (Coelho *et al*., 2013; O'Rourke, 2009). Both over and underproduction of adipose tissue may result in severe health implications. Excessive adipose tissue leads to obesity plays a role as primary *in–vo* site of inflammation.

Inflammatory diseases may cause failure in CNS leptin signaling (O'Rourke, 2009; Ritchie, 2007; Coelho *et al*., 2013). Following signal failure, the body will experience a decrease in energy usage. This condition is referred to as leptin resistance and, also known as, hormonal defect. A few studies have reported that leptin level was higher in females compared to males. This could be due to an excessive production of leptin caused by stimulation of estrogens in obese females and a reduction in the development of androgens in obese male (Isidori *et al*., 1999; Gao & Horvath, 2008).

Adiponectin is responsible to glucose and lipid metabolism. Adiponectin mediates insulin, sensitizing and plays as protector to pathogenesis of Type 2 diabetes. Obese individual has minimal adiponectin production compared to lean individuals. Both adults and children who suffered abdominal obesity have a greater risk to this disease compared to peripheral fat distribution (Hurt *et al*., 2010). A reduction in adiponectin level in obese individuals may lead to insulin resistant (Yadav *et al*., 2013). Adiponectin also has anti–atherogenic feature which can play a protective role in cardiovascular disease. Thus, obese individuals with low adiponectin level may have a significant increased risk of coronary disease (Berg & Scherer, 2005).

Adipose tissue also secretes adipokines and cytokines. Among all, tumor necrosis factor–α (TNF–α) is one of pro–inflammatory cytokines secreted by adipose tissue (Ritchie, 2007), and the association between obesity and TNF–α has been reported (Genco *et al*., 2005). It has been reported that TNF–α level in serum and saliva was found higher in obese patients with chronic periodontitis (75.15, 50.11 pg/mL) compared with non–obese patients with healthy periodontal (32.14, 22.62 pg/mL) (Kose *et al*., 2015) respectively. Circulating TNF level was more in chronic periodontitis (Bretz *et al*., 2005) and was significantly associated with severity of chronic periodontitis (Engebretson *et al*., 2007).

Thus, obesity and chronic periodontitis has been associated with the increasing level of TNF–α. It has been suggested that obesity may a play role in modulating TNF level; and may influence the pathogenesis of periodontal disease. It was hypothesized that obesity contributes to an overall inflammatory burden of an individual and subsequently this may alter the course of the periodontal disease (Saito *et al*., 2005).

2.1.5 Relationship between Obesity and Chronic Periodontitis

The relationship between obesity and chronic periodontitis is well documented in clinical and epidemiological studies (Zuza *et al*., 2011; Dias Gonçalves *et al*., 2015; Khan *et al*., 2015; Vitolo, 2015). Various studies have demonstrated high prevalence of chronic periodontitis among obese populations, including the European, American, Japanese, Jordanian, and Malaysian (Linden *et al*., 2007; Al-Zahrani *et al*., 2003; Ekuni *et al*., 2008; Khader *et al*., 2009; Khan *et al*., 2015). European men (between the 60 to 70 age range) presented with high BMI (≥30 kg/m^2) have been associated with chronic periodontitis. However, the prevalence was significant with low threshold chronic periodontitis which is at least 2 teeth present with non–contiguous interproximal sites with 1 pocket ≥ 5 mm and ≥ 6 mm loss of attachment (Linden *et al*., 2007).

Other studies investigated the correlation between periodontal disease status and obesity reported that younger adults population (age 18 to 34 years old) was associated with an increased in prevalence of abdominal obesity (Al-Zahrani *et al*., 2003). Similar findings were also found among Japanese university students. These students demonstrated high risk for chronic periodontitis and it was dependent on the BMI level. The risk of chronic periodontitis has increased by 16% for each 1-kg/m^2 of BMI (Ekuni *et al*., 2008).

A study among Jordanian population reported 51.9% of obese participants had periodontal disease compared to overweight (29.6%) and normal weight (14%) participants. Moreover, the obese group showed significantly higher average of VPI ($p = 0.002$), GBI ($p = 0.001$), PPD ($p = 0.002$), CAL ($p < 0.0005$) (Khader *et al*., 2009).

A study among Malaysian population showed that 74% of obese Malaysians had chronic periodontitis and 40% of them were adults (30 to 39 years age group). Almost 80% of the obese population had VPI of ≥ 20% and GBI of ≥ 30%, thus supported the notion that there is a strong relationship between obesity and chronic periodontitis ($p < 0.001$) (Khan *et al*., 2015).

2.2 Periodontal Disease

2.2.1 Definition of Periodontal Disease

Periodontal disease is a group of conditions that cause gingival inflammation, destruction of periodontal tissues and if untreated leads to tooth loss (Strohm & Alt, 1998). The two common types are chronic gingivitis and chronic periodontitis.

Chronic gingivitis refers to inflammation of the soft tissue namely the gingival tissue. The clinical features include changes in color, consistency, contour and texture of the gingival tissues. The inflamed gingival tissues appeared erythema and edematous, shiny and soft or spongy texture as a result of plaque accumulation (Figure 2.1). Usually, the inflamed tissues bleed spontaneous upon probing (Preshaw, 2014).

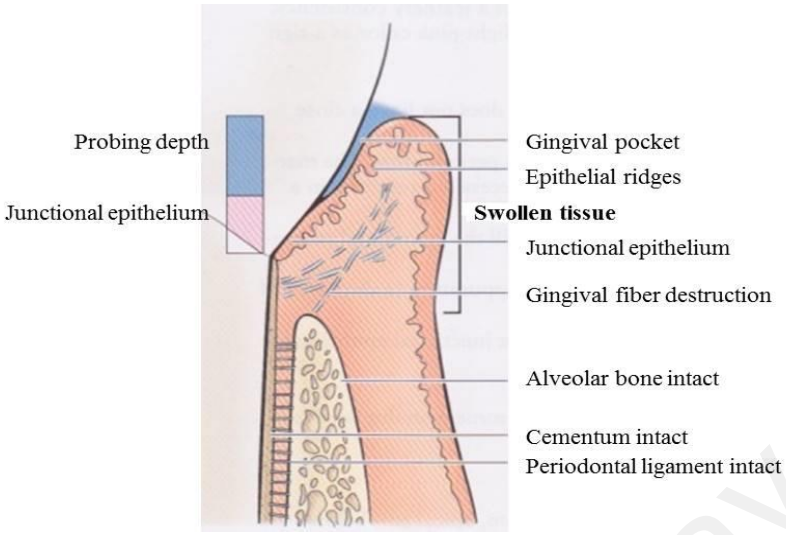


Figure 2.1: Illustration diagram of gingivitis (Nield-Gehrig & Willmann, 2011)

Chronic periodontitis is referring to inflammation extending to the hard tissues including alveolar bone and cementum. The clinical features of chronic periodontitis include PPD formation (Figure 2.2), recession, CAL, increased in mobility and reduced vertical height. If the condition is left untreated, it results in tooth loss (Preshaw, 2014).

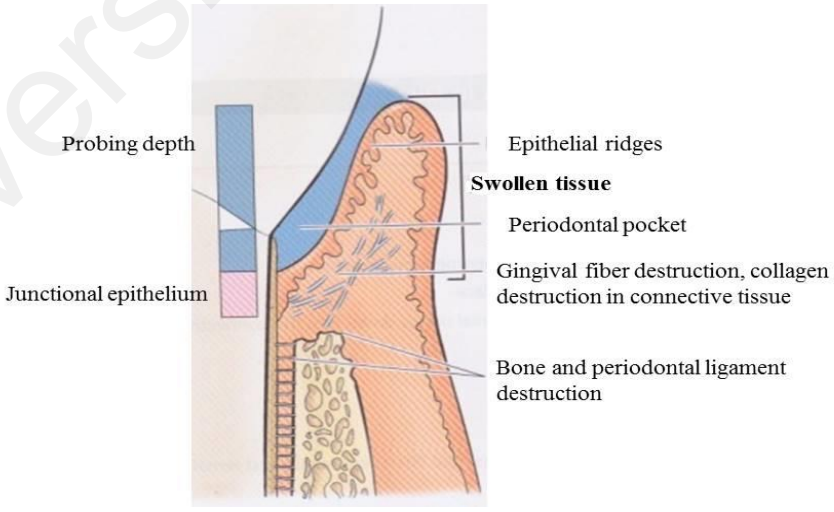


Figure 2.2: Illustration diagram of periodontitis (Nield-Gehrig & Willmann, 2011)

2.2.2 Etiology of Chronic Periodontitis

Decades ago, dental plaque was well accepted as the etiology of periodontal disease. In the same experimental gingivitis study, Löe *et al.*, (1965) also confirmed plaque accumulation caused gingivitis and reinstitution of oral hygiene can reverse gingivitis into healthy periodontal condition. Subsequently, a study also confirmed that dental plaque is the etiology for chronic periodontitis (Lindhe *et al*., 1973). Interaction between dental plaque and host related factors as genetics, environment and acquired conditions, such as diabetes mellitus and smoking contribute to the progression of chronic periodontitis (Garcia *et al*., 2001; Bergström, 2004).

2.2.3 Prevalence of Chronic Periodontitis

(a) Prevalence of Chronic Periodontitis Worldwide

The worldwide prevalence of chronic periodontitis among adults population was 30 to 35% (WHO, 2007). A national study in the USA reported about 64.7 million adults (aged 30 years and older) suffered chronic periodontitis (Eke *et al*., 2012). Similar finding was observed among Finnish population (Mattila *et al*., 2010). About 64% subjects (aged 30 years and above) had mild chronic periodontitis (PPD at least 4 mm) while about 21% had severe chronic periodontitis (PPD $\geq$ 6 mm). A study in South Brazil showed that the prevalence of chronic periodontitis was ranged between 18.2% and sharply increased up to 72.0% (Susin *et al*., 2011). However, this study focussed on adolescence (14 to 19 years old) and young adults (24 to 29 years old) subjects.

In Asia, a cross sectional study involved multi–center research in North Jordan has identified high prevalence of chronic periodontitis among subjects aged ≥ 40 years old (74.5%) compared to subjects aged < 40 years old (2.3%) (Ababneh *et al*., 2012). Similarly, approximately 65.3% prevalence of chronic periodontitis was observed among adults aged above 30 years old in rural district of India with 18.1% had ≥ 6 mm PPD (Jayakrishnan *et al*., 2005). Second national survey in China demonstrated prevalence of chronic periodontitis has increased with age. Adults (35 to 44 years old) had 12.8% shallow PPD and 2.1% deep PPD while elderly (65 to 74 years old) had 18.4% shallow PPD and 3.8% deep PPD (Hong-Ying *et al*., 2002).

(b) Prevalence of Chronic Periodontitis in Malaysia

In Malaysia, several oral health surveys in adults were carried out from 1970s till present. In general, the results showed that periodontal health decreased as age increased. The National Oral Health Survey (NOHSA) on Malaysian adults showed high prevalence (10.6%) of deep PPD (≥ 6 mm) was found in those between 45 to 54 years age group compared to those between 15 to 19 years age group (0.1%). The lowest prevalence of shallow PPD was found in those between 15 to 19 years age groups but higher in 55 to 64 years age group with 3.2% and 32.3% respectively (NOHSA, 2000).

A study investigating the elderly in East Malaysia estimated approximately 98.4% subjects was affected with chronic periodontitis. The highest proportion of chronic periodontitis was among aged group 50 to 59 years age group (96.9%), male gender (94.2%) and subjects with moderate PPD (4 to 5 mm) (36.8%) (Mohd-Saleh & Abdul-Kadir, 2010).

Meanwhile, study of oral diseases among urban poor population in Malaysia reported that chronic periodontitis was the commonest (97.1%) dental problem. The prevalence of deep PPD was 22.9% and 19.6% for pockets 4 to 5 mm and ≥ 6 mm, respectively. As expected, more than 90% of adults (≥ 30 years old) had higher deep PPD (≥ 6 mm) compared to young adults (≤ 29 years old). This study also showed that chronic periodontitis was significantly higher in male (95.9%) than females (92.7%) (Jaafar *et al*., 2014).

2.2.4 Pathogenesis of Chronic Periodontitis

Periodontal diseases result from the interaction of etiology factor, formation of subgingival dental plaque in microenvironment and additional influenced by host's immune and inflammatory response (Hernandez *et al*., 2011). Bacteria and host response were the primary factors that lead the occurrence and progression of chronic periodontitis (Figure 2.3). In response to bacteria challenge during periodontitis, the host's immune and inflammatory were activated. It caused the major destruction of soft and hard tissue (Hernandez *et al*., 2011). Generally, both bacteria and host tissue will release their products which are responsible in bone resorption and destruction of connective tissue during inflammation and immune reaction in chronic periodontitis (Yucel-Lindberg & Båge, 2013).

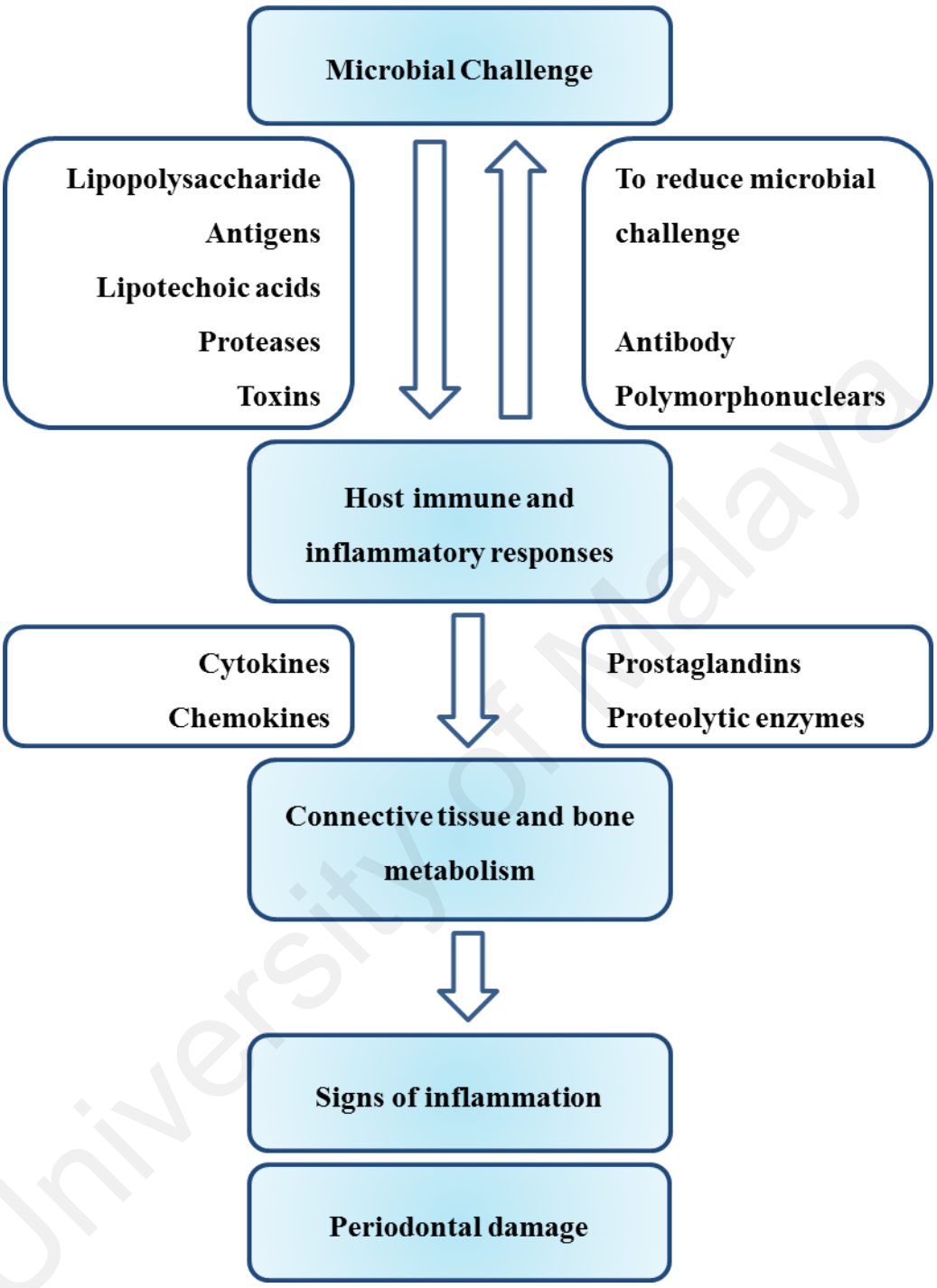


Figure 2.3: Schematic overview of the pathogenesis of periodontitis (Adapted and modified from Yucel-Lindberg & Båge, 2013)

(a) Bacterial Factors in Periodontal Destruction

Main products that are released in the pathogenesis of chronic periodontitis from the bacterial factor were lipopolysaccharides, capsular materials and bacterial enzymes. These products were secreted by Gram-negative bacteria. In agreement with Löe *et al*., (1965) study, Gram-positive bacteria were shifted to complex Gram-negative bacteria soon after the clinical signs of gingival inflammation appeared.

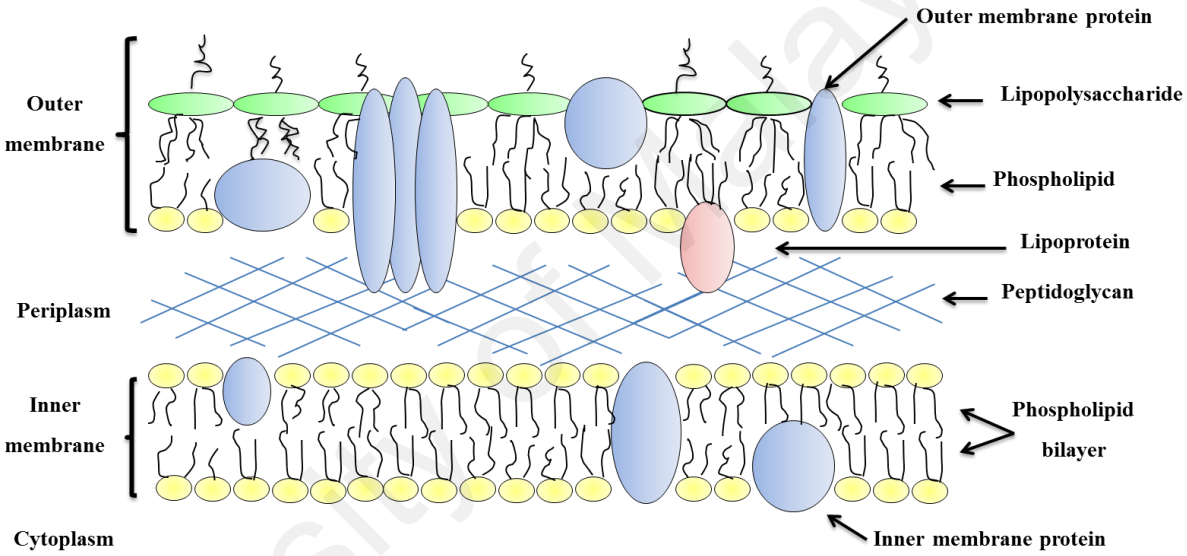


Figure 2.4: Schematic diagram of surface structure of Gram–negative bacteria (Adapted and modified from Dumitrescu & Ohara, 2010)

Lipopolysaccharide (Figure 2.4) is a cell wall component and played an important role in alveolar bone loss during chronic periodontitis. The ability of lipopolysaccharide in periodontal destruction was observed when lipopolysaccharide stimulates the bone resorption. Bone resorption occurred when osteoclasts break down bone and release the minerals, such as, calcium into the blood. Animal study using rats had showed the severe alveolar bone loss and proinflammatory cytokine were induced by lipopolysaccharide derived from *A. actinomycetemcomitans* (Rogers *et al*., 2007).

Capsular materials consist of hyaline polysaccharide which coats a bacterial cell. Gram negative bacteria include *P. gingivalis* (Laine & Winkelhoff, 1998) and *A. actinomycetemcomitans* (Wahasugui *et al*., 2013) that involved in the chronic periodontitis pathogenesis are encapsulated. It could be explained that capsule of *P. gingivalis* blocking the activation of host immune response system (Singh, 2011). In this condition, *P. gingivalis* would survive within host cells and increase the virulence. In addition, this capsule polysaccharide of *P. gingivalis* would interfere in host–pathogen interaction and decreases the immune response of human gingival fibroblasts (Brunner *et al*., 2010)

Enzymes released by bacterial also contain factor that might be responsible in the proteolytic activity and pathogenesis of chronic periodontitis. The secretion of bacterial enzyme, such as, proteases and peptidases by *P. gingivalis* act as source of nutrient for growth (Dumitrescu & Ohara, 2010). Furthermore, protease secreted by *A. actinomycetemcomitans* may prevent the proliferation of human gingival epithelial cells (Wang *et al*., 2001). Meanwhile, protease in *T. forsythia* showed its virulence through degradation host periodontal tissue and modifying host cell proteins for colonization of bacteria (Sharma, 2010). This could explain how the bacterial enzymes could be a factor of pathogenesis of chronic periodontitis.

(b) Host Factors of Periodontal Tissue Destruction

Host response mediated mainly by B and T lymphocytes, neutrophils and monocytes or macrophage also lead to the occurrence of chronic periodontitis. The inflammation reaction showed the response towards periodontal pathogens and this could trigger the destruction of periodontal tissues. During inflammation and immune reaction, these are activated to produce inflammatory mediators, such as, cytokines. Cytokines are soluble proteins mediator, responsible in tissue degradation and bone resorption. During inflammation, cytokines stimulates bone resorption and act as host–derived none resorbing factor to alveolar bone loss in chronic periodontitis (Kinane *et al*., 2008).

Interleukins are the one part of cytokine group that engaged in the inflammatory process. Interleukin (IL)–1a, IL–1b and tumor necrosis factor (TNF)–alpha were capable to stimulate bone resorption and inhibit bone formation (Kinane *et al*., 2008). Furthermore, increased activity of IL–1 and TNF were significantly found with reduction of osteoclasts formation and excessive bone loss (Assuma *et al*., 1998).

The periodontium comprised of fibrous elements and extracellular matrix components. Matrix metalloproteinase (MMPs) are capable for remodeling and degradation of the matrix components. MMPs component was expressed high in aggressive and chronic periodontitis (Garlet *et al*., 2004).

2.2.5 Case Definition of Chronic Periodontitis

The existence of periodontal disease for more than a century gives rise to the clinicians to recognize the need to classify them. A systematic classification will allow the clinicians to provide effective treatments. To date, there are various classifications of periodontal disease.

In the 19th century, classification was based on clinical characteristics and etiology of the periodontal disease. However, the classification was not evidence based. Later, American Academy of Periodontology (AAP) proposed classification scheme based on the principle of basic pathology (Wiebe & Putnins, 2000). Periodontal disease was classified as either (i) simple characterized by pocket formation or (ii) complex by means of advanced tissue destruction. Later, AAP developed with a new concept of classification which was based on rate of disease progression as well as response to treatment. In general, this classification has been accepted with minor changes in term of age during onset of the disease.

The use of various case definitions has given an impact on the published data on the prevalence of periodontal disease (Costa *et al*., 2009; Kassab *et al*., 2011). This has discouraged scientific comparisons across different populations worldwide. Hence, a standard case definition of periodontal disease is necessary for the use in epidemiology and clinical based study.

Page and Eke (2007) proposed a case definition which was based on clinical diagnosis of periodontitis for population–based surveillance. Measurements of PPD, CAL, GBI was taken individually or with combination into this classification. This classification defined moderate and severe chronic periodontitis based on the number of affected sites with PPD and CAL.

The case definition for population-based surveillance of periodontitis was updated (Eke *et al*., 2012). A new definition of mild periodontitis was added for better understanding of the prevalence of periodontitis. Overall, case definition updated for periodontitis is stated as Table 2.1.

Table 2.2: Case definition of periodontitis (Eke *at al*., 2012)

Case Definition	Descriptions
Severe periodontitis	• 2 or more interproximal sites with CAL ≥ 6 mm (not on same tooth) And • 1 or more interproximal site(s) with PPD ≥ 5 mm
Moderate periodontitis	• 2 or more interproximal sites CAL ≥ with 4 mm (not on same tooth) Or • 2 or more interproximal sites with PPD ≥ 5 mm (not on same tooth)
Mild periodontitis	• 2 or more interproximal sites with CAL ≥ 3 mm And • 2 or more interproximal sites with PPD ≥ 4 mm (not on same tooth) Or • 1 site with PPD ≥ 5 mm

2.3 Periodontal Pathogen

2.3.1 Dental Plaque Formation

Bacteria colonized body surfaces including oral cavity. Teeth structures comprises of hard surfaces which enable the development of bacterial deposits which is known as dental plaque. The stages of dental plaque formation is summarised in Figure 2.5.

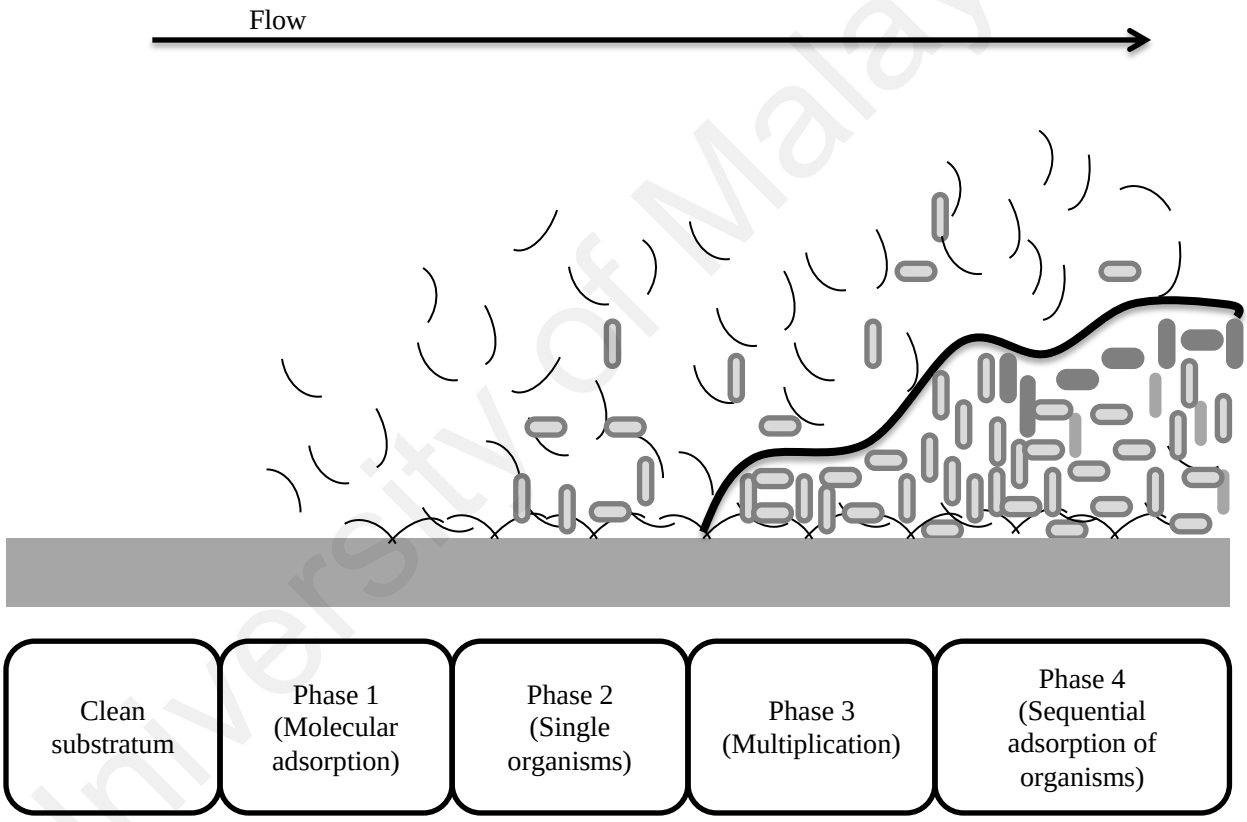
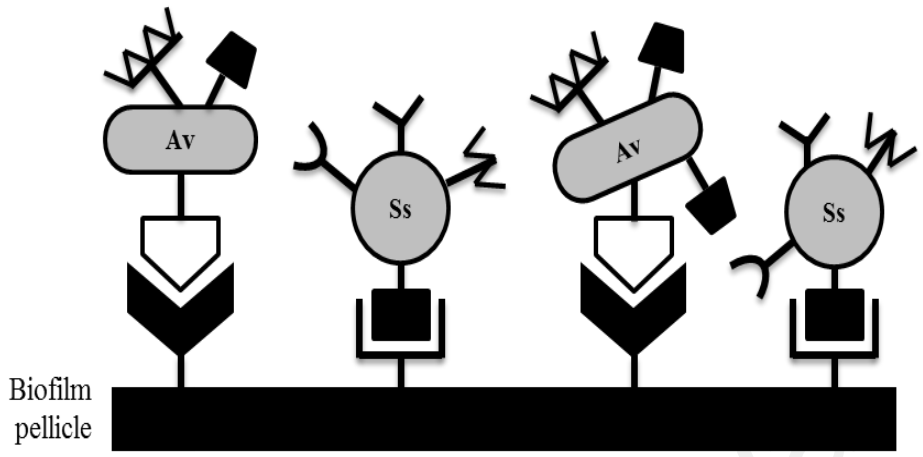


Figure 2.5: Stages in the formation of dental plaque (Adapted and modified from Marshall, 1992)

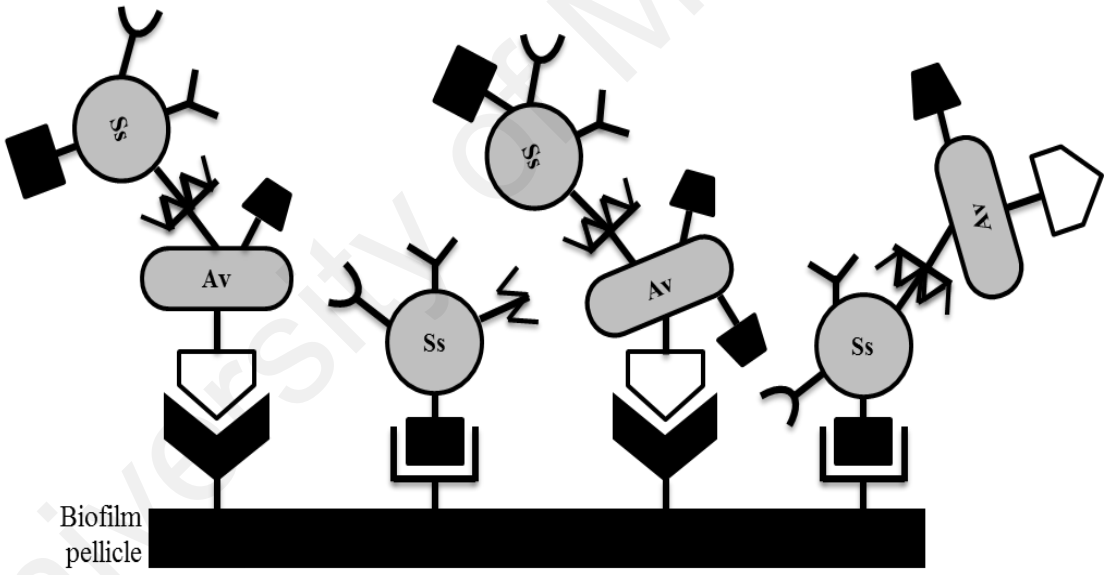
The dental plaque formation is initiated when a solid substratum is immersed into the oral cavity full with fluid media or following tooth cleaning. The hydrophobic and macromolecules start to adsorb the tooth surface, forming a conditioning film, term the acquired pellicle (Phase 1). The acquired pellicle is composed of salivary glycoprotein

which alters charges and free energy between the conditioning film and tooth surface. This in return increases the efficiency of bacterial adhesion and subsequently, this leads to adhesion of single bacterium to the tooth surface (Phase 2). Bacteria adhere to these coated surfaces; with some bacteria possess specific attachment structures which allow them to bind firmly. The continued growth of the adhering bacteria, adhesion of new bacteria and growth of extracellular matrix production results in an increases in bacterial mass (Phase 3 and 4). At this phase, the conditioning film becomes thicker and this results in poor diffusion of oxygen into and out of the biofilm matrix. This has an impact on the ecology of bacteria, where completely anaerobic conditions eventually emerge in the deeper layers (Kinane *et al*., 2008).

Figure 2.6 summarized the process of bacterial colonization on the pellicle surface. The colonization of bacteria onto pellicle–coated surface is dominated by facultative anaerobic Gram–positive cocci such as *Streptococcus sanguinis*. In the next phase, initially low numbers of Gram–positive rods turn to exceed the number of streptococci. Gram–negative has poor ability to adhere on pellicle surface. However, filaments attached on the outer surface of Gram–positive cocci and rod allow preceding adherence of more Gram–negative organism. A complex heterogeneity of dental plaque and exchange nutritional between species form a stable bacterial community (Kinane *et al*., 2008).

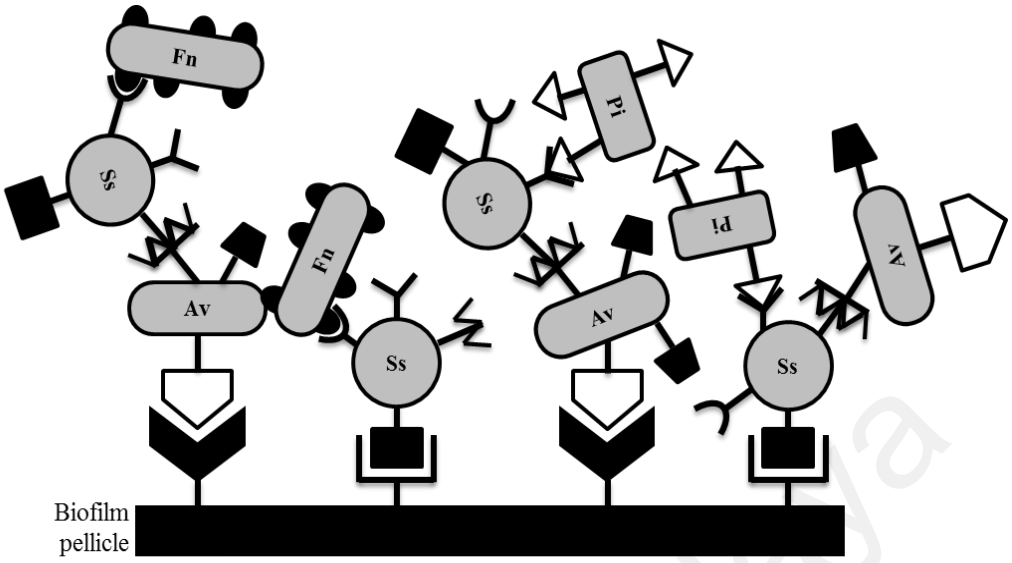


(a) Adhesion of Gram–positive facultative bacteria on pellicle (Ss: *Streptococcus sanguinis*, Av: *Actinomyces* sp.)

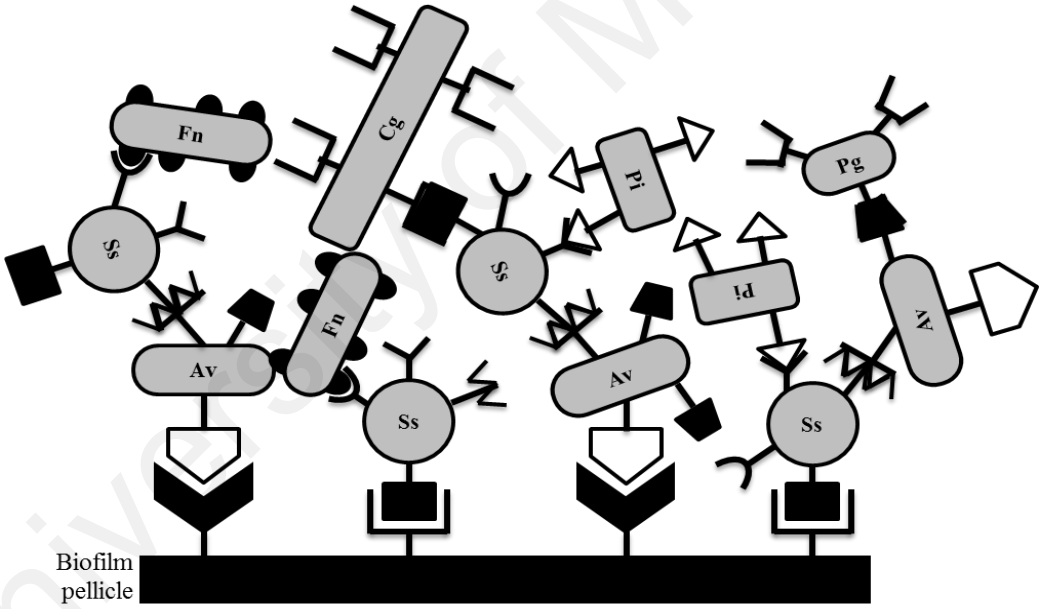


(b) Adhesion of Gram–positive facultative cocci and rods. The bacteria initiate to coaggregate and multiply

Figure 2.6: Stages of bacterial colonization on the pellicle surface (Adapted and modified from Kinane *et al.*, 2008)



(c) Adhesion of Gram–negative organism on surface receptors of the Gram–positive facultative cocci and rods (*Fn*: *Fusobacterium nucleatum, Pi*: *P. intermedia*)



(d) The heterogeneity increases as plaque ages and matures. As a result of ecologic changes, more Gram–negative strictly anaerobic bacteria colonize secondarily and contribute to an increased pathogenicity of the dental plaque (*Pg*: *P. gingivalis*, Cg: *Capnocytophaga* sp.)

Figure 2.6 (continued): Stages of bacterial colonization on the pellicle surface (Adapted and modified from Kinane *et al.*, 2008)

2.3.2 Periodontal Pathogens

The most important and most prevalence anaerobic Gram–negative bacteria in subgingival area are *P. gingivalis, T. forsythia* and *A. actinomycetemcomitans* (Tomita *et al*., 2013; Heller *et al*., 2012). These bacteria play an important role in the onset and subsequent development of chronic periodontitis starting with (1) formation of periodontal pocket, (2) destruction of connective tissue, and (3) resorption of alveolar bone.

The microorganism could produce disease either as direct invasion on the tissue or indirectly by bacterial enzymes and toxins. In order to be periodontal pathogens, the organism must present at higher number in disease active sites, possess virulence factor relevant to the disease and elicit cellular response.

(a) *P. gingivalis*

P. gingivalis also known as *Bacteroides gingivalis* is a Gram–negative, black–pigmented with coccobacillus shape. It is also characterizes as encapsulated, non–motile, non–saccharolytic and obligate anaerobe environment is necessary for growth. *P. gingivalis* is classified under the red complexes pathogen which is the most pathogenic to the host (Haffajee & Socransky, 1994; Dosseva-Panova *et al*., 2014).

P. gingivalis possesses several virulence factors such as lipopolysaccharides, polysaccharide capsule, fimbria, hemagglutinins and extracellular proteolytic enzymes that facilitate their colonization in oral cavity and can affect directly the periodontium or host function (Dosseva-Panova *et al*., 2014) and result in damages to the gingival tissue and bone.

In vitro has the ability to bind and to invade oral gingival epithelial cells, as well as able to spread from cell to cell into periodontal tissue makes *P. gingivalis* one of the most virulent periodontal pathogens (Tribble *et al*., 2013). The attachment between *P. gingivalis* and host cell surfaces is mediated by long filamentous appendages called fimbriae. The major fimbriae gene which is associated with specific virulence alleles and its initiated diseases is called as gene *fimA*. In details, most common types of gene *fimA* found in periodontal diseases are *fimA* type II and IV due to their cytotoxic.

(b) *T. forsythia*

T. forsythia is formerly known as *Bacteroides forsythus* is a Gram–negative with rod shape bacterium. It is also characterizes as non-pigmented saccharolytic and need to obligate anaerobes environment for growth (Dosseva-Panova *et al*., 2014). *T. forsythia* colonizes the human oral cavity which is also known as to be associated with chronic periodontitis. These bacteria are classified as "red complexes". It can be isolated from various parts in mouth area including periodontal pockets, tonsils, at the back of tongue and saliva.

T. forsythia also secretes proteolytic enzymes which are responsible in destruction of immunoglobulin and complement system, results in apoptosis or cell death (Dosseva-Panova *et al*., 2014). The apoptotic inducing activity could eliminate host immune cells, losing of these cells from developing periodontal pocket would allow the colonization of bacteria (Holt & Ebersole, 2005). Thus, the ability of *T. forsythia* induced apoptosis can be considered as part of progression of periodontitis.

The surface layer (S–layer) of *T. forsythia* is the outermost cell enveloped component known to play a role in adhesive or invasion of surface recognition. Furthermore, the S–layer contributed to its survival in serum and associated with congregation of oral bacteria. The survival ratio of the mutant (lacking of S–layer) *T. forsythia* in non-heat-inactivated of calf serum was significantly lower than wild type *T. forsythia* which contain encoded protein TfsA and TfsB of S–layer (Shimotahira *et al*., 2013). Thus, it proves that the S–layer of *T. forsythia* is virulence and maybe associated with periodontitis.

(c) *P. intermedia*

P.intermedia is a Gram–negative with black pigmented rod bacteria. *P.intermedia* is also characterized as non–motile and obligate anaerobe bacteria. *P.intermedia* is frequently found to be associated with chronic periodontitis. The bacteria contain virulence factor proteases which are able to act various actions. It provides substrate for bacterial growth, able to degrade matric components and alter vascular permeability (Eley *et al*., 2010).

(d) *A. actinomycetemcomitans*

A. actinomycetemcomitans is formerly known as *Actinobacillus actinomycetemcomitans* and the major pathogens of the genus *Aggregatibacter*. *A. actinomycetemcomitans* is a gram negative with small rod bacterium. These non–motile and encapsulated pathogens required capnophilic facultative anaerobe environment for its growth (Haffajee & Socransky, 1994; Dosseva-Panova *et al*., 2014).

It is commensal of the human mouth and can be recovered on culture of oral secretions in up to 20% of healthy people and in the great majority of those with aggressive periodontitis. Previously, localized juvenile periodontitis is genetic or inheritance with rapid destruction of tissue in incisors and first molar. *A. actinomycetemcomitans* with serotype B strain is reported to be associated with this disease.

A. actinomycetemcomitans produce a lot of virulence factors, such as, leukotoxin, endotoxin, collagenase and protease (Dosseva-Panova *et al*., 2014). These factors have the ability to invade periodontal tissues. Most studies showed that virulence factor of *A. actinomycetemcomitans* are leukotoxins of repeats in toxins (RTX) and lipopolysaccharide (Raja *et al*., 2014). Leukotoxins of RTX is responsible to modulate by inactive or invade host immune system. Meanwhile, lipopolysaccharide is able to induce tissue destruction through secretion of cell stress proteins. Among all serotype (a–f) species of *A. actinomycetemcomitans*, serotype 'a' and 'c' were associated with periodontal health. Meanwhile, serotype 'b' was found more frequently in periodontitis subjects under the age of 18 years and significantly found more often in aggressive than in chronic periodontitis (van der Reijden *et al*., 2008).

2.3.3 Distribution of Periodontal Pathogens

(a) Distribution of Periodontal Pathogens among Obese

A study carried out by Haffajee & Socransky, (2009) reported that BMI was significantly associated with periodontal status, even after adjusted to age, gender and smoking status. In this study, *T. forsythia* was reported as the most prevalent periodontal pathogens observed in those obese individuals with healthy periodontium or gingivitis compared to those with normal BMI. Other members of the red complex, such as, *P. gingivalis* were not significantly found.

Similar finding was found among obese adolescent. In addition, the obese adolescents were reported to be significantly associated with bacterial cells in subgingival biofilm (Zeigler *et al*., 2012). They reported that the amount of bacterial cells among obese subjects were threefold higher than in those non–obese subjects. Nevertheless, this finding suggested that the link between oral microbiota among obese with healthy peridontium.

Interestingly, it was reported that there is a relationship between red complexes and BMI among Japanese population. The study demonstrated that the amount of red complex oral microbe was higher in those with high BMI or WC independent on chronic periodontitis (Matsushita *et al*., 2015).

(b) Distribution of Periodontal Pathogens in Chronic Periodontitis

A study done by Heller, (2012), showed that microbial profiles could be distinguished between chronic periodontitis and generalized aggressive. In this study, *P. gingivalis* was found to be associated with chronic periodontitis. A similar concept study reported that *T. forsythia* was most frequently detected in the subgingival plaques sample from both aggressive (75%) and chronic periodontitis (80%) but not statistically significant ($p > 0.05$). However, when the bacterial counts were compared, mean value for total *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythia* appeared to be greater in aggressive group than in the chronic periodontitis group. No statistically significant difference was observed (Tomita *et al*., 2013).

In a study that evaluated the detection of periodontal pathogens in chronic periodontitis showed that among 116 collection sites were positive for *P. gingivalis* (46.6%), *T. forsythia* (41.4%) and *A. actinomycetemcomintans* (27.6%). Moreover, *P. gingivalis* and *T. forsythia* showed significant association ($p < 0.05$) with deeper pocket (≥ 8 mm) (Farias *et al*., 2012).

A study conducted among Italians with chronic periodontitis reported that *T. forsythia* has the highest load (12×10^5 cells/plaque sample). Meanwhile *A. actinomycetemcomitans* was the less bacteria load (0×10^5 cells/plaque sample) (Gatto *et al*., 2014). Interestingly, bacterial load was significantly increased by the increasing numbers of bleeding sites and mean PPD. A strong correlation ($r = 0.52$, $p = 0.0001$) was observed between *T. forsythia* and mean PPD. Meanwhile, moderate correlation was reported between *Treponema denticola* and bleeding on probing ($r = 0.39$, $p = 0.001$) (Gatto *et al*., 2014).

2.3.4 Identification of Periodontal Pathogens

Several lab techniques are available to identify periodontal pathogens including the microbiological, biochemical and molecular methods. Microbiological method used microscopic appearances to identify and describe the pathogens. Thus, this method allows physical overview of the characteristic of pathogens in terms of shape, colour and size. However, this method does not allow cell count. The microbiological method requires biochemical test to support the results. A series of biochemical tests are needed to be performed in order confirm the pathogen. These microbiological and biochemistry methods only provide qualitative results. A molecular method is required to quantify the pathogens in a more specific and sensitive manner. This will yield results rapidly and with high accuracy. However, molecular method is costly in terms of instruments and reagents.

(a) Anaerobic Culture

Anaerobic culture is the most common and traditionally used method to detect and quantify subgingival plaque. This method was also used to determine the *in vitro* antimicrobial susceptibility of oral pathogens. However, this method requires very specific growth requirements and the growth is extremely slow. It is less favourable as it has low level of sensitivity, time consuming, laborious and limit the numbers of samples that can be evaluated (Boutaga *et al*., 2003).

A few studies reported that this anaerobic culture has a low level of sensitivity. Boutaga *et al*., (2003), carried out a comparison study of *P. gingivalis* in detection by using qPCR and anaerobic culture methods. Out of 259 samples, (i) Three samples were positive of *P. gingivalis* in the detection by using qPCR but negative of *P. gingivalis* in detection by

using anaerobic culture method. Even after the culturing process was repeated, the negative sample of *P. gingivalis* in detection by using anaerobic culture method remained non–growth, (ii) seven samples had been found positive of *P. gingivalis* in detection by using qPCR method but negative detection to anaerobic culture method. Then, the culturing process was repeated for sample with negative detection results. Fortunately, four out of seven samples were confirmed of positive *P. gingivalis* in detection after second attempt of prolonged anaerobic culture method, (iii) twenty samples with low dilution of *P. gingivalis* were positively detected using qPCR method but negative detection to anaerobic culture method, and (iv) all samples with positive of *P. gingivalis* in detection using anaerobic culture method also appeared to be positive detection by the qPCR method. This study demonstrated limitation in *P. gingivalis* using culture method. The study also demonstrated the advantages of qPCR method in term of time efficiency and its sensitivity.

(b) Quantitative Real–Time Polymerase Chain Reaction (qPCR)

Molecular biology methods have been widely used to detect periodontal pathogens. Various diagnostic assays using immunoassays, enzyme assay and molecular techniques have been developed to analyze deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein.

Conventional PCR method used to be a tool of choice for specific detection of periodontal pathogens due to its rapid and sensitive detection ability. However, conventional PCR has been shown to be less accurate in term of quantification (Nonnenmacher *et al*., 2004). A previous study has compared the sensitivity of periodontal pathogens detection using both conventional PCR and qPCR (Soleimani *et al*., 2013). It

was concluded that the qPCR showed a ten-fold increase in sensitivity in detecting *T. forsythia* and *A. actinomycetamcomitans* (Soleimani *et al*., 2013).

The disadvantage of using conventional PCR also includes the ability to provide qualitative instead of quantitative results. Furthermore, conventional PCR allows detection of PCR product in the final phase or at end–point of PCR reaction. On the other hand, qPCR allows for the detection of PCR product during the early phases of the reaction. The end–point detection of PCR product had to counter with several problems, such as, low resolution, low sensitivity, poor precision and a need for post PCR processing.

Nowadays, several alternative methods have been developed and molecular methods such as checkboard DNA–DNA hybridization are widely used in detection and quantification of periodontal pathogens. DNA–DNA hybridization was developed by Socransky *et al*., (1994) in processing of large numbers of samples and profiling multiple species within the same sample simultaneously. However, this method was not appropriate for small number of different species of periodontal pathogens.

The qPCR method offers accurate quantitative assays for detection of periodontal pathogens compared to other molecular methods. The progression of qPCR was monitored by the fluorescence emitted by the reporter molecule manifold as the PCR product accumulates with each cycle of amplification. The qPCR technique can be classified under (1) non–specific detection using DNA binding dyes and (2) specific detection target specific probes.

For non–specific detection, DNA binding dyes (intercalating dye) are used as fluorescent reporter to monitor the qPCR reaction. SYBR® Green is widely used as double strand DNA specific dye reporter for qPCR. Once the SYBR® Green binds to the minor

groove of the DNA double helix, the reporter emitted the fluorescence. The fluorescence of SYBR® Green increases as the products accumulate with each successive cycle of amplification. This means that qPCR creates more DNA, more dye can bind and more fluorescence would be generated.

Meanwhile for the specific detection, hydrolyze oligonucleotide probes labeled with both a reporter fluorescence dye and a quencher dye is used to monitor the qPCR reaction. The probe yields low level of fluorescence output when reporter and quencher close to one another and inverse when they separated. TaqMan® probes are designed to bind downstream of one of the primers during the qPCR reaction and then cleaved by the polymerase enzyme. By cleaving the probe, reporter and quencher are separated and in return the level of fluorescence was increased. This means that qPCR creates more DNA, more probes can bind and separated, the more fluorescence would be generated.

Intercalating dye is more reliable option for qPCR as it is less expensive for experimental design involved with lots of genes or target compared to hydrolyze probe. However, intercalating dye is non–specific because it is able to bind at the wrong target and report on any double stranded DNA that is formed during qPCR reaction regardless of what it is. On the other hand, hydrolyze probe is inherent specifically as the fluorescence comes from the genuine amplification of target sequence.

The fluorescence probe aided with advance instrumentation is able to measure the amplified PCR product in the log phase of the reaction (Nonnenmacher *et al*., 2004). It was reported that the sensitivity of qPCR was measured by range of 10^8 till 10^0 of plasmids copies of periodontal pathogens. Approximately, the sensitivity was established by a minimum range of 10^2 in the qPCR reaction. The specificity of qPCR could be evaluated

through primers and probes tested with total DNA from different periodontal pathogens. The primers demonstrated a specific amplification of periodontal pathogens and did not amplify other DNA sequence. However, there was still a possibility for the assays to yield a false–positive result but in a low probability (Nonnenmacher *et al*., 2004).

2.4 Periodontal Therapy

2.4.1 Non–Surgical Periodontal Therapy (NSPT)

As discussed earlier, plaque is etiology of chronic periodontitis. Periodontal therapy is then necessary to remove the plaque and preparing the root surfaces for healing. Periodontal therapy is divided into NSPT and surgical therapy.

Basically, NSPT is directed at the removal of supragingival and subgingival plaque and any predisposing factor, for example calculus deposits. In general, all chronic periodontitis patients will be subjected to NSPT and the outcome will be assessed. Following NSPT, those sites that failed to respond to NSPT (persistent or increase PPD) may be suggested for surgical intervention (Clerehugh *et al*., 2013; Eley *et al*., 2010; Nield-Gehrig & Willmann, 2011).

NSPT will include supragingival and subgingival scaling, as well as root planing in PPD ≥ 5 mm (Eley *et al*., 2010; Nield-Gehrig and Willmann, 2011). It can be carried out using ultra sonic scaler and hand instruments. Patients need to maintain good oral hygiene practice to control the accumulation of the dental plaque. Proper tooth brushing techniques together with interdental cleaning were the best combination for optimal plaque control.

2.4.2 Response of NSPT towards Clinical Parameters

NSTP which includes scaling and root planing is considered as a gold standard treatment for periodontitis due to its effectiveness. Several studies had demonstrated its effectiveness following 3, 6, 12 and 24 months post NSPT. At 3 months post NSPT, the clinical parameters has decreased, mean PPD from 3.9 to 3.0 mm, CAL 4.1 to 3.8 mm and also mean VPI (Ivić-Kardum *et al*., 2001). Both partial mouth scaling and full mouth scaling with root planing have shown significant reduction in PPD and CAL after 12 months post NSPT in diabetics subjects (Santos *et al*., 2012).

2.4.3 Response of NSPT towards Periodontal Pathogens Profiles

The NSPT response could also be measured through changes in the periodontal pathogens by comparing quantitatively before and after treatment. Socransky *et al*., (2013) investigated subgingival microbiological counts in chronic periodontitis subjects up to 2-years post treatment. Microbial count of *T. forsythia* reduced rapidly at 3 months post NSPT and the count reduction remained up to 24 months post NSPT. In addition, the microbial count of *P. intermedia* had shown reduction at 3 months post NSPT but the count reduction only until 24 months post NSPT. However, the microbial count of *P. gingivalis* and *A. actinomycetemcomitans* possessed slow but consistent reduction rate over 24 months posts NSPT. In this case, NSPT is able to induce a shift from a pre–dominant gram–negative to a gram–positive subgingival microbiota.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Reagents

Basic chemicals used were, 70% ethanol, absolute alcohol, 1X Phosphate Buffered Saline (PBS) as plaque buffer and nuclease–free water. Meanwhile, reagents used were DNA extraction kit (DNeasy® Blood & Tissue Kit) (Figure 3.1), TaqMan® Fast Advanced Master mix and custom TaqMan Gene Ex Assays. Primers sequence for *P. gingivalis*, *T. forsythia*, *P. intermedia* and *A. actinomycetamcomitans* are customized based on previous study (Nonnenmacher *et al*., 2004; Boutaga *et al*., 2005). Primers and fluorogenic probes for the specific detection of the four bacteria as stated in Table 3.1.

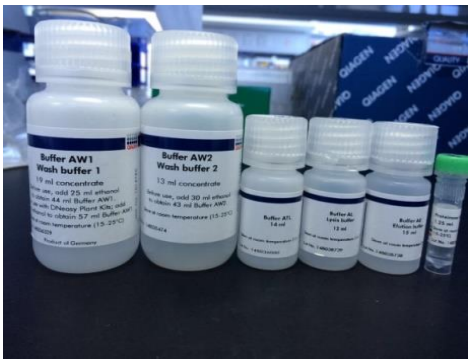
3.1.2 Instruments and Equipment

Instruments used were biological safety cabinet class II, automated DNA extraction (QIAcube DNA extractor, QIAGEN) (Figure 3.1), spectrophotometer analyzer (NanoDrop 2000 Spectrophotometer, Thermo Scientific) (Figure 3.2), quantitative real–time polymerase chain reaction (qPCR) (7500 Fast RT–PCR System, Applied Biosystems®) (Figure 3.3), micro centrifuge (Mini Spin Eppendorf 5415 R) (Figure 3.3) plate centrifuge (Hermle Z 300 K) (Figure 3.4), vortex mixer (VELP Scientifica), refrigerator 4°C and freezer –20°C.

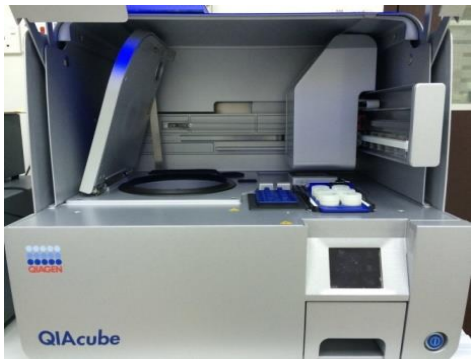
Laboratory equipment used were micropipettes (i) 0.2–2.5 µl, (ii) 1–10 µl, (iii) 10–100 µl and (iv) 200-1000 µl, sterile pipette tips (i) white, (ii) yellow and (iii) blue, conical flask (250 ml), measuring cylinder (i) 100 ml and (ii) 1000 ml, parafilm and ice box

Table 3.1: Sequence of primers and probes for periodontal pathogens

Primer and probe	**Sequence (5' – 3')**	
P. gingivalis		
Forward	TGC AAC TTG CCT TAC AGA GGG	Nonnenmacher *et al*., (2004)
Reverse	ACT CGT ATC GCC CGT TATTC	
Probe	FAM-AGC TGT AAG ATA GGC ATG CGT CCC ATT AGCTA-TAMRA	
T. forsythia		
Forward	GGG TGA GTA ACG CGT ATG TAA CCT	Boutaga *et al*., (2005)
Reverse	ACC CAT CCG CAA CCA ATA AA	
Probe	FAM-CCC GCA ACA GAG GGA TAA CCC GG-TAMRA	
P. intermedia		
Forward	CGG TCT GTT AAG CGT GTT GTG	Boutaga *et al*., (2005)
Reverse	CAC CAT GAA TTC CGC ATA CG	
Probe	FAM-TGG CGG ACT TGA GTG CAC GC-TAMRA	
A. actinomycetemcomitans		
Forward	GAA CCT TAC CTA CTC TTG ACA TCC	Boutaga *et al*., (2005)
Reverse	TGC AGC ACC TGT CTC AAA GC	
Probe	FAM-AGA ACT CAG AGA TGG GTT TGT GCC TTAGGG-TAMRA	



(a) DNeasy® Blood & Tissue Kit Reagent



(b) QIAcube DNA extractor, QIAGEN

(c) NanoDrop 2000 Spectrophotometer, Thermo Scientific

(d) NanoDrop 2000 spectrophotometer software

(e) Mini Spin Eppendorf 5415 R

(f) 7500 Fast Real-Time PCR System, Applied Biosystems®

(g) Hemle Z 300 K

Figure 3.1: Equipment used for DNA extraction (a–d) and qPCR (e–g) procedure

3.2 Methods

3.2.1 Flow Chart of the Study

(a) Flow Chart of Clinical Procedures

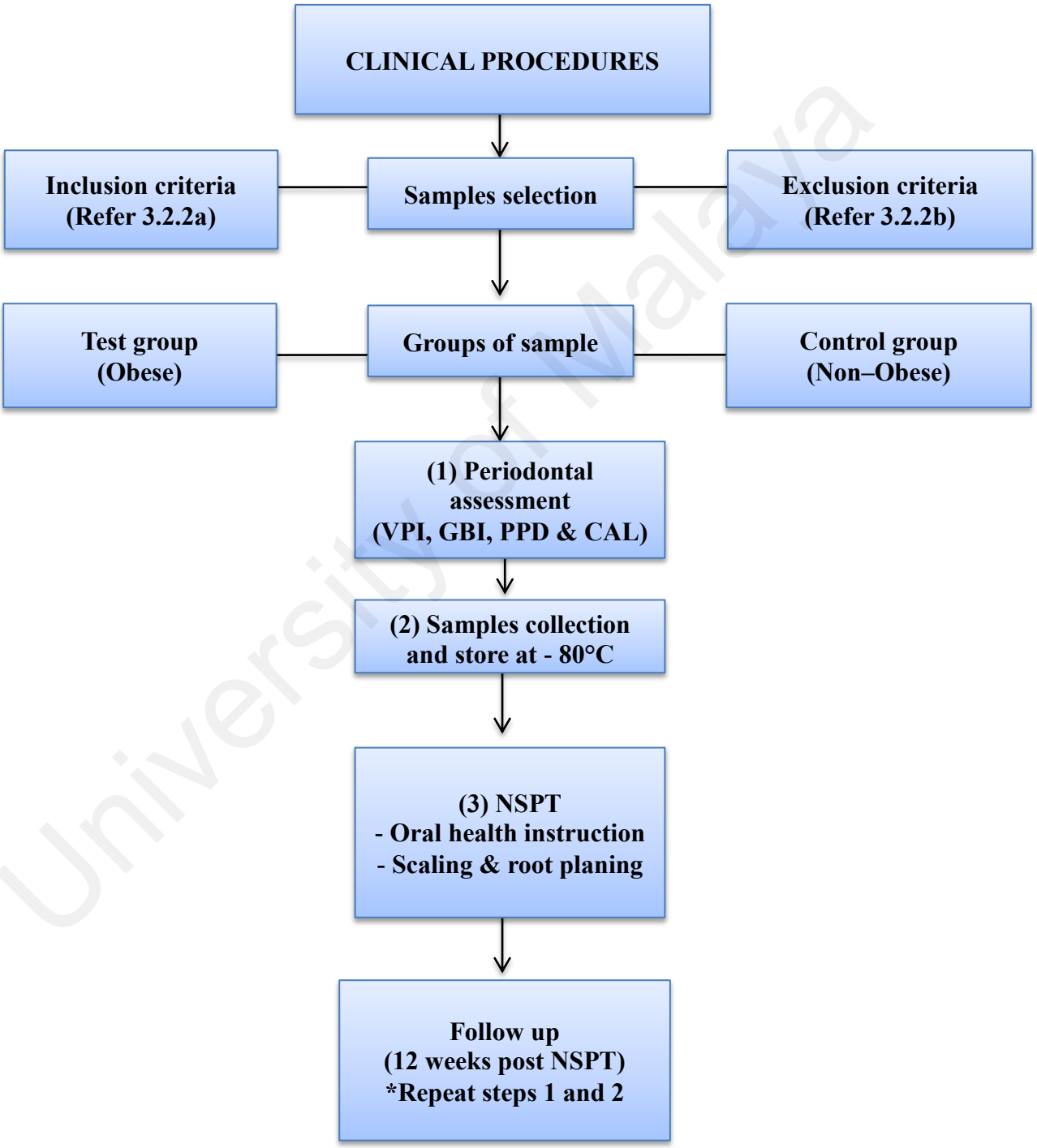


Figure 3.2: Flow chart of clinical procedure in this study

(b) Flow Chart of Laboratory Procedures

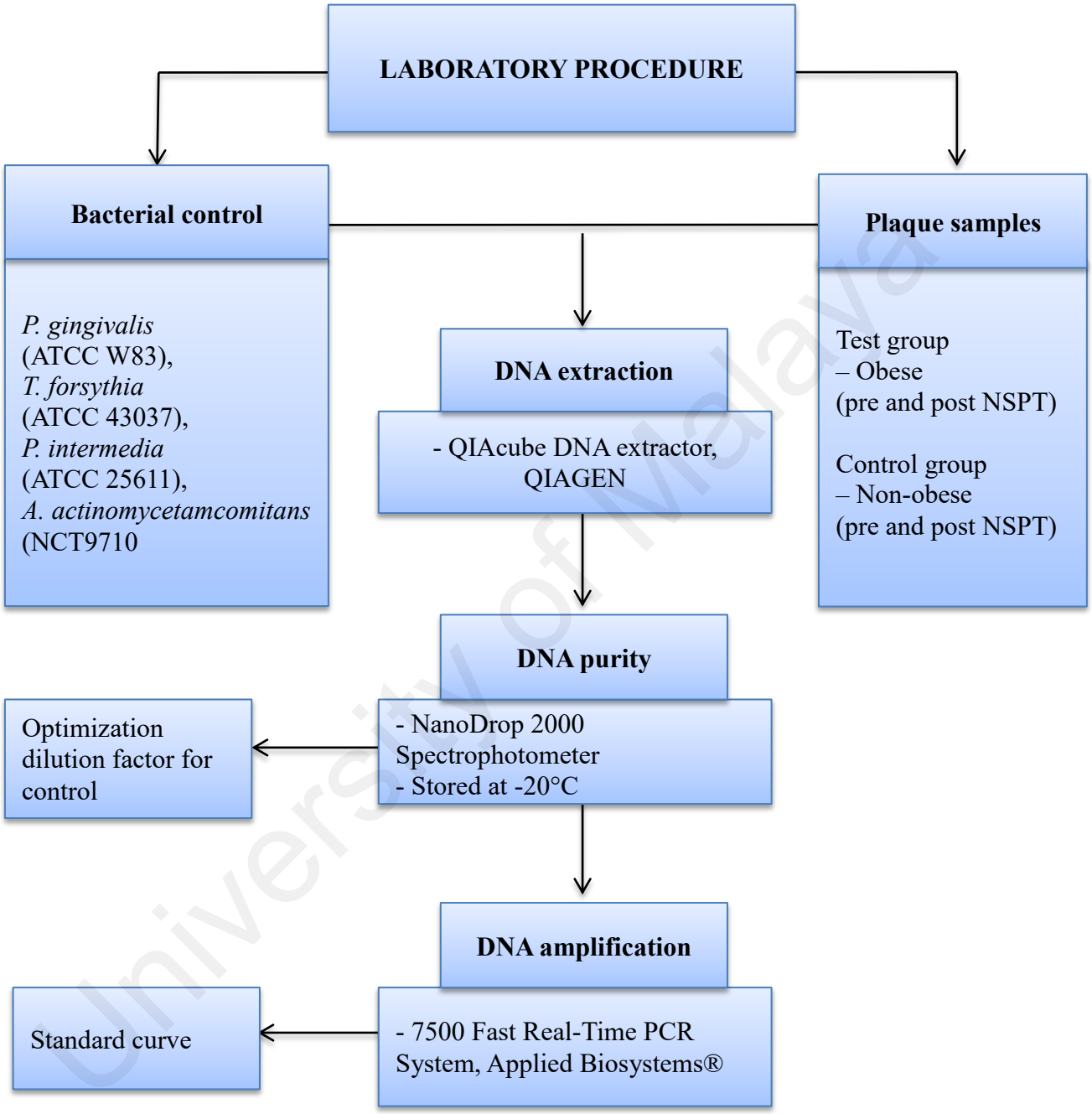


Figure 3.3: Flow chart of laboratory procedure in this study

3.2.2 Study Design

This is a prospective, before and after clinical trial study conducted at the Faculty of Dentistry, University Malaya. This study was a part of a large scale study under the University Malaya Research Grant (UMRG). Clinical sessions, samples collection and periodontal treatments were done by calibrated clinicians. All of the data and sample was managed under Malaysian Periodontal Database and Biobank System (MPDBS). This is the first periodontal database and biobank in Malaysia with the aim to facilitate multicenter periodontal research (Vaithilingam *et al*., 2015). Ethical approval for this study was obtained from the Medical Ethics Faculty of Dentistry, University of Malaya (DF PE1501/0085(L)). This study abided with the Consolidate Standards of Reporting Trials (CONSORT) Statement and registered at clinicaltrias.gov, number NCT02618486.

3.2.3 Samples Selection

A total of 120 subgingival plaque samples were used in this study. Based on the inclusion and exclusion criteria, the samples were grouped into obese (test, n=30) and non–obese (control, n=30). For both groups, the subgingival plaque collections were carried out at baseline (prior to NSPT) and at 12 weeks post NSPT.

The inclusion and exclusion criteria for the samples were as follows:

(a) Inclusion Criteria

1. Patients who are Malaysians
2. Patients who were obese with BMI $\geq$ 30.0 kg/m^2 and those non–obese with BMI < 25.0 kg/m^2 (WHO, 2012)
3. Patients who are 30 years old and above
4. Patients who were diagnosed with chronic periodontitis
5. Patients who have at least 12 teeth present

(b) Exclusion Criteria

1. Patients who had periodontal treatment within the past 6 months
2. Patients who was under antibiotics, topical/systemic steroid treatment for the past 4 months
3. Patients who were pregnant or lactating mothers
4. Patients who were mentally handicapped patients
5. Patients who had history of valve replacement and rheumatic heart disease which require antibiotic coverage

3.2.4 Sample Size

The sample size calculation was determined based on a previous international study on clinical and microbial changes following periodontal treatment (Buzinin *et al*., 2014). The sample size calculation was using G*Power software version 3.1.9.2. By considering the mean count of *P. gingivalis* of two related groups, the ideal sample size to achieve 95% power of study with an α of 0.05 is at least 30 samples per group. After considering 10% drop off, each group needs at least 33 samples per group. Due to time limitation, only 30 samples met the criteria and included in the study. So far, no samples were withdrawn in the middle of the study was reported.

3.2.5 Sample Characteristics

Sample characteristics include (i) age, (ii) gender, (iii) ethnicity, (iv) level of education, (v) smoking status and (vi) diabetic status were obtained from MPDBS database. Smoking status was further labeled as smoker and non–smoker. Those who are current and social smokers were considered as smokers; while those non–smokers and former smokers were categorized as non–smokers. For systemic disease, the samples were divided into diabetic or non–diabetic. This was based on the close relation between obesity and diabetes mellitus.

3.2.6 Clinical Parameters

The clinical parameters data were obtained from the MPDBS database. The clinical parameters measurements were performed by trained and calibrated clinicians between 2013 and 2015. A full mouth periodontal examination (FEME) was carried out on all teeth except third molar. Periodontal measurements were carried out on the same appointment date and before plaque samples collection. For both groups, the measurements were carried out at baseline and at 12 weeks post NSPT.

The periodontal clinical measurements include the followings:

(a) Visible Plaque Index (VPI)

VPI was recorded on the basis of the presence of visible plaque on the tooth surface or while scrapping with the probe some plaque were recorded on the tip of the probe. It was scored using dichotomous method.

(b) Gingival Bleeding Index (GBI)

The GBI was assessed by using William's probe (Hu-Friedy, Chicago USA). If the bleeding was recorded within 10 seconds after probing it was recorded as bleeding (score 1), where as if no bleeding occurred it was recorded as no bleeding (score 0) (Ainamo & Bay, 1975).

(c) Periodontal Pocket Depth (PPD)

PPD is the measure of the distance from the gingival margin to the base of the pocket. The measurement was carried out using a William's probe (Hu-Friedy, Chicago USA) by placing the probe parallel to the long axis of the tooth. The William's probe (Hu-Friedy, Chicago USA) had the calibrated markings at every 1 mm for the length of 10 mm. The PPD was assessed at 6 sites per tooth for all teeth.

(d) Clinical Attachment Loss (CAL)

CAL is a distance from the cemento-enamel junction to the base of the pocket. The levels of CAL are dependent upon 2 things.

3.2.7 Non–Surgical Periodontal Therapy (NSPT)

(a) Oral Hygiene Instructions

All patients were given the same oral hygiene instruction and advice. They were suggested to use a soft bristled toothbrush, fluoride content of toothpaste and dental floss for interproximal plaque removal. The patients were also guided with the right techniques of teeth brushing and flossing. On subsequent techniques, the clinicians had to show the patients where sites of teeth were exhibited of inflammation, bleeding on probing and accumulation of plaques. Hence, the patients would get a better clinical picture and verbal information, as well as to motivate the patients to improve their oral hygiene practices.

In addition, all patients were also provided with oral hygiene home care kits (contain with toothbrush, toothpaste, dental floss and mouth rinse). This point of view would be marked and compared when the patients came for the next visit of post 12 weeks NSPT.

(b) Scaling and Root Planing

Full mouth scaling and root planing were done for all patients in both test and control groups at sites with PPD ≥ 5 mm (Swierkot *et al*., 2009) using an ultrasonic scaler (Sirona C8 03653) and Gracey curette (Hu Friedrich). The patient's mouth was irrigated with 0.12% chlorhexidine. The patient was advised to continue mouth rinse three times daily for 14 days using 15 ml of 0.12% chlorhexidine gluconate mouthwash (Oradex®) (Raman *et al*., 2014).

3.2.8 Plaque Samples Collection

The subgingival plaque samples were collected by trained clinicians between 2013 and 2015. For both groups, the subgingival plaque collections were carried out at baseline and at 12 weeks post NSPT. The area adjacent to the sample collection was cleaned from supragingival plaque prior to subgingival plaque collection. Then, the samples were collected from the deepest site at each quadrant of teeth using a curette and were pooled together in a microcentrifuge tube. The plaque samples obtained were re–suspended in 1.5 ml of Phosphate Buffered Saline (PBS) and immediately stored at -80°C freezers till microbiological identification.

3.2.9 Bacterial Culture

The American Type Culture Collection (ATCC) bacteria were premier source for microbial reference strains since 1920s. *P. gingivalis* (ATCC® W83™), *T. forsythia* (ATCC® 43037), *P. intermedia* (ATCC® 25611™) and *A. actinomycetemcomitans* (NCT9710) were used in the study.

The strains were cultured in tryptic soy broth and grown as recommended in the ATCC manual. All of these anaerobic Gram–negative bacteria required an anaerobic environment of 80% N_2, 10% CO_2, and 10% H_2. The anaerobic environment for culturing and incubation were carried out using the anaerobic jar. The jar works on evacuation and replacement principle. The lid of the jar comprised gases meter, inlet and outlet tube to a vacuum for the exchange of air and mixture gases. The air inside the chamber is evacuated and replaced with 80% N_2, 10% CO_2, and 10% H_2. Methylene blue is used as indicator for anaerobic environment when it is remained colourless. The uncultured plate for the contaminants purpose was placed on the jar with similar anaerobic environment. The optimum temperature for growth maintained at 37°C and incubated for 48 to 72 hours.

3.2.10 Deoxyribonucleic Acid (DNA) Extraction

(a) Principle of DNA Extraction

The main purpose of DNA extraction is to obtain the purified form of DNA for further investigation. Nucleus is surrounded with cell membrane and cell wall which need to be removed in other to get the purified DNA. At least four steps are required (i) lysis, (ii) bind, (iii) wash and (iv) elute to purify the DNA from the rest of cell.

Briefly, lysis refers to breaking the cell wall and cellular membrane which can be achieved by incubation of the sample material in Proteinase K supplemented lysis buffer. The precipitated or binding of DNA is washed with ethanol solution to remove soluble impurities. Finally, the elution of purified DNA is stored in a buffer to ensure the stability and for long term storage.

(b) DNA Purification and Quantifications

The purity and quantity of the extracted DNA samples can be measured through absorbance reading of DNA using spectrophotometer analyzer (NanoDrop 2000 Spectrophotometer.

The ratio absorbance of OD 260 and OD 280 was used to assess the purity of DNA. Generally, a ratio of ~1.8 is accepted as "pure" for DNA and ~1.8 to 2.0 considered as acceptance range for DNA purity. If the ratio is noticeably lower or higher than acceptable range, it may indicate the presence of phenol, protein or other contaminants.

The spectrophotometer analyzer could also be used to quantify the concentration of DNA in nanograms per microliter (ng/µl). In this study, the range concentration of DNA 15–20 ng/µl has been put as standardize concentration. Even though qPCR could be analysed, the DNA sample in a low concentration due to its sensitivity, this condition would produce biased results towards DNA sample which had too low or too high DNA concentration. For sample with high concentration of DNA, it will undergo the dilution process while sample with low concentration DNA will undergo DNA concentrate.

3.2.11 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

(a) Preparation Custom TaqMan Assays

Each custom TaqMan Assays contain sequence of primer and probes representing different bacteria. The assays were stored at freezer –20°C. 360 µl volumes in each assay may cover 360 reactions. The sequence of primer and probes are shown on Table 3.1.

(b) Preparation of qPCR Master Mix

There are several main components for qPCR master mix, such as, TaqMan fast advanced master mix, TaqMan Assay (primers and probes), nuclease–free water and DNA samples. The qPCR reaction was prepared in 20 µl volumes containing the following final concentration of each component as shown in Table 3.2

Table 3.2: Components of qPCR master mix

Components	1X Reaction (µl)
TaqMan Fast Advanced Master Mix	10.0
TaqMan Assay	1.0
Nuclease–free water	8.0
DNA sample	1.0
Final volume	20.0

(c) Setup qPCR

All DNA extraction sample were analysed using qPCR (7500 Fast RT–PCR System, Applied Biosystems®). The 7500 fast RT–PCR System was setup (Figure 3.4) with initial stage of enzyme activation of at 95°C for 20 seconds. Then, next 40 cycles for denaturation at 95°C for 3 seconds, continued with annealing and extension phases at 60°C for 30 seconds each. This setup was modified from the previous methods done by Boutaga *et al*., (2003). By using qPCR fast reaction, it would take almost 45 minutes per run to complete. The data were analyzed with 7500 fast RT–PCR system software.

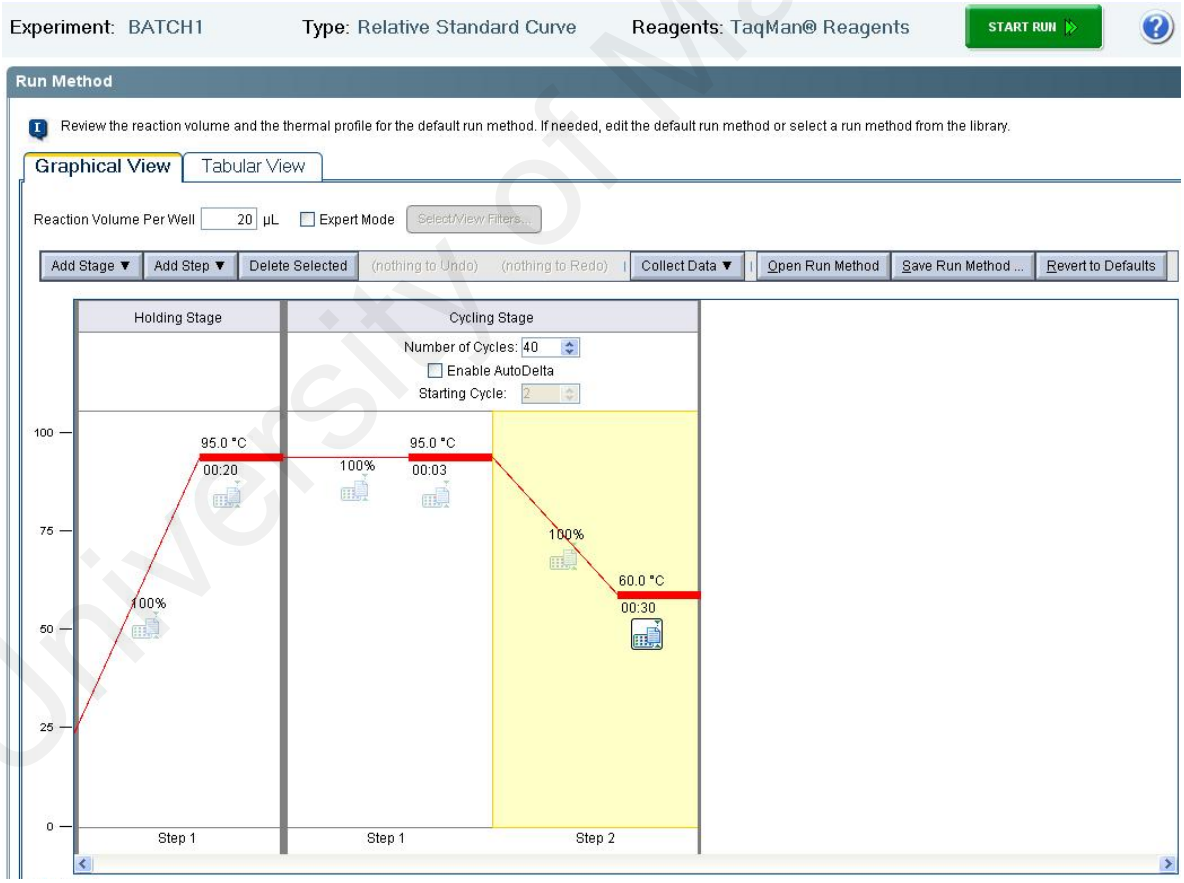


Figure 3.4: The temperature, time and cycle setting of qPCR

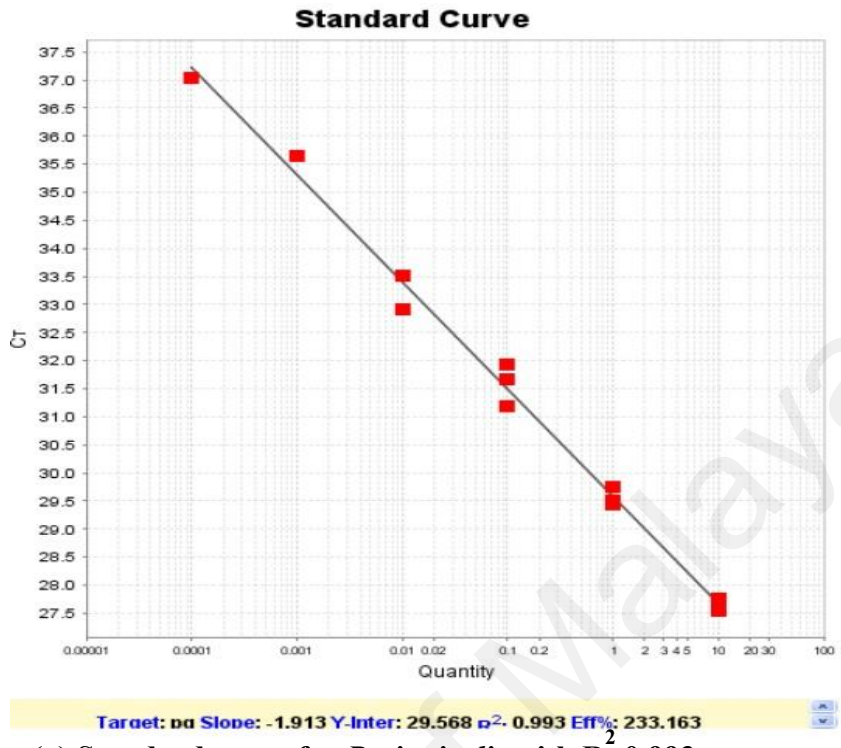
(d) Optimization of qPCR

In order to get reliable results, the applied nucleic acid quantification and working procedures should be highly optimized. There are several ways to optimize the qPCR assays and one of them is quantification of DNA by looking at standard curve method. Dilution of known amounts of reference strains of DNA were used to design the standard curve. This method focussed on relationship between input copy numbers with increased of fluorescence in the exponential phase.

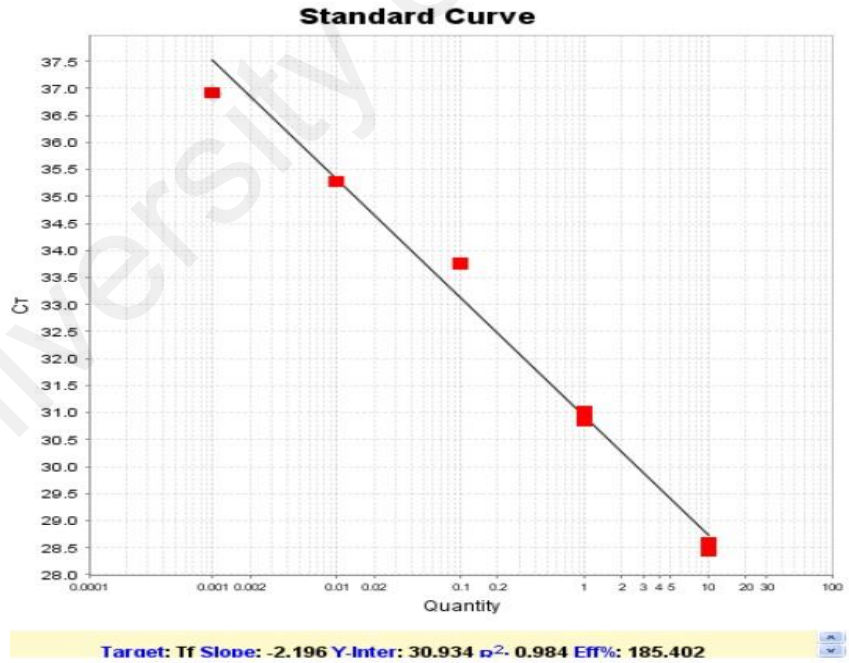
All bacteria have their own standard curve as references for unknown samples. DNA of all reference strains namely *P. gingivalis, T. forsythia, P. intermedia* and *A. actinomycetemcomitans* were extracted and diluted to 2 folds, 5 folds and 10 folds before being used in qPCR. The analysis DNA of each reference strains with series of dilution was run thrice to confirm the reproducibility of the data. Triplicate data from threshold cycle (C_T) values were then calculated for average. Between these three different dilution factors, 10 folds concentration produced the reliable C_T values and ending curve.

(e) Standard Curve of qPCR

Standard curve (Figure 3.6) was constructed by plotting the C_T values against the logarithm of the initial copy numbers of standards with known concentration. The copy numbers can be calculated after linear regression of standard curve by correlation coefficient (R^2) of $\leq$ 0.99 with the efficiency range 90% to 100%. This absolute quantification is able to determine the exact value of copy number per cell, per total DNA concentration or per sample matrix.

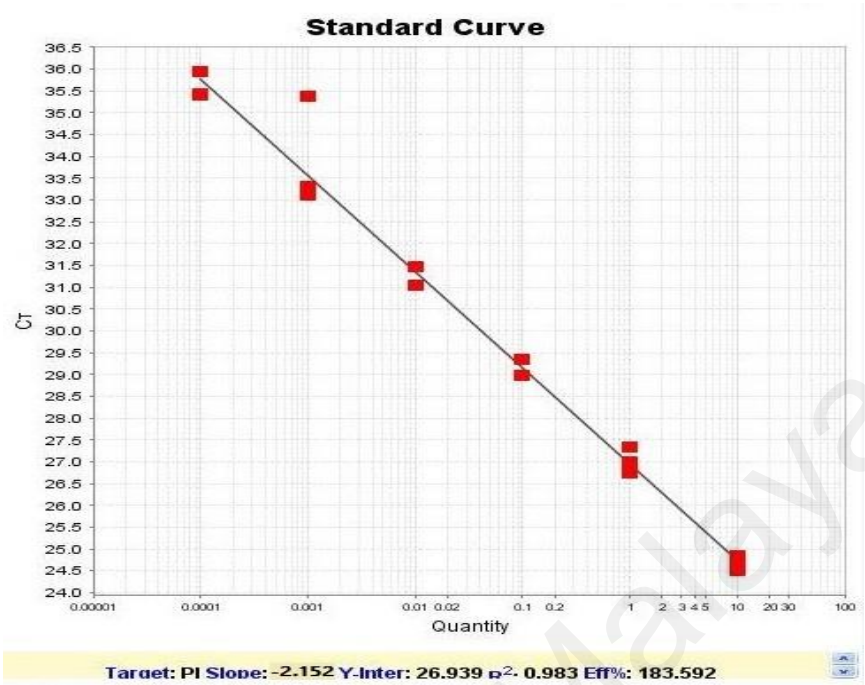


(a) Standard curve for *P. gingivalis* with R^2 0.993.

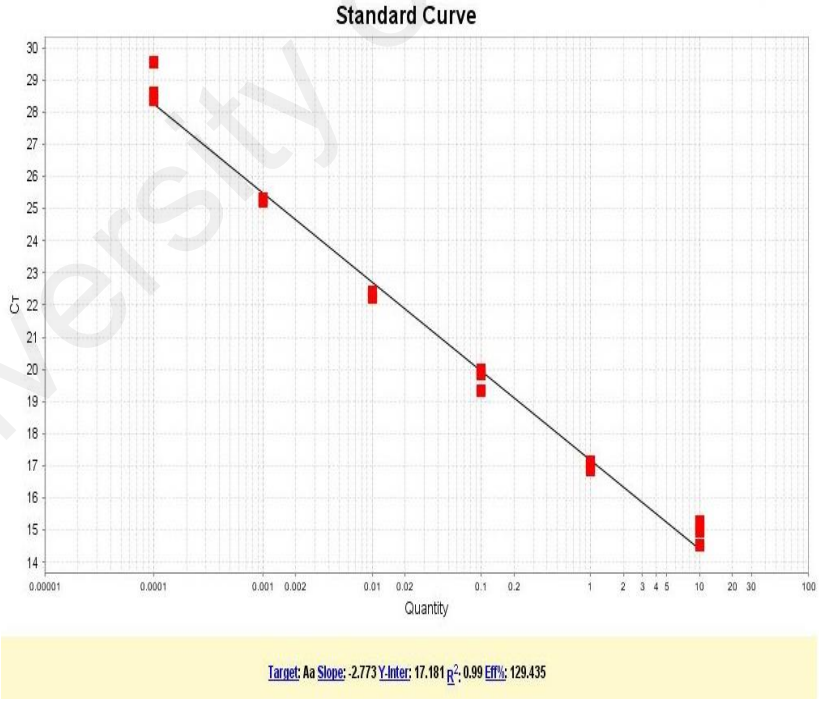


(b) Standard curve for *T. forsythia* with R^2 0.984.

Figure 3.5: Standard curve for (a) *P. gingivalis*, (b) *T. forsythia*, with R^2 value is closer to 1.0 showed the data fits the line



(c) Standard curve for *P. intermedia* with R^2 0.983.



(d) Standard curve for *A. actinomycetemcomitans* with R^2 0.99.

Figure 3.5 (continued): Standard curve for (c) *P. intermedia*, (d) *A. actinomycetemcomitans*, with R^2 value is closer to 1.0 showed the data fits the line

(f) Calculation of Mean Count of Periodontal Pathogens

All the information on standard curve of bacteria control would be important to calculate the copy numbers of other unknown samples, for example, based on standard curve of *P. gingivalis* plotted on Figure 3.6 (a):

$$y = mx + c$$

y = Y coordinate (average of C_T value)

m = 1.913 (slope of gradient)

c = 29.568 (y–intercept)

x = X coordinate (amount of DNA in ng)

According to the formula, all the samples would get their copy number values by using the value of m, x, and c from the standard curve control bacteria and value of y from C_T value reading during qPCR. After getting the copy number of target DNA for each bacteria, the exact amount of DNA present will be calculated. The formula as followed:

$$\text{Number of copies} = (\text{amount of (ng)} * 6.022\text{x}10^{23}) / (\text{length} * 1\text{x}10^{9} * 650)$$

Amount (ng) = amount of DNA in nanograms

6.022×10^{23} = Avogadro's number molecules/mole

Length = length of DNA fragment in base pair

$1\text{x}10^{9}$ = conversion to nanograms

650 = molecular weight of bp

The lengths of the DNA fragments in kilo base pair were available in the Table 3.3:

Table 3.3: Length of DNA fragment in base pair (kb)

Periodontal Pathogens	**Length of template (kb)**	
P. gingivalis	2 343	Dashper *et al*., (2008)
T. forsythia	3 405	Posch *et al*., (2012)
P. intermedia	2 699	Microbewiki, (Retrieved April 21, 2015)
A. actinomycetemcomitans	2 105	Najar *et al*., (2009)

3.5.12 Statistical Data Analysis

Data entry and statistical analysis were carried out using Statistical Package for the Social Sciences (SPSS), version 18.0 (SPSS Inc. Chicago, IL, USA). The data was tested for normality of distribution using Shapiro–Wilk test before the test of hypothesis analysis. *p*–value of less than 0.05 will be set as statistically significant. Independent T–test was used to analyze the significant difference in age and BMI distribution while Chi Square test was used to analyze significant difference of the distribution of subject characteristics for both obese and non-obese.

The distributions of clinical parameters at baseline were measured using either Independent Sample T–test or Mann Whitney test. Meanwhile, Chi–Square test is used to measure the frequency of periodontal pathogens between two groups at baseline.

The test namely as repeated measure ANOVA was used to compare mean clinical parameter and periodontal pathogens within group based on time and to compare mean

difference between groups regardless of time. The significant level was determined when 95% CI showed no overlapping values.

Further, multiple regression test was carried out to determine associated factors that predict the changes of dependent variables. The relationship between mean changes of clinical parameters and mean count changes of periodontal pathogens were measured using Pearson's coefficient correlation and Spearman coefficient correlation. The strengths of correlation coefficients values are shown in Table 3.4.

Table 3.4: The strength of the correlation coefficient values (Piaw, 2013)

Correlation coefficient size (r)	Correlation strength
0.71 to 1.00 or − 0.71 to – 1.00	Strong
0.51 to 0.70 or – 0.51 to – 0.70	Moderate
0.01 to 0.50 or – 0.01 to – 0.50	Weak
0.00	No correlation

CHAPTER 4: RESULTS

4.1 Subject Characteristics Data of Study Population

Table 4.1 summarized the mean and standard deviation (SD) for age and BMI of the study population. The mean age was 45.77 (± 10.42) for the obese group and 47.23 (± 10.42) for the non–obese group, with no significant difference (p = 0.588) between groups. As expected, mean BMI between obese and non–obese groups showed statistical significance (p = 0.000) with mean difference 9.30 kg/m^2.

In addition, table 4.1 also summarized the subject characteristics data (gender, ethnicity, education level, smoking and diabetic status) of the study population. The Malay ethnicity was dominant among the obese group. The non–obese group showed an almost equal distribution among the 3 ethnic groups. The non–obese group also had almost 50% ratio of female to male participants. There were significant differences (p = 0.037) in terms of gender and ethnicity for both obese and non–obese groups.

Furthermore, approximately about 60 to 80% of the participants in both groups had at least secondary education level. The Chi–Square analysis showed no significant difference in terms of education level (p = 0.285) for obese and non–obese groups. The obese group was significantly associated with non–smoking (p = 0.003) but non–diabetic was associated (p = 0.044) for the obese and non–obese groups.

Table 4.1: Distribution of subject characteristics data of the study population

Variables	Obese N = 30	Non–obese N = 30	*p*–value
	Mean (± SD)		
Age (years)	45.77 (10.42)	47.23 (10.42)	0.588[a]
BMI (kg/m^2)	33.18 (3.82)	23.88 (2.56)	0.001[a]*
	N (%)		
Gender			
Male	9 (30.00)	17 (56.70)	0.037[b]*
Female	21 (70.00)	13 (43.30)	
Ethnicity			
Malay	21 (70.00)	12 (40.00)	0.037[b]*
Indian	2 (6.70)	8 (26.70)	
Chinese	7 (23.30)	10 (33.30)	
Education level			
Primary	11 (36.70)	6 (20.00)	0.285[b]
Secondary	13 (43.30)	14 (46.70)	
Tertiary	6 (20.00)	10 (33.30)	
Smoking status			
Smoker	5 (16.70)	16 (53.30)	0.003[b]*
Non–smoker	25 (83.30)	14 (46.70)	
Diabetic status			
Diabetic	6 (20.00)	1 (3.30)	0.044[b]*
Non–diabetic	24 (80.00)	29 (96.70)	

[a] Independent T–Test; [b] Chi–Square test; * Significant *p*–value < 0.05

4.2 Clinical Parameter Data of Study Population

4.2.1 Distribution of Mean Clinical Parameters at Baseline

Table 4.2 showed the means and SDs for VPI, GBI, PPD and CAL at baseline for the obese and non–obese groups. The mean percentage of VPI was higher in the obese with 78.44 (± 24.93) compared to the non–obese 45.54 (± 20.03) groups. Similar observation was noted for mean GBI. The obese group had higher mean percentage of GBI compared to non–obese with 70.23 (± 27.63) and 40.95 (± 13.83) respectively. Moreover, the difference was statistical significant ($p < 0.001$) in terms of VPI and GBI. In general, the mean PPD and CAL were higher for the non–obese compared to the obese group. The mean PPD was 2.95 (± 0.58) mm for the non–obese compared to 2.32 (± 0.41) mm in the obese group. The mean CAL was 3.69 (± 1.14) mm for the non–obese compared to 2.95 (± 0.68) mm in the obese group. There was significant difference for ($p < 0.05$) both PPD and CAL.

Table 4.2: Distribution of clinical parameters at baseline

Clinical parameter	Obese N = 30	Non–obese N = 30	*p*-value
	Mean (± SD)		
VPI (%)	78.44 (24.93)	45.54 (20.03)	< 0.001[b*]
GBI (%)	70.23 (27.63)	40.95 (13.83)	< 0.001[a*]
PPD (mm)	2.32 (0.41)	2.95 (0.58)	< 0.001[a*]
CAL (mm)	2.95 (0.68)	3.69 (1.14)	0.004[b*]

[a] Independent Sample T–Test; [b] Mann Whitney Test; * Significant *p*–value < 0.05

4.2.2 Comparison of Mean Clinical Parameters within Group and Mean Difference between Groups

Table 4.3 summarized the comparison of means VPI, GBI, PPD and CAL within the group based on time, as well as the mean difference between groups. At baseline, the obese group showed means of 78.44% for VPI and 70.23% for GBI. At 12 weeks post NSPT, the means of VPI and GBI were reduced to 9.56% and 17.18% respectively. Moreover, the reduction was statistically significant with 95% CI (69.130, 87.748; 4.760, 14.350) for VPI and 95% CI (59.918, 80.549; 11.189, 23.160) for GBI. For the non–obese, the means was 45.54% and 40.95% for VPI and GBI respectively at baseline. At 12 weeks post NSPT, the means VPI and GBI were reduced to 30.51% and 25.94% respectively. The reduction was statistically significant with 95% CI (38.056, 53.018; 24.019, 36.991) for VPI and 95% CI (35.784, 46.111; 20.595, 31.277) for GBI.

At baseline, the obese had means of 2.32 mm for PPD and 2.95 mm for CAL. At 12 weeks post NSPT, the means of PPD and CAL were reduced to 1.91 mm and 2.47 mm respectively. The reduction was statistically significant with 95% CI (2.169, 2.479; 1.752, 2.077) for PPD and 95% CI (2.700, 3.205; 2.255, 2.691) for CAL. In contrast, non–obese group had means 2.95 mm for PPD and 3.69 mm for CAL during baseline. These means were decreased at 12 weeks post NSPT, of 2.33 mm and 3.14 mm respectively. However, only means PPD at baseline and at 12 weeks post NSPT were significantly reduced with 95% CI of PPD (2.734, 3.166; 2.127, 2.525).

Comparisons mean differences of VPI, GBI, PPD and CAL between groups showed that only VPI and GBI had significant differences. Between two groups, obese was associated with the changes of VPI [(95% CI (58.607, 79.160; 9.299, 20.765)] and changes of GBI [95% CI (43.536, 62.581; 9.163, 20.860)].

Mean clinical parameters based on the severity of the disease were shown in Table 4.4. PPD were divided into percentage sites of PPD < 4 mm, PPD 4–6 mm and PPD > 6 mm as well as CAL. Overall, subjects in obese and non–obese showed the positive response when percentage sites of PPD 4–6 mm and PPD > 6 mm was decreased at 12 weeks post NSPT. However, the percentage sites with PPD 4–6 mm decreased significantly in obese group from 8.29% to 2.89% while non–obese group from 21.88% to 10.29% in both obese and non–obese respectively. Meanwhile, percentage sites with PPD < 4 in obese and non–obese increased significantly at 12 weeks post NSPT with 91.19% to 96.77% and 73.97% to 87.79% respectively. Comparison between groups showed that PPD measurement had significant difference in all site with PPD < 4 mm, PPD 4–6 mm and PPD > 6. On the other hand, percentage sites of CAL < 4 mm, CAL 4–6 mm and CAL > 6 showed no significant changes at 12 weeks post NSPT as well as no significant difference between obese and non – obese group.

Table 4.3: Overall mean of clinical parameters within group based on time and mean difference between groups

Variables	Group vs Time		Mean	95%, CI	Mean difference	95%, CI
VPI (%)	**Obese**	Baseline	78.44	69.130, 87.748*	68.88	58.607, 79.160*
		12 weeks	9.56	4.760, 14.350		
	Non–obese	Baseline	45.54	38.056, 53.018*	15.03	9.299, 20.765
		12 weeks	30.51	24.019, 36.991		
GBI (%)	**Obese**	Baseline	70.23	59.918, 80.549*	53.06	43.536, 62.581*
		12 weeks	17.18	11.189, 23.160		
	Non–obese	Baseline	40.95	35.784, 46.111*	15.01	9.163, 20.860
		12 weeks	25.94	20.595, 31.277		
PPD (mm)	**Obese**	Baseline	2.32	2.169, 2.479*	0.41	0.286, 0.534
		12 weeks	1.91	1.752, 2.077		
	Non–obese	Baseline	2.95	2.734, 3.166*	0.62	0.449, 0.799
		12 weeks	2.33	2.127, 2.525		
CAL (mm)	**Obese**	Baseline	2.95	2.700, 3.205*	0.48	0.349, 0.610
		12 weeks	2.47	2.255, 2.691		
	Non–obese	Baseline	3.69	3.269, 4.118	0.55	0.381, 0.721
		12 weeks	3.14	2.743, 3.542		

Repeated measures ANOVA; *Significant difference with no overlapping between 95% CI

Table 4.4: Severity of mean clinical parameters within group based on time and mean difference between groups

Variables	Group vs Time		Mean	95%, CI	Mean difference	95%, CI
% Sites with PPD < 4mm	**Obese**	Baseline	91.19	88.01, 94.36*	5.59	3.85, 7.32*
		12 weeks	96.77	94.54, 99.01		
	Non–obese	Baseline	73.97	68.51, 79.42*	13.82	9.54, 18.11
		12 weeks	87.79	83.89, 91.69		
% Sites with PPD 4-6 mm	**Obese**	Baseline	8.29	5.29, 11.28*	5.40	3.70, 7.10*
		12 weeks	2.89	0.88, 4.90		
	Non–obese	Baseline	21.88	17.66, 26.11*	11.60	8.21, 14.99
		12 weeks	10.29	7.36, 13.22		
% Sites with PPD > 6 mm	**Obese**	Baseline	0.49	-0.11, 1.08	0.15	-0.07, 0.37*
		12 weeks	0.34	-0.11, 0.79		
	Non–obese	Baseline	4.14	2.42, 5.86	2.21	0.87, 3.56
		12 weeks	1.93	0.63, 3.22		
% Sites with CAL < 4 mm	**Obese**	Baseline	73.08	66.81, 79.35	12.14	8.14, 16.13
		12 weeks	85.22	79.95, 90.49		
	Non–obese	Baseline	59.28	50.56, 68.00	9.64	5.51, 13.77
		12 weeks	68.93	60.44, 77.41		
% Sites with CAL 4-6 mm	**Obese**	Baseline	24.56	19.19, 29.93	10.97	6.94, 15.00
		12 weeks	13.59	9.08, 18.10		
	Non–obese	Baseline	28.83	23.78, 33.89	4.57	0.80, 8.34
		12 weeks	24.26	18.63, 29.90		
% Sites with CAL > 6 mm	**Obese**	Baseline	2.11	0.66, 3.55	0.92	0.30, 1.54
		12 weeks	1.19	0.05, 2.33		
	Non–obese	Baseline	11.77	7.29, 16.26	4.96	2.96, 6.96
		12 weeks	6.81	3.39, 10.23		

Repeated measures ANOVA; *Significant difference with no overlapping between 95% CI

4.2.3 Multiple Regression Clinical Parameters with Subjects' Characteristics

A linear regression was run to find out the possible predictor(s) for mean changes in VPI and GBI. After adjusted with subjects' characteristics, the predictors of mean changes of VPI were (i) group and (ii) smoking status while the predictor of mean changes GBI are (i) group, (ii) gender, (iii) ethnicity and (iv) smoking status. Further analysis was carried out using multiple regressions were summarized in Table 4.5. Generally, only the obese group was found significantly as a potential associated factor to influence the mean changes of clinical parameters of VPI with $p < 0.0001$. It means that for every individual added in obese group, the mean change of VPI is expected to increase by 53%. Meanwhile, after adjusted with multiple regression analysis, gender, ethnicity and smoking status were not considered as potential associated factors ($p > 0.05$) to predict the mean changes of GBI. Likewise, for mean changes of VPI, for every individual added to the obese group, the mean changes of GBI is expected to increase by 38%.

Table 4.5: Multiple regression clinical parameters with subjects' characteristics

Variables	Model	B	t	*p* - value
Mean changes				
VPI (%)	1 (constant)			
	Obese	53.32	8.484	< 0.0001*
	Smoker	− 1.456	− 0.221	0.826
GBI (%)	1 (constant)			
	Obese	38.05	6.964	< 0.0001*
Adjusted gender, ethnicity and smoking status were not associated to predict the changes of clinical parameters				

* Significant *p*–value < 0.05

4.3 Periodontal Pathogens Data of Study Population

4.3.1 Distribution of Frequency Periodontal Pathogens

Table 4.6 presented the frequency of periodontal pathogens in both groups. Detection level of all periodontal pathogens in each individual was considered if the level reached a minimum detection level; *P. gingivalis* ($\geq 10^4$ copy cells) (Boutaga *et al*., 2003), *T. forsythia* ($\geq 10^5$ copy cells) (Suzuki *et al*., 2004), *P. intermedia* ($\geq 10^2$ copy cells) (Nagashima *et al*., 2005) and *A. actinomycetemcomitans* ($\geq 10^2$ copy cells) (Jervee-Storm *et al*., 2005).

At baseline, more than 76% of samples from the obese and non–obese groups have been detected with the presence of *P. gingivalis*, *T. forsythia* and *P. intermedia*. However, there was no significant difference between *P. gingivalis* ($p = 0.085$), *T. forsythia* ($p = 0.071$) and *P. intermedia* ($p = 0.781$) between groups.

Meanwhile, *A. actinomycetemcomitans* was only identified in 43.3% of samples in the non–obese group. No detection (0%) was recorded in the obese group at baseline. The Chi–Square analysis showed that the difference of periodontal pathogens between groups was statistically significant ($p < 0.001$).

Table 4.6: Frequency of periodontal pathogens at baseline

Periodontal pathogens	N (%)		*p*–value
	Obese	Non–obese	
P. gingivalis	25 (83.30)	29 (96.70)	0.085
T. forsythia	23 (76.70)	28 (93.30)	0.071
P. intermedia	25 (83.30)	26 (86.70)	0.781
A. actinomycetemcomitans	0 (0.00)	13 (43.30)	0.000*

Chi–Square Test; * Significant *p*–value < 0.05

4.3.2 Comparison Mean Counts of Periodontal Pathogens within Group and Mean Difference between Groups

The comparison means count of periodontal pathogens within group based on time and mean difference between groups was shown on Table 4.7.

In the obese group, means counts of *P. gingivalis* and *T. forsythia* were 1.38×10^6 copy cells and 0.48×10^6 copy cells, respectively, at baseline. At 12 weeks post NSPT, the means counts decreased to 1.36×10^6 copy cells and increased 0.55×10^6 copy cells respectively. For both periodontal pathogens, the changes were not statistically significant. The mean count of *P. intermedia* was 0.78×10^6 copy cells at baseline, and the mean count decreased almost half (0.40×10^6 copy cells) after 12 weeks post NSPT. The reduction was statistically significant with 95% CI (0.57, 0.98; 0.25, 0.55). No mean count was recorded for *A. actinomycetemcomitans* at baseline and 12 weeks post NSPT.

Furthermore, in non–obese group, means counts of *P. gingivalis, T. forsythia, P. intermedia* and *A. actinomycetemcomitans* were reduced with means differences of 0.48×10^6 copy cells, 0.16×10^6 copy cells, 0.19×10^6 copy cells and 0.79×10^6 copy cells, respectively at 12 weeks post NSPT. However, the mean count reduction for *A. actinomycetemcomitans* was only statistically significant after 12 weeks NSPT with 95% CI (0.39, 1.18; 0.00, 0.00).

Comparison in mean difference of periodontal pathogens between groups showed that only *A. actinomycetemcomitans* demonstrated a statistically significant difference. Between the two groups, the non–obese was associated with the changes of *A. actinomycetemcomitans* [95% CI (0.00, 0.00; 0.40, 1.18)].

Table 4.7: Overall mean of periodontal pathogens within group based on time and mean difference between groups

Periodontal pathogens	Group vs Time		Mean count ($\times 10^6$) (copy cells)	95%, CI ($\times 10^6$)	Mean difference ($\times 10^6$) (copy cells)	95%, CI ($\times 10^6$)
P. gingivalis	**Obese**	Baseline	1.38	1.10, 1.66	0.02	− 0.30, 0.33
		12 weeks	1.36	1.15, 1.57		
	Non–obese	Baseline	1.63	1.36, 1.90	0.48	0.17, 0.79
		12 weeks	1.15	0.85, 1.46		
T. forsythia	**Obese**	Baseline	0.48	0.35, 0.61	0.07	− 0.06, 0.19
		12 weeks	0.55	0.40, 0.69		
	Non–obese	Baseline	0.80	0.67, 0.93	0.16	− 0.01, 0.32
		12 weeks	0.64	0.49, 0.79		
P. intermedia	**Obese**	Baseline	0.78	0.57, 0.98*	0.38	0.21, 0.54
		12 weeks	0.40	0.25, 0.55		
	Non–obese	Baseline	0.99	0.80, 1.18	0.19	− 0.06, 0.44
		12 weeks	0.80	0.57, 1.04		
A. actinomycete-mcomitans	**Obese**	Baseline	0.00	0.00, 0.00	0.00	0.00, 0.00*
		12 weeks	0.00	0.00, 0.00		
	Non–obese	Baseline	0.79	0.39, 1.18*	0.79	0.40, 1.18
		12 weeks	0.00	0.00, 0.00		

Repeated measures ANOVA; *Significant difference with no overlapping between 95% CI

4.3.3 Multiple Regression Periodontal Pathogen with Clinical Parameters and Subjects' Characteristics

Linear regression was carried out to identify possible predictor(s) of mean count changes of *A. actinomycetemcomitans*. After been adjusted for the clinical parameters and the subjects' characteristics, predictors of mean count changes of *A. actinomycetemcomitans* were (i) group, (ii) mean changes of VPI and (iii) smoking status.

Further analysis using multiple regressions was presented in Table 4.8. Obese group was the associated factor to predict the mean count changes of *A. actinomycetemcomitans* with $p < 0.0001$. Generally, for every individual added in the obese group, mean count changes of *A. actinomycetemcomitans* decreased by 0.789×10^6 copy cells. Moreover, the combination of obese and smoking status was also significantly influenced in the mean count changes of *A. actinomycetemcomitans* ($p < 0.05$) after adjusted with the other predictors. It could be concluded that, any addition of an obese individual and a smoker individual will cause a decrease by 0.607×10^6 copy cells and an increase by 0.497×10^6 copy cells means count changes of *A. actinomycetemcomitans* respectively.

Table 4.8: Multiple regression periodontal pathogen with clinical parameters and subjects' characteristics

Variables	Model	Beta ($\times 10^6$)	t	*p*–value
Mean count changes				
A. actinomycete-mcomitans **($\times 10^6$) (copy cells)**	1 (constant)			
	Obese	− 0.789	− 4.091	< 0.0001*
	2 (constant)			
	Obese	− 0.607	−3.017	0.004*
	Smoking	0.497	2.354	0.022*
Adjusted mean changes of VPI was not associated to predict the changes of *A. actinomycetemcomitans*				

* Significant *p*–value < 0.05

4.4 Correlation between the Mean Changes of Clinical Parameters with Mean Count Changes of Periodontal Pathogens for Both Groups

Table 4.9 indicated the correlation between the mean changes of clinical parameters with mean count changes of periodontal pathogens for both groups. The results indicated the distribution of Pearson's correlation coefficient and Spearman correlation coefficient where both tests were used to determine the correlation between variables. The strengths of the correlation coefficient (r) values were shown in the table.

Overall, obese group showed no significant correlation with weak strength ($r < 0.32$; $p > 0.05$) between all mean changes of clinical parameters (VPI, GBI, PPD and CAL) and mean count changes of periodontal pathogens (*P. gingivalis, T. forsythia, P. intermedia* and *A. actinomycetemcomitans*).

In the non–obese group, mean changes of GBI were significantly correlated with mean count changes of *P. gingivalis* ($p = 0.013$) and *T. forsythia* ($p = 0.018$). However, weak correlation was demonstrated between mean changes of GBI with mean count changes of *P. gingivalis* ($r = 0.450$) and *T. forsythia* ($r = 0.429$).

Similar findings were found in this study in terms of clinical parameters. In non–obese group, similar periodontal pathogens i.e., *P. gingivalis* and *T. forsythia* showed correlation with the clinical parameters. Changes mean count of *P. gingivalis* was significantly correlated by weak strength ($p = 0.034$; $r = 0.388$) with mean changes of PPD. Moreover, weak correlation was observed significantly ($p = 0.015$; $r = 0.439$) between mean count changes of *T. forsythia* and mean changes of PPD.

Table 4.9: Correlation between the changes of clinical parameters with periodontal pathogens for both groups

Mean/mean count changes	Obese N = 30		Non–obese N = 30	
	r	***p*–value**	**r**	***p*–value**
VPI				
P. gingivalis	0.322	0.082[a]	0.009	0.962[a]
T. forsythia	0.009	0.962[b]	0.016	0.933[a]
P. intermedia	0.026	0.891[a]	− 0.106	0.576[a]
A. actinomycetemcomitans	−	−	0.227	0.227[a]
GBI				
P. gingivalis	0.135	0.478[a]	0.450	0.013[a]*
T. forsythia	0.092	0.628[b]	0.429	0.018[a]*
P. intermedia	0.161	0.396[a]	0.103	0.590[a]
A. actinomycetemcomitans	−	−	0.290	0.121[a]
PPD				
P. gingivalis	0.167	0.377[a]	0.388	0.034[a]*
T. forsythia	0.186	0.324[b]	0.439	0.015[a]*
P. intermedia	0.299	0.108[a]	0.203	0.283[b]
A. actinomycetemcomitans	−	−	0.219	0.246[a]
CAL				
P. gingivalis	0.153	0.420[a]	0.316	0.088[a]
T. forsythia	0.201	0.287[a]	0.359	0.052[a]
P. intermedia	0.205	0.276[a]	0.059	0.756[a]
A. actinomycetemcomitans	−	−	0.261	0.164[a]

[a] Pearson's correlation ; [b] Spearman correlation; * Significant *p*–value < 0.05

CHAPTER 5: DISCUSSIONS

5.1 Study Design and Sample Population

The current study was designed as a prospective before and after clinical trial aimed at to investigate the effect of obese population towards NSPT, in terms of changes in clinical and microbiological parameters after 12 weeks.

Due to cost and time constraints, convenience sampling method was used. This method enables one to achieve correct sample size in an inexpensive and relatively fast manner. However, the convenience sampling method may introduce inherent bias as it does not represent the general population. It is acknowledged that this method could either be an over– or under representation of a particular population. Thus, the results should be interpreted with care to avoid generalization.

Ideal sample should involve a small set of individual representing the selected population (Kadam & Bhalerao, 2010). However, if too few individuals were involved, it would introduce bias in results interpretation as the sample does not represent the target population. Meanwhile, if more individuals present were required, the study would be unethical as more individuals were put at risk of intervention. Thus, calculation of adequate sample size was carried out to ensure optimum number of individuals to generate ethically and scientifically valid results (Kadam & Bhalerao, 2010).

In the present study, one of the inclusive criteria was participants who were ≥ 30 years old. This age limit was important as to reduce the likelihood of recruiting participants with aggressive periodontitis which are common among younger adults.

There were significant differences in gender, age, smoking status and diabetic status for both test and control groups. Ideally, the study should have matched age, gender and ethnicity for the population as to control the confounding factors. This is relevant in cases with substantial differences in the occurrence of possible cofounders between the test and control groups (Faresjö & Faresjö, 2010). Any cofounders may introduce bias and reduce the efficiency of a result. However, in this study, other factors such as participants' compliance and time constrains were the factors that might lead to the gender disproportion among participants.

Smoking has been accepted as a risk factor for chronic periodontitis and its negative effects on periodontium have been well established. Smokers are more susceptible to develop periodontal disease compared to non–smokers (Bergström, 2004). In this study, neither smokers nor those with diabetes mellitus were excluded during sample recruitment. This was because it was anticipated it would be difficult to achieve a proper sample size due to high prevalent of smokers among Malaysians.

Previous studies have confirmed the association between obesity and diabetic (Nguyen *et al*., 2011; Tan *et al*., 2011) and diabetic and periodontal disease (Kaur *et al*., 2009). This is due to obesity as the strongest risk factor, especially for type 2 diabetes, which is in turn is a risk factor for periodontal disease. Thus, it is difficult to diassociate diabetes mellitus from obese individuals. Due to that, it was agreed that both smoking status and diabetic status could be excluded at the analysis levels if necessary.

5.2 Biometric Assessment

In this study, the Asian classification was used to allocate participants into obese (BMI $\geq$ 27.5 kg/m^2) and non–obese groups (BMI $\leq$ 27.5 kg/m^2). The classification was chosen based on Malaysian Clinical Practice Guidelines (CPG) recommendation (Ismail *et al*., 2004) which was more relevant among the Asian population. As an obesity measure, its advantages outweigh its disadvantages, that, it is inexpensive, simple to perform, does not require special equipment and it can be used in community based studies (Prentice & Jebb, 2001). The authors are aware that using BMI alone to classify for obesity could introduce bias. The standard setting of obesity through BMI does not take into actual frame size. Furthermore, BMI did not reflect whether weight is consisted of fat or muscle. Thus, it would be relevant to add on other measurements to factor in abdominal obesity which include waist circumference, waist hip ratio and body fat percentage to eliminate the inconsistency of BMI.

5.3 Clinical Parameters Assessment

In this study, Basic Periodontal Examination (BPE) was used as a screening tool to identify individuals with chronic periodontitis. BPE also assists clinicians in deciding for radiograph for further investigations. It is the initial step to achieve a homogenous sample in research i.e. to achieve a diagnostic tool which enable us to define disease threshold in a population.

FEME is the gold standard in diagnosing periodontal status. It involves recording the clinical parameters at 6 points for all teeth except the third molar. Thus, FEME process led to patients' fatigue, time consuming and can be costly. Alternatively, partial mouth examination could be employed. Many epidemiological studies have employed the partial recording methodologies for practical reasons to reduce cost and time, as well as reduced the patient–examiner fatigue. Studies that attempted to quantify accurately the amount of information lost through the different partial recording systems have revealed that the discrepancy between the findings obtained may be substantial (Hunt, 1987; Kingman *et al*., 2008). Partial mouth recording technique may result in over or underestimation of the chronic periodontitis prevalence (Owens *et al*., 2003). In addition, FEME has been accepted as a better technique in estimating periodontal disease status as compared to partial mouth recordings (Thomson & Williams, 2002).

5.4 Periodontal Pathogens Assessment

Dental plaque was taken from the deepest periodontal pocket (Boutaga *et al*., 2007) at least at two different teeth. Mix of plaque collected at different site might control any bias of the particular of periodontal pathogens localized in oral cavity at that time.

DNA extraction for dental plaque sample was the important stage to ensure the DNA product yield was purified. Hence, the automated DNA extraction was used in this study. By using this method, several manual steps and direct contact during DNA extraction process were removed. Thus, the contamination on DNA product could be reduced.

The qPCR method was used to quantify detection of periodontal pathogens. This automated instrument has the ability to monitor continuously the fluorescence, detection and quantification of amplified bacterial products in the log phase of the reaction (Boutaga *et al*., 2003). It was chosen because it used laser to scan 96 well plate containing sample and target gene load and TaqMan fluorescence probes to measures the accumulation of PCR products. The high specificity of primer and probes in TaqMan enabled us to distinguish target pathogens from other close related sample. Furthermore, the cubicle for preparation of qPCR also was applied with ultraviolet (UV) light in a few minutes before working session as the UV radiation is useful in the inactivation of microorganism (Chang *et al*., 1985). The combination of disinfection with 70% alcohol and UV exposure may provide the sterile environment during working with DNA sample.

5.5 Changes in Clinical Parameters Following NSPT

The present study reported significantly higher means VPI and GBI for the obese group (> 70%) compared to the non–obese group (> 40%) at baseline. This finding was similar to previous studies in various obese populations (Khader *et al*., 2009; Saxlin *et al*., 2010; Zeigler *et al*., 2012; Ababneh *et al*., 2012). Following 12 weeks post NSPT, both groups also showed reduction in means VPI (up to 31%) and GBI (up to 26%) . These findings were in agreement to previous study when Dias Goncalves *et al*., (2015) reported improvement in VPI (up to 35%) and GBI (up to to 29%) following NSPT. In addition, other studies reported significant reduction in VPI and GBI (Zuza *et al*., 2011; Duzagac *et al*., 2015) for both groups obese and non–obese with chronic periodontitis.

Obesity is a low chronic inflammatory condition, thus it is speculated that the obese condition could have contributed to the increased existing burden of inflammation associated with chronic periodontitis (Genco *et al*., 2005). Suvan *et al*., (2014) also suggested that obesity could trigger greater inflammatory burden in other co–morbidities including periodontitis. Dental plaque is the etiology for chronic periodontitis (Loe *et al*., 1965) and thus chronic periodontitis patients are expected to have high VPI. Meanwhile, GBI is a measure of gingival inflammation level. Plaque accumulation contributes to gingival inflammation. It is anticipated that those with high VPI would have high GBI too. Furthermore, NSPT has been shown to effectively provide immediate and long term improvement in clinical parameters including reduction in plaque level and gingival inflammation (Cugini *et al*., 2000). Thus, it is expected that following NSPT, the means VPI and GBI will be reduced. Due to its effectiveness, the higher the initial means, the more reduction would be expected following NSPT.

In the current study, the obese group showed greater reduction in VPI and GBI mean differences (69% and 53%) between baseline and 12 weeks post NSPT compared to the non–obese group (15% and 15%) respectively. Interestingly, a study done by Al-Zahrani & Alghamdi, (2012) also reported similar reduction with mean difference of VPI and GBI among obese group (33% and 35%) was higher than non–obese group (14%, 20%) respectively. Following adjustment of cofounding factors i.e. gender, ethnicity, group, smoking habit and diabetic status, only group (obesity) was considered as the potential factor to predict the mean percentages changes of VPI and GBI. This supports and confirms the notion that obese condition contributes and adds on burden of inflammation to the existing inflammatory condition of chronic periodontitis.

In the current study, the means of PPD and CAL for the obese groups (2.32 mm and 2.95 mm) were lower than the non–obese groups (2.95 mm and 3.69 mm) respectively, with statistically significant difference, at baseline. This finding was in contrast to the study done by Dias Goncalves *et al*., (2015), where the means of PPD and CAL in obese group (3.60 mm and 4.90 mm) were significantly higher than those of non–obese (3.40 mm and 4.40 mm) respectively. Previous study also reported percentages of PPD and CAL ≥ 4 mm among obese populations were 25% and 64% respectively (Khan *et al*., 2015). Prevalence of chronic periodontitis among adolescents was 72% with CAL ≥ 3 mm and ≥ 5 mm affected 50.4% and 17.4% subjects respectively (Susin *et al*., 2011).

The differences could be due to the difference in case definition used. The present study used case definition suggested by Eke *et al*., (2012), which defined the chronic periodontitis as CAL ≥ 3 and PPD ≥ 4 mm affecting two or more teeth. Meanwhile, the study done by Dias Goncalves *et al*., (2015) defined chronic periodontitis as > 30% of the site with CAL and PPD ≥ 4 mm. In this case, the use of low thresholds may result in recruitment of more localized chronic periodontitis than generalized chronic periodontitis. The means of PPD would have diluted after dividing the total PPD or CAL by the total sites which may include healthy periodontal sites. Thus, less extensive periodontal involvement would have contributed to lower overall means of PPD and CAL.

Following NSPT, the means of PPD and CAL in both groups were significantly reduced after 12 weeks except CAL in the non–obese group. This finding was paralleled with previous study when obese and non–obese groups with chronic periodontitis as subjects of study (Zuza *et al*., 2011; Dias Goncalves *et al*., 2015; Duzagac *et al*., 2015). Obese groups exhibited lower means PPD and CAL at baseline and lower reduction (up to 0.48 mm) at 12 weeks post NSPT than the non–obese group (up to 0.62 mm) respectively.

In contrast, other studies reported that non–obese presented with lower mean PPD and CAL at baseline but more reduction (up to 0.40 mm) after treatment than the obese group (up to 0.30 mm) (Dias Goncalves *et al*., 2015). On the other hand, obese groups showed higher PPD at baseline and greater reduction (1.55 mm) at 12 weeks following NSPT, compared to non–obese groups (0.68 mm) (Duzagac et al., 2015).

The findings may suggest that obese individuals from the current study are protected from chronic periodontitis. Interestingly, these findings could be explained by the choice of case definition for chronic periodontitis used. The case definition by Eke *et al*., (2012) allows recruitment of individuals with localized chronic periodontitis condition into the sample. Following that, the severity of the disease in terms of means PPD and CAL could have been diluted when measured out. In this study, the sample population was mainly those with localized chronic periodontitis and this did not reflect the actual prevalence of chronic periodontitis in the population.

The findings of the current study also supports the notion that regardless of the state of obesity, exposure to periodontal disease depends upon the susceptibility of an individual's genetic makeup (Löe *et al*., 1986; Michalowicz *et al*., 2000). It is also acknowledged that chronic periodontitis is a continuous process which under natural condition, irrespective of its quiescence and reminiscence progresses steadily over time (Löe *et al*., 1986). In addition, PPD is the periodontal measurement of active disease and provides information on current disease status (Haffajee *et al*., 1983). However, the measurement of PPD is not as reliable as the CAL. It depends on various factors, such as inflammatory condition of the marginal gingiva.

It could also suggest that maybe the obese subjects was currently diagnosed as chronic periodontitis compared with the non–obese subjects which already had past history of chronic periodontitis. However, this condition showed there is an association between obesity and means PDD and CAL. A study done by Suvan *et al*., (2014) approved that BMI and obesity were independent predictors of poor response following NSPT. Analysis of this study stated that obese subjects have 3.2% more sites with PPD ≥ 4 mm and 0.14 mm greater at two months after treatment. This study also proposed possible mechanism that clarify association between obesity and chronic periodontitis; (1) obesity itself associated with a systemic and locally increased response, (2) obesity's influence on quantity and composition of dental plaque (inflammation burden), (3) combination of both.

5.6 Changes in Periodontal Pathogens Profile Following NSPT

The current study reported more than 76% prevalence of *P. gingivalis, T. forsythia* and *P. intermedia* and low prevalence (43%) of *A. actinomycetemcomitans* was detected in both obese and non–obese groups with chronic periodontitis. Similar periodontal pathogens profiles were reported whereby the obese group showed more than 80% of *P. gingivalis, P. intermedia* and *A. actinomycetemcomitans* but low prevalence (66%) of *T. forsythia* was documented (Fadel *et al*., 2014).

In contrast, non–obese subjects with healthy periodontium showed low median percentages of *P. gingivalis, T. forsythia*, *P. intermedia* and *A. actinomycetemcomitans* (up to 1.44%) compared to obese group (up to 3.11%) (Goodson *et al*., 2009). Meanwhile, the prevalence of *P. gingivalis* and *T. forsythia* was found independently higher (61%–95%)

(Yang *et al*., 2004; Wara-aswapati *et al*., 2009) and relatively frequent detected together (Tomita *et al*., 2013) in adult chronic periodontitis subjects.

It could be suggested that the prevalence of *P. gingivalis* and *T. forsythia* was frequently detected with chronic periodontitis subjects and no large difference between obese and non–obese with or without chronic periodontitis. The association between *P. gingivalis* and *T. forsythia* and chronic periodontitis was found in previous studies (Mayanagi *et al*., 2004; da Silva-Boghossian *et al*., 2011). The risk of chronic periodontitis is significantly increased with high abundance of red complex periodontal pathogens, such as *P. gingivalis* (Gohler *et al*., 2014) and *T. forsythia* (Gatto *et al*., 2014). Besides the presence of red complex, orange complex species like *P. intermedia* also showed high prevalence in chronic periodontitis subjects (Ximénez-Fyvie *et al*., 2000); Papapanou *et al*., 2002). However, the presence of *A. actinomycetemcomitans* was varied in chronic periodontitis subjects (Schlegel-Bregenzer *et al*., 1998; Tomita *et al*., 2013) and spotted high prevalence in aggressive periodontitis (Tomita *et al*., 2013; da Silva-Boghossian *et al*., 2011). In this study, the high prevalence of periodontal pathogens associated with chronic periodontitis reported for both groups supported and confirmed the accuracy of subject selection.

After the completion of 12 weeks NSPT, the obese group showed slight reduction on mean count of *P. gingivalis* (0.02×10^6 copy cells) and significant decrease of *P. intermedia* (0.38×10^6 copy cells). For non–obese group, all periodontal pathogens showed a reduction but statistically significant only on *A. actinomycetemcomitans*. Large reduction means count of *P. gingivalis*, *T. forsythia* and *A. actinomycetemcomitans* (up to 100%) in non–obese group and *P. intermedia* (up to or 49%) in obese group was documented.

In this case, the obese group may have more conducive environment for the periodontal pathogen compared with the non–obese group. However, no data for comparison in terms of periodontal pathogens profile post NSPT between obese and non–obese with chronic periodontitis subjects was reported in previous study.

Studies in chronic periodontitis subjects showed that *P. gingivalis, T. forsythia, P. intermedia* and *A. actinomycetemcomitans* was reduced by 7% (Ivić-Kardum *et al*., 2001) and up to 45% (Predin *et al*., 2014) following the 12 weeks NSPT. Other studies showed reduction in *P. gingivalis, T. forsythia, P. intermedia* and *A. actinomycetemcomitans* ranged from 18% up to 99% after NSPT in diabetic subjects with chronic periodontitis (Christgau *et al*., 1998; Buzinin *et al*., 2014). A few studies reported also showed a reduction of *P. gingivalis* and *T. forsythia* (Haffajee *et al*., 2008; Mdala *et al*., 2013) as well as *P. intermedia* (Socransky *et al*., 2013). However, this could be due to these studies used adjunctive antibiotics (systemic or local) with NSPT.

These findings concurred with the fact that mean count of *P. gingivalis, T. forsythia, P. intermedia* and *A. actinomycetemcomitans* in obese or non–obese groups were effectively reduced by using NSPT. The treatment was capable of lowering these periodontal pathogens but not eliminating them. However, NSPT has limitations i.e. inability to reach periodontal pathogens in deep PPD as well as to remove periodontal pathogens within the tissue lining deep periodontal pocket (Lowenguth & Greenstein, 1995). Thus, in some cases, adjunctive antimicrobial agents or even surgical intervention are necessary. In other studies, NSPT was speculated to control not only single species of periodontal pathogens but the alteration of ecosystem which pathogens remained at level that are unable to initiate or continue disease progression (Socransky *et al*., 2013).

In some instances, the host itself does not response well towards the therapy. Haffajee *et al*., (1997) came out with the postulated NSPT have additional effects that influence host–parasite interaction. The postulates characterized as (i) NSPT decreased inflammation (local environment) accompanied decreased in PPD could markedly affect the milieu of the organism and alter the interaction with the host (ii) Introduction of periodontal pathogens during NSPT could illicit alteration in host response including local or systemic antibody production, that might be protective to the host periodontal tissue. In addition, reduction in local inflammation would lead to reduction in gingival crevicular fluid and subgingival taxa had limited prominent source of nutrients for the growth or colonizing (Socransky *et al*., 2013).

Surprisingly, mean count of *T. forsythia* in the obese group was slightly increased at the 12 weeks post NSPT. It could not be explained whether mean count of *T. forsythia* in this study declined during the first few weeks and turn to increase or slowly increase post in the NSPT. However, previous studies showed similar pattern whereby the prevalence of *P. gingivalis* and *T. forsythia* among adults with chronic periodontitis reduced up to 6 months and showed slight increased at 9 and 12 months post therapy (Haffajee *et al*., 1997; Cugini *et al*., 2000). However, the rebound in periodontal pathogens may suggest worsening chronic periodontitis clinical condition. Unfortunately, our study had no sufficient duration to monitor the changes. The data suggested that the complex of periodontal pathogens that diminished during therapy may have included those that were responsible for the observed tissue loss.

The inconsistency results among the obese with chronic periodontitis at post NSPT suggested that obese condition may have contributed and may have a link to the prevalence of periodontal pathogens. Obesity, as a chronic inflammatory disease, is associated with

increased levels of proinflammatory cytokine such as TNF–α, interleukin–6 and decrease adiponectin levels. These proinflammatory cytokines was responsible for the over production of reactive oxygen and nitrogen by macrophages and monocytes, then leading to increase oxidative stress (Furukawa *et al*., 2004). The modified inflammatory background and hyperoxidative state is, in return, susceptible to bacterial infection which might initiate or continues progression of chronic periodontitis.

Comparison of mean differences of *A. actinomycetemcomitans* showed that the non–obese group had the most significant difference. Further analysis was carried out to determine whether the significant change of *A. actinomycetemcomitans* was associated with other predictor factors. After adjustment to confounding factors, group (obesity) and smoking status were identified as the predictors of changes of *A. actinomycetemcomitans* and rejected other factors, such as VPI, GBI, gender, ethnicity and diabetic status. It was suggested that non–smoking status ($B = 0.497$) may cause greater mean count changes of *A. actinomycetemcomitans*. The relation of smoking history to changes of periodontal pathogens was also examined. Similar finding was observed but only on *P. gingivalis* and *T. forsythia.* Both pathogens decreased significantly in non-smokers and former smokers but increased somewhat in current smokers (Haffajee *et al*., 1997). In contrast, smoking significantly reduced counts of *Actinomyces* by 10% (Mdala *et al*., 2013). It has been shown that smokers response less favourably to NSPT (Bergström, 2004). A study done by Bagaitkar *et al*., (2009) suggested that tobacco might induce alterations to periodontal pathogens–host interactions. Periodontal pathogens will adapt and changes its DNA and membrane protein in response to cigarette smoke. Thus, smokers are more likely to be resistant to NSPT and more susceptible to infection by periodontal pathogens.

5.7 Correlation between the Mean Changes of Clinical Parameters with Mean Count Changes of Periodontal Pathogens for Both Groups

In the current study, mean count changes of *P. gingivalis* and *T. forsythia* was correlated with mean changes of GBI and PPD in non–obese group. It was noted that the strength correlation between mean changes of GBI and mean count changes of *P. gingivalis* and *T. forsythia* was significantly weak. In agreement, the significant reduction of GBI score indicates that the full mouth periodontal pathogens loads had an even greater decrease (Cugini *et al*., 2000). The relationship between PPD and *P. gingivalis* and *T. forsythia* had been documented by a number of studies (Haffajee *et al*., 1997; Cugini *et al*., 2000). The prevalence and level of *P. gingivalis* was higher in deeper PPD but major decrease of *T. forsythia* was observed at shallow and intermediate pocket depth (Haffajee *et al*., 1997). However, it was not well defined whether a decrease in *T. forsythia* led to an improved clinical outcome or, whether a decrease in PPD affected colonization by *T. forsythia*.

CHAPTER 6: CONCLUSIONS

6.1 Conclusions

Within the limitation of the study, the following conclusions can be drawn:

1. Higher VPI and GBI but lower PPD and CAL was observed in the obese group compared to the non–obese groups with chronic periodontitis following 12 weeks NSPT.
2. Lower *P. gingivalis*, *T. forsythia*, and *A. actinomycetemcomitans* but higher *P. intermedia* were observed in the obese groups compared to the non–obese groups with chronic periodontitis following 12 weeks NSPT.
3. No correlation was found between clinical parameters (VPI, GBI, PPD and CAL) and periodontal pathogens profiles (*P. gingivalis, T. forsythia, P. intermedia* and *A. actinomycetemcomitans*) in obese groups with chronic periodontitis following 12 weeks NSPT.

6.2 Recommendations

1. Distribution of chronic periodontitis cases according to its severity which are include moderate to severe and exclude mild chronic periodontitis.
2. Increase sensitivity of study by improvement with selection specific control groups, such as, obese without chronic periodontitis and non–obese without chronic periodontitis to compare with test group obese with chronic periodontitis.

3. Extend the experimental period up to at least 12 months post NSPT with consistent review for each 3 months for better outcomes monitoring.
4. Combination therapy for both local inflammations of chronic periodontitis and systemic inflammation of obesity, such as NSPT parallel with weight management program for participants may present more definite outcomes on the effect of obesity on clinical parameter and periodontal pathogens profile.

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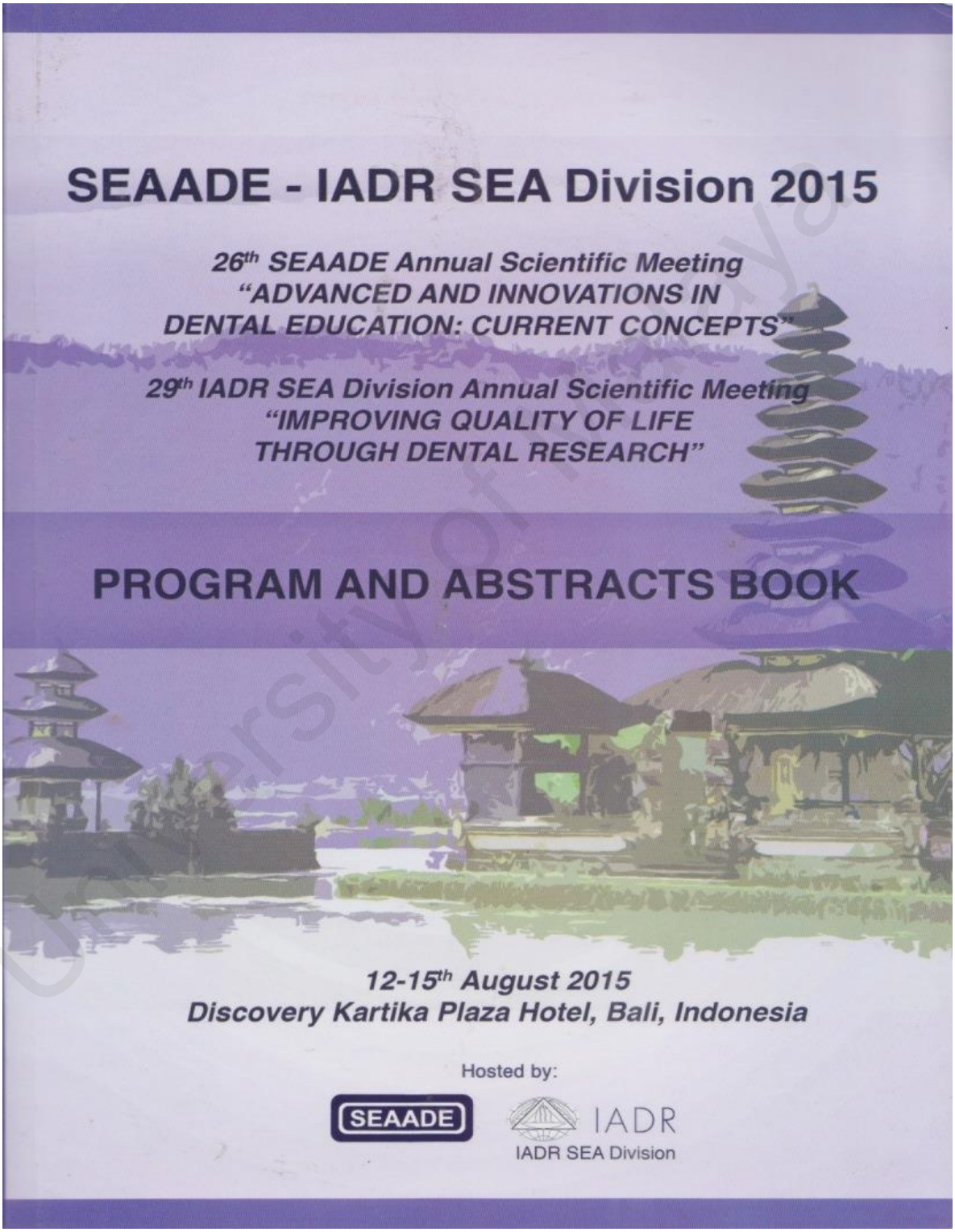
LIST OF PUBLICATIONS AND PAPER PRESENTED

POSTER PRESENTATION: IADR SEA DIVISION 2015



Objectives *Porphyromonas gingivalis* is regarded as a 'keystone' periodontopathogen, preferentially acquiring iron-based metabolism for its growth. Bismuth as a classical drug against *Helicobacter pylori* is able to replace the iron in transferrin, while its effects on *P. gingivalis* remains unknown. This study explored the potential effects of bismuth on *P. gingivalis* and the underlying molecular mechanisms.

Methods The MICs of ranitidine bismuth citrate and metronidazole for *P. gingivalis* were determined with its initial CFU at 10^6/ml. The UV-vis spectra of 10 μM of hemin was scanned from 700-200 nm, and the bismuth nitrilotriacetic acid was titrated in aliquots to monitor the replacement of ferrous ion in hemin. At the early log phase of *P. gingivalis* culture, 25 μM of ranitidine bismuth citrate was added for 24 h treatment.

Results The MIC of ranitidine bismuth citrate for *P. gingivalis* was 50 μM, and the MIC of metronidazole was 0.25 μg/ml. Initial data showed that the combination of bismuth compounds with metronidazole could decrease the dosages of both drugs against *P. gingivalis* by over 4 folds, indicating the synergistic effects of bismuth on metronidazole. Titration of bismuth compound into hemin solution led to the decrease of the intensity of the characteristic peak at 388 nm for hemin (iron-bound) on UV-vis spectra and the increase of the peak at 425 nm, suggesting that bismuth could successfully extract ferrous ion from hemin. Moreover, *P. gingivalis* accumulated 523 μg of bismuth per gram of dry cells after 24 h treatment with ranitidine bismuth citrate.

Conclusions This novel study suggests that the absorbed bismuth could inhibit the growth of *P. gingivalis* by replacing ferrous ion in hemin and other iron-containing compounds and proteins. Further study is highly warranted to determine the clinical implications of the current findings.

110

Identification of Periodontal Microbial in Obese With Chronic Periodontitis

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Objectives A positive association between obesity and chronic periodontitis (CP) has been documented. The aim of the study is to quantify and compare the frequency of detection of periodontal pathogens; *P. gingivalis*, *T. forsythia*, *P. intermedia* and *A. actinomycetamcomitans* in obese and non-obese participants with CP.

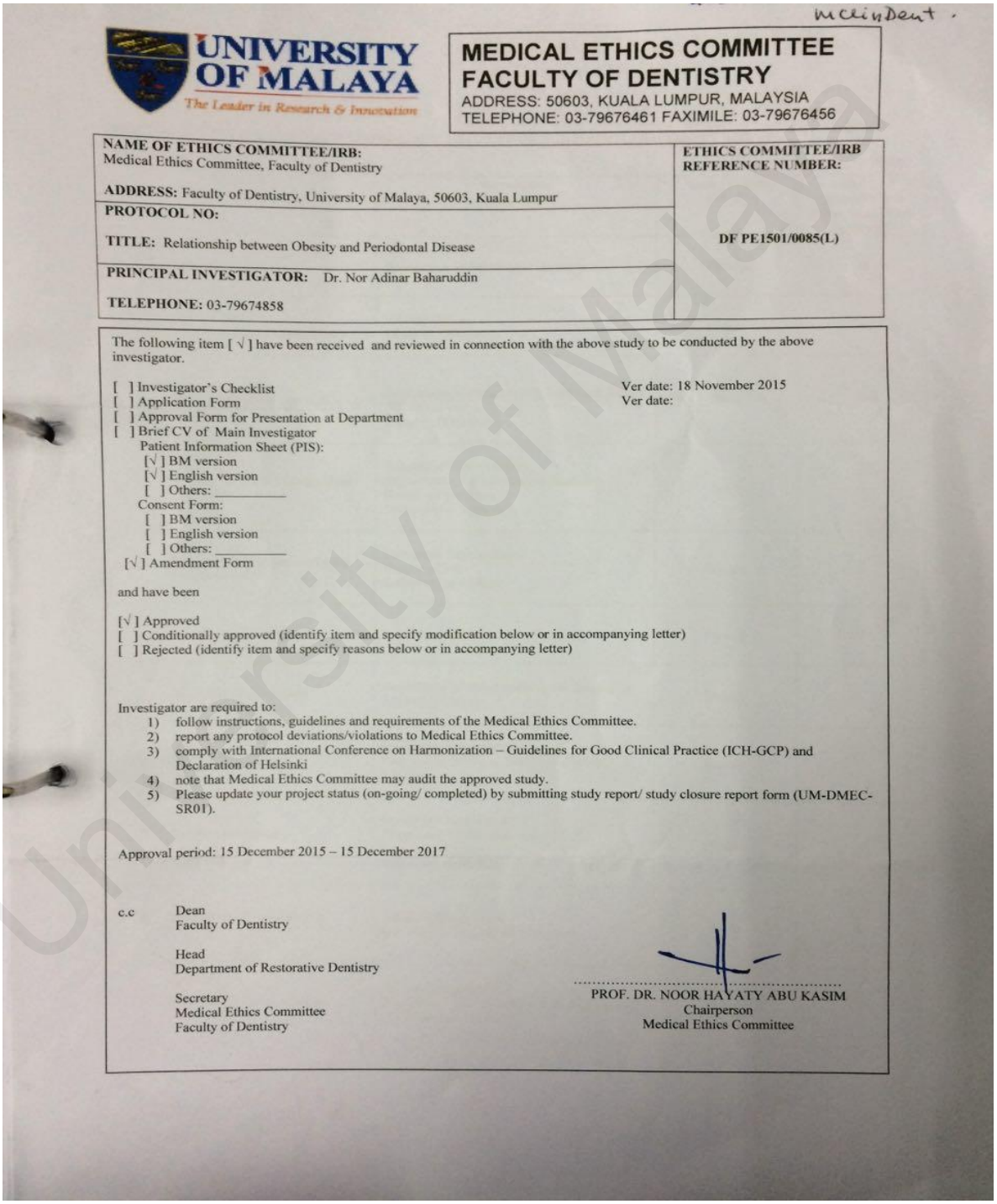
Methods This was a cross sectional study with 52 participants who were diagnosed with moderate to advanced CP, divided into obese (n=28) and non-obese (n=24) groups. Obese is defined as individuals with BMI≥27.5 kg/m^2. CP is defined as those with at least ≥2 interproximal sites with CAL ≥3 mm, and ≥2 interproximal sites with PPD ≥4 mm (not on same tooth) or one site with PPD ≥5 mm. The inclusion criteria included those who were ≥30 years old and had at least 12 teeth. Those who had periodontal treatment, antibiotic therapy and systemic anti-inflammatory drugs in the last 4 months were excluded. For each patient, sub-gingival plaque sampling was carried out from the deepest pockets in each quadrant using curettes and pooled. Identification and quantification of periodontal microbial was performed using real-time Polymerase chain reaction (qPCR).

Results *P. gingivalis*, *T. forsythia* and *P. intermedia* were identified in 93-100% participants, regardless group. Lower frequency of detection of *A. actinomycetamcomitans* was observed in both groups. However, the obese group (25%) had much lower frequency of detection than the non-obese group (54.2%). A higher mean count for *P. gingivalis*,*T. forsythia* and *P. intermedia* was observed in the non-obese group compared to the obese group (p>0.05). In contrast, mean count for *A. actinomycetamcomitans* was higher in the obese group (p>0.05).

Conclusions Frequency of detection of *P. gingivalis*, *T. forsythia* and *P. intermedia* was high for both groups. There was a higher trend of detection of *A. actinomycetamcomitans* in the non-obese CP group.

APPENDIX I

Appendix I: Ethical Approval from Medical Ethics Faculty of Dentistry, University of Malaya



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MEDICAL ETHICS COMMITTEE
FACULTY OF DENTISTRY
ADDRESS: 50603, KUALA LUMPUR, MALAYSIA
TELEPHONE: 03-79676461 FAXIMILE: 03-79676456

NAME OF ETHICS COMMITTEE/IRB: Medical Ethics Committee, Faculty of Dentistry **ADDRESS:** Faculty of Dentistry, University of Malaya, 50603, Kuala Lumpur	**ETHICS COMMITTEE/IRB REFERENCE NUMBER:**
PROTOCOL NO: **TITLE:** Relationship between Obesity and Periodontal Disease	**DF PE1501/0085(L)**
PRINCIPAL INVESTIGATOR: Dr. Nor Adinar Baharuddin **TELEPHONE:** 03-79674858	

The following item [√] have been received and reviewed in connection with the above study to be conducted by the above investigator.

[ ] Investigator's Checklist
[ ] Application Form — Ver date: 18 November 2015
[ ] Approval Form for Presentation at Department — Ver date:
[ ] Brief CV of Main Investigator
Patient Information Sheet (PIS):
[√] BM version
[√] English version
[ ] Others: _____
Consent Form:
[ ] BM version
[ ] English version
[ ] Others: _____
[√] Amendment Form

and have been

[√] Approved
[ ] Conditionally approved (identify item and specify modification below or in accompanying letter)
[ ] Rejected (identify item and specify reasons below or in accompanying letter)

Investigator are required to:
1) follow instructions, guidelines and requirements of the Medical Ethics Committee.
2) report any protocol deviations/violations to Medical Ethics Committee.
3) comply with International Conference on Harmonization – Guidelines for Good Clinical Practice (ICH-GCP) and Declaration of Helsinki
4) note that Medical Ethics Committee may audit the approved study.
5) Please update your project status (on-going/ completed) by submitting study report/ study closure report form (UM-DMEC-SR01).

Approval period: 15 December 2015 – 15 December 2017

c.c Dean
Faculty of Dentistry

Head
Department of Restorative Dentistry

Secretary
Medical Ethics Committee
Faculty of Dentistry

PROF. DR. NOOR HAYATY ABU KASIM
Chairperson
Medical Ethics Committee

APPENDIX II

Appendix II: DNA Extraction Protocol

QIAcube Principle

The principle of automated QIAcube DNA extractor was followed as manual procedure of DNA extraction (i.e., lyse, bind, wash and elute). The samples are lysed in the heated orbital shaker. Then, each lysate is transferred to a spin column in a rotor adapter. The nucleic acid was binding to the silica membrane or purification resin of the spin column and washed to remove contaminants. The spin columns are transferred to a microcentrifuge tube for elution of purified nucleic acid.

Preparation DNA Extraction Reagents

DNA extraction kit (DNeasy® Blood & Tissue Kit) (Figure3.1) containing buffer, Proteinase K, DNeasy Mini Spin Columns and elution tube RB (1.5 ml). While rotor adapter holder and RB collection tube (2 ml) have to purchase separately. Various buffer provided which are wash buffer AW1 and AW2, lysis buffer AL, elution buffer AE and ATL. Concentrated wash buffer AW1 and AW2 have to be dissolved with absolute ethanol before use. The rest reagents were ready to use directly and can be stored in room temperature.

Setup Automated DNA Extractor

Isolation of DNA from plaque sample was done using fully automated QIAcube DNA extractor. This automated system can maximize at least 12 samples per run. All the prepared reagents and filter tips were placed onto the reagent and tips (200 µl and 1000 µl) column (Figure A).

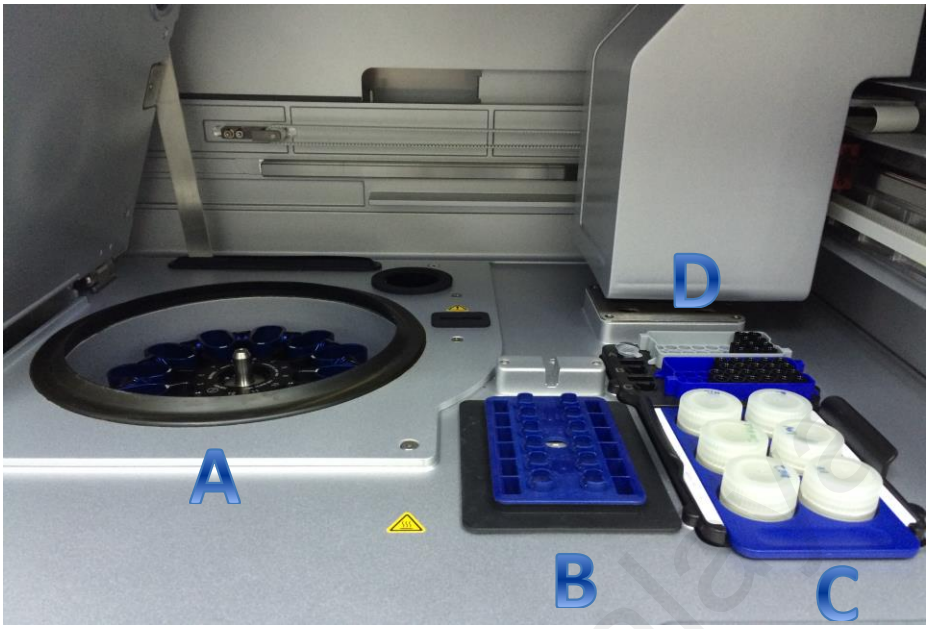

Figure A: Overview the component of QIAcube DNA extractor. Indicator (A) QIAcube centrifuge, (B) QIAcube shaker, (C) Reagent column and (D) Filter tips column

Plaque samples were thawed and vortexed shortly for 10 seconds to mix up the dense plaque. Plaque sample was taken out about 400 µl and transferred into RB tube. Further, plaque samples in RB tube were centrifuged for 5 minutes at 13, 000 rpm. Supernatant from each RB tube was discarded and the pellet was left intact at the bottom of tube. RB tubes containing pellet were loaded at QIAcube shaker (Figure A). Elution tubes used to collect the final extracted DNA were placed on rotor adapter holder together with DNeasy Mini Spin Columns in QIAcube centrifuge (Figure A). The arrangement RB tubes loaded in QIAcube shaker and elution tube in QIAcube centrifuge is shown in Figure B. The process of DNA extraction using QIAcube DNA extractor would take about 70 minutes to be completed for all the 12 samples per run.

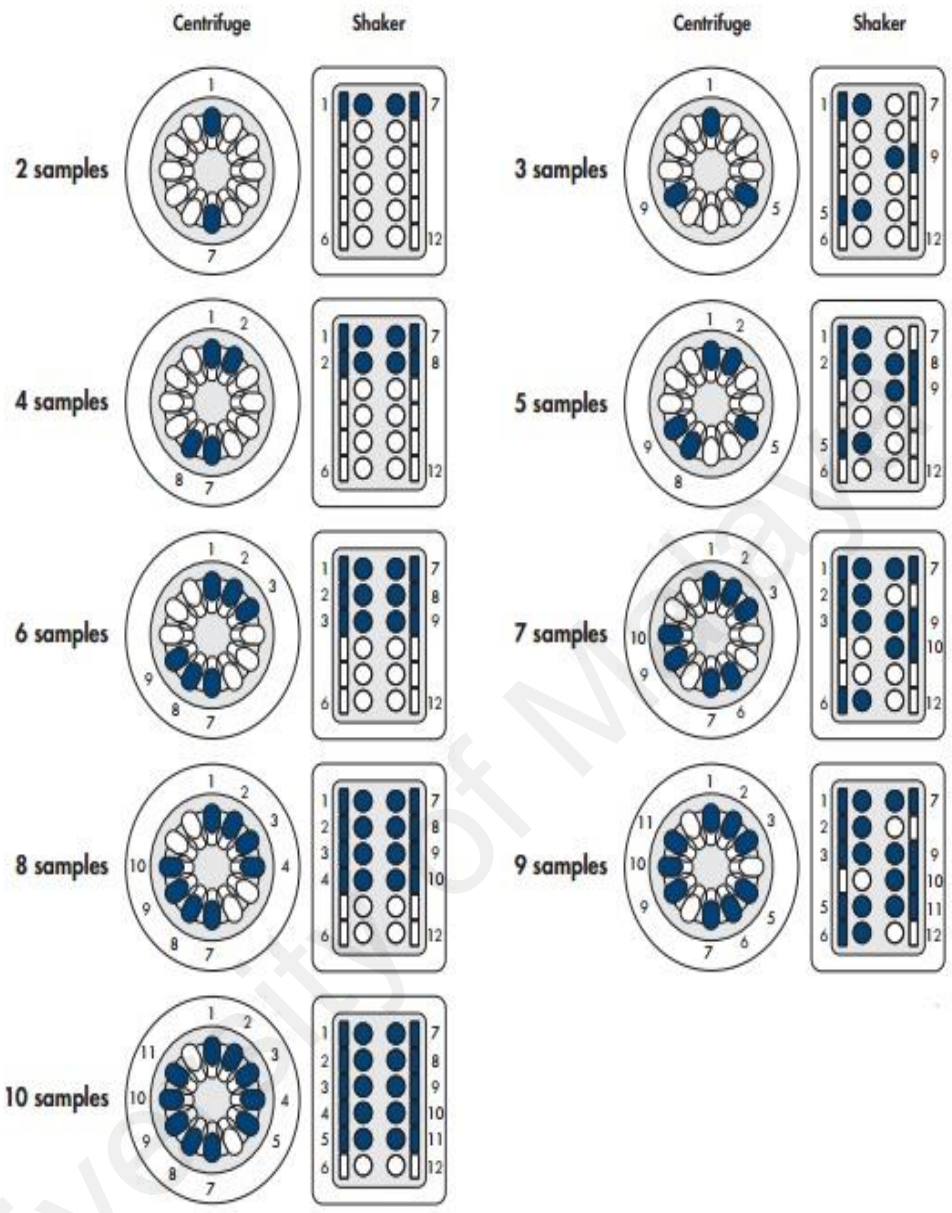

Figure B: The arrangement of samples in QIAcube centrifuge and QIAcube shaker

APPENDIX III

Appendix III: qPCR Operation

Setup qPCR

All DNA extraction sample will undergo qPCR using 7500 Fast RT–PCR System, Applied Biosystems®). This instrument came with installation software which able to measure quantitatively the concentration of DNA of specific target. The preparation plate setup in microplate had to be similar as plate setup in the system. Microplate fast optical (Figure C) consists of 96 well reactions and able to run 31 samples plus 1 negative control in a time which one sample may take 3 wells of reaction.

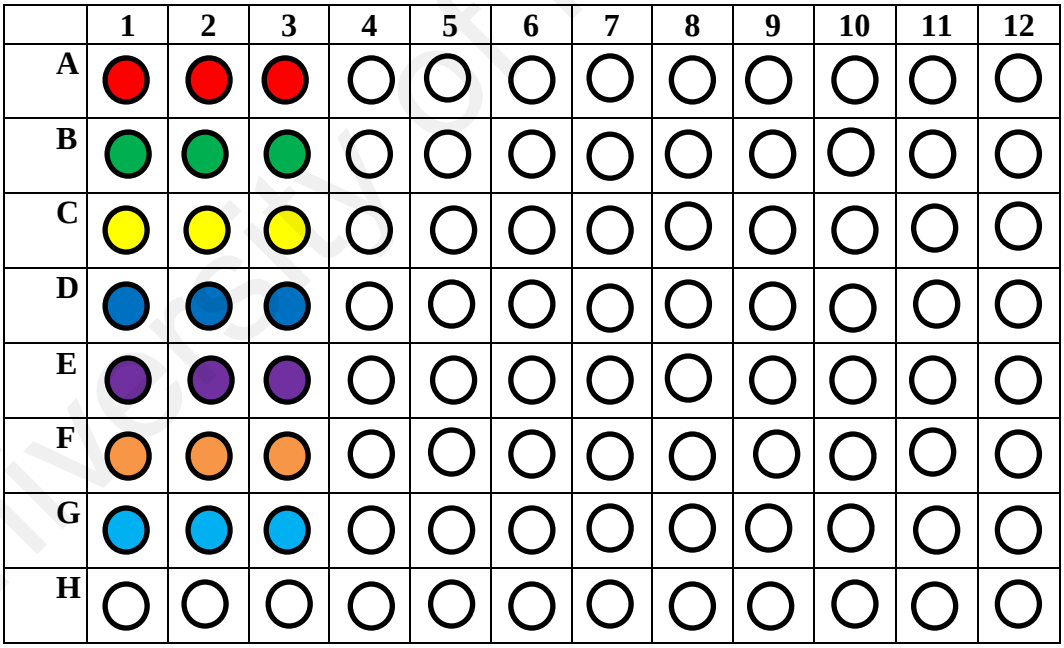

Figure C: Schematic diagram of microplate with 96 well. Same colour of well A1, A2 and A3 indicated replicates of the same samples and different samples occupied well B1–B3 till G1–G3 respectively. The last three well H1–H3 were reserved for negative control which consist of the same element as other wells without the addition of DNA.