Management of periodontitis in patients with type 2 diabetes – the clinical and biological response

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List of Abbreviations

A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
AGE	advanced glycation end product
ANCOVA	analysis of covariance
ANOVA	analysis of variance
BMI	body mass index
BOP	bleeding on probing
BP	blood pressure
BSA	bovine serum albumin
β-cell	beta cell
CAL	clinical attachment loss
CPITN	community periodontal index of treatment need
CRP	C-reactive protein
CVD	cardiovascular disease
DNA	deoxyribonucleic acid
GCF	gingival crevicular fluid
GI	gingival index
ELISA	enzyme-linked immunosorbent assay
FFA	free fatty acid
FMI	full mouth instrumentation
HbA1c	percentage glycated haemoglobin
HDL	high density lipoproteins
hsCRP	high sensitive C-reactive protein
IDF	International Diabetes Federation
IL-1β	interleukin 1 beta
IL-6	interleukin 6
IMD	index of multiple deprivation
IQR	inter-quartile range
LDL	low density lipoproteins
LLOD	lower limit of detection
LOA	loss of attachment
LPS	lipopolysaccharide
MAMP	microbe-associated molecular pattern

mGI	modified gingival index
MMP	matrix metalloproteinases
mRNA	messenger ribonucleic acid
n	number
non-HDL	non high density lipoproteins
NS	not significant
NSM	non-surgical periodontal management
OHI	oral hygiene instruction
PAG	periodontal associated genotype
PBS	phosphate buffered saline
PD	probing depth
PESA	periodontal epithelial surface area
PI	plaque index
PISA	periodontal inflamed surface area
P.gingivalis	*Porphyromonas gingivalis*
PMN	polymorphonuclear leukocytes
T.forsythia	*Tannerella forsythia*
F. nucleatum	*Fusobacterium nucleatum*
RAGE	advanced glycation endproducts receptor
RCT	randomised controlled trial
RIA	radioimmunoassay
ROS	reactive oxygen species
SD	standard deviation
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TLR	toll-like receptor
TNF-α	tumour necrosis factor alpha
WHO	World Health Organisation

Abstract

Type 2 diabetes (T2DM) is a risk factor for periodontal disease, however the pathogenic links between the two disease are not completely understood. Both diseases are considered to be inflammatory conditions and, therefore, cytokines are likely to play a role in the shared susceptibility between the two diseases. Therefore, the study evaluated, longitudinally over 12 months, the impact of periodontal therapy on clinical outcomes, glycaemic control, hsCRP, lipids and local and systemic levels of IL-6, TNF-α, IL-1β and IFN-γ in patients with T2DM.

101 T2DM and 83 non-diabetic subjects were recruited and, of these, periodontitis was diagnosed in 47 T2DM and 48 non-diabetic subjects. Pre-treatment, subjects with T2DM had significantly higher BMI and significantly lower systolic BP and cholesterol compared to non-diabetic subjects. Serum levels of TNF-α, IL-1β and IFN-γ were significantly higher in subjects with T2DM compared to non-diabetic subjects. Regardless of diabetic status, GCF and salivary levels of IL-1β were significant predictors of the clinical periodontal condition.

In T2DM and non-diabetic subjects, all clinical periodontal outcomes were significantly improved at 3, 6 and 12 months following NSM and both groups demonstrated significant reductions in GCF IL-1β levels at 3, 6 and 12 months. In non-diabetic subjects, a significant reduction in non-HDL and cholesterol was seen 6 months after NSM. In subjects with T2DM, serum TNF-α was significantly reduced 3 and 6 months after NSM. In subjects with T2DM, HbA1c showed 0.45% and 0.40% reductions at 3 and 12 months after NSM, although these reductions did not achieve statistical significance.

In conclusion, periodontal therapy led to significant reductions in GCF IL-1β in all subjects, and also produced a significant reduction in circulating levels of TNF-α in subjects with T2DM. Furthermore, IL-1β in saliva and GCF appear to be good prognostic markers for periodontitis regardless of diabetes status.

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Chapter 1 Introduction

1.1 Periodontal disease

1.1.1 Definition and classification of periodontal disease

Periodontal disease is a bacterially-induced chronic inflammatory disorder affecting the supporting structures of the teeth. Accumulation of bacterial plaque and the development of a dysregulated, destructive host immune-inflammatory response leads to pocket formation, loss of connective tissue attachment, alveolar bone destruction and tooth loss.

A classification for periodontal disease is necessary in order to provide a framework for both the effective clinical management of patients and the implementation of robust research methodologies. The provision of a clinical diagnosis, based on a widely accepted classification system, guides the allocation of treatment. In this way, a classification provides the basis for the differentiation of clinical treatment, influencing the way in which patients are managed. Similarly, a classification system used for research, allocates subjects into groups based on the presence or absence of disease. This also affects the clinical management of the patient but importantly may also influence the conclusions drawn from research. In this way, the dogma relating to the aetiology, pathogenesis and management of periodontal disease is intimately influenced by the classification of periodontal disease within research studies.

In line with developments and changes to the understanding of the aetiology and pathogenesis of periodontal disease, a classification system for periodontal disease must be revised and updated. Published classifications will at best represent a consensus view

of experts within the field of periodontology. Currently, well recognised criteria are available to assist in the clinical management of patients (Armitage, 1999).

The current clinical classification system was formulated and published over a decade ago following the International Workshop for a Classification of Periodontal Disease and Conditions (Armitage, 1999). This extensive and detailed classification system, although cumbersome, provides clinicians with a way to begin to organise the healthcare needs of patients (Table 1.1).

With regards to the classification of periodontal disease for research there is, however, a lack of consistency within the literature of what constitutes periodontal disease. Some studies have very low thresholds for defining the presence of periodontitis whereas others have much higher thresholds. The recognition of the need for a clear definition of what constitutes a periodontal case for research purposes is not new. Indeed, two separate criteria for periodontal case definition have been proposed by two different working groups (Table 1.2), firstly, the 5th European Workshop in Periodontology (Tonetti and Claffey, 2005) and secondly the American Academy of Periodontology (Page and Eke, 2007). However, inconsistent use ensures this continues to be a contentious and currently unresolved issue (Meisel and Kocher, 2009; Preshaw, 2009).

Table 1.1 Overview of the classification of periodontal diseases

I. Gingival Diseases
   A. Dental plaque-induced gingival diseases
   B. Non-plaque-induced gingival lesions
II. Chronic Periodontitis
III. Aggressive Periodontitis
IV. Periodontitis as a Manifestation of Systemic Diseases
V. Necrotising Periodontal Diseases
VI. Abscesses of the Periodontium
VII. Periodontitis Associated with Endodontic Lesions
VIII. Developmental or Acquired Deformities & Conditions

The classification of periodontal disease and conditions provides a framework for management of patients and case definition for research purposes. The table shows a classification of periodontal disease that is modified from the International Workshop for a Classification of Periodontal Disease and Conditions (Armitage, 1999).

Table 1.2 Case definition of periodontal disease in research

	5th European Workshop (Tonetti and Claffey, 2005)	**American Academy** (Page and Eke, 2007)
Incipient / moderate periodontitis	Presence of proximal attachment loss of ≥ 3mm in ≥2 non-adjacent teeth.	Presence of ≥ 2 interproximal sites with attachment loss of ≥4mm (not on same tooth) **or** ≥ 2 interproximal sites with probing depths ≥ 5mm (not on same tooth).
Substantial / severe periodontitis	Presence of proximal attachment loss of ≥ 5mm in ≥30% of teeth present.	Presence of ≥ 2 interproximal sites with attachment loss of ≥6mm (not on same tooth) **and** ≥ 1 interproximal site with probing depths ≥ 5mm

Currently within the published literature a lack of consistency exists with regards to the definition of what constitutes a periodontal case. The table shows two different case definitions of periodontal disease that have been proposed for use in research (Tonetti and Claffey, 2005; Page and Eke, 2007).

Although, both these case definitions use loss of attachment (LOA) at interproximal sites of non-adjacent teeth as the main measurement, there is recognition that periodontal disease cannot be detected by measurement of a single variable. LOA, whilst a measure of cumulative lifetime experience of periodontitis, provides little information of the current condition of the periodontal tissue attachment loss and therefore, must be supplemented by additional measurements of bleeding on probing and probing depth. In addition, both criteria recognise the need to take into account the potential error inherent in measuring attachment loss, to avoid including cases without periodontitis. Thus, the threshold for interproximal LOA was set at ≥ 6 mm (Page and Eke, 2007) or ≥ 5 mm (Tonetti and Claffey, 2005).

Given that the American Academy of Periodontology's definition of 'severe periodontitis' only requires a minimum of two teeth with 6mm attachment loss and one tooth with a probing depth of 5 mm (Page and Eke, 2007), it would seem possible that based on this definition, a subject could be included into a study with only minimal levels of disease or attachment loss caused by overhanging restorations or the distal aspect of second molars where a third molar has been extracted. Obviously, having a high threshold of inclusion into a study must be weighed against an ethical issue of missing periodontal cases, however, if the main aim of a periodontal case definition is to provide a robust basis for research, there is a clear need to ensure that the cases that are included within studies have suitable levels of disease to ensure valid conclusions can be made. With regards to identifying subjects with substantial extent and severity of periodontal disease, it would appear that the 5th European Workshop in Periodontology (Tonetti and Claffey, 2005) provides more robust inclusion criteria, requiring subjects to have interproximal attachment loss of ≥ 5 mm in ≥ 30% of teeth present.

To supplement conventional classification of periodontal disease, studies investigating the links between periodontitis and systemic disease have also attempted to quantify the surface area of inflamed periodontal tissue using clinical attachment level, recession and bleeding on probing (BOP) (Hujoel *et al.*, 2001), with previous studies using calculated values for periodontal epithelial surface area (PESA) and periodontal inflamed surface area (PISA) to investigate the condition of the periodontal tissues (Nesse *et al.*, 2008; Nesse *et al.*, 2009). In this way, quantifying periodontal disease on a continuous scale not only avoids the use of an arbitrary cut off point for periodontal case definition but also fits within the biological model that the greater the inflammatory burden from the periodontal tissues, the greater the contribution to systemic conditions beyond the oral cavity (D'Aiuto *et al.*, 2004; Nesse *et al.*, 2009).

In a recent study, the accuracy of periodontitis prevalence determined by partial mouth periodontal examinations compared to full mouth 'gold standard' periodontal examinations was evaluated. It was found that the partial examinations substantially under-estimated the prevalence of periodontal disease by at least 50%, leading to high levels of misclassification of periodontal cases (Eke et al., 2010). Furthermore, the impact of using different periodontal definitions was recently investigated within a study that reanalysed data from a cohort study of 1296 pregnant women (Manau et al., 2008). Within the original study, logistic regression analyses for variables influencing adverse pregnancy outcomes showed that after adjusting for confounding variables, a statistically significant association was found between preterm birth and the presence of periodontal disease (odds ratio 1.77; 95% confidence interval: 1.08–2.88) (Agueda et al., 2008). Within the reanalysis of data, 14 different periodontitis definitions were applied. Using the same analysis, only 5 of the 14 tested definitions of periodontitis resulted in a statistically significant association with premature birth (Manau et al.,

2008). This clearly shows that the variation in odds ratios can be attributed to the use of different definitions of what constitutes a periodontal case, thus, highlighting the importance of having a robust and widely accepted definition of periodontal disease for research. In addition to ensuring valid conclusions can be drawn from individual studies, this will also facilitate meta-analyses of studies, allowing more powerful conclusions to be drawn from a body of published research.

1.1.2 Epidemiology of periodontitis

Epidemiological studies over the past twenty years have attempted to provide information concerning the extent and severity to which periodontal disease affects the population. The most recent 2009 UK Adult Dental Health Survey (ADHS) reported that approximately 66% of the adults aged 55 and above have moderately advanced chronic periodontitis (with attachment loss ≥ 4 mm) and 25% have severe periodontitis (attachment loss ≥ 6 mm) (White et al., 2011). This highlights that periodontal disease remains common at a low level, with more severe disease concentrated in a relatively small proportion of the UK population (Steele and O' Sullivan, 2011). The same study found that visible plaque and calculus were present in 66% and 68% of adults, respectively (Chadwick *et al.*, 2011; Steele and O' Sullivan, 2011). Similar data have been reported in studies in different populations. For example, a study of 7,447 dentate individuals in the USA found that, whilst over 90% of people aged 13 or over had experienced some loss of attachment, only 15% exhibited more severe destruction (attachment loss ≥ 5 mm) (Brown et al., 1996). A study of Tanzanian adults found that, whilst there were abundant plaque and calculus deposits, with ubiquitous gingivitis, pockets > 3 mm and attachment loss > 6 mm occurred at less than 10% of tooth surfaces. In addition, 75% of the tooth sites with attachment loss ≥ 7 mm were found in 31% of subjects (Baelum et al., 1986).

Data from a large study of 9698 subjects in the USA, using pre-defined criteria for case definition, supports the view that although mild periodontitis is widespread, moderate to severe periodontitis affects a relatively small proportion of the population 3.1-9.5% (Albandar et al., 1999). Interestingly, however, a different study of 853 dentate individuals in Brazil employing full mouth probing assessments at six sites per tooth (Susin et al., 2004) reported a much higher prevalence of advanced periodontal disease, with 52% exhibiting severe destruction (attachment loss $\geq$ 7 mm). Also, a more recent epidemiological study indicates that since the 1980s, the prevalence of severe periodontitis in the USA may have decreased from 7.3% to 4.2% (Borrell et al., 2005). Despite this being lower than the prevalence estimates for advanced periodontal disease cited in the 1950s and 1960s (Marshall-Day et al., 1955), it still represents a significant number of adults who suffer from periodontal disease and who may ultimately experience tooth mobility and tooth loss.

Differences between prevalence rates for periodontal disease reported over the last 60 years may be related to methodological differences, for example, full mouth versus part mouth assessments and the use of various case definitions. There have also been clear improvements in periodontal health awareness and improved provision of dental care (Steele and O' Sullivan, 2011). In addition, within Western populations, the last 40 years has seen a decline in the percentage of people who smoke (Pierce, 1989; Molarius *et al.*, 2001). Given the importance of smoking as a risk factor for periodontal disease (Kinane and Chestnutt, 2000) with 50% of periodontal disease being attributed to smoking (Tomar and Asma, 2000), it is likely that a reduction in the number of smokers will have contributed to decreasing prevalence rates of periodontitis.

Data gathered from large cross-sectional studies have shaped our understanding of the prevalence of periodontal disease in the population. Notwithstanding the impact

of methodology and disparities in the definition of periodontitis, it would appear that whilst gingivitis and mild-moderate periodontitis are relatively common, severe periodontitis is less prevalent, usually not exceeding 10% of the population, despite plaque being a common finding in the majority of people.

1.1.3 Pathogenic mechanisms in periodontitis

Within periodontitis, the microbial dental plaque biofilm initiates a host inflammatory immune reaction within the periodontal tissues. The inflammatory reaction is not only visible clinically in the affected periodontium but the histopathological features have also been well described (Page and Schroeder, 1976). Within the periodontium, the inflammatory and immune processes primarily function to protect the tissues against microbes and their products, which persist on the tooth surface within a complex biofilm community. The host defence reactions, however, also cause damage within the surrounding tissue, contributing to the tissue destruction observed in periodontitis.

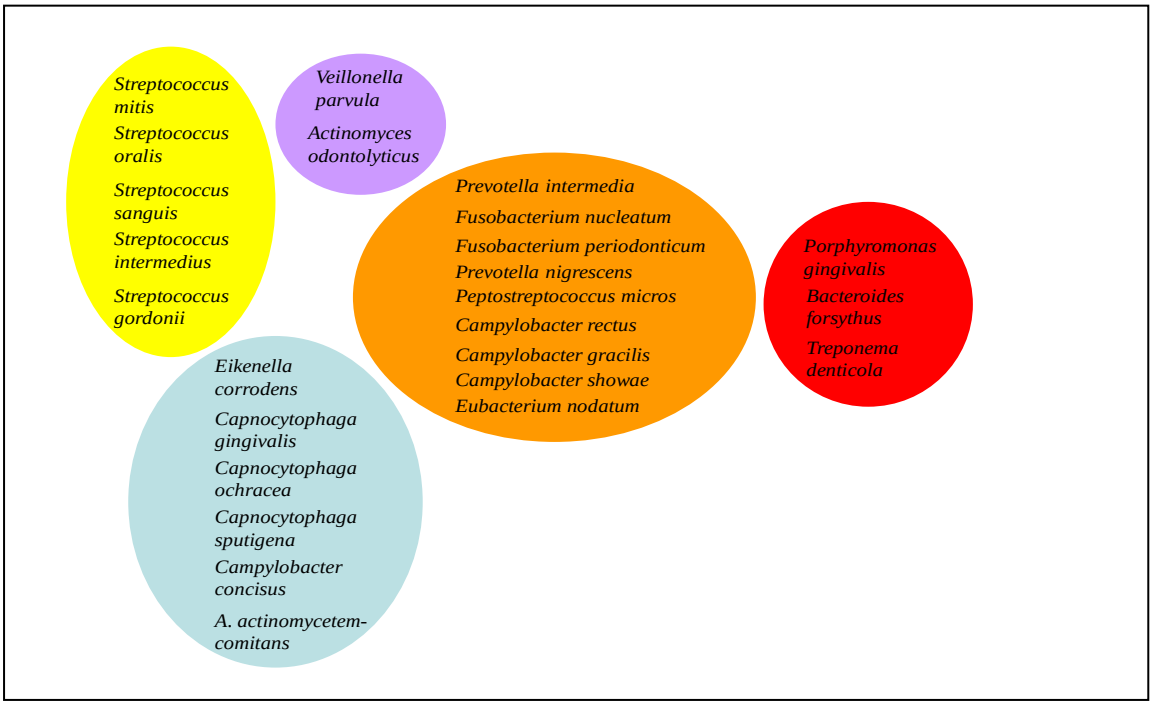
1.1.3.1 Initiating role of dental plaque biofilm

Dental plaque is a microbial biofilm. Biofilms are defined as “matrix-enclosed bacterial populations adherent to each other and/or to surfaces” (Costerton *et al.*, 1994; Socransky and Haffajee, 2002). Oral bacteria bind to a pellicle formed from saliva and gingival crevicular fluid and to one another in a highly specific succession of species. In periodontally healthy sites, the dental plaque biofilm consists mostly of Gram-positive bacterial species, with approximately 15% Gram-negative species found and, in contrast, periodontally diseased sites show an increase in the proportion of Gram-negative species to up to 50% (Tanner et al., 1996). Accompanying this compositional microbial transition from health to disease is an increase in total bacterial numbers from

approximately 10^2-10^3 bacteria during health, 10^4-10^6 during gingivitis and escalating as high as 10^5-10^8 during periodontitis (Tanner et al., 1996).

It is widely accepted that bacteria in the dental plaque biofilm initiate the inflammatory immune response seen within the periodontal tissues. However, not all dental biofilms result in periodontal destruction and to highlight this a consensus report of the 1996 World Workshop on Clinical Periodontics concluded that *Porphyromonas gingivalis (P.gingivalis)*, *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans)* (previously known as *Actinobacillus actinomycetemcomitans*) and *Tannerella forsythia (T.forsythia) (previously known as Bacteroides forsythus*) should be considered as major periodontal pathogens (Zambon, 1996) with the subsequent recognition that *Fusobacterium nucleatum (F. nucleatum)* is also part of this group (Teles et al., 2006). Indeed, molecular techniques and cluster analysis of subgingival plaque have demonstrated that certain species frequently occur together in 'complexes' (Figure 1.1) and confirmed a strong association between *P.gingivalis* (described as a red complex microorganism) and deeper pocket depths and increased levels of bleeding on probing in periodontal disease (Socransky et al., 1998). In a further study, plaque samples from patients with periodontitis tend to contain an increased proportion of red and orange complex species of bacteria compared to plaque from periodontally healthy patients (Ximenez-Fyvie et al., 2000). In addition, the relationship between orange and red complex species clinical measures of periodontitis is also mirrored for supragingival plaque samples (Haffajee et al., 2008). However, these named periodontal pathogens have also been detected in periodontally healthy subjects (Loomer, 2004; Sanz and Quirynen, 2005), highlighting the complex interplay between the bacterial challenge and the host response that is involved in periodontal disease.

Figure 1.1 Overview of microbial Complexes in subgingival plaque



Certain microbial species in subgingival plaque have been found to frequently occur together in ‘complexes’ The figure shows a diagrammatical representation of these ‘complexes’ (Socransky et al., 1998).

The dental plaque biofilm provides a diverse source of antigens, including lipopolysaccharides (LPS), leukotoxin, lipoteichoic acid and peptidoglycan, fimbriae and extracellular enzymes (Travis *et al.*, 1997; Fives-Taylor *et al.*, 1999). This bacterial challenge causes direct damage and stimulates the immune-inflammatory response within the periodontal tissues. For example, cysteine proteinases (gingipains) produced by *P.gingivalis* contribute to tissue destruction and facilitate the invasion of bacteria into the host tissues (Genco *et al.*, 1999; Imamura, 2003; Andrian *et al.*, 2004), additionally, LPS of gram-negative bacteria, such as *P.gingivalis*, stimulates the host response via specific host receptors (Dixon et al., 2004).

Clearly, studies over the past 20 years have confirmed the initiating role of bacteria in the pathogenesis of periodontal disease, identifying a limited number of specific bacterial species that have been associated with severe disease (Tanner *et al.*, 1996; Socransky *et al.*, 1998). However, differences in disease experience between individuals are not always matched with microbiological factors and individuals may harbour organisms without showing progressive periodontal destruction (Cullinan et al., 2003). Therefore, although periodontitis appears to be related to the presence of certain pathogenic species in the subgingival microflora (Socransky *et al.*, 1998; Haffajee *et al.*, 2008) the presence of a pathogenic microflora alone is insufficient to cause periodontal disease. The bacteria-host interaction and the nature of the subsequent host immune-inflammatory response play a critical role in the development and progression of periodontal disease.

1.1.3.2 Host response

The histological characteristics of the inflammation that develops in the periodontal tissues following the formation of the dental plaque biofilm has been well defined (Page and Schroeder, 1976). Blood vessels within the periodontal tissues dilate,

become more permeable, fluid and migrating defence cells accumulate within the tissues at the site of infection. In an attempt to remove the bacterial challenge, large numbers of neutrophils and later lymphocytes accumulate in the periodontal tissues and migrate through the junctional epithelium into the periodontal pocket (Page and Schroeder, 1976). The persistence of the dental plaque biofilm, however, leads to a continuing cycle of microbial challenge and host inflammatory immune responses. Therefore, in addition to the tissue damage caused directly by bacteria, resident tissue cells and infiltrating host defence cells contribute to connective tissue breakdown and alveolar bone loss (Bartold and Narayanan, 2006).

In vitro experiments indicate that host cells respond to bacteria by activating intra-cellular signalling pathways leading to cytokine secretion (Handfield et al., 2008). The activation of the host response to the dental plaque biofilm is dependent on the host cells' ability to recognise the presence of bacteria and their products. A diverse collection of specific host receptors enables host cells within the periodontal tissues to recognise microbe-associated molecular patterns (MAMPs). Consequently, the host is able to orchestrate an immune-inflammatory response that reflects the bacterial challenge (Dixon and Darveau, 2005). Examples of MAMPs, important in periodontal disease pathogenesis, include bacterial LPS and fimbriae which are recognised via receptors such as soluble LPS binding protein, membrane-associated CD14 and Toll-like receptors (TLRs).

In vitro experiments have shown that whole periodontal bacteria stimulate the secretion of a range of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-8 and IL-12 (Sandros *et al.*, 2000; Kusumoto *et al.*, 2004). Furthermore, activation of TLRs by periodontal pathogens has been shown to induce the release of a similar range of inflammatory cytokines and antimicrobial proteins from host cells (Jotwani *et al.*, 2003;

Dixon *et al.*, 2004; Eskan *et al.*, 2007; Eskan *et al.*, 2008), highlighting the important role of host cell receptors in the inflammatory immune response within periodontal disease. Therefore, the activation of specific receptors by bacterial MAMPs allows the periodontal tissues to direct an inflammatory-immune response that is appropriate to the bacterial challenge present within the dental plaque biofilm. It is however paradoxical that these defensive processes result in the majority of tissue damage leading to the clinical manifestations of periodontal disease.

Neutrophils [polymorphonuclear leukocytes (PMNs)] are critical components of the innate immune system and are essential to maintain periodontal health in the face of a constant bacterial challenge from the dental plaque biofilm. PMNs serve a protective function through their ability to phagocytose and kill microorganisms. The vital role of PMNs in innate immunity is highlighted by congenital defects such as Chediak-Higashi syndrome and leukocyte adhesion deficiency syndrome, in which genetic abnormalities alter PMN functional responses, leading to recurrent microbial infection and also severe periodontal disease (Lekstrom-Himes and Gallin, 2000). However, along with protecting the periodontium from microbial invasion, PMNs release potent lysosomal enzymes, cytokines and reactive oxygen species (ROS) that can be destructive to the periodontal tissues (Van Dyke and Vaikuntam, 1994; Johnstone *et al.*, 2007). Furthermore, it has been suggested that contributing to the destructive process in periodontitis is neutrophil hyperactivity leading to overproduction and release of antimicrobial and potentially tissue-damaging reactive oxygen species (ROS) (Fredriksson et al., 2003). In the absence of exogenous stimulation, peripheral blood neutrophils from persons with chronic periodontitis demonstrate an increase in extracellular ROS release in vitro (Matthews et al., 2007).

The persistent nature of the dental plaque biofilm results in the activation of the adaptive immune response, leading to the recruitment T and B cells into the periodontal tissue (Page and Schroeder, 1976). An appropriate adaptive immune response to the continued microbial challenge relies on a balanced production, by the host tissues, of different subsets of T cells. The production of Th1 cells leads to cell mediated immune response, with the activation of macrophages and the induction of B cells to produce opsonising antibodies, which facilitates bacterial killing. On the other hand, the production of predominately Th2 cells provides humoural immunity, with activation of B cells to produce neutralising antibodies.

Th1 and Th2 cells release different but overlapping sets of cytokines, however, despite extensive research, the contribution of different Th1 and Th2 cells populations to periodontal destruction has yet to be clearly defined. Some studies support the hypothesis that Th1 cells are associated with stable periodontal sites and Th2 cells are associated with disease progression (Gemmell and Seymour, 1994; Bartova *et al.*, 2000). However, other studies have reported a predominance of Th1 responses or reduced Th2 responses in periodontal disease (Salvi *et al.*, 1998; Takeichi *et al.*, 2000). In addition, there are studies demonstrating the involvement of both Th1 and Th2 cells in periodontal disease (Gemmell *et al.*, 1999; Berglundh *et al.*, 2002). Despite a lack of consensus about the role of different T cell populations in periodontal tissue destruction it remains clear that the balance of cytokines, produced by the innate and adaptive immune responses, within inflamed periodontal tissues is a contributing factor in whether the disease remains stable or leads to progression and tissue destruction (Okada and Murakami, 1998).

1.1.3.3 Cytokines

Cytokines are central to the pathogenesis of many chronic inflammatory diseases, including periodontal disease (Seymour and Taylor, 2004). Within the periodontal tissue, cytokines are produced not only by infiltrating host defence cells, such as lymphocytes, macrophages and neutrophils but also by resident periodontal tissue cells, such as epithelial cells and fibroblasts (Takashiba et al., 2003). Many cytokines are self-regulatory, able to induce their own expression in an autocrine or paracrine manner and have pleiotrophic actions on a number of cell types. Cytokines act on their target cells by binding to specific receptors and initiating intracellular messengers resulting in phenotypic changes in the cell via altered gene regulation (Birkedal-Hansen, 1993; Taylor *et al.*, 2004).

In addition to their involvement in the host response against the microbial challenge of the dental plaque biofilm, cytokines are also able to mediate connective tissue and alveolar bone destruction through the induction of fibroblasts and osteoclasts to secrete tissue degrading enzymes (Bartold and Narayanan, 2006). Therefore, although primarily involved in protection, it can be seen that cytokines, for example interleukin-1 beta (IL-1β), tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), have biological activity that underpin tissue damage in chronic inflammation, including periodontitis (Okada and Murakami, 1998).

The key role that cytokines play in the host inflammatory-immune response in periodontal disease is supported by the analysis of human samples as well as studies in animals (Gemmell *et al.*, 1997; Landi *et al.*, 1997; Okada and Murakami, 1998). Despite the complex nature of the cytokine response that is involved in both the maintenance of periodontal health and periodontal tissue destruction, it is possible to identify cytokines that appear fundamental to this host response. For example, pro-inflammatory

cytokines, such as IL-1β and TNF-α have an important role in the initiation, regulation and perpetuation of innate immune responses (Birkedal-Hansen, 1993; Alexander and Damoulis, 1994). Early studies of human periodontal tissue biopsy samples highlight the importance of both IL-1β and TNF-α (Honig *et al.*, 1989; Stashenko *et al.*, 1991b), with higher levels of IL-1β and TNF-α in periodontal disease sites compared to healthy sites (Stashenko et al., 1991b) and higher IL-1β levels in samples from sites undergoing active attachment loss in comparison to stable disease sites or healthy sites (Stashenko et al., 1991a). Furthermore, studies have found increased levels of IL-1β in gingival crevicular fluid (GCF) in samples from subjects with periodontitis (Preiss and Meyle, 1994; Figueredo *et al.*, 1999; Engebretson *et al.*, 2002; Zhong *et al.*, 2007) and following non-surgical periodontal therapy, improvements in periodontal health are accompanied by statistically significant reductions in GCF levels of IL-1β (Engebretson *et al.*, 2002; Thunell *et al.*, 2010).

As a pro-inflammatory cytokine capable of stimulating bone resorption, interleukin-6 (IL-6) may also play an important role in periodontal pathogenesis. In vitro, IL-6 stimulates bone resorption (Palmqvist et al., 2002) and concentrations of IL-6 appear increased in inflamed periodontal tissues (Irwin and Myrillas, 1998). Studies have also demonstrated elevated IL-6 levels in GCF from diseased sites compared with control samples (Mogi *et al.*, 1999; Lin *et al.*, 2005). Furthermore, significantly higher levels of IL-6 were found systemically in patients with periodontitis compared to periodontally healthy controls (Marcaccini *et al.*, 2009; Sun *et al.*, 2009) and circulating levels of IL-6 were shown to decrease 3 months after non-surgical periodontal therapy (Marcaccini et al., 2009). The interplay between IL-6, IL-1β and TNF-α is highlighted by a cell culture study of primary gingival fibroblasts that demonstrated a constitutive mRNA expression of IL-6 and dose-dependent up-regulation of IL-6 mRNA and IL-6

protein levels following stimulation by IL-1β and TNF-α (Palmqvist et al., 2008). Conversely, IL-6 has also been shown to induce IL-1 receptor antagonist (Tilg et al., 1994), thereby potentially providing a degree of control over upregulated inflammatory responses. This highlights the increasing appreciation that periodontal disease progression depends on the balance between pro-inflammatory and anti-inflammatory responses maintained by a network of cytokines (Graves and Cochran, 2003).

Cell culture studies have been used to investigate the production of cytokines by different cell types and investigate the molecular cell responses important to cytokine secretion. Studies have demonstrated that following stimulation with periodontal pathogens or their components a range of cytokines, including IL-1β, TNF-α, IL-8 and IL-6, are secreted by primary human gingival epithelial cells (Eskan *et al.*, 2008; Stathopoulou *et al.*, 2009). This is also highlighted by stimulation studies using epithelial cell lines (Bodet *et al.*, 2006; Gursoy *et al.*, 2008). However, given the non-oral origin of the cell lines, caution must be used when drawing conclusions from such studies.

The functioning of the adaptive immune response through the production of T cell populations is controlled by the cytokines that are present within the periodontal tissues (Gemmell et al., 1997). For example, interleukin-2 (IL-2) is essential for T cell proliferation and differentiation and activated T cells secrete and respond to IL-2 in an autocrine fashion. Early studies have demonstrated a lack of IL-2 production and reduced IL-2 receptor (IL-2R) expression within T cells from patients with chronic periodontitis, providing a possible explanation for the dysregulated T cell proliferation observed in these studies (Seymour *et al.*, 1985; Kimura *et al.*, 1991). Furthermore, IL-1β and TNF-α, cytokines primarily involved in inflammation also have the ability to act on T cells as co-activators of IL-2 production (Dinarello, 2007).

Dysregulated cytokine networks may result in a variety of biological effects including up-regulation of adhesion molecules on leukocytes and endothelial cells, local production of chemokines, osteoclastic bone resorption and increased production and release of prostaglandins and matrix metalloproteinases (MMPs). Indeed, IL-1β, TNF-α, IL-6 have the potential to play a role in the propagation of a destructive inflammatory response, bone resorption and connective tissue destruction (Graves and Cochran, 2003). Furthermore, multiple feedback loops develop; for example, cytokines induce the production of prostaglandins, and increased prostaglandin concentration results in increased cytokine secretion (Noguchi et al., 2001).

In addition to the local immune inflammatory response within the periodontal tissues there is evidence that periodontal inflammation is associated with a heightened systemic inflammatory state. A meta-analysis of 10 cross-sectional studies (Paraskevas et al., 2008) provides evidence that C-reactive protein (CRP) is consistently elevated in patients with periodontitis compared to healthy controls. A recent cohort study, following patients over a 10 year period, confirms an association between advanced periodontitis and elevated CRP levels (Linden et al., 2008).

Periodontal research has begun to elucidate the mediators that are important within periodontal tissues during health and disease. However, there are limitations inherent within the sampling and analytical techniques of currently published studies. Due to the small fluid volume available when assessing GCF, the use of conventional enzyme-linked immunosorbent assay (ELISA) limits the number of cytokines that can be analysed. Furthermore, a lack of sensitivity, particularly at the lower detection level, is problematic for the accurate quantification of cytokines that may play a role within periodontal disease pathogenesis. The recognition that individual cytokines do not act in isolation but rather as a complex network (Taylor *et al.*, 2004; Preshaw and Taylor,

2011) highlights a requirement for simultaneous analysis of a range of cytokines important within periodontal disease.

1.1.4 Quantification of cytokines

Sensitive methods for the precise quantification of cytokines are necessary when assessing cytokine levels in clinical samples, particularly when cytokine levels may be low and, as in the case of gingival crevicular fluid (GCF), sample volumes are very small. Various techniques for detecting and quantifying cytokines in biological samples have been used including bioassay, radioimmunoassay (RIA), ELISA, and more recent multiplex assays for the simultaneous quantification of multiple cytokines.

Bioassays have been used to monitor the effects of cytokines on biological systems *in vitro*, for example the impact of adding specific cytokines on responses of cells in culture. For example, primary human gingival fibroblasts, cultured with various concentrations of IL-1β or TNF-α, demonstrated that the production of both IL-6 messenger ribonucleic acid (mRNA) and IL-6 protein were concentration-dependently stimulated by IL-1β and TNF-α (Palmqvist et al., 2008). Furthermore, the impact of IL-6 on the osteoblastic differentiation of primary human periodontal ligament cells in culture was assessed by quantifying alkaline phosphatase staining histochemically (Iwasaki et al., 2008). The advantage of bioassay is that a biological response is measured to confirm the potential biological relevance of a given level of a cytokine. However, biological samples potentially contain many cytokines and contamination by other more active substances will influence results. This technique is not adequately sensitive for cytokine quantification as part of a clinical periodontal treatment study.

RIAs are based on the principle of competition between an antigen (in the clinical sample) and a radio-labelled homologous antigen for a limited number of specific antibody binding sites. Subsequently, a liquid scintillation counter quantifies

the amount of radio-labelled homologous antigen. This is inversely proportional to the mediator concentration in the sample, which is calculated from a standard curve generated from known amounts of mediator. RIAs have been developed for various mediators. For example, using RIA it was demonstrated that patients with periodontitis had significantly higher mean GCF concentrations of prostaglandin-E_2 than patients with gingivitis (Offenbacher et al., 1981). Despite the sensitivity of this assay, the impractical length of RIA and the availability of more rapid assays preclude the use of RIA for cytokine quantification in clinical studies.

ELISAs are non-competitive immunoassays based on the capture of test antigen (or standards of known quantity) by antibody coated onto the wells of microtitre plates. After a washing step to remove any free antigen, a second antibody is added and this binds to the antigen already present on the plate. The plate is then washed to remove unbound antibody and then, a ligand is added. The ligand is a molecule which binds to the antibody bound onto the plate, and itself is covalently coupled to an enzyme such as peroxidase. Free ligand is washed away, then bound ligand is visualised by the addition of a chromogen, a colourless substrate which is acted on by the enzyme portion of the ligand to produce a coloured end product. The colour intensity in the reaction wells is determined by optical density scanning of the plate, and the quantity of the test antigen is determined by comparison with a standard curve. ELISA have been used to quantify GCF levels of cytokines in patients with periodontitis (Zhong et al., 2007) and to assess changes in GCF cytokine levels following periodontal management (Engebretson et al., 2002). However, the GCF volumes obtained from patients are very small and low cytokine levels preclude the use of dilutions. Therefore, ELISA can only be used to quantify a single cytokine per GCF sample. This is an important limitation given the

increasing recognition of the importance of cytokine networks in periodontal disease pathogenesis (Preshaw and Taylor, 2011).

More recently, high-throughput multiplex immunoassays have been developed allowing simultaneous quantification of multiple cytokines. Using ELISA technology, two basic assay formats have been developed for simultaneous quantification of multiple cytokines; planar array assays and micro-bead assays. In planar assays, different capture antibodies are spotted at defined positions on a 2-dimensional array, such as a pre-coated microtitre plate. In the micro-bead assays the capture antibodies are conjugated to different populations of micro-beads, which can be distinguished by the fluorescence intensity in a flow cytometer. Both formats use a standard curve of known concentrations of cytokines to quantify unknown cytokine levels. Based on an assessment of the quality of the calibration curves for 5 common cytokines a comparison of 5 multiplex immunoassay platforms, concluded that the MULTI-ARRAY (Meso Scale Discovery) and the Luminex-based Bio-Plex (Bio-Rad Laboratories) platforms were the most suitable for cytokine analysis or quantification (Fu *et al.*, 2010). Using a micro-bead assay, multiple cytokines were quantified to assess changes in serum levels in patients with diabetes following periodontal therapy (O'Connell et al., 2008). Furthermore, also using micro-bead assays to quantify 16 cytokines in GCF samples, a small study of 6 patients demonstrated a significant post treatment reduction in 13 cytokines in GCF samples taken from diseased sites (Thunell et al., 2010). The high cost of these multiplex assays could, however, limit their use in large clinical studies.

1.1.5 Factors influencing the susceptibility to periodontitis

Key to the development of periodontal disease is a chain of events involving microbial challenge from the plaque biofilm, stimulation of the host inflammatory

immune responses and destruction of connective tissue and alveolar bone breakdown within the periodontium leading to the clinical signs of disease. All stages within this chain of events are open to the influence of other factors including genetic, acquired or environmental factors (Page and Kornman, 1997). Such factors influence disease phenotype and are specific to individual patients. The relative influence of different factors is not yet fully understood and whilst the role of tobacco smoking is well defined, other factors such as genetic susceptibility remain to be fully elucidated. In addition, the systemic health of patients appears to influence the development of periodontal disease with, for example, the presence of diabetes increasing the prevalence, incidence and severity of periodontitis (Taylor, 2001). However, the mechanistic links between periodontal disease and other systemic diseases are not fully defined. The chronic and multi-factorial nature of both periodontal disease and diabetes highlights the complex nature of the interplay between systemic health and periodontal disease.

Tobacco smoking is a recognized risk factor for periodontal disease (Tomar and Asma, 2000). Risk calculations suggest that 40 percent of chronic periodontitis may be attributed to smoking (Brothwell, 2001). Smokers are up to 4 times more likely to have periodontal disease compared with non-smokers (Tomar and Asma, 2000; Calsina *et al.*, 2002) and those with a longer smoking history have an increased risk of developing periodontal disease (Linden and Mullally, 1994; Hyman and Reid, 2003). In addition to the well recognised systemic health benefits of quitting smoking, research has shown improved treatment outcomes, with greater reductions in probing depths, in patients who were supported to quit smoking as part of periodontal management (Preshaw et al., 2005).

When considering the mechanisms by which smokers are at an increased risk for periodontitis, numerous authors have reported the potential effects of smoking on the bacterial challenge, the periodontal tissues and the immune-inflammatory response. For example, smoking has a long term chronic effect to impair gingival circulation (Bergstrom and Bostrom, 2001; Dietrich *et al.*, 2004). Smoking appears to have a deleterious impact on the dental plaque biofilm, increasing those bacteria commonly associated with periodontal disease (Zambon *et al.*, 1996; Umeda *et al.*, 1998; Eggert *et al.*, 2001; Haffajee and Socransky, 2001). Interestingly, a recent study demonstrated that following non-surgical management, the microbial profile in smokers remained similar to baseline whereas quitters demonstrated significantly different profiles (Fullmer et al., 2009). Tobacco smoking affects multiple functions of PMNs, including migration and chemotaxis (Seow et al., 1994) and an increase in PMN elastase suggests enhancement of degranulation in the neutrophils of smokers (Soder et al., 2002), with increased concentrations of macrophage-derived TNF-α from smokers suggesting a more destructive inflammatory process (Fredriksson et al., 2002). Studies, however, have also reported lower IL-1β (Rawlinson et al., 2003) and interleukin alpha (IL-α) (Petropoulos et al., 2004) levels in the GCF of smokers. Thus, the effect of smoking on host immune-inflammatory responses, as a mechanism for increased susceptibility to periodontitis, remains unclear.

A body of evidence now supports the theory that genetic factors play an important role in determining susceptibility to periodontitis. Periodontal disease is part of the phenotype of inherited conditions such as Papillon-Lefèvre syndrome (Firatli et al., 1996) and twin studies indicate that about 50% of the variance in attachment loss is due to the influence of heredity (Michalowicz et al., 2000). In order to study the precise genetic factors associated with susceptibility to periodontal disease, variations in

cytokine genes have been investigated. Based on the paradigm that cytokine gene polymorphisms could affect the transcription and subsequent release of cytokines, and because of the fundamental role of IL-1β in the pathogenesis of periodontal diseases, polymorphisms in the IL-1 gene have been the main focus of research (Taylor et al., 2004). Data from Korman et al that suggested that non-smokers with periodontitis who were positive for a combination of two defined IL-1 single nucleotide polymorphisms (SNPs), known as the periodontal associated genotype (PAG), had a 7-fold increased chance of having severe periodontal disease compared to those who where PAG negative (Kornman et al., 1997). Subsequent studies confirmed that PAG correlates with severity of periodontitis (Kornman *et al.*, 1999; Papapanou *et al.*, 2001) although such results are not replicated in other studies (Taylor et al., 2004). In a 5 year longitudinal study, Cullinan et al (2001) showed that this specific IL-1 genotype was a contributing but nonessential factor in the progression of periodontal disease. Equally, they showed smokers with *P. gingivalis* had significantly more periodontal disease compared with smokers without *P. gingivalis* and IL-1 genotype positive smokers had more disease than IL-1 genotype negative smokers (Cullinan et al., 2001). This study clearly demonstrates the interplay between bacteria, host and environmental factors in the pathogenesis of periodontal disease. Furthermore, although genetic associations between polymorphism in the IL-1 gene and periodontal disease appear to exist, unambiguous results are not yet apparent and firm conclusions about the genotype of patients with periodontitis cannot be made.

1.1.6 Management of periodontitis

The main aim of NSM, including instrumentation and effective oral hygiene instruction (OHI), is to prevent tooth loss via the effective and continued prevention of periodontal disease progression, achieved by the reduction of the microbial burden that

is present around the periodontal tissues. Effective periodontal therapy disrupts the subgingival plaque biofilm, allowing a shift in the microbial populations to those more commonly associated with health. For example, periodontal instrumentation resulted in a significant decrease in the deoxyribonucleic acid (DNA) probe counts of subgingival microbial species commonly associated with periodontal disease (Haffajee *et al.*, 1997; Cugini *et al.*, 2000). Given that the presence of calculus above or below the gingival margin impedes effective oral hygiene practices, the removal of calculus remains a primary aim for periodontal instrumentation. The concept of complete removal of all subgingival calculus is, however, viewed as unrealistic, with periodontal healing occurring despite the presence of residual calculus being detected microscopically (Nyman *et al.*, 1986; Cobb, 2002).

When considering the methodology used for the non-surgical approach to periodontal instrumentation, a recent meta-analysis concluded that, for both moderate pockets (5-7 mm) and deep pockets (≥ 7mm), no significant differences in probing depth reduction, clinical attachment gain and bleeding on probing were found when comparing traditional quadrant-based instrumentation with full mouth instrumentation (FMI) on a single visit (Farman and Joshi, 2008). Studies also highlight that the full mouth approach required significantly less instrumentation time to achieve similar results than quadrant therapy (Koshy *et al.*, 2005; Wennstrom *et al.*, 2005), although, a higher level of pain may be experienced following the full mouth approach (Apatzidou and Kinane, 2004; Wennstrom *et al.*, 2005). Furthermore, studies demonstrate that, in patients with chronic periodontitis, sonic or ultrasonic instrumentation achieves treatment outcomes that are comparable with hand instrumentation (Wennstrom *et al.*, 2005; Aslund *et al.*, 2008). Also, no significant difference was found in the incidence of recurrence of diseased periodontal pockets, defined as pockets with probing depths of

≥5mm and bleeding on probing, between the full-mouth ultrasonic debridement approach and quadrant by quadrant hand instrumentation (Tomasi et al., 2006).

Clinical treatment outcomes for non-surgical periodontal management (NSM) include reduction in probing depths, gain in clinical attachment and reduction in bleeding on probing. The reduction in probing depths results from a combination of gain in clinical attachment and gingival recession. Numerous studies have shown the outcomes that can be expected following nonsurgical periodontal therapy are remarkably consistent and the magnitude of both probing depths reduction and gains in clinical attachment are influenced by the initial probing depth measurements. For example, a review of periodontal treatment outcomes showed that for those pockets initially 4-6 mm deep, mean probing depth reductions of approximately 1.0-1.5 mm and mean attachment gains of 0.5-1.0 mm are consistently seen (Cobb, 2002). For pockets that are 7 mm or greater, mean probing depth reductions of 2.0-2.5 mm and mean attachment gains of 1.0-1.5 mm can be expected (Cobb, 2002).

The presence or absence of BOP is widely interpreted to represent the presence of an inflammatory infiltrate within the periodontal tissues and despite BOP being more reliable than other clinical signs for detection and monitoring of periodontal disease (Lang et al., 1996), BOP has been shown to have a low predictive value, being a poor predictor for periodontal disease progression (Lang et al., 1986). In contrast, the absence of BOP provides a high negative predictive value and is therefore an important indicator of periodontal health and stability (Lang *et al.*, 1990; Joss *et al.*, 1994).

In research, the use of tooth loss as a marker of disease is complicated, not only by tooth loss due to caries but also by the long follow up time required. Therefore, a patient’s response to periodontal treatment is presented in terms of probing depth reduction, gain in attachment and change in BOP, all of which are surrogate markers of

periodontal disease (Hujoel, 2004). Furthermore, studies also lack predefined goals for periodontal treatment against which patients can be assessed (Hujoel, 2004; Armitage, 2008). There appears to be an assumption that simply because a subject attends the desired number of appointments their treatment needs have been met. Obviously, within research trials it would be difficult to manage patients to a given endpoint, providing therapy until that point is reached, however, having stated treatment goals or clinical endpoints would allow for subjects to be categorised at the end of the study period into responders and non-responders (Hujoel, 2004; Armitage, 2008).

1.2 Diabetes

1.2.1 Definition and classification of diabetes

The term ‘diabetes’ encompasses a group of metabolic disorders, characterised by hyperglycaemia, resulting from defects in insulin secretion, insulin action, or both. Diabetes is classified based on the underlying aetiological cause and the vast majority of cases of diabetes fall into two broad categories, type 1 and type 2 (Table 1.3). Type 1 diabetes mellitus (T1DM) is caused by an absolute deficiency of insulin secretion and can be identified by the presence of serological markers indicating autoimmune destruction of the β cells of the pancreas. In type 2 diabetes mellitus (T2DM) the main causal processes are insulin resistance (i.e. an inability of the body to respond normally to insulin) and failure of pancreatic β cells to produce sufficient insulin. The subsequent hyperglycaemia has wide ranging molecular and cellular effects that predispose individuals to the classic diabetes complications of retinopathy, nephropathy, neuropathy, macrovascular disease, including cardiovascular disease. Diabetes is diagnosed on the basis of World Health Organisation (WHO) recommendations from 1999, that incorporate criteria for fasting plasma glucose levels and also plasma glucose levels 2 hours after a 75g glucose load (WHO, 1999) (Table 1.4).

Conditions that predispose to overt diabetes, including impaired fasting glucose and impaired glucose tolerance are also defined in the WHO recommendations (WHO, 1999) (Table 1.4). If untreated, about 7% of people with impaired fasting glucose and impaired glucose tolerance will progress to overt diabetes every year (Tuomilehto et al., 2001) and impaired glucose tolerance itself carries an increased risk of macrovascular disease (DECODE, 1999).

Table 1.3 Overview of the classification of diabetes

Type 1 diabetes mellitus

Type 2 diabetes mellitus

Gestational diabetes

Other Specific Types of Diabetes

- Genetic defects
- Diseases of exocrine pancreas
- Endocrinopathies
- Drug or infection induced
- Others

The table shows a classification of the main types of diabetes based on the underlying aetiological cause.

Table 1.4 Overview of the diagnostic criteria of diabetes mellitus

	Glucose concentration in venous plasma (mmol/L)
Diabetes mellitus	Fasting ≥ 7.0 or 2-h post glucose load ≥ 11.1
Impaired glucose tolerance	Fasting < 7.0 and 2-h post glucose load ≥ 7.8 and < 11.1
Impaired fasting glucose	Fasting ≥ 6.1 and < 7.0 and 2-h post glucose load < 7.8

The table shows the diagnostic criteria of diabetes mellitus and other categories of hyperglycaemia (WHO, 1999)

1.2.2 Epidemiology of diabetes

Diabetes has reached epidemic proportions, the International Diabetes Federation (IDF) estimates that currently diabetes affects more than 246 million individuals worldwide and this is expected to rise to 380 million people by 2025 (IDF, 2006). Between 2007 and 2025, the global predicated growth is 55%, with the greatest increases in the developing countries of Africa, Asia and South America (Figure 1.2) (IDF, 2006). Within Europe, it is estimated that diabetes affects 53.2 million or 8.4% of the adult population and this is predicted to rise by 21% to 64.1 million or 9.8% in 2025, with the greatest increase being seen in the older age group (Table 1.5) (IDF, 2006).

In recent years, research within the UK has demonstrated a rise in the prevalence (Newnham *et al.*, 2002; Fleming *et al.*, 2005; Lusignan *et al.*, 2005; Gonzalez *et al.*, 2009) and incidence (Ryan *et al.*, 2005; Forouhi *et al.*, 2007; Gonzalez *et al.*, 2009) of diabetes cases. Furthermore, the rise of diabetes cases in the UK is largely due to T2DM, with the incidence of T1DM remaining relatively constant over the past decade and the proportion of obese individuals newly diagnosed with T2DM has increased from 46% to 56% (Gonzalez et al., 2009).

In Western developed countries, the prevalence of T2DM is closely linked to socio-economic status (Ismail *et al.*, 1999; Evans *et al.*, 2000) and there are increasing numbers of diabetic individuals in deprived areas of affluent countries, creating implications for the delivery of targeted health care and prevention.

Figure 1.2 Estimates for 2007 & 2025 for the number of people with diabetes

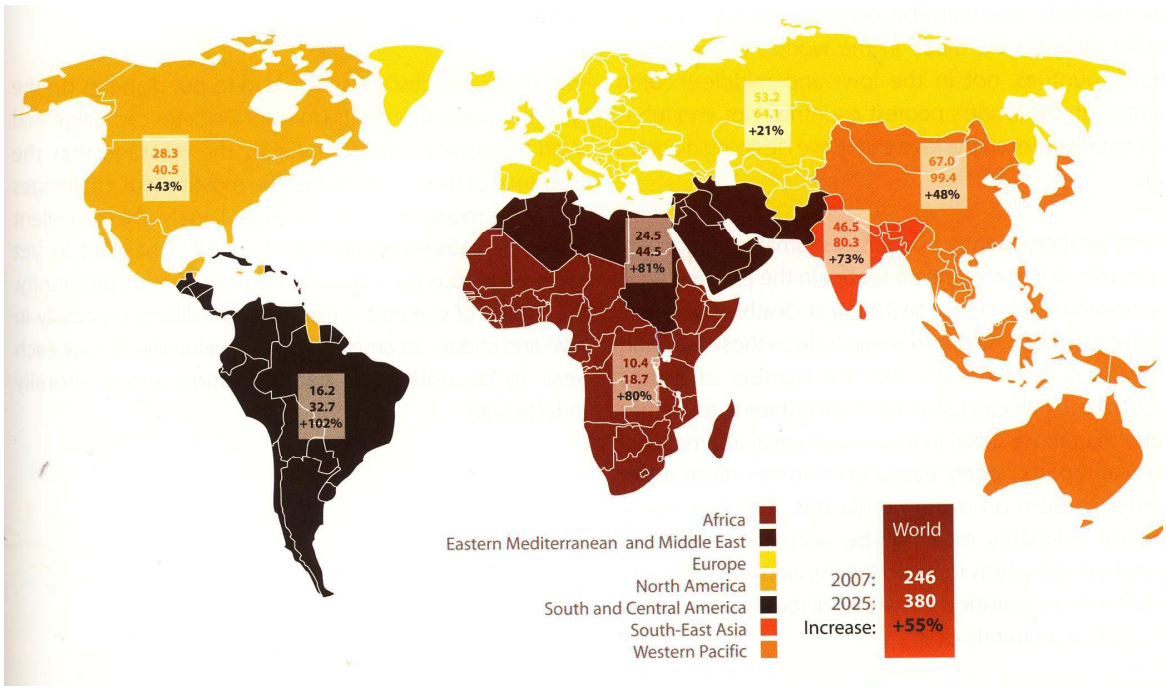


Figure shows worldwide estimates for 2007 & 2025 for the number of people with diabetes (IDF, 2006)

Table 1.5 European estimates for people with diabetes

	2007	2025
People with diabetes aged 20-79 (millions)	53.2	64.1
People with diabetes aged 20-39 (millions)	3.7	3.4
People with diabetes aged 40-59 (millions)	19.6	20.9
People with diabetes aged 60-79 (millions)	29.8	39.8
Prevalence (%)	8.4	9.8

Table shows the estimates for people with diabetes in Europe (IDF, 2006)

1.2.3 Pathogenesis of Type 2 Diabetes

Insulin secretion from the pancreas normally reduces glucose output by the liver, enhances glucose uptake by the liver and skeletal muscle, and suppresses fatty acid release from adipose tissue. T2DM is defined and diagnosed on the basis of hyperglycaemia, although the extent of the metabolic dysfunction is much broader than glucose metabolism (Home and Pacini, 2008). Hyperglycaemia is seen when the normal balanced interplay between insulin secretion and insulin action is disrupted. When insulin action decreases, the body usually compensates by increasing pancreatic beta-cell (β-cell) function and insulin production. T2DM, however, develops when β-cell functioning is inadequately low for a specific degree of impaired insulin action (Stumvoll et al., 2005). Decreased insulin secretion will reduce insulin signalling in its target tissues. Insulin resistance pathways affect the action of insulin in liver and muscle, the major target tissues, leading to increased circulating fatty acids and the hyperglycaemia of diabetes. Therefore, the underlying pathogenic defects in T2DM involve multiple pathways, each contributing to the underlying disease state (Stumvoll et al., 2005). Although evidence for familial clustering points to the contribution of genetic mechanisms, recent rapid increases in diabetes incidence and prevalence indicate that environmental and lifestyle factors are also of major relevance (Zimmet *et al.*, 2001; Stumvoll *et al.*, 2005).

1.2.3.1 Insulin resistance

Insulin resistance is seen when the effects of insulin are less than expected for both glucose disposal in skeletal muscle and liver and the suppression of hepatic endogenous glucose production.

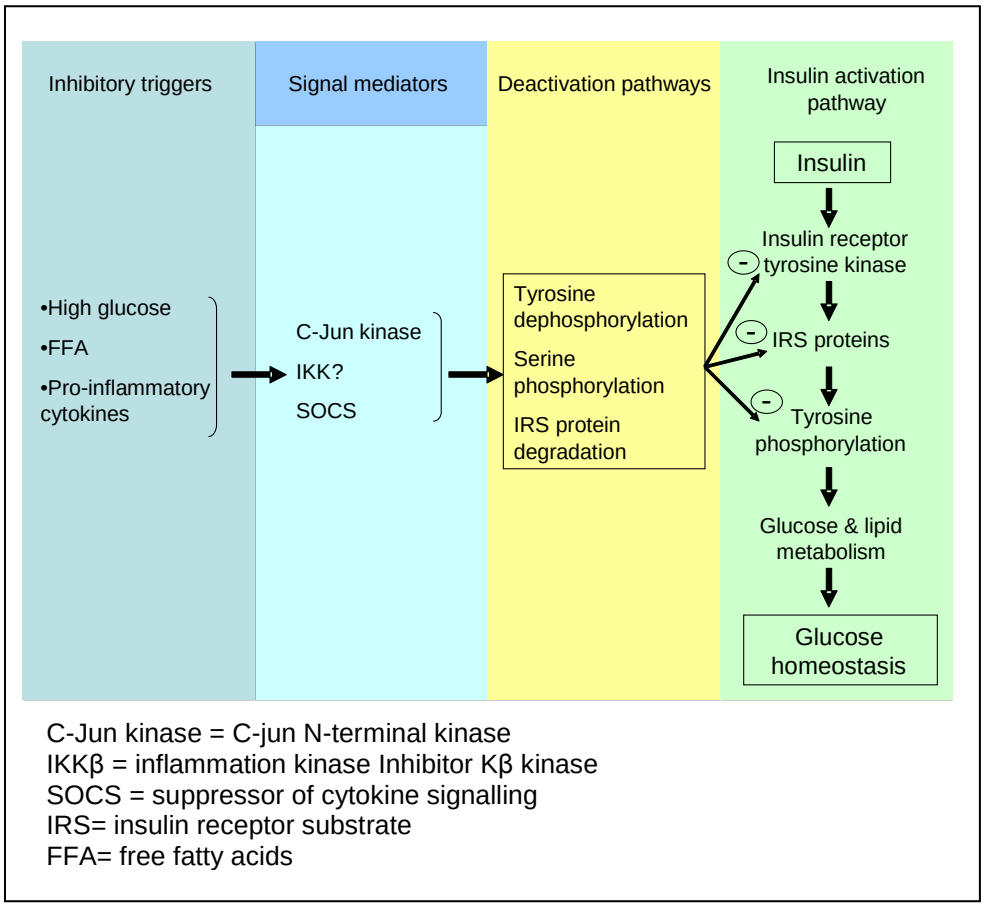
On a molecular level, hyperglycaemia, free fatty acids (FFA) and inflammatory cytokines can down-regulate intracellular insulin signalling and this contributes to

insulin resistance (Figure 1.3). (Griffin *et al.*, 1999; Itani *et al.*, 2002; Gao *et al.*, 2004). Furthermore, TNF-α has been shown to enhance adipocyte lipolysis, which increases FFA, in addition to contributing to intracellular insulin deactivation pathways (Paz *et al.*, 1997; Aguirre *et al.*, 2000). TNF-α, IL-1β and IL-6 also have a direct inhibitory effect on the intracellular insulin signalling cascades (Hotamisligil, 2000; Senn *et al.*, 2003; Jager *et al.*, 2007). Therefore, by targeting the insulin signalling cascade, inflammatory cytokines are capable of impairing insulin action, contributing to the development of insulin resistance (Tilg and Moschen, 2008).

1.2.3.2 Beta-cell dysfunction

In health, the adaptive response of pancreatic β-cells to insulin resistance involves an increase in insulin release that is sufficient to overcome the reduced efficiency of insulin action. Patients develop T2DM when β-cells are unable to compensate fully for decreased insulin sensitivity (Kahn et al., 2001). Indeed, deterioration of insulin secretion over time is the usual course in most patients, and many patients will have deficient insulin secretion after 10 years of diabetes (Wallace and Matthews, 2002). The rate of deterioration in diabetes control is around 1.5% HbA1c per 10 years and it thought to be a result of a decline in β-cell function (Home, 2008b).

Figure 1.3 Overview of insulin signalling and insulin resistance



On a molecular level, hyperglycaemia, FFA and inflammatory cytokines can down-regulate intracellular insulin signalling and this contributes to insulin resistance. The figure shows and overview of insulin signalling and insulin resistance. Adapted from (Stumvoll et al., 2005)

In chronic hyperglycaemia, increased amounts of ROS are generated in β-cells with subsequent damage to cellular components or apoptosis (Stumvoll *et al.*, 2005; Kahn *et al.*, 2006). Elevated plasma FFA levels also contribute to progressive loss of β-cell function (Zhou and Grill, 1994; Robertson *et al.*, 2004; Kahn *et al.*, 2006). In addition, it has long been recognised that IL-1β has the ability to damage β-cells (Bendtzen *et al.*, 1986; Mandrup-Poulsen *et al.*, 1986). Furthermore, recent clinical trials have highlighted the role of IL-1β in β-cell dysfunction and diabetes. In a placebo-controlled randomised trial, administration of a competitive antagonist of IL-1β, interleukin-1 receptor antagonist, improved glycaemic control of patients with poorly controlled T2DM (Larsen et al., 2007). Furthermore, the use of neutralising antibodies to IL-1β, also improves glycaemic control and β-cell function in patients with T2DM (Donath et al., 2008). The β-cell dysfunction that contributes to T2DM therefore appears, at least in part, to involve IL-1β.

1.2.3.3 Obesity

An imbalance between nutritional intake and energy expenditure culminates in obesity. Obesity has long been recognised as a powerful risk factor for T2DM (Lundgren *et al.*, 1989; Chan *et al.*, 1994; Carey *et al.*, 1997; Wang *et al.*, 2005). Indeed, general obesity, as measured by body mass index (BMI), and abdominal adiposity strongly and independently predicts risk of T2DM (Wang et al., 2005). It has been said that the leading cause for developing insulin resistance is obesity (Kahn et al., 2006).

The classical view of adipose tissue as an energy storage depot has now been replaced by the perception that adipose tissue is an active endocrine organ playing a central role in lipid and glucose metabolism, and producing various hormones and

cytokines involved in the development of insulin resistance, T2DM and vascular diseases (Hajer *et al.*, 2008; Taube *et al.*, 2009).

Due to changes in function of adipocytes and infiltrating macrophages, obesity is associated with the appearance of a chronic, low grade inflammatory state (Ross, 1999). An increase in adipose tissue and adipocytes volume is accompanied by a rise in plasma adipokine levels, with the exception of adiponectin which is lower in obesity (Skurk *et al.*, 2007; Wannamethee *et al.*, 2007). Macrophages are more prevalent in the adipose tissue of obese subjects compared to lean subjects and the macrophage quantity correlates with measures of insulin resistance (Otto and Lane, 2005). Furthermore, the number of macrophages in adipose tissue is reduced by weight loss (Cancello et al., 2005). The interplay between macrophages and adipocytes by a paracrine effect is proposed to be central to adipose tissue dysfunction (Hajer et al., 2008). Enlarged adipocytes are shown to release increased levels of FFA which bind to TLRs in macrophages, resulting in activation of intracellular signalling and augmented TNF-α production (Suganami *et al.*, 2005; Suganami *et al.*, 2007). In turn, TNF-α activates human adipocytes, thereby further inducing lipolysis and FFA production in addition to enhancing the expression of IL-6 and mediators that facilitate the accumulation of macrophages in adipose tissue (Ruan *et al.*, 2002; Permana *et al.*, 2006). Therefore, the FFA, TNF-α and IL-6 released from adipose tissue (Hotamisligil *et al.*, 1995; Wellen and Hotamisligil, 2005; Scherer, 2006) have an inhibitory effect on intracellular insulin signalling pathways, contributing both to insulin resistance in hepatic tissue, skeletal muscle and adipose tissue and reduced insulin production from β-cells (Kahn *et al.*, 2006; Goldstein, 2008; Hajer *et al.*, 2008).

1.2.3.4 Genetic factors

Although lifestyle factors and obesity seem to be important pathogenic factors, genetic elements are also involved in the pathogenesis of T2DM. A positive family history gives a 2.4 fold increased risk for T2DM, with up to 25% of first degree relatives of people with T2DM developing impaired glucose tolerance or diabetes (Pierce et al., 1995). The lifetime risk (at age 80 years) for T2DM is calculated at 38% if one parent has T2DM (Pierce et al., 1995) and if both parents have T2DM, the prevalence of T2DM in the offspring is estimated to be around 60% by the age of 60 years (Tattersal and Fajans, 1975).

Research has attempted to identify causative factors among many candidate genes that have a plausible role in T2DM pathogenesis (Prokopenko et al., 2008). In most instances, initial associations were not replicated in subsequent analyses and only a few candidate gene associations have been confirmed by meta-analysis (Parikh and Groop, 2004).

1.2.3.5 Inflammation

Although the cellular mechanisms contributing to the pathogenesis of T2DM are complex and not fully elucidated, it appears inflammation plays a key role. Inflammatory cytokines, such as TNF-α, IL-1β and IL-6, contribute to the development of T2DM through insulin resistance and β-cell dysfunction (Donath *et al.*, 2003; Pickup, 2004). Inflammatory cytokines such as TNF-α and IL-6 have been linked to insulin resistance, and their expression is increased in adipose tissue in obese subjects (Kern et al., 2001). In response to elevated concentrations of glucose and FFA monocytes, macrophages, endothelial cells and adipocytes release pro-inflammatory cytokines, including IL-6, TNF-α and IL-1β (Morohoshi *et al.*, 1996; Guha *et al.*, 2000; Maedler *et al.*, 2002; Donath *et al.*, 2003; Shanmugam *et al.*, 2003). Cytokines such as TNF-α, IL-

1β and IL-6 contribute to insulin resistance through the down-regulation of specific aspects of intracellular insulin pathways (Figure1.3) (Hotamisligil *et al.*, 1996; Stumvoll *et al.*, 2005).

Furthermore, the intracellular signalling cascades that contribute to insulin resistance are also involved in inflammatory signalling cascades (Shoelson *et al.*, 2006; Goldstein, 2008). Recent research has focused on modulating inflammatory pathways as a potential target in the management of T2DM. Studies investigating salicylates as potential therapeutic agents for the management of T2DM have shown that, *in vivo*, administration of salicylates improved both inflammatory parameters and glucose and lipid homeostasis, highlighting the involvement of the inflammatory signalling cascade in the pathogenesis of T2DM (Fleischman *et al.*, 2008; Goldfine *et al.*, 2008). IL-1β blockade has also been shown to improve glycaemic control and β-cell function (Larsen *et al.*, 2007; Donath *et al.*, 2008).

1.2.4 Complications of diabetes

Diabetes is associated with reduced life expectancy, increased morbidity and increased mortality. UK patients with T2DM have a mortality rate almost twice as high as those without diabetes (Mulnier et al., 2006). Diabetes has an irreversible and detrimental effect on the micro- and macro-vasculature, with cardiovascular disease (CVD), myocardial infarction and stroke being the leading cause of mortality in patients with T2DM (Dale et al., 2008). Furthermore, diabetic nephropathy, retinopathy and neuropathy are major causes of renal failure, blindness and sensory loss (Frank, 2004; Gilbertson *et al.*, 2005). Type 2 diabetes is notable for the increased cardiovascular risk that it carries, clinically presenting as myocardial infarctions, angina, peripheral artery disease (leg claudication, gangrene), and carotid artery disease (strokes, dementia)

(NICE, 2008). The duration of diabetes and the degree of glycaemic control are important factors for all diabetic complications (UKPDS, 1998a).

Within the pathological processes of diabetic vascular injury, the molecular consequences of hyperglycaemia superimpose onto existing contributing factors for vascular disease, such as raised levels of serum lipids and hypertension. In patients with diabetes, increased oxidative stress and inflammation are recognised pathogenic mechanisms for vascular damage, with altered gene expression in the vasculature, providing a pro-inflammatory and thrombogenic environment (Feng *et al.*, 2005; Crimi *et al.*, 2007).

The molecular pathways that contribute to diabetic complications are complex and incompletely understood. Hyperglycaemia leads to the formation of advanced glycation end products (AGEs), leading to protein cross linking and activation of the AGE receptor (RAGE) (Brownlee et al., 1988). Animal studies have confirmed the role of RAGE activation in the development of diabetic neuropathy, nephropathy and vascular disease (Soro-Paavonen et al., 2008). RAGE activates numerous signalling pathways leading to activation of pro-inflammatory and pro-coagulatory genes (Hudson et al., 2005) and studies in rodent models of vascular disease have shown that blockade of RAGE can prevent development of disease (Park *et al.*, 1998; Bucciarelli *et al.*, 2002). Furthermore, activation of RAGE contributes to diabetic-induced damage through increased oxidative stress, with RAGE activation contributing, along with hyperglycaemia, to excessive production of ROS and the down-regulation of antioxidant activity (Calcutt et al., 2009). Increased ROS activate a range of intracellular signalling pathways and transcription factors thought to influence phenotypic changes and release of mediators that contribute to tissue damage (Nishikawa *et al.*, 2000; Lambeth, 2007; Calcutt *et al.*, 2009).

1.2.5 Management of Type 2 diabetes

The management of T2DM is focused on controlling risk factors for both microvascular and macrovascular diabetic complications. Therefore, current targets for T2DM management include weight control, smoking, plasma glucose control, blood pressure control, blood lipid control, reduction of thrombogenicity, laser therapy for eye damage, drug therapy to delay kidney damage, foot care, and symptomatic treatments for nerve damage (Home *et al.*, 2008; NICE, 2008).

T2DM is predominately managed by the patient as part of their daily life, with structured education being an integral and ongoing part of diabetes care, to ensure patients understand diabetes and their key role in its management (Deakin *et al.*, 2006; Davies *et al.*, 2008). As part of the management of risk factors for diabetes-related complications it is recommended that dietary advice, following the principles of healthy eating, is integrated within a personalised diabetes management plan, including other aspects of lifestyle modification, such as increasing physical activity, losing weight and smoking cessation (Home *et al.*, 2008; NICE, 2008).

The UK Prospective Diabetes Study (UKPDS), a large (n=3,867) randomised, prospective, multicenter study with a 10 year follow-up period compared the effect of intensive therapy (either sulfonylurea or insulin or, in overweight patients, metformin) and conventional therapy (dietary restriction) (UKPDS, 1998a). The UKPDS demonstrated that the risk of microvascular and macrovascular complications of T2DM is strongly associated with hyperglycaemia as measured by percentage glycated haemoglobin (HbA1c), with a 1% reduction in HbA1c being associated with a 21% risk reduction in any diabetic complication clinical endpoint (Stratton et al., 2000). In the initial 10 year trial, intense therapy, achieving tighter glycaemic control, resulted in an lower incidence of microvascular complications, but not macrovascular complications

(UKPDS, 1998a; Stratton *et al.*, 2000). Recently published data, however, from the 10 year post trial follow-up of the UKPDS showed that if intensive glucose control was started at the time of diagnosis, a sustained legacy effect on diabetic complications was demonstrated. Therefore, even when between group differences in HbA1c were lost, the intensely managed group continued to be associated with a significant decreased risk of myocardial infarction and death, in addition to the well-established reduction in the risk of microvascular disease (Holman et al., 2008a). Consequently, national guidelines have therefore set an ideal target HbA1c of 6.5%, using dietary and lifestyle interventions with medications, as required, to achieve and maintain this target HbA1c level, with monitoring at a minimum of 6 monthly intervals (Home *et al.*, 2008; NICE, 2008).

The impact of tight blood pressure control on the risk of developing diabetic complications has also been extensively studied. Analysis of data from the UKPDS has shown the risk of complications in T2DM is associated independently and additively with hyperglycaemia and hypertension. Intensive treatment of both these risk factors is required to minimise the incidence of complications (Stratton et al., 2006). The UKPDS achieved a risk reduction for both micro and macro vascular disease with tight control of blood pressure (Turner *et al.*, 1998a). Furthermore, the 10 year post trial follow-up demonstrated that good blood pressure control must be continued if the positive effect on diabetic complications is to be maintained (Holman et al., 2008b). consequently, national guidelines recommend a target blood pressure level of 140/80 mm Hg and lifestyle advice, anti-hypertensive medications and at least annual monitoring are advocated to enable this goal to be reached (Home *et al.*, 2008; NICE, 2008).

It is also recommended that for patients with T2DM, blood lipid control should be an integral part of cardiovascular risk management, including an annual measurement of a full lipid profile (including total cholesterol, high density lipoprotein

cholesterol, low density lipoprotein cholesterol and triglyceride estimations) and simvastatin prescribed for patients not reaching the recommended targets (total cholesterol < 4.0 mmol/l, high density lipoprotein cholesterol ≤ 1.4 mmol/l, serum triglyceride < 4.5 mmol/l) (Home *et al.*, 2008; NICE, 2008).

1.2.5.1 Glycaemic control

The widespread clinical use of HbA1c to monitor long-term glycaemic control in patients with diabetes was established following publication of data from the UKPDS demonstrating the relationship between HbA1c and diabetic complications caused by hyperglycaemia (Stratton *et al.*, 2000). HbA1c provides clinicians with an indication of a patient's long-term glycaemic control and is used to set appropriate management goals to enable patients to achieve adequate plasma glucose control. HbA1c is, however, a surrogate marker of plasma glucose (Home, 2008a). HbA1c is formed in a non-enzymatic pathway by haemoglobin's normal exposure to elevated plasma levels of glucose. HbA1c concentrations, relative to circulating haemoglobin A concentrations, therefore represents the amount of glycated haemoglobin within erythrocytes, reflecting the average level of glucose to which the cell has been exposed during the preceding months. However, the relationship between HbA1c and plasma glucose is complicated by the fact that HbA1c taken at a specific time point is contributed to by the total population of erythrocytes, from the oldest (120 days) to the youngest. Additionally, recent plasma glucose levels have a greater impact on HbA1c than more distant plasma glucose levels. Therefore, HbA1c is viewed as a weighted average of plasma glucose in the preceding 120 days (Rohlfing et al., 2002).

Analysis of data from a large prospective, multicenter study demonstrated a strong correlation between plasma glucose and HbA1c, confirming a predictable relationship between HbA1c and hyperglycaemia (Rohlfing et al., 2002). Understanding

this relationship allows clinicians and patients to set appropriate day to day plasma glucose targets to achieve the recommended HbA1c goal of 6.5% (NICE, 2008).

1.2.5.2 Drug therapy in the management of Type 2 diabetes

For those who are inadequately controlled with diet and lifestyle management, metformin and sulfonylureas form the mainstay of pharmacological treatment. Metformin can also be combined with insulin therapy, resulting in a reduction in required insulin dose and body weight (Douek et al., 2005). Improvements in hyperglycaemia coupled with unaltered plasma insulin concentrations indicate that metformin is an insulin-sensitizing drug, suppressing endogenous glucose production and improving insulin-stimulated glucose uptake, with no direct effect on β-cells in stimulating insulin secretion (Matthaei *et al.*, 2000; Holstein and Beil, 2009). Sulfonylureas, on the other hand, act at the β-cell membrane by closing ATP-sensitive potassium channels, leading to enhanced insulin secretion (Holstein and Beil, 2009). The efficacy and safety of both metformin and sulfonylureas have been clearly demonstrated by large prospective studies (UKPDS, 1998b; UKPDS, 1998a; Patel *et al.*, 2008) and confirmed by meta-analysis of observation studies (Rao et al., 2008).

Currently, thiazolidinediones are recommended only as a second line therapy when glycaemic control is inadequate and sulphonurea, metformin or insulin are not tolerated (NICE, 2008). This reflects the relatively modest benefits demonstrated with thiazolidinediones and the potential long-term side effects on the cardiovascular system and bone (Dormandy *et al.*, 2005; Singh *et al.*, 2007; Loke *et al.*, 2009). The thiazolidinediones sensitise and enhance the effect of insulin in skeletal muscle, adipose and hepatic tissues without increasing pancreatic secretion of insulin. Thiazolidinediones primarily activate receptors in adipose tissue and affect adipose metabolism and distribution. Thiazolidinediones may also preserve β-cell function

(Kendall et al., 2006), an important consideration following the recognition that a progressive decline in glycaemic control is seen in T2DM with increasing therapy requirements. There is a suggestion in the literature that thiazolidinediones may ameliorate insulin resistance by reducing circulating concentrations of pro-inflammatory cytokines and increasing concentrations of adiponectin (Stumvoll et al., 2005), however, supporting evidence remains sparse.

As part of the management of T2DM, statins are extensively used for lowering cholesterol and reducing cardiovascular events. Recent studies have shown that statins have additional beneficial anti-inflammatory effects (Ridker and Silvertown, 2008). In a recent randomised controlled trial, in apparently healthy subjects without hyperlipidemia but with elevated high sensitive C-reactive protein (hsCRP), the administration of statins reduced circulating hsCRP levels and also reduced the incidence of major cardiovascular events (Ridker *et al.*, 2008). In vitro studies have also begun to demonstrate the inhibitory effect of statins on cytokine release from monocytes and macrophages (Krysiak *et al.*, 2011).

1.3 Periodontal disease & diabetes

1.3.1 The epidemiological association between diabetes & periodontitis

A number of case reports, cross-sectional studies, and a few longitudinal studies have reported an increased prevalence of periodontal disease in patients with T2DM. Indeed, it is accepted in the periodontal community that T2DM is an important risk factor for periodontal disease and, indeed, periodontal disease is viewed as a complication of diabetes (Loe, 1993; Salvi *et al.*, 1997b). Epidemiological evidence supports associations between diabetes and increased prevalence and severity of periodontal disease (Taylor, 2001). Although the majority of studies have been cross-

sectional in design, typically describing findings from small convenient samples, there is a smaller subset of population-based studies which strongly support associations between diabetes and periodontal disease (Nelson *et al.*, 1990; Shlossman *et al.*, 1990; Emrich *et al.*, 1991; Taylor *et al.*, 1998b).

Several of these studies have focused on the Pima Indians, a population suffering from very high prevalence rates of T2DM. In this population, diabetic subjects were 2.8 times and 3.4 times more likely to have periodontal disease compared to non-diabetic controls when periodontitis was defined by clinical attachment loss or radiographic bone loss, respectively (Emrich et al., 1991). In another study of the Pima Indians, the incidence of periodontal disease over 2.6 years was 2.6 times higher in the subjects with T2DM than that observed in the non-diabetic controls (Nelson et al., 1990). It has also been reported that T2DM patients with poor glycaemic control have a much greater risk of progressive alveolar bone loss (odds ratio 11.4) compared to non-diabetic subjects (odds ratio 2.2) (Taylor *et al.*, 1998b). Interestingly, this study also provided evidence to support a possible negative effect of periodontitis on glycaemic control, as subjects with moderate or well controlled T2DM at baseline who also had severe periodontitis were approximately 6 times more likely to have poor glycaemic control at 2-years follow-up than subjects who did not have severe periodontitis at baseline (Taylor *et al.*, 1998b).

Similar findings of increased prevalence of periodontal disease have also been reported in European populations with type 2 diabetes (Sandberg et al., 2000). Additionally, the NHANES data from the USA, found that adults with poorly controlled diabetes had an almost threefold increased risk of having periodontitis compared with adults without diabetes. Furthermore, adults with diabetes under good glycaemic control had no significant increase in risk for periodontal disease (Tsai et al., 2002). When

viewed from the wider perspective of maintaining systemic health, one study has shown that diabetics with severe periodontal disease are six times more likely to have poor glycaemic control (Taylor et al., 1996). Furthermore, patients with severe periodontal disease have a significantly increased risk for microalbuminaemia and end stage renal disease compared to those who do not (Shultis et al., 2007). It has also been reported that diabetic Pima Indians with severe periodontal disease have an increased risk of death from diabetic nephropathy and ischemic heart disease (Saremi et al., 2005). Furthermore, in a large study that explored data from the NHANES study and follow-up evaluations for 9296 subjects, it was demonstrated that subjects with moderate levels of periodontal disease had a 2-fold increased odds for developing T2DM, and this increased risk was maintained even in those who had never smoked (Demmer et al., 2008).

Observations such as these lend support to the concept of a ‘bi-directional’ relationship between T2DM and periodontal disease, with T2DM being associated with increased prevalence and severity of periodontal disease, and periodontitis being associated with poorer glycaemic control or increased risk of diabetic complications. The principal findings of key studies that have investigated associations between periodontal disease and T2DM are summarised in Table 1.6. Taken collectively, the epidemiological evidence confirms that diabetes is a risk factor for periodontal disease. The risk for developing periodontitis is greater if diabetes control is poor and those with good diabetic control do not have a greater risk of periodontal disease than non-diabetics (Tsai et al., 2002).

Table 1.6 Principal and recent studies investigating links between periodontal disease & T2DM

Author, Year	Subjects	Age	Study design	Principal findings
(Nesse et al., 2009)	40 T2DM	58 ± 9.5	Cross-sectional	In patients with T2DM a dose-response relationship was shown between HbA1c and the surface area of inflamed periodontal tissue (PISA). An increase of PISA by 333mm^2 was associated with a 1.0% increase of HbA1c.
(Shultis et al., 2007)	529 T2DM	25-79	Prospective	In patients with severe periodontal disease the incidence of developing end stage renal disease was 3.5 times as high compared to those with none/mild periodontitis.
(Engebretson et al., 2007)	46 T2DM	54	Cross-sectional	Serum TNF-α correlated with attachment loss (r=0.40, p=0.009). A dose-response relationship was observed between periodontitis severity and TNF-α (p=0.012).
(Mattout et al., 2006)	71 T2DM, 2073 controls	35-65	Cross-sectional	Significantly more attachment loss in T2DM patients. More gingival inflammation in T2DM after controlling for plaque levels
(Jansson et al., 2006)	191 T2DM	55 ± 5	Cross-sectional	20% of the T2DM patients had periodontal disease. These subjects also had significantly higher HbA1c (7.1%) than periodontally healthy T2DM patients (6.5%)
(Peck et al., 2006)	23 T2DM	59 ± 10	Cross-sectional	Periodontal disease affected 42% of those with poor glycaemic control (HbA1c > 8.0%) compared to 18% of those with good control (HbA1c < 8.0%)
(Campus et al., 2005)	71 T2DM 141 controls	60 ± 10	Cross-sectional	T2DM patients had significantly more missing teeth, pockets > 4 mm, bleeding on probing and plaque than control patients
(Saremi et al., 2005)	628	≥35	Prospective	T2DM patients with severe periodontal disease had 3.2 times the risk of death from diabetic nephropathy and ischemic heart disease compared to those with none/mild/moderate periodontitis.
(Lu and Yang, 2004)	72 T2DM	54	Cross-sectional	Gingival inflammation and attachment loss were significantly higher in the T2DM

	92 controls			patients
(Engebretson et al., 2004)	45 T2DM	32-69	Cross-sectional	T2DM patients with poor glycaemic control (HbA1c > 8.0%) had significantly higher GCF IL-1β levels than better controlled T2DM patients
(Tsai et al., 2002)	4343 T2DM	45-90	Cross-sectional	Poorly controlled T2DM patients had a significantly higher prevalence of periodontal disease than those without T2DM (OR: 2.9, 95% CI: 1.4, 6.0)
(Sandberg et al., 2000)	102 T2DM 102 controls	65 ± 8	Cross-sectional	Significantly more T2DM patients (44.8%) had interproximal alveolar bone loss > 1/3 root length compared to control subjects (25.5%)
(Cutler et al., 1999a)	28 T2DM 7 controls	~28-66	Cross-sectional	Probing depths, attachment loss and gingival inflammation were all significantly elevated in T2DM patients compared to controls
(Taylor *et al.*, 1998b)	24 T2DM 338 controls	15-57	Prospective	Radiographic bone loss (up to 1/4 root length) was more prevalent in T2DM patients (67%) compared to controls (44%)
(Emrich et al., 1991)	254 T2DM 930 controls	15-55+	Cross-sectional	Prevalence of periodontal disease was higher in T2DM patients compared to non-diabetic controls or subjects with impaired glucose tolerance
(Shlossman et al., 1990)	736 T2DM 2483 controls	5-45+	Cross-sectional	Periodontal disease was more prevalent in T2DM patients than controls
(Nelson et al., 1990)	720 T2DM 1553 controls	15-55+	Cross-sectional and prospective	83% of T2DM patients demonstrated interproximal bone loss compared to 19% of controls. In follow-up appointments, the incidence of periodontitis was 60 new cases per 1000 person-years in T2DM patients compared to 17 new cases in controls

1.3.2 Mechanistic links

The observed epidemiological associations between periodontitis and T2DM may arise from common pathological defects that result in increased susceptibility to both diseases. Furthermore, T2DM or specifically hyperglycaemia may also modify inflammatory processes contributing to local dysregulated immune-inflammatory responses, causing increased periodontal destruction.

1.3.2.1 Inflammation

It is increasingly recognised that inflammation plays a role in the development of T2DM. Low grade systemic inflammation precedes the development of T2DM (Freeman *et al.*, 2002; Duncan *et al.*, 2003; Bertoni *et al.*, 2010) and plasma concentrations of IL-6 and TNF-α are increased in obese individuals and in those with T2DM (Ziccardi *et al.*, 2002; Dandona *et al.*, 2004). Further, prospective studies suggest that higher levels of systemic inflammation, such as serum IL-6 and CRP, predict future occurrence of T2DM (Pradhan *et al.*, 2001; Bertoni *et al.*, 2010).

In response to hyperglycaemia, a variety of cells, including monocytes, macrophages, β-cells, endothelial cells and adipocytes, release pro-inflammatory cytokines, such as IL-6, TNF-α and IL-β (Morohoshi *et al.*, 1996; Guha *et al.*, 2000; Maedler *et al.*, 2002; Donath *et al.*, 2003; Shanmugam *et al.*, 2003). Through the inhibition of intracellular insulin signalling cascades, TNF-α (Aguirre *et al.*, 2000; Hotamisligil, 2000), IL-1β (Jager et al., 2007) and IL-6 (Senn et al., 2003) impair insulin action and contribute to the development of insulin resistance. Furthermore, blocking IL-1β has been shown to improve β-cell function and glycaemic control in patients with T2DM (Larsen *et al.*, 2007; Donath *et al.*, 2008). Also, research focusing on the modulation of inflammatory pathways has shown that, in patients with T2DM,

salsalate improved both inflammatory parameters and glucose homeostasis, highlighting the role of the inflammatory signalling cascade in the development of T2DM (Goldfine et al., 2008). Furthermore, cell culture experiments have demonstrated that in basal and LPS stimulated conditions, purified neutrophil cell preparations isolated from venous blood samples, released higher amounts of IL-1β and TNF-α in subjects with T2DM compared to non-diabetic controls (Hatanaka et al., 2006).

Within the pathogenesis of periodontal disease, the importance of inflammation and the production of inflammatory cytokines is well recognised. A recent systematic review concluded that there was strong evidence from cross-sectional studies that plasma CRP was elevated in periodontitis-affected subjects compared with controls (Paraskevas et al., 2008). Cytokines, such as IL-1β, TNF-α and IL-6, are involved in the immune-inflammatory host reaction to the bacterial plaque biofilm with cell culture studies confirming that these cytokines are secreted by primary human epithelial cells following stimulation with periodontal pathogens (Eskan *et al.*, 2008; Stathopoulou *et al.*, 2009). Clinical research has also demonstrated elevated cytokine levels in tissue and GCF samples in disease compared to health (Stashenko *et al.*, 1991b; Irwin and Myrillas, 1998; Engebretson *et al.*, 2002; Lin *et al.*, 2005; Zhong *et al.*, 2007) with reductions following successful periodontal management (Engebretson et al., 2002). Circulating levels of IL-6 were higher in patients with periodontitis compared to periodontally healthy controls (Marcaccini *et al.*, 2009; Sun *et al.*, 2009) and systemic levels of IL-6 decreased following periodontal therapy (Marcaccini et al., 2009).

Clearly, inflammatory cytokines contribute to the pathogenesis of both T2DM and periodontitis. Additionally, inflammation also provides a possible scientific basis for the increased susceptibility to periodontal disease seen in people with T2DM. Alterations in immunologically active molecules as a result of T2DM may alter the

complex cytokine network within the periodontium and thus contribute to local periodontal tissue destruction.

Given the importance of inflammation, relatively few studies have, until recently, investigated the role of inflammatory cytokines in patients with T2DM and periodontitis. In one study, a trend for increasing GCF IL-1β concentrations was identified as diabetes control decreased (Cutler et al., 1999a). Also, poorly controlled T2DM patients with untreated periodontitis have significantly higher GCF IL-1β levels than T2DM patients with moderate or good glycaemic control (Engebretson et al., 2004). A cell culture study of human monocytes, showed that the production of prostaglandin-E_2 and IL-1β in response to challenge with LPS from *P. gingivalis* was significantly higher in monocytes from diabetic patients compared to non-diabetics with periodontitis (Salvi et al., 1997c). A further study demonstrated that in patients with T2DM, serum TNF-α correlated with attachment loss and a dose-response relationship was observed between periodontitis severity and circulating TNF-α (Engebretson et al., 2007). More recently, a number of small studies have begun to document the impact of periodontal therapy on cytokines (see section 1.3.5 of the introduction) (Correa *et al.*, 2008; Dag *et al.*, 2009; Correa *et al.*, 2010; Kardesler *et al.*, 2010; Santos *et al.*, 2010; Kardesler *et al.*, 2011). Table 1.7 summarises principal and recent studies investigating links between periodontal disease and T2DM.

1.3.2.2 Adipokines

Obesity is a risk factor for several chronic diseases, most notably hypertension, T2DM, dyslipdaemia and coronary heart disease (Gregg *et al.*, 2005; Flegal, 2006; Pischon *et al.*, 2007). Furthermore, a recent meta-analysis of data from cross-sectional studies demonstrate a positive association between periodontal disease and obesity (Chaffee and Weston, 2010) . Currently, a lack of longitudinal data limits understanding

of the causal relationship between obesity and periodontal disease and further studies would be required to clarify whether obesity is a risk factor for periodontal disease or that periodontitis increases the risk of weight gain (Chaffee and Weston, 2010). However, inflammation is described as the mechanism that links these two diseases (Pischon *et al.*, 2007).

In patients with T2DM, one potential source of cytokines is the adipose tissue, a recognised endocrine organ that secretes molecules involved in appetite regulation, metabolism and inflammation. Increased adipose mass associated with obesity is linked with a low-grade, chronic inflammatory response, characterised by altered production of adipokines and increases in biological markers of inflammation (Neels and Olefsky, 2006; Galic *et al.*, 2010). Furthermore, the increased mass of adipose tissue in obesity is associated with an increased infiltration with macrophages, capable of producing pro-inflammatory cytokines (Coenen *et al.*, 2007; Lumeng *et al.*, 2007). Indeed, expression of TNF-α has been shown to be increased in adipose tissue in obesity (Hotamisligil et al., 1993) with increased serum concentrations of both TNF-α and IL-6 seen in obese individuals (Ziccardi et al., 2002) and a positive correlation between IL-6 and body mass demonstrated in T2DM (Lazar, 2005). In addition to the contribution TNF-α and IL-6 have to the development of T2DM through their roles in both insulin resistance and β-cell dysfunction, they also have direct pro-inflammatory effects on inflammatory cells, including those in the periodontal tissues.

The adipose tissue also secretes adipokines, such as leptin and adiponectin. Leptin is a major regulator of body weight by repressing food intake and promoting energy expenditure (Rabe et al., 2008). In addition to these actions, leptin is also involved in glucose homeostasis, modulating β-cell function (Niswender and

Magnuson, 2007) and improving insulin sensitivity in the liver and skeletal muscle (Minokoshi *et al.*, 2002; Rabe *et al.*, 2008).

In addition to regulating appetite and energy storage, leptin is an important stimulator of inflammatory responses. It stimulates neutrophil chemotaxis and stimulates cytokine release by monocytes (Sanchez-Margalet et al., 2003). Furthermore, obesity appears to have an impact on leptin production and action. Leptin production is increased in obese compared to lean subjects (Fried et al., 2000). Therefore, it is plausible that elevated leptin levels in obese patients with T2DM may contribute to enhanced periodontal tissue destruction by activation of pro-inflammatory responses in the periodontium. However, the limited data on the role of leptin in periodontitis presents a confusing picture, with leptin concentrations in gingival tissue being inversely correlated with probing depths (Johnson and Serio, 2001) and higher GCF leptin levels measured in non-smokers than smokers, and higher in shallow pockets than deep pockets in non-smokers (Bozkurt et al., 2006).

A further adipokine that may be relevant in the context of periodontitis and T2DM is adiponectin. Adiponectin regulates energy expenditure, stimulates glucose uptake and fatty acid oxidation in skeletal muscle and adipose tissue whilst also suppressing hepatic glucose output (Galic *et al.*, 2010). Prospective and longitudinal studies indicate that lower levels of adiponectin are associated with a higher incidence of T2DM (Lindsay *et al.*, 2002; Daimon *et al.*, 2003; Spranger *et al.*, 2003a; Duncan *et al.*, 2004; Mather *et al.*, 2008; Tabak *et al.*, 2009), with a recent meta-analysis confirming that across diverse populations, higher adiponectin levels are associated with a lower risk of T2DM (Li et al., 2009). In obesity adiponectin levels are reduced and weight reduction leads to increases in circulating adiponectin (Ouchi *et al.*, 1999; Matsubara *et al.*, 2002). Furthermore, adiponectin is generally considered to have anti-

inflammatory effects, and low levels of adiponectin have been associated with obesity, diabetes and cardiovascular disease (Kadowaki and Yamauchi, 2005). However, to date, there is no published research investigating the role of adiponectin in periodontitis.

1.3.2.3 Altered neutrophil function

Impaired or dysregulated PMN function may contribute a further mechanistic link between T2DM and periodontitis. The role of PMNs in the maintenance of periodontal health is highlighted by studies that have reported impaired chemotaxis in aggressive forms of periodontitis (Sigusch et al., 2001). Reduced PMN function has also been found in patients with T2DM, including impaired chemotaxis (Mowat and Baum, 1971; Gustke *et al.*, 1998), adherence (Bagdade et al., 1978), and phagocytosis (Marhoffer et al., 1992). Studies have correlated both periodontitis and diabetes with defective PMN chemotaxis; diabetic patients with severe periodontitis had depressed PMN chemotaxis compared to both diabetic patients with mild periodontitis and non-diabetics with mild or severe periodontitis (Manouchehr-Pour *et al.*, 1981; Bissada *et al.*, 1982).

Diabetes may also result in increased periodontal susceptibility via impaired PMN apoptosis (Graves et al., 2006). Although, PMNs are key components of the defence mechanism against periodontal plaque bacteria in periodontitis, if apoptosis is delayed this may lead to increased retention of PMN in the periodontal tissues. This in turn could lead to increased tissue damage by the release of destructive MMPs and ROS by the PMNs.

1.3.2.4 Hyperlipidaemia

In T2DM hyperglycaemia is often accompanied by hyperlipidaemia. As part of the prevention of diabetes related complications, the management of hyperlipidaemia forms a major part of the management of T2DM (NICE, 2008).

Research demonstrates an association between raised serum lipids and periodontitis. Studies have found that subjects with periodontal disease have hyperlipidaemia when compared with subjects with a healthy periodontium (Cutler *et al.*, 1999b; Losche *et al.*, 2000) and patients diagnosed with hyperlipidaemia have significantly higher values of periodontal parameters than control subjects with a normal metabolic status (Noack *et al.*, 2000; Fentoglu *et al.*, 2009). Recently, a small interventional study also demonstrated that improved periodontal health, following periodontal treatment, produced a positive effect on lipid parameters in patients with hyperlipidaemia and periodontitis (Fentoglu et al., 2010).

Hyperlipidaemia is described as one of the factors associated with diabetes-induced immune cell alterations (Iacopino and Cutler, 2000), however, only a few studies have investigated the effect of improved periodontal health on serum lipid profiles in subjects with T2DM (Kiran *et al.*, 2005; Kardesler *et al.*, 2010) .

1.3.2.5 Advanced glycation endproducts

In a hyperglycaemic environment, numerous proteins including collagen undergo a non-enzymatic glycosylation process to form AGEs. There is evidence that supports a role for AGEs via their interaction with cellular AGE receptor (RAGE) in exacerbating diabetic complications including periodontal disease (Jakus and Rietbrock, 2004; Takeda *et al.*, 2006).

Binding of AGE to RAGE on monocytes and macrophages has been associated with an up-regulation of pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-6 (Lalla et al., 2001). Monocytes from patients with diabetes also produce significantly greater amounts of TNF-α, IL-1β and PGE_2 *in vitro* than non-diabetic controls (Salvi *et al.*, 1997a; Salvi *et al.*, 1997c). The presence of RAGE in the gingival tissues has been

confirmed by immunohistochemistry in both diabetic and non-diabetic subjects (Katz et al., 2005).

AGE formation also results in the production of ROS, and AGEs detected in the gingival tissues of diabetic patients have been shown to increase oxidant stress in these tissues when compared to non-diabetic individuals (Schmidt et al., 1996). Enhanced oxidant stress and the subsequent endothelial cell changes that occur may lead to vascular injury common to diabetic complications, including periodontal disease (Schmidt *et al.*, 1994; Vlassara, 2001). AGEs also enhance the respiratory burst of PMNs (Wong et al., 2003). This mechanism may contribute to local tissue damage in the periodontium of patients with T2DM, given the known importance of PMNs in periodontal tissue breakdown resulting from extracellular release of lysosomal contents.

The irreversible nature of AGE formation and the interaction with RAGE creates environments in which cells are constantly exposed to these products, thereby creating heightened inflammatory responses. Studies support the view that AGE-mediated events are of primary importance in the pathogenesis of diabetic complications such as retinopathy, nephropathy, neuropathy and atherosclerosis. They may also be involved in changes within the periodontium, rendering T2DM patients with poor glycaemic control and elevated AGE production more susceptible to periodontal disease. Thus, increased susceptibility to periodontal disease in patients with poorly controlled T2DM may be partly attributed to increased AGE deposition as a result of hyperglycaemia and subsequent increased activation of immune-inflammatory events via RAGE, and also the enhanced respiratory burst in PMNs. This lends further support to the concept of the diabetic hyper-responder who mounts an upregulated periodontal immune-inflammatory response to plaque bacteria, leading to increased tissue destruction.

1.3.3 Association between periodontal health and glycaemic control

In a cross-sectional study, 46 patients with T2DM and chronic periodontitis were studied to determine the relationship between plasma TNF-α levels and clinical periodontal status, GCF levels of IL-1β and HbA1c levels (Engebretson et al., 2007). Plasma TNF-α showed a significant positive correlation with attachment loss (r=0.40, p=0.009) and GCF IL-1β (r=0.33, p=0.035). A dose-response relationship was observed between periodontitis severity and plasma TNF-α (p=0.012) (Engebretson et al., 2007). This study highlighted that chronic periodontitis is associated with TNF-α levels in subjects with T2DM, supporting the hypothesis that periodontal inflammation may contribute to systemic inflammation and insulin resistance seen in patients with T2DM.

To explore the possible dose-response relationship between periodontitis and HbA1c levels, a cross-sectional study assessed HbA1c and the clinical periodontal status in 40 patients with T2DM using PISA (Nesse *et al.*, 2008; Nesse *et al.*, 2009). Traditional definitions of periodontal disease are categorical and the calculation of PISA provides a composite numerical score to define periodontitis whilst also attempting to quantify the inflammatory burden from the periodontal tissues. Multiple linear regression analysis demonstrated a dose-response relationship between PISA and HbA1c in patients with T2DM and a PISA increase of 333 mm^2 was associated with a clinically significant increase in HbA1c level of 1% (Nesse et al., 2009). A prospective treatment study will be required to further explore the impact of periodontal management on PISA and HbA1c.

1.3.4 Periodontal treatment outcomes in people with diabetes

In recent years, a number of studies have published clinical periodontal treatment outcomes in patients with T2DM (Stewart *et al.*, 2001; Kiran *et al.*, 2005; Promsudthi *et al.*, 2005; Jones *et al.*, 2007; Correa *et al.*, 2008; O'Connell *et al.*, 2008;

Dag *et al.*, 2009; Kardesler *et al.*, 2010; Kardesler *et al.*, 2011) (Table 1.7). The clinical treatment outcomes assessed in these studies include reduction in probing depths, gain in clinical attachment and reduction in bleeding on probing and overall, these studies have confirmed that positive clinical periodontal treatment outcomes to non-surgical periodontal management in patients with T2DM (Stewart *et al.*, 2001; Kiran *et al.*, 2005; Promsudthi *et al.*, 2005; Jones *et al.*, 2007; Correa *et al.*, 2008; O'Connell *et al.*, 2008; Dag *et al.*, 2009; Kardesler *et al.*, 2010; Kardesler *et al.*, 2011). Furthermore, studies that evaluated periodontal treatment outcomes in subjects with and without T2DM have confirmed similar improvements in periodontal health for both T2DM and non-diabetic subjects (Correa *et al.*, 2008; Dag *et al.*, 2009; Kardesler *et al.*, 2010; Santos *et al.*, 2010; Kardesler *et al.*, 2011) (Table 1.7).

More recently, composite assessments have also been used to evaluate the periodontal condition of patients with T2DM, incorporating within a numerical score, both periodontal epithelial surface area and BOP (Nesse *et al.*, 2008; Nesse *et al.*, 2009) and thus providing an evaluation of the inflammatory burden produced from the periodontium. However, to date, there are no published data exploring the impact of periodontal therapy on such composite measures.

1.3.5 Impact of periodontal treatment on inflammation in T2DM

Periodontal inflammation may contribute to the systemic inflammatory component of insulin resistance and T2DM. Furthermore, alterations in immunologically active molecules as a result of T2DM may alter the cytokine network within the periodontium and thus contribute to local periodontal tissue destruction (Preshaw et al., 2007).

Recently, a number of small prospective treatment studies have begun to document the impact of periodontal therapy on markers of inflammation, with some

studies evaluating local cytokine levels (Correa *et al.*, 2008; Santos *et al.*, 2010; Kardesler *et al.*, 2011) and other studies evaluating circulating cytokine levels (Dag *et al.*, 2009; Correa *et al.*, 2010; Kardesler *et al.*, 2010) (Table 1.7). However, to date, the impact of periodontal therapy on both local and systemic cytokines has not been investigated within a study (Table 1.7).

The impact of periodontal management on serum levels of multiple mediators was assessed in a prospective treatment study of patients with T2DM (n=30), with a significant reduction in IL-6 levels were demonstrated following treatment, but not for other relevant mediators, such as TNF-α, IL-1β (O'Connell et al., 2008). As part of a small prospective treatment study, 13 subjects with T2DM were provided with mechanical periodontal debridement and local delivery of minocycline for 4 weeks. Serum TNF-α levels pre- and post-treatment were then assessed. Following treatment a significant reduction in serum TNF-α was demonstrated ($p<0.015$) (Iwamoto et al., 2001). This study also showed a strong correlation between reduction in serum TNF-α concentration and in the reduction in HbA1c following periodontal treatment, with the authors concluding the improvements in metabolic control following the periodontal treatment may possibly be mediated by reduced serum TNF-α (Iwamoto et al., 2001). Recently, a small prospective treatment study assessed the impact of periodontal therapy on systemic inflammatory mediators. In 13 well controlled (HbA1c <7%) and 12 poorly controlled (HbA1c ≥7%) patients with T2DM and 15 non-diabetic subjects, serum levels of TNF-α, IL-6 and CRP were quantified by ELISA before and then 1and 3 months after non-surgical periodontal management (Kardesler et al., 2010). At 3 months post-treatment, the only significant reduction was in the serum IL-6 levels in well controlled patients with diabetes and non-diabetics. Interestingly, this was also the first study to publish the impact of periodontal management on serum levels of 2

adipokines, leptin and adiponectin. Compared to pre-treatment levels, the non-diabetic group had increased adiponectin levels at month 3 ($p<0.05$) and leptin levels were increased in well-controlled patients with diabetes at month 1 ($p<0.05$) (Kardesler et al., 2010). In a similar study, the impact of periodontal therapy on serum TNF-α levels was assessed in T2DM and non-diabetic subjects, and a significant reduction in serum TNF-α was demonstrated in both groups at 3 months post periodontal treatment compared to baseline levels (Dag et al., 2009).

The impact of non-surgical periodontal therapy on levels of IL-1β and MMP-8 and -9 was assessed in patients with (n=23) and without (n=26) T2DM. Following periodontal therapy, a significant reduction in GCF volume and the total amount of IL-1β and MMP-8 and -9 was seen in deep sites and significant reductions in GCF volume and the total amount of IL-1β were seen in shallow sites. No significant differences in total amount of the mediators in both deep and shallow sites were found between diabetes and non-diabetic controls at baseline and follow-up (Correa et al., 2008). The reduction in GCF IL-1β levels following periodontal treatment would confirm data from earlier studies investigating non-diabetics (Engebretson et al., 2002).

Given the wide clinical use of CRP to quantify systemic inflammatory state and assess the related cardiovascular risk, it is surprising that few studies document the impact of periodontal therapy on CRP levels in patients with T2DM. In a small pilot study hsCRP levels were assessed before and 4 weeks after non-surgical periodontal therapy in subjects with diabetes (n=10) and showed that following treatment, mean hsCRP levels were significantly decreased from 2.3 to 1.5 mg/L ($p<0.01$) (Lalla et al., 2007). Unfortunately, a lack of non-diabetic control group precludes conclusions being drawn about the importance of diabetes. In a further study of 33 patients with diabetes and 20 non-diabetic controls, there were no significant differences found in the median

CRP values either before or 4 months after periodontal management (Christgau et al., 1998). Both of these studies are limited by the inclusion of both T2DM and T1DM patients, given their differing pathology. In a more recent study of 23 patients with T2DM, a non-significant decrease in hsCRP was found following improved periodontal health (Correa et al., 2010) however, interpretation of this result is difficult given the lack of a non-diabetic control group.

Among the prospective treatment studies investigating the impact of periodontal therapy on cytokine levels, there is considerable heterogeneity in terms of the methodology. For example, the inclusion criteria for periodontal case definition and the choice of cytokines studied lacks consistency and varying sampling and elution methods are used for GCF. Drawing simple, clear conclusions from these studies is therefore difficult. As a general rule, many of these studies have used very small numbers of subjects, and focussed on one or two mediators at a time which limits our understanding of the complexity of cytokine networks is important in both T2DM and periodontitis. Furthermore, none of the studies have simultaneously assessed local and systemic inflammatory cytokine levels following periodontal therapy and thus the conclusions that can be drawn from the literature are compromised.

The interplay between local inflammation within the periodontal tissues, systemic inflammation and glycaemic control is complex and not fully understood. To clarify the impact of periodontal treatment on systemic inflammation in patients with T2DM, further studies are required to assess glycaemic control, CRP and explore the relevance of relevant cytokines and adipokines both locally within the GCF and systemically.

1.3.6 The impact of periodontal treatment on glycaemic control and lipid profiles

In addition to the observational evidence highlighting the relationship between periodontal disease and T2DM, increasing numbers of interventional studies have been conducted to explore the effects of periodontal disease on glycaemic control in patients with diabetes. In patients with T2DM the impact of periodontal management on glycaemic control, as measured by HbA1c, has been investigated using controlled and non-controlled interventional studies (Table 1.7).

A randomized clinical trial was carried out in the USA to study the efficacy of periodontal management on glycaemic control of 165 veterans over 4 months (Jones et al., 2007). HbA1c was the main outcome measure when comparing the treatment group (n=82), that received periodontal instrumentation, a 2 week course of systemic doxycycline and daily chlorhexidine rinse, against the control group (n=83) that received no periodontal management as part of the research. To enable improvements in glycaemic control to be studied, only subjects with poorly controlled diabetes, indicated by a HbA1c score of ≥8.5%, were included. Mean baseline HbA1c levels were 10.2% and 9.9% for control and treatment group respectively. However, 4 month mean HbA1c data for each group were not published. Between group differences in the mean change in HbA1c of the study period were not significant, even after controlling for baseline HbA1c, duration of diabetes and age. The number of subjects achieving clinically significant reductions in HbA1c of 0.5% and 1% (Stratton et al., 2000) was not significantly different between groups. Therefore, the study concluded that no significant benefit in glycaemic control was found at 4 months post-periodontal therapy. Unfortunately, the inclusion criteria were based on subjects having a community periodontal index of treatment need (CPITN) score of ≥3 in at least two sextants.

Furthermore, despite the central role of periodontal inflammation in the biological rationale supporting periodontal management and glycaemic control, data to clarify the periodontal status of each group were not published. This precludes conclusions being made about the relative success of periodontal management and its influence on glycaemic control.

In a smaller randomised control trial (RCT), the effect of improved periodontal health on metabolic control was investigated in 44 subjects with T2DM (Kiran et al., 2005). Between baseline and the 3 month review, HbA1c levels in the treatment group showed a significant decrease whereas the control group had a non-significant increase in HbA1c. Caution on the interpretation of this result must be used since only subjects with HbA1c values of 6-8% were included. Therefore, excluding subjects with poor glycaemic control and artificially restricting the standard deviations of the mean values for each group, could potentially have enabled significant differences to be achieved more easily. Additional metabolic data including triglyceride, total cholesterol, high density lipoproteins and low density lipoproteins showed no significant changes following periodontal treatment. The study also describes significant improvement in plaque index (PI), gingival index (GI), probing depths (PD), LOA and BOP in the treatment group but not in the control group. However, only mean periodontal data were presented, thus simplifying and missing valuable PD, recession or LOA data and failing to adequately describe the tissue response occurring following periodontal treatment. Therefore, although the study concludes that non-surgical periodontal treatment is associated with improved glycaemic control in patients with T2DM, the limitations of the study in reporting periodontal data and using restricted HbA1c values as inclusion criteria weaken this conclusion (Kiran et al., 2005).

Consideration of additional intervention studies both controlled (Stewart *et al.*, 2001; Promsudthi *et al.*, 2005; O'Connell *et al.*, 2008) and non-controlled (Grossi *et al.*, 1997; Iwamoto *et al.*, 2001) also demonstrate inconsistent results regarding the impact of periodontal management on HbA1c. More recently, meta-analyses have been conducted in an attempt to clarify whether periodontal treatment has an effect on glycaemic control (Janket *et al.*, 2005; Darre *et al.*, 2008; Simpson *et al.*, 2010). In the earlier meta-analysis of 10 intervention studies to quantify the effects of periodontal therapy on HbA1c, a weighted mean HbA1c decrease of 0.66% was observed, though this did not achieve statistical significance (Janket et al., 2005). Darré et al used a systematic review to identify 9 controlled interventional studies, providing a total of 485 patients, to be included into the meta-analysis (Darre et al., 2008). This meta-analysis indicated that periodontal treatment effectively reduced HbA1c levels with a calculated standardised mean difference (SMD) in HbA1c of 0.46 which was found to be significant (95% confidence interval (CI), 0.11, 0.82; p=0.01). Additionally, when the SDM was back-transformed, by multiplying by it by a 'typical' standard deviation (SD), into the original HbA1c units, the findings suggested that periodontal treatment could lead to a clinically significant 0.79% reduction in HbA1c values. However, the authors expressed caution regarding the interpretation of this result following the finding that exclusion of a single study (Stewart et al., 2001) decreased the SMD to a non-significant value of 0.27 (95% CI, -0.01, 0.60).

In the most recent, meta-analysis, the effect of the mean % difference in HbA1c for NSM, with or without antibiotic therapy, against no treatment or usual treatment was -0.40% (95% CI, -0.78, 0.01), representing a statistically significant reduction in HbA1c (p=0.04) for NSM and the authors concluded that some evidence exists for improvements in metabolic control in people with diabetes after periodontal therapy,

although larger studies are required to fully understand the potential of periodontal therapy to improve glycaemic control in people with diabetes (Simpson *et al.*, 2010).

Clearly, there is a lack of conclusive evidence to recommend periodontal management as an effective means to reduce HbA1c levels in people with diabetes. Darré and co-workers highlighted that a RCT with sufficient statistical power would be invaluable, and their post-hoc calculation showed that a minimum sample size of 150 participants would be required (Darre *et al.*, 2008; Garcia, 2009). Subsequently, a study protocol for a randomised, controlled trial has recently been published (Vergnes et al., 2009). This study aims to assess whether periodontal treatment could lead to a decrease in HbA1c in diabetic patients with periodontitis. The target sample size is 150 participants, with half in the immediate treatment group and half in a delayed treatment group. Periodontal treatment will include NSM with systemic antibiotic therapy and chlorhexidine mouthwash. The difference in change of HbA1c and quality of life between the two groups will be assessed at a 13 week follow-up appointment.

In patients with T2DM, the effective management of dyslipidaemia, including raised serum levels of total cholesterol, low density lipoproteins (LDL) and triglycerides (TG) is an integral part of diabetic management. It has been proposed that dyslipdaemia plays an integral part, along with systemic inflammation, in the relationship between periodontal tissue inflammation and diabetes (Iacopino and Cutler, 2000). However, studies that investigated the impact of periodontal management on lipid profiles in patients with periodontitis and T2DM have failed to demonstrate significant changes in lipid levels (Kiran *et al.*, 2005; Kardesler *et al.*, 2010).

Table 1.7 Studies investigating the impact of periodontal therapy on periodontal health, glycaemic control and cytokine levels

Author, Year	Sample size (n)	Duration (weeks)	Treatment provided	Changes in periodontal health	Changes in cytokines	Changes in HbA1c
(Kardesler *et al.*, 2011)	T2DM: 20 non-diabetic: 22	12	NSM	Significant improvement in PI, GI, PD, CAL and BOP for both groups.	A non-significant significant reduction in total amount of IL-6 in GCF in T2DM and non-diabetic subjects (data values not published)	T2DM reported a significant reduction in HbA1c in T2DM subjects (data values not published)
(Santos *et al.*, 2010)	Well controlled T2DM: 18 Poorly controlled T2DM: 20	24	NSM	Significant improvement in PI, GI, PD, CAL and BOP for both groups.	No significant change in total amount of TNF-α, IL-4, IL-17 and IL-23 in GCF in both well and poorly controlled T2DM subjects. Total amount of INF-γ in GCF showed a significant increased in well controlled T2DM but no change in poorly controlled T2DM subjects (data values not published)	Mean HbA1c showed a non-significant increase of 0.3% and 1.3 % in poorly and well controlled T2DM subjects respectively
(Correa *et al.*, 2010)	T2DM: 23	12	NSM	Significant improvement in PI, GI, PD, CAL and BOP for both groups	Median plasma TNF-α levels showed a significant reduction of 1.1pg/ml. Plasma IL-4, IL-6, IL-8 & Il-10 levels showed non-significant reductions of 3.7, 0.8, 0.6, 3.3pg/ml respectively.	Non significant reduction in median HbA1c of 0.4%
(Kardesler *et al.*, 2010)	Well controlled T2DM: 13 Poorly controlled T2DM: 12 Non-diabetic: 15	12	NSM	Similar improvements in PI, PD, CAL and BOP for all groups for	Serum TNF-α levels showed non-significant reductions in all groups. Serum IL-6 levels showed a significant reduction in well controlled T2DM and non-diabetic subjects and a non-significant reduction in poorly controlled T2DM subjects. (data values not published)	Poorly controlled T2DM showed a significant reduction in HbA1c 3 months after periodontal treatment ($P<0.05$)

(Dag *et al.*, 2009)	Well controlled T2DM: 15 Poorly controlled T2DM: 15 Non-diabetic: 15	12	NSM	Significant improvement in PI, GI, PD, CAL and BOP for all groups	Mean serum TNF-α levels showed a significant reduction of 2.1, 2.17 and 2.70pg/ml in well controlled T2DM, poorly controlled T2DM and non-diabetic subjects respectively	Mean HbA1c showed a significant reduction of 0.21% in well controlled T2DM subjects and a non-significant reduction of 0.19% in poorly controlled T2DM subjects
(Correa et al., 2008)	T2DM: 23 non-diabetic: 26	13	NSM	Significant improvement in PI, GI, PD, CAL and BOP for both groups.	Median total amount of IL-1β showed significant reduction of 83.4 and 38.7pg/ml in T2DM and non-diabetic subjects respectively	In T2DM group, following periodontal treatment the mean HbA1c showed a non significant reduction of 0.31± 1.81%
(O'Connell et al., 2008)	treatment A: 15 treatment B: 15	12	NSM +/- doxycycline	At follow-up significant improvement in PI, GI, PD, CAL and BOP for both treatment groups. No significant difference between groups.	Multiple serum cytokines levels assessed (n=30). Significant reduction in mean serum IL-6 levels of 1pg/ml. No change in serum TNF-α and IL-1β levels and a non-significant increase in serum IFN-γ.	Following treatment a significant reduction in HbA1c of 1.5% for the group with doxycycline. No significant difference between groups.
(Jones et al., 2007)	treatment: 82 usual care: 83	16	NSM + doxycycline	Data not published	Data not published	Both groups had mean HbA1c reduction that was not significant. Between group difference in HbA1c was clinically and statistically not significant. Number of subjects achieving reductions in HbA1c of 0.5% and 1% was not significantly different between groups.
(Kiran et al., 2005)	treatment: 22 no treatment: 22	12	NSM	At follow-up significant improvement in PI, GI, PD, CAL and BOP for treatment group	Data not published	HbA1c levels in the treatment group decreased significantly from 7.31 ± 0.74 to 6.51 ± 0.80, whereas the control group showed a slight but insignificant increase in HbA1c.

(Promsudthi et al., 2005)	treatment: 27 no treatment: 25	12	NSM+ doxycycline	At follow-up significant improvements in PI, PD, CAL and BOP for treatment group with shallower PD and less CAL compared to the control group	Data not published	Following treatment, the reduction in HbA1c did not reach significance. Although more patients in the treatment group had a decrease in HbA1c this difference was not significant.
(Rodrigues et al., 2003)	treatment A: 15 treatment B: 15	12	NSM+/- amoxicillin	Following treatment a significant reduction in PD, BOP and PI was recorded in both groups. No significant changes in CAL.	Data not published	HbA1c reduced in both groups, although only changes in group receiving instrumentation without antibiotics reached significance
(Stewart et al., 2001)	treatment: 36 control: 36	43.5	NSM	Periodontal treatment outcomes not published and periodontal status of control group not known	Data not published	Statistically significant reductions in HbA1c in both the treatment group (from 9.5% to 7.6%) and control group (from 8.5% to 7.7%).
(Iwamoto et al., 2001)	treatment: 13	4	NSM +local minocycline	No significant change in mean PD following treatment. Further periodontal data not published.	Data not published	Following periodontal treatment HbA1c levels reduced significantly 7.96 ± 1.94 to 7.12 ± 1.48

Table shows and overview of interventional studies investigating changes in glycaemic control, periodontal health and cytokine levels following periodontal management in patients with T2DM

1.4 Aims of the study

1. To clinically characterise the periodontal status of a local North East population of patients with T2DM by measuring the extent and severity of periodontal disease.

2. To explore the local levels in GCF and saliva of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) in patients with T2DM compared to non-diabetic patients.

3. To explore the systemic levels in serum of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) in patients with T2DM compared to non-diabetic patients.

4. To investigate associations between clinical periodontal status, inflammatory cytokine levels and markers of diabetes control.

5. To investigate the clinical periodontal healing in patients with T2DM and periodontitis following NSM and evaluate the impact of improved periodontal health on markers of diabetes control and local and systemic inflammatory cytokine levels.

Chapter 2 Materials and methods

2.1 Ethical approval

Ethical approval for this research was obtained from Sunderland Local Research Ethics Committee (ref 06/Q0904/8). The application included a protocol of the study that highlighted particular ethical issues pertaining to the study, with the main ethical problems being related to the samples that were collected (GCF, saliva, blood) which were purely for research purposes and would otherwise not be collected. This was made clear to prospective participants in the information sheet. Whereas collection of GCF and saliva samples was non-invasive, quick and painless, collection of blood samples could be associated with discomfort and there was the potential for unwanted events (e.g. bruising). The risks of venepuncture were minimised by the use of trained, experienced clinicians to obtain the blood samples. The periodontal examinations and treatment that was provided as part of the study constituted routine clinical care and one positive aspect of the study was that the T2DM patients received an oral and periodontal examination, and if they are found to have periodontal disease, treatment was offered. Patients were also given information and instruction on how to better maintain good oral and periodontal health. All samples collected and all data recorded were stored anonymously, using a coding system. No information that was generated as part of this research had an impact on the patients' clinical care and no feedback of the results of analysis of individual samples was given to individual patients.

2.2 Patient recruitment and discharge

T2DM patients were recruited from both secondary care databases, held by Dr. Weaver at the Queen Elizabeth Hospital in Gateshead and Professor Roy Taylor at Newcastle University, and databases held within primary care settings, such as general

medical practices that are part of the North East Diabetes Research Network. The information held on the databases in both primary and secondary care settings, allowed T2DM patients matching the age criteria to be identified. These patients were sent a letter informing them about the research and inviting them to participate. Patient participation was on an 'opt-in' basis. If patients then contacted the research team to indicate that they would like to participate, a short telephone screening took place to ensure they fitted within the inclusion and exclusion criteria. Patients were then sent the full information sheet to read, and arrangements were made for them to attend the Dental Hospital for the pre-treatment screening appointment. Recruitment of non-diabetic patients involved the identification of suitable patients from either those referred from general dental practice into the restorative department within the School of Dental Sciences or patients seen on student treatment clinics within the School of Dental Sciences. Each non-diabetic subject was matched to a previously recruited T2DM subject. Subjects were matched based on age (within 5 years), gender, smoking status and periodontal diagnosis. Suitable non-diabetic subjects were approached during an appointment at the dental hospital, informed about the study and given the opportunity to decide whether to participate.

Patients recruited fulfilled the following inclusion criteria: 30-55 years old, male or female, with a minimum of 20 natural teeth. Exclusion criteria included pregnancy, immunosuppression, any condition requiring prophylactic antibiotics prior to dental treatment, bleeding disorders, prolonged bleeding as a result of medication, drug-induced gingival overgrowth, any medical condition that could compromise safe participation in the study, or any patient who had undergone non-surgical therapy for periodontal disease in the last 6 weeks.

At the pre-treatment screening appointment, patients who were identified as having periodontal disease were offered the necessary treatment and these patients were monitored as part of the longitudinal component of the study. The patient's general medical practitioner (GMP) and general dental practitioner (GDP) were informed via letter of their involvement in the research. Following completion of the study, patients were discharged back to their GDP for long-term periodontal maintenance care, with a written discharge letter that detailed the maintenance plan that the GDP should follow. For those patients identified at the screening appointment as not having periodontal disease, their participation in the study was limited to the screening visit only. For these patients without periodontal disease they were offered oral hygiene advice and a prophylaxis and this was provided at the screening appointment. If the clinical examination identified other dental or oral problems, such as caries, appropriate management was arranged within general dental practice or in the dental hospital, for patients not registered with a GDP.

2.3 Consent

Written informed consent was obtained when the patient first attended, at the start of the pre-treatment screening appointment. This process involved a clinician confirming that the patient had received and understood the written information about the study. The clinician then verbally confirmed the background and aims of the study as well as the potential benefits and risks of participation in the study. Each subject had the opportunity to ask questions and not to participate in the study. Subjects wishing to participate in the study then signed two copies of the consent forms, one copy was retained within the patient's individual case report form and one copy was given to the patient.

2.4 Power calculation and estimation of sample size

Providing a definitive power calculation at the planning stage of the study was difficult given the paucity of studies that had investigated this area at that time. Using data (Kiran et al., 2005) from a study published at the time of planning, a power calculation provided an estimate of the number of patients required with periodontitis for detecting significant changes in HbA1c over 12 months. Therefore, to provide a 85% power for detecting significant changes in HbA1c over 12 months, assuming α=0.05, δ=0.6% and σ=1.0%, it was estimated that 30 patients with periodontitis and diabetes would be required to be followed for the duration of the study. To compensate for an estimated 20 % drop out of subjects, the aim was to recruit 36 T2DM subjects with periodontitis into the study. Using an estimate of the prevalence of periodontitis in diabetic populations of approximately 30% (Nelson et al., 1990), it was estimated that 140 T2DM subjects would be required to be screened in order to identify 36 T2DM patients with periodontitis.

2.5 Periodontal disease case definition

At the first visit (pre-treatment screening), all subjects received a full periodontal examination, including PI, modified Gingival Index (mGI), PD, recession and BOP. Radiographs were obtained as clinically indicated, and clinical and radiographic examinations were used to confirm the periodontal diagnosis based on diagnostic criteria (Table 2.1). Difficult diagnoses were resolved by discussion between two clinicians.

Table 2.1 Periodontal Diagnostic Criteria

Healthy Periodontium	BOP ≤ 15% No probing depth sites >4mm No attachment loss – disregard localised recession (e.g. due to tooth brush trauma) No bone loss
Gingivitis	BOP > 15% No sites with probing depths > 4mm, except for up to 5 sites with 5mm probing depths (e.g. at distal surface of last standing molars) No attachment loss - disregard localised recession (e.g. due to tooth brush trauma) No bone loss
Periodontitis	≥ 6 sites with probing depths of ≥ 5mm Loss of attachment and / or bone loss

Robust case definitions for periodontal status are required to avoid misclassification of subjects. Table shows the case definitions used in the current study to define periodontitis, gingivitis and healthy periodontium.

2.6 Clinical protocol

2.6.1 Plaque scores

The plaque index (PI) of Silness and Loe was used (Silness and Loe, 1964) as follows:

0 No plaque.

1 A film of plaque adhering to the free gingival margin and the adjacent area of the tooth. The plaque may be seen in situ only after application of disclosing solution or by using the probe on the tooth surface.

2 Moderate accumulation of soft deposits within the gingival pocket, or the tooth and gingival margin which can be seen with the naked eye.

3 Abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin.

The tooth was dried with a gentle stream of air and disclosing solution was not used. Visual examination was sufficient to identify sites with a score of 2 or 3. If no plaque could be seen with the unaided eye, the probe was swept along the gingival margin to differentiate between a score of 1 (if plaque was present on the probe tip) or 0 (with no plaque present). The presence or absence of plaque was recorded at 6 sites per tooth on the four target teeth from which GCF samples were taken. The scores were recorded immediately by an assistant (as were gingival scores, probing depths, recession and bleeding scores).

2.6.2 Modified Gingival Index

The modified gingival index (mGI) (Lobene et al., 1986) was used to clinically assess gingival inflammation as follows:

0 Absence of inflammation.

1 Mild inflammation; slight change in colour, little change in texture of any portion but not the entire gingival margin or papillary gingival unit.

2 Mild inflammation; criteria as above but involving the entire marginal or papillary gingival unit.

3 Moderate inflammation; glazing, redness, oedema and/or hypertrophy of the marginal or papillary gingival unit.

4 Severe inflammation; marked redness, oedema and/or hypertrophy of the marginal or papillary gingival unit, spontaneous bleeding, congestion or ulceration.

The gingival tissue was dried with a gentle stream of air. Visual examination was used to allocate a score at 6 sites per tooth on the four target teeth, from which GCF samples were taken. A score of 0 was allocated if no inflammation was seen and a score of 4 was given if the tissues were severely inflamed displaying spontaneous bleeding, ulceration or marked swelling/redness. A score of 1 was given if only part of the area was inflamed. Scores 2 and 3 indicated mild and moderate inflammation that affected the entire area.

2.6.3 Probing depth

The University of North Carolina (UNC) 15 probe (Dentsply, Addlestone, UK) was used to measure probing depths (PD), taken as the distance from the gingival margin to the base of the pocket. The probe was inserted into the pocket and advanced apically, along the long axis of the tooth, until the resistance of the gingival tissue at the base of the pocket was felt. The probing depth recording was made in mm by direct visualisation of the probe markings. Probing depths were recorded at 6 sites per tooth for all teeth present, excluding the 3rd molars.

2.6.4 Bleeding on probing

Following the completion of probing depths within one aspect of a quadrant (for example, buccal aspect of the upper right quadrant) the probing sites were re-examined to determine whether post-probing bleeding occurred. Bleeding status was recorded dichotomously at 6 sites per tooth depending on the presence or absence of bleeding from the base of the pocket following probing.

2.6.5 Recession

The UNC 15 probe was used to measure recession, taken as the distance from the cemento-enamel junction (CEJ) to the gingival margin. Recession was measured whilst the probe was inserted into the pocket during the probing depth assessment. When the CEJ could be seen above the gingival margin, the recession recording was made in mm by direct visualisation of the probing markings. When the CEJ was below the gingival margin, the trained clinician estimated the position of the CEJ in relation to the gingival margin and made a negative recording in mm. Recession was recorded at 6 sites per tooth for all teeth present, excluding the 3rd molars.

2.6.6 Loss of attachment

The value for loss of attachment (LOA) was the sum of probing depth and recession, and is therefore, the distance from the CEJ to the base of the pocket. LOA was calculated at 6 sites per tooth for all teeth present, excluding the 3rd molars.

2.6.7 Periodontal epithelium surface area and periodontal inflamed surface area

Using a previously published Excel spread sheet, PESA (mm^2) and PISA (mm^2) were calculated for each subject (Nesse et al., 2008). Firstly, LOA and recession values at 6 sites per tooth were entered, allowing PESA for each tooth to be calculated. The

PESA for each tooth was multiplied by the proportion of sites around the tooth that was affected by BOP to calculate the PISA for each tooth. The sum of all individual PISAs around individual teeth gave the total PISA (mm^2) within each subject's mouth and similarly the sum of all individual PESA gave total PESA (mm^2).

2.6.8 Smoking status

Pre-treatment and at month 12 smoking habits were assessed, according to whether the subjects were current, non, or ex-smokers and the smoking extent of the current and ex-smokers further quantified according to the standardised measure of pack years. This equates the number of cigarettes smoked by each patient to the number of pack years smoked, a pack year being equal to smoking 1 pack of 20 cigarettes per day for 1 year.

2.6.9 Demographic data

At the pre-treatment screening visit, demographic data including age and gender was recorded. Socio-economic status was assessed using index of multiple deprivation (IMD). A number of indicators, covering a range of economic, social and housing issues are combined to produce a single IMD each small area in England. Each area can also be ranked relative to another according to their level of deprivation. The Office of National Statistics' online neighbourhood statistics tool was used to calculate IMD from a subject's 6 digit postcode.

2.6.10 Diabetes history

For all subjects with T2DM a diabetes history was taken at the screening visit. This included years since diagnosis, age at diagnosis, family history of diabetes, method of diabetes control, level of glycaemic control in the last year (good/moderate/poor), presence of macro- and microvascular diabetes complications and medications.

2.6.11 Physical examination

At the pre-treatment screening, month 6 and month 12 visits, subject's blood pressure was recorded using the subject's right upper arm, with the patient seated using an automated machine. Height and weight were also recorded allowing body mass index (BMI) to be calculated by dividing the weight (in kilograms) by the square of the height (in metres).

2.6.12 Oral examination

At the pre-treatment screening visit, oral soft tissues were examined to identify any lesions, oral candidosis or xerostomia. Denture use was also recorded. The dentition was then examined, teeth were assessed on a pre-defined dental examination criteria (Kelly, 2000), recording the number of teeth in each category (Table 2.2).

2.6.13 Oral health behaviour

At the pre-treatment screening appointment, subjects were asked closed questions regarding oral health behaviour. Questions included frequency of tooth brushing and interproximal cleaning as well as timing and reason for last dental visit.

Table 2.2 Diagnostic criteria for hard tissue examination

Sound & Untreated	No evidence of caries into dentine or restorations Including caries restricted to enamel
Restored (1 to 3 surfaces)	Amalgam, composite, GIC, F/S, onlays, inlays and ¾ crowns – up to and including 3 surfaces Including veneers, shims and adhesive retainers for resin retained bridges
Extensively Restored (4 or more surfaces)	Amalgam, composite, GIC, onlays or inlays – 4 surfaces or more Permanent or temporary crowns, including full coverage crowns and conventional bridge abutments
Carious	Visual – usually manifests as shadowing under an occlusal surface or marginal ridge Cavitated – but no pulpal involvement Includes temporary dressing placed for treatment of caries
Broken down / pulpal involvement	Teeth so broken down that it is inconceivable that there is not pulpal involvement

The hard tissues were examined and assessed against on a pre-defined dental examination criteria (Kelly, 2000)

2.6.14 Non-surgical periodontal management and follow-up

Following pre-treatment screening, comprehensive targeted non-surgical periodontal therapy was provided for subjects with periodontitis (the treatment day constituted the baseline time point, and was within 2 months of the pre-treatment screening appointment), using oral hygiene instruction (OHI) and a full mouth instrumentation (FMI) approach (Quirynen et al., 2000). A combination of ultrasonic instrumentation, using a Cavitron Select machine (Dentsply, Addlestone, UK), and hand instrumentation, using flexichange scalers and gracey curettes (Dentsply, Addlestone, UK), was used. Pockets were irrigated with 0.2% chlorhexidine gluconate (Kent express, Kent, UK). Effective tooth brushing and interproximal cleaning was demonstrated to the patient.

The subjects were then seen at week 3 and week 6 after initial treatment for OHI and further prophylaxes to disrupt reforming plaque deposits. For these subjects, a periodontal examination was subsequently undertaken at 3, 6 and 12 months after the initial instrumentation to evaluate periodontal healing following the treatment. Additional periodontal treatment to eliminate periodontal inflammation was undertaken at months 3, 6 and 12 months as clinically indicated (Figure 2.1). Pre-treatment screening was undertaken on all participants, however, only those subjects with periodontitis proceeded beyond screening. At the pre-treatment screening appointment, subjects with gingivitis received oral hygiene instruction and full mouth instrumentation and prophylaxis, as required. Patients with gingivitis or healthy periodontal tissues were not followed up after the screening visit.

2.6.15 Data collection & storage

Patients were allocated a subject identification number and each patient had a case report form (CRF) in which all clinical periodontal data were recorded at the time of examination by the clinician or an assistant. The results from the clinical biochemistry laboratory were also recorded within the patient's CRF.

2.7 Statistical analysis

Statistical analyses were conducted using the statistical software SPSS 15. All variables were assessed for normality using the Kolmogorov-Smirnov test, supplemented with histograms. Where there was no evidence to reject normality, means and standard deviations (SD) of these parametric variables were calculated. Where the assumption of normality was rejected, medians and interquartile ranges (IQR) of these non-parametric variables were calculated. Discrete variables were analysed using chi-squared tests. The significance of all tests was assessed at the 5%.

Cross-sectional pre-treatment data (categorised based on diabetes status) were analysed with independent samples t-tests or Mann-Whitney tests for parametric or non-parametric variables, respectively. Cross-sectional data (categorised based on periodontal status and diabetes status) were analysed with one-way analysis of variance (ANOVA) or the Kruskal-Wallis test for parametric and non-parametric variables, respectively. Independent samples t-tests or Mann-Whitney tests were applied for *post hoc* analysis of parametric or non-parametric data, respectively. A P-value of <0.05 was considered significant. P-values were corrected for multiple comparisons with the Bonferroni-Holm test. The associations between cytokine levels and clinical data were assessed using Pearson's correlation coefficients (r), if both variables were normally

distributed, or Spearman's correlation coefficients (rho), if both variables were not normally distributed. Scatter diagrams were constructed to illustrate these associations.

Longitudinal non-parametric data were analysed with the Friedman test, with the Wilcoxon Mann-Whitney test applied for *post hoc* analyses. Longitudinal parametric data were analysed with repeated measures ANOVA and paired samples t-tests for post hoc analyses. A p-value of <0.05 was considered significant. P-values were corrected for multiple comparisons with the Bonferroni-Holm test. At each time-point, for subjects with periodontitis only, cross-sectional data were analysed using independent samples t-tests or Mann-Whitney tests for parametric or non-parametric data, respectively. Also, to evaluate the impact of any pre-treatment differences between subjects with T2DM and non-diabetic subjects, analysis of covariance (ANCOVA) was performed, using diabetes status as a factor and pre-treatment parameter as covariate to reveal whether adjusted month 3, month 6 and month 12 parameters still differed significantly between subjects with and without diabetes.

Multinominal logistic regression analyses were applied to test the value of PISA as a predictor of periodontal status categorised as periodontal health, gingivitis or periodontitis. Linear regression analyses were used to determine the ability of cytokine levels in GCF and saliva to predict PISA.

Figure 2.1 Overview of study protocol

		Pre-treatment screening	Treatment* (month 0)	Week 3*	Week 6*	Month 3*	Month 6*	Month 12*
Informed consent		●						
Demographic data		●						
Medical history		●					●	●
Diabetes history #		●						
Smoking status		●						
Physical exam		●					●	●
Oral health history		●						
Oral examination		●		●	●	●	●	●
GCF samples		●				●	●	●
Saliva samples		●				●	●	●
Periodontal examination	PI	●				●	●	●
	mGI	●				●	●	●
	PD	●				●	●	●
	Recession	●				●	●	●
	BOP	●				●	●	●
Blood samples	HbA1c	●				●	●	●
	hsCRP	●				●	●	●
	Lipids	●				●	●	●
Initial periodontal therapy	OHI & FMI		●					
Prophylaxis				●	●	●	●	●
Additional periodontal therapy	(As required)					●	●	●

Table shows an overview of the study protocol to highlight the procedures undertaken at each time point
not used in non-diabetic subjects
* only in subjects with periodontitis

2.8 Sampling, elution and storage of GCF

GCF was sampled with Periopaper strips (Oraflow Inc, New York) and the volume quantified using a calibrated Periotron 6000 (Preshaw et al., 1996). Prior to use, the Periotron was allowed to 'warm up' according to the manufacturers' instructions, and then zeroed with a blank (dry) Periopaper. The reading dial was adjusted until the digital display indicated zero.

To minimise contamination by blood, GCF samples were collected prior to periodontal probing. At screening, 4 GCF samples were collected from each patient, from the mesio-buccal aspects of the four 1st molars. If the 1st molar was absent in a quadrant, the 2nd molar was used, then the 2nd premolar, then the 1st premolar, then the canine or incisor teeth (the sampled teeth were designated target teeth). The site was isolated with cotton rolls and a saliva ejector, and dried with a gentle stream of air. Supragingival plaque, if present, was carefully removed with a curette prior to sampling. A Periopaper was placed carefully into the sulcus until mild resistance was felt and was held there for 30 seconds.

The paper was transferred immediately to the jaws of the Periotron to minimise evaporation errors. Care was taken to ensure that Periopapers were positioned in a standardised position between the jaws, so that the black line on the paper was at the outer rim of the jaw plate. After 'mode II' illuminated on the Periotron display, the GCF volume (in Periotron units) was recorded by an assistant. The Periopaper was then placed into a sterile plastic 0.5ml micro-tube (Sarstedt, Leicester, UK) containing 150µl autoclaved and filtered phosphate buffered saline (PBS). Each GCF sample was stored in a separate micro tube and each tube was labelled with the patient's study number, tooth number and date. Between samples, the jaws of the Periotron were cleaned with

an alcohol swab and allowed to dry. The GCF samples were kept on ice at the chair side and transferred, within 20 minutes of sampling, to the laboratory and frozen at -80^{o}C (Cutler et al., 1999a) to await subsequent elution & analysis.

This process was used to collect GCF samples from the same 4 sites at months 3, 6 and 12 in those patients who were diagnosed with periodontal disease and who were entered into the longitudinal phase of the study.

Elution of GCF from the Periopaper was carried out on the same day the samples were analysed used the Multi-spot cytokine assay. All samples were thawed on ice for 15 minutes and then 50µl of 1% bovine serum albumin (BSA) was added. GCF samples were centrifuged (Sigma 3K10 centrifuge) at 300rpm for 60 minutes at 4^{o}C and then at 12000rpm for 2 minutes at 4^{o}C. The Periopapers were then removed with college tweezers, with the ends of the tweezers being rinsed with phosphate buffered saline PBS between samples.

2.9 Collection of venous blood sample

A tourniquet was applied 8 cm above either the antecubital fossa or the hand, and the veins were allowed to engorge with blood. Using a 21 gauge and 1.5 inch Vacutainer needle (NHS Supply Chain, Derbyshire, UK) and a needle holder for 16mm diameter tubes (NHS Supply Chain, Derbyshire, UK), venous access was achieved and a venous blood sample was taken to fill the following three plastic, Vacutainer tubes (BD, Oxford, UK): 3ml EDTA (lavender top), 5ml serum separation (gold top) and 9ml serum separation tube (gold top). Once all blood was sampled, the tourniquet was loosened, the needle removed and pressure applied to the sample site with cotton wool until haemostasis was achieved. All tubes containing the blood samples were slowly

inverted at least five times and, keeping the tubes upright, the samples were left to stand for 30 minutes prior to transferring to the appropriate laboratory.

2.10 Clinical biochemistry analysis

The 3ml EDTA (lavender top) and 5ml serum separation (gold top) tubes were labelled with a adhesive labels from the patient's hospital notes and these samples were subsequently sent to the Clinical Biochemistry Department of the Newcastle Royal Victoria Infirmary for analysis of the HbA1c, hsCRP, triglyceride, cholesterol, high density lipoprotein (HDL) and non-high density lipoproteins (non-HDL) levels according to the standard operating procedure of the Newcastle Hospital NHS Trust Clinical Biochemistry Laboratory.

2.11 Serum separation and storage

The 9ml serum separation tube (gold top) was kept on ice at the chair side and transferred, within 2 hours of sampling, to the laboratory. The sample was centrifuged at 1500 x g for 15 minutes at 4^{o}C to separate the serum to the top of the tube via the formation of a polymer barrier. A Pasteur pipette was used to transfer the serum into six 0.5ml micro tubes, labelled with subject number and date and samples were then frozen at -80^{o}C to await subsequent elution & analysis.

2.12 Collection and storage of saliva

10ml of sterile saline was syringed onto the upper gingival margins, with the patient sat upright in the dental chair. The patient was asked to rinse around for 30 seconds and then expectorate into a polystyrene cup. This expectorated saliva wash was transferred into sterile 15ml centrifuge tubes (Sarstedt, Leicester, UK) The saliva wash sample was kept on ice at the chair side and transferred, within 20 minutes of sampling, to the laboratory. The sample was centrifuged at 1500 x g for 15 minutes at 4^{o}C to

separate any particulate matter to the bottom of the tube. A Pasteur pipette was used to transfer the fluid into four 0.5ml micro tubes, labelled with subject number and date and samples were then frozen at -80^{o}C to await subsequent elution & analysis.

2.13 Calculating GCF volume

Firstly, a quadratic equation was generated as part of the calibration of the Periotron 6000 which was undertaken every 12 weeks during this study. Then the software package Excel was used to solve the quadratic equations in order to calculate GCF volume from the Periotron units.

2.14 Multi-spot cytokine assay

IL-6, TNF-α, INF-γ and IL-1β concentrations in serum, saliva and GCF samples were determined with a commercially available human pro-inflammatory 4-plex I Ultrasensitive kit (Meso Scale Discovery, Maryland, USA). All assays were performed at room temperature. Details of sensitivity ranges for each cytokine are listed in table 2.3. The multi-spot assay employs a sandwich immunoassay format within a 96 well plate, in which 4 specific capture antibodies are immobilized on 4 different spots at the base of each well. The standard solution or samples are incubated in the plate, and each cytokine binds to its corresponding capture antibody spot. Cytokine levels are quantified using a cytokine-specific detection antibody labelled with MSD SULFO-TAG reagent (Figure 2.2).

Nonspecific binding, on the pre-coated 96-well multi-spot assay plate (Meso Scale Discovery, Maryland, USA) was blocked with 25 µl of Human Serum Cytokine Assay Diluent (Meso Scale Discovery, Maryland, USA). The plate was sealed with an adhesive plate seal and incubated for 30 minutes at room temperature on a plate shaker at 300 rpm. The Human Serum Cytokine Assay Diluent was aspirated out of each well

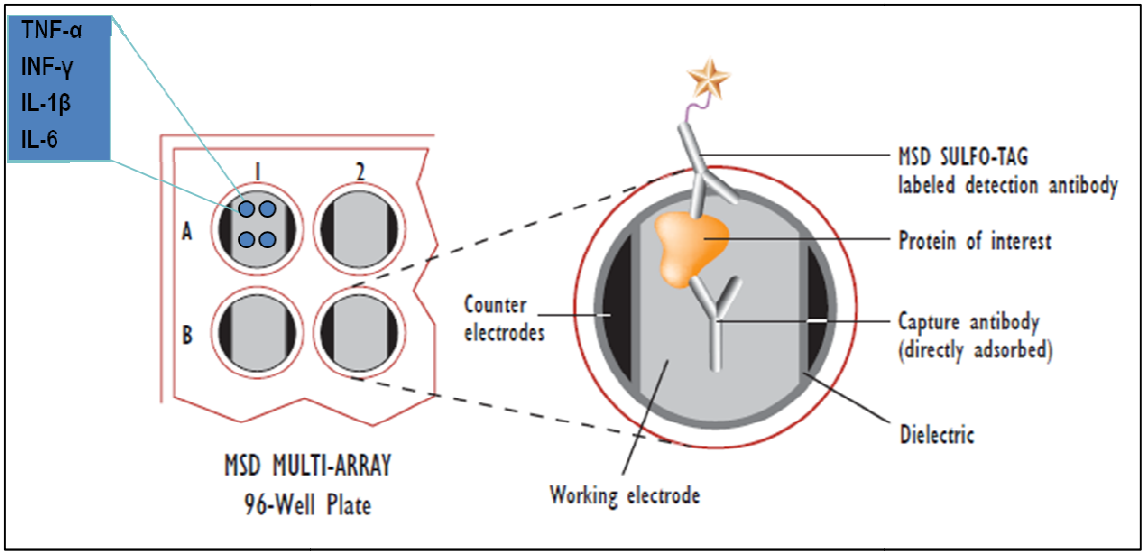
and 25 µl of the sample (in duplicate) or 25 µl of standard (in duplicate, serial dilution prepared in Human Serum Cytokine Assay Diluent) were added in accordance with a pre-defined plate map. A duplicate Human Serum Cytokine Assay Diluent sample was included as a negative control. Standard and samples were sealed and incubated for 2 hours on a plate shaker at 300 rpm. The sample or standard was aspirated off and the plate was washed three times with wash buffer (0.05% Tween 20 in PBS), aspirating the contents of each well between each wash. 25 µl of the prepared detection antibody solution (60 µl detection antibody blend, Meso Scale Discovery, diluted into 2.94 ml of Human Serum Cytokine Assay Diluent) was added to each well. The plate was sealed and incubated for 2 hours on a plate shaker at 300 rpm, avoiding direct light. The detection antibody solution was aspirated off and the washing step was repeated. 150 µl of the prepared read buffer (8ml read buffer solution, Meso Scale Discovery, in 8ml of de-ionized water) was added using reverse pipetting technique, to avoid air bubbles. The plate was immediately analysed using the SECTOR imager.

To utilize the quantitative value of the electrochemiluminescent detection, a titration curve was produced for each plate using a known standard. Using the software provided with the SECTOR instrument, a 4-parameter logistic model was used to fit the data generated from the known standards. The algorithms used by the SECTOR instrument allowed signals from standard samples with known levels of the cytokine to calculate the concentration of the cytokine in the sample. The SECTOR instrument calculates the lower detection limit for each plate, based on 2.5 standard deviations above the value for the negative control (0 pg/ml standard) (Table 2.3).

Table 2.3 Sensitivity range for multi-spot cytokine assay

Multi-spot cytokine assay	Sensitivity range [pg/ml]
IL-1β	2500 – 0.78
IL-6	2500 – 0.76
TNF-α	2500 – 0.96
INF-γ	2500 – 1.8

Figure 2.2 Diagram of multi-spot cytokine assay



The diagram represents the multi-spot cytokine assay which employs a sandwich immunoassay format within a 96 well plate. At the base of each well, 4 specific capture antibodies are immobilized on 4 different spots enabling the simultaneous quantification of IL-6, TNF-α, IL-1β and IFN-γ from a 25 µl clinical sample.

Chapter 3 Calibration and verification studies

3.1 Clinical Calibration

3.1.1 Background

In clinical periodontal research, periodontal probes are used to make direct, site-specific measurements of PD and recession. LOA is then calculated with LOA= PD + recession. LOA has been described as the gold standard measurement for assessing periodontal disease progression and therapeutic outcomes (Jeffcoat, 1992; Pihlstrom, 1992; Reddy, 1997).

Manual probing has been shown to accurately assess LOA (Hull et al., 1995). However, the validity of periodontal probing to assess PD is dependent upon the accuracy and reproducibility of probing measurements. Factors, including probing pressure, probe tip diameter, variations in probe markings and probe insertion angle may contribute to increased variability and decreased reproducibility in PD measurements (Pihlstrom, 1992; Reddy, 1997).

A low level of examiner reproducibility in periodontal probing assessments leads to higher measurement variance independent of the actual biological component of the probing variance (Jeffcoat, 1992). This would potentially increase the need for larger sample size to demonstrate statistical significance between outcomes variables and would raise the minimum threshold of clinical change needed to reliably identify periodontal sites that experience progression or healing in longitudinal analysis. Assessment of examiner reproducibility and calibration training are used to identify and minimise sources of examiner variation (Polson, 1997).

3.1.2 Aim

The aim of this phase of the research was to examine inter- and intra-examiner reproducibility for the measurement of PD, recession, plaque and gingival inflammation.

3.1.3 Methods

The clinicians responsible for performing the periodontal examinations underwent calibration training, including a discussion of the principles of periodontal data collection and procedures focused on the use of manual probing forces, identification of interproximal sites and appropriate reference points, LOA calculation and the use the PI and mGI to score plaque and gingival inflammation. Following this, both slides (PI and mGI) and patients (mGI, PD and recession) were examined to assess examiner reproducibility. Each clinician independently made an initial evaluation to provide measurements of mGI, PI, PD and recession at 6 sites per tooth. Following a 30 minute break, measurements were repeated by each examiner. During the repeat assessment, the examiners were blind to both the initial measurements and the measurements of the other examiner. To reflect the diversity of patients, a variety of patients were used to assess reproducibility, including those with healthy periodontal tissues, gingivitis and periodontitis.

The percent exact agreement and agreement with ±1.0 mm were determined between initial and replicate assessments of PD, recession, PI and mGI. Kappa statistics (Hunt, 1986) were used to quantify inter- and intra-examiner agreement beyond chance for site-based replicate assessments of PD, recession, PI and mGI by employing all examined sites and ignoring the contribution of subjects. A $\geq$ 80% level of exact agreement and a $\geq$ 90% level of within 1 agreement was considered satisfactory and kappa values between 0.40 and 0.75 were considered to represent fair to good

agreement, with kappa > 0.75 indicating excellent agreement (Landis and Koch, 1977; Petrie, 2009).

3.1.4 Results

Table 3.1 reveals that in Nov 2006, at the start of the study, the exact agreement for mGI, PI, PD and recession was found to be above the 80% level and the within 1 agreement was above the 90% level for both examiners. The kappa values for exact intra-examiner agreement all exceeded the threshold required (> 0.75) to indicate excellent intra-examiner agreement except for mGI in examiner 2 with a kappa value of 0.68 indicated good intra-examiner agreement (Table 3.1).

Table 3.1 demonstrates that at this same time point, the kappa values for exact inter-examiner agreement for PI and recession were 0.61 and 0.50, indicating fair inter-examiner agreement. However, for PD inadequate inter-examiner agreement was indicated by a kappa value of 0.30. The kappa value for mGI could not be computed due to the lack of symmetry in the categories of the 2-way table from which kappa value is calculated. The exact agreement was found to be above the 80% level for PI, but not for mGI, PD and recession and the within 1 agreement was above the 90% level for mGI, PI and recession. Inter-examiner agreement could be considered adequate for mGI, PI and recession but not PD (Table 3.1)

Table 3.1 reveals that in Dec 2006, following further calibration training, the exact agreement (within 1) for PD was found to be 82.9% (97.6%) between examiners and the kappa value for exact inter-examiner agreement for PD of 0.75 indicated excellent inter-examiner agreement (Table 3.1).

Table 3.1 also presents data at a further 3 time points carried out within the timeframe of the clinical trial. Despite variation in kappa values, all the measurements

demonstrated kappa values that indicated fair to good (0.40 and 0.75) or excellent (> 0.75) agreement. Furthermore, ≥ 90% level of within 1 agreement was seen for all measurements demonstrating good intra-examiner agreement with variations mostly occurring within 1 (Table 3.1).

3.1.5 Conclusion

The findings demonstrate that, using a manual probe, appropriately trained examiners produce kappa values for exact agreement that indicate acceptable inter- and intra-examiner agreement. Furthermore, a high (≥ 90%) level of within 1 agreement confirms that when agreement is not seen the magnitude of differences is small.

Table 3.1 Intra- and inter-examiner reproducibility data

		Nov 2006				Dec 2006	June 2007				May 2008				Sept 2009		
		mGI	PI	PD	Rec	PD	mGI	PI	PD	Rec	mGI	PI	PD	Rec	mGI	PD	Rec
Examiner 1 (PG)	κ	0.86	0.83	0.80	0.90												
	Exact agreement	90.6%	88.9%	85.5%	93.6%												
	Within 1	100%	100%	98.8%	98.8%												
	Mean bias	-0.04	0.03	0.02	-0.01												
	Measurement error	0.03	0.01	-0.004	0.01												
Examiner 2 (HF)	κ	0.68	0.77	0.79	0.82		0.46	0.69	0.55	0.65	0.77	0.82	0.54	0.54	0.45	0.63	0.65
	Exact agreement	81.3%	89.2%	83.7%	86.6%		64.9%	77.1%	75.0%	95.8%	85.4%	90.0%	70.5%	76.3%	69.0%	79.5%	80.8%
	Within 1	100%	100%	97.1%	99.4%		93.5%	100%	98.6%	100%	99.3%	96.3%	97.4%	94.2	99.4	100	98.1
	Mean bias	-0.02	0.05	-0.12	0.01		-0.39	-0.15	-0.04	-0.01	-0.10	0.01	0.10	-0.02	-0.17	-0.03	-0.04
	Measurement error	0.04	0.02	-0.04	0.02		0.27	-0.03	-0.03	-0.01	0.05	-0.001	0.10	-0.03	0.12	-0.03	0.07
Inter-examiner	κ	-	0.61	0.30	0.50	0.75											
	Exact agreement	74.5%	83.1%	47.7%	65.4%	82.9%											
	Within 1	100%	100%	86.9%	93.3%	97.6%											
	Mean bias	-0.06	0.03	0.02	-0.18	-0.04											
	Measurement error	0.02	0.01	-0.27	-0.001	-0.02											

κ: kappa statistic
Exact agreement: exact agreement between replicates on measurement scale
Within 1: agreement between replicates to within 1 unit on measurement scale
Mean bias: mean of differences between replicates
Measurement error: standard deviation of differences between replicates

3.2 Periotron calibration

3.2.1 Background

The Periotron 6000 (ProFlow Inc., Amityville NY, USA) is used to determine GCF volumes sampled on Periopaper strips, and has been shown to be reproducible and accurate when operated correctly (Chapple et al., 1995). The Periotron 6000 quantifies the volume of GCF collected on a filter paper by measuring the electrical capacitance of the wet paper strip. The Periotron must be calibrated prior to use by the placement of known volumes of test fluid onto Periopapers and recording the digital output to produce a calibration curve. In the present study, the Periotron was calibrated every 12 weeks. Previous research has demonstrated that the calibration curve for the Periotron 6000 is quadratic in nature (Preshaw et al., 1996). Thus, by solving a quadratic equation, a Periotron unit can be converted into a GCF fluid volume.

3.2.2 Methods

The Periotron 600 fluid analyser was allowed to warm up for 10 minutes prior to use, then zeroed with a dry Periopaper. A Hamilton 7000 series microlitre syringe (maximum volume 2 μl with 0.02 μl gradations) was used to dispense known volumes of calibration liquid (human serum) onto Periopapers. Ideally, GCF should be used for calibration purposes, although it would be probably impossible to collect sufficient volumes. It is generally agreed that serum is the most appropriate alternative, being similar to GCF in both viscosity and composition (Cimasoni, 1974).

Whole human venous blood samples were collected in a 9 ml serum separation tube (gold top). After 30 minutes standing at room temperature, the sample was centrifuged at 1500 x g for 15 minutes at 4 ^{o}C to separate the serum to the top of the tube via the formation of a polymer barrier. The Hamilton syringe was mounted in a

retort stand positioned at eye level to minimise parallax errors. A piece of lightly coloured card placed behind the syringe facilitated visualisation of the meniscus. Serum was repeatedly drawn into the syringe then dispensed to ensure that the inner walls of the syringe barrel were coated prior to calibration. 16 different volumes of serum (ranging from 0.1 to 1.6 μl) were used, and each volume was measured in triplicate. Serum was dispensed from the syringe onto the Periopaper strip which was transferred rapidly to the jaws of the Periotron to minimise evaporation errors. Between each sample the jaws of the Periotron were cleaned with an alcohol swab and allowed to dry. The Periotron was then re-zeroed with a dry Periopaper strip.

3.2.3 Results

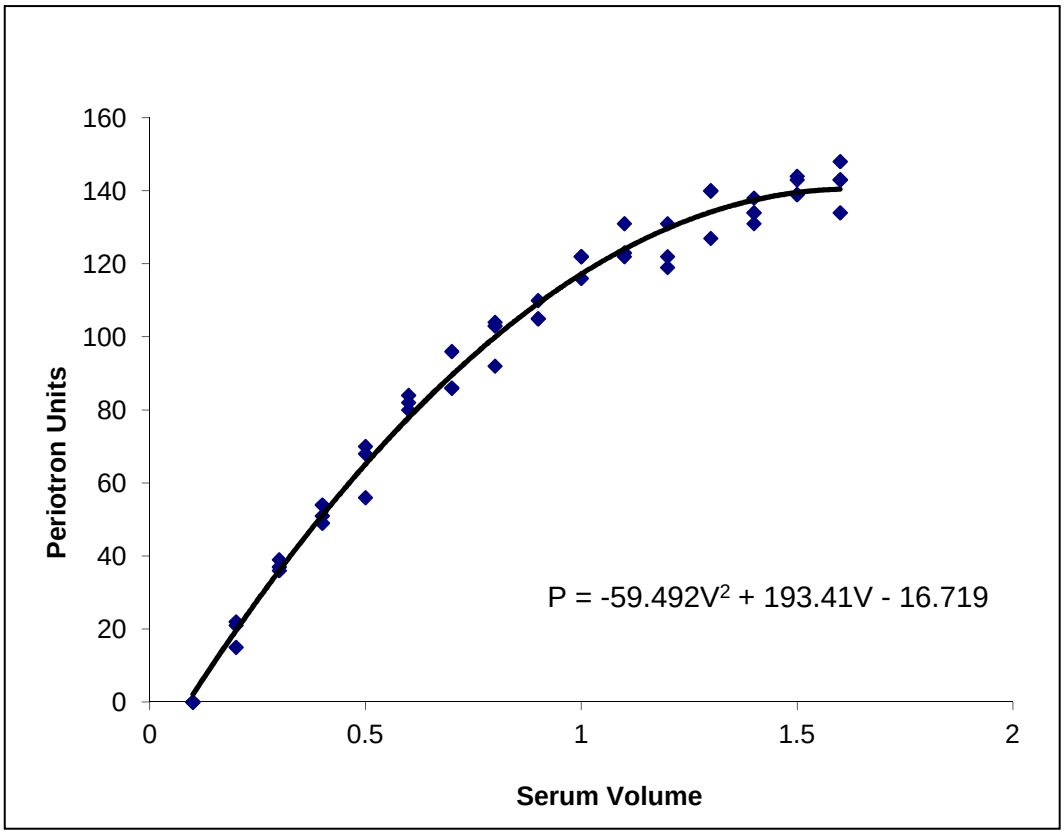
The calibration curve generated for the Periotron 600 using serum is shown in Figure 3.1. The quadratic regression equation is $P = AV^2 + BV + C$ (where P = Periotron units, V = fluid volume and A, B and C are constants determined by the regression). In this case, $P = -59.49V^2 + 193.41V – 16.719$ (Figure 3.1).

3.2.4 Discussion

Ensuring accuracy is important when evaluating and presenting cytokine levels in GCF. Within the literature, cytokine levels are presented as ‘total amount of cytokine protein’ per 30 second GCF sample and/or concentration of cytokine, in which the total amount of cytokine is divided by the GCF volume (Correa *et al.*, 2008; Santos *et al.*, 2010; Kardesler *et al.*, 2011). However, when presenting GCF levels as a concentration there should be an appreciation that very low GCF volumes could have a dramatic affect on a calculated GCF cytokine concentration. This would have particular consequences in prospective treatment studies that are evaluating the impact of improved periodontal health, following periodontal therapy, on GCF cytokine levels. For example, in recent studies, reductions in both GCF volume and total amounts of IL-

6, IL-1β and IFN-γ in GCF have been shown to accompany improvements in periodontal health following periodontal treatment in T2DM subjects, however, when the same changes in cytokine levels are presented as concentrations they appear to demonstrate increases in cytokine levels following periodontal treatment (Correa *et al.*, 2008; Santos *et al.*, 2010; Kardesler *et al.*, 2011). It is possible that this apparent increase in GCF cytokine levels is an artefact of the reductions in GCF volume. To aid interpretation of results and improve the clarity of conclusions drawn from prospective studies such as these, it would seem more appropriate to present changes in GCF volume and changes in total amount of GCF cytokine as separate treatment outcomes.

Figure 3.1 Example Periotron 6000 calibration curve



Scatter plot for Periotron units generated for 16 different volumes of serum (ranging from 0.1 to 1.6 µl) undertaken in triplicate with quadratic trend line. This was undertaken as part of the Periotron 6000 calibration studies.

3.3 Verification of methods for GCF elution

3.3.1 Background

The clinical GCF sampling technique, using filter paper strips placed within the gingival crevice for 30 seconds is consistently described within the literature (Engebretson et al., 2007). However, the method used to elute mediators from the filter paper strips into supernatant for analyses is rarely described, with variations in elution method having a potential impact on mediator levels. In an attempt to devise a standard methodology that maximised cytokine protein elution, verification studies were conducted to compare 4 different elution methods.

3.3.2 Methods

1µl of known concentration of IL-6 was spiked onto a Periopaper strip and then placed into 150 µl PBS. The chosen elution method was used (A, B, C or D). The Periopaper was removed and the recovered IL-6 concentration quantified using an IL-6 ELISA (DuoSet human IL-6, R&D systems).

A: Centrifuge 60 minutes at 300 rpm (8 g) and then 2 minutes at 12000 rpm (13201 g), 4 ^{o}C.

B: Centrifuge 2 minutes at 12000 rpm (13201 g), 4 ^{o}C.

C: Addition of 50µl 1% BSA, centrifuge 60 minutes at 300 rpm (8g) and then 2 minutes at 12000 rpm (13201 g), 4 ^{o}C.

D: Addition of 50 µl 1% BSA, centrifuge 2 minutes at 12000 rpm (13201 g), 4 ^{o}C.

3.3.3 Results

Table 3.2 shows the mean recovered IL-6 concentrations and the % recoverability for the 4 methods, based on 2 independent experiments assayed in triplicate. Both the

addition of 50 µl 1% BSA, to reduce non-specific binding, and centrifugation of samples for 60 minutes at low speed, improved the recoverability of IL-6 from Periopapers, particularly at lower concentrations (Table 3.2).

3.3.4 Conclusion

The elution protocol for clinical GCF samples was informed by these results. This will ensure maximal recoverability of cytokines present at low physiological levels within GCF samples. Therefore, prior to quantification of cytokines, GCF samples were taken out of storage in the -80 ^{o}C freezer and defrosted on ice. 50µl 1% BSA was then added and samples were centrifuged for 60 minutes at 300 rpm (8 g) and then for 2 minutes at 12000 rpm (13201 g) at 4^{o}C (method C).

Table 3.2 Recoverability of IL-6 following elution from Periopapers

Total IL-6 Conc (pg/ml)	Method A		Method B	
	Mean recovered IL-6 Conc (pg/ml)*	**% Recoverability**	**Mean recovered IL-6 Conc (pg/ml)***	**% Recoverability**
233.33	146.59	62.83	123.97	53.13
116.67	26.89	23.05	26.09	22.36
58.33	8.07	13.83	6.05	10.37

Total IL-6 Conc (pg/ml)	Method C		Method D	
	Mean recovered IL-6 Conc (pg/ml)*	% Recoverability	Mean recovered IL-6 Conc (pg/ml)*	% Recoverability
175	92.88	53.07	78.76	45.01
87.5	34.63	39.57	17.28	19.75
43.75	14.17	32.39	8.11	18.53

The method used to elute mediators from the filter paper strips into supernatant for analyses is rarely described in the literature, with variations in elution method having a potential impact on mediator levels. The table shows the mean recovered IL-6 concentration and % recoverability for 4 elution methods. Results are based on 2 independent experiments assayed in triplicate. The initial total concentration of IL-6 is lower for methods C & D because these samples were diluted, as part of the method, by the addition of 50 µl 1% BSA.

3.4 Verification of multi-spot cytokine assay for clinical GCF samples

3.4.1 Background

The accurate quantification of cytokines in biological samples is dependent on the accuracy of the analytical technique. In an attempt to assess the suitability of the multiplex cytokine assay to analyse GCF samples the lower limit of detection (LLOD) and inter-plate variation was assessed.

3.4.2 Method

The multi-spot assay was performed according to the previously described method and the LLOD, which is the calculated concentration of the signal that is 2.5 standard deviations over the zero standard, was recorded for IL-6, IL-1β, TNF-α and IFN-γ on 4 plates undertaken on different days (Table 3.3). Additionally, GCF was sampled and immediately eluted according to the previously described methods. To avoid repeated freeze-thaw cycles the supernatant was aliquoted into 3 separate 500µl microtubes prior to being stored in the -80 freezer until analysis. The assay was performed according to the previously described method on 3 plates undertaken on different days (Table 3.4). All GCF samples were thawed once. The inter-plate variation was calculated as follows:

$$\frac{SD}{mean} \times 100$$

3.4.3 Results

The inter-plate variation for the calculated LLOD for IL-6, IL-1β and TNF-α was 14.8 %, 10.8 % and 16.0 % respectively. The inter-plate variation for the LLOD for

IFN-γ was 51.4% (Table 3.3). The inter-plate variation for GCF IL-6, IL-1β, TNF-α and IFN-γ was 19%, 17.5%, 23.8% and 67.8 % respectively (Table 3.4).

3.4.4 Conclusion

In summary, for the calculated LLOD the inter-plate variation was less than 20% for IL-6, IL-1β and TNF-α. Similarly for GCF samples the inter-plate variation was less than 25% for IL-6, IL-1β and TNF-α. These data indicate a reasonable level of reproducibility and increases the confidence of using this multi-spot assay to quantify IL-6, IL-1β and TNF-α in GCF, particularly as the calculated LLOD values were all very low (typically < 1 pg/ml). However, for IFN-γ, the inter-plate variation for the calculated LLOD and GCF samples was 51.4% and 67.8% respectively. This indicates a low level of reproducibility for IFN-γ and reduces the confidence for using the multiplex assay to quantify IFN-γ in GCF.

Table 3.3 Inter-plate variation of LLOD for multi-spot cytokine assay

Plate number	IL-6 (pg/ml)	IL-1β (pg/ml)	TNF-α (pg/ml)	IFN-γ (pg/ml)
I	0.818	0.885	1.06	2.68
II	0.656	0.808	0.779	1.26
III	0.673	0.697	1.13	2.69
IV	0.885	0.729	0.936	0.936
Mean	0.758	0.780	0.963	1.8
SD	0.112	0.084	0.154	0.926
%CV	14.8	10.8	16.0	51.4

Results are based on 4 plates (assayed on separate days) in duplicate

Table 3.4 Inter-plate variation of multi-spot cytokine assay for GCF samples

Plate number	IL-6 (pg/ml)	IL-1β (pg/ml)	TNF-α (pg/ml)	IFN-γ (pg/ml)
I	4.54	211	1.06	2.6
	5.16	229	0.96	2.49
II	4.76	263	1.68	4.82
	5.4	286	1.56	5.11
III	3.43	186	1.09	0.76
	3.46	192	1.13	0.82
Mean	4.45	227.8	1.25	2.77
SD	0.85	39.85	0.30	1.88
Inter-plate variation	19.0	17.5	23.8	67.8

Results are based on 3 plates (assayed on separate days) in duplicate

3.5 Verification of stability of samples within -80 ^{o}C freezer

3.5.1 Background

Within biological samples, the degradation of cytokine proteins during storage and prior to analysis could negatively impact on the accuracy of results for the quantification of cytokine levels in clinical samples. In an attempt to evaluate the stability of cytokines within clinical samples, variations in GCF, saliva and serum cytokine levels following storage for 6 months at -80^{o}C was assessed.

3.5.2 Method

GCF was obtained and eluted according to the previously described method. To avoid repeated freeze-thaws, the supernatant was aliquoted into 2 separate 500 µl microtubes. Samples were then stored at -80 ^{o}C for either 0 or 6 months. The multi-spot assay was performed according to the previously described method. All samples were freeze-thawed only once. The inter-plate variation was calculated as follows:

$$\frac{SD}{mean} \times 100$$

3.5.3 Results

For GCF sample A, the inter-plate variation, between month 0 and month 6, for GCF IL-6, IL-1β, TNF-α and IFN-γ was 44.8%, 26.8%, 15.6% and 52.5% respectively (Table 3.5). At both month 0 and month 6, the levels for IL-6 were below the mean LLOD and the IFN-γ cytokine levels at month 0 was below the mean LLOD (the mean LLOD was previously demonstrated in Table 3.3). For all cytokines, the month 6 analysis produced higher levels for all 4 cytokines in comparison to the analysis at month 0.

For GCF sample B, the inter-plate variation, between month 0 and month 6, for GCF IL-6, IL-1β, TNF-α and IFN-γ was 21.5%, 29.3%, 33.68% and 30.8% respectively (Table 3.4). At month 6, the differences between the duplicates, for all 4 cytokines, appear higher than previous verification data (Table 3.4). For all cytokines, the month 6 analysis produced higher levels for all 4 cytokines in comparison to the analysis at month 0.

The mean inter-plate variation (for samples A and B), between month 0 and month 6, for GCF IL-6, IL-1β, TNF-α and IFN-γ was 33.1%, 28.0%, 24.7% and 41.7 % respectively.

3.5.4 Conclusions

In summary, for GCF samples stored for 6 months in a -80 ^{o}C freezer, the inter-plate variation for IL-6, TNF-α and IL-1β appears higher than the inter-plate variation seen previously without the extended storage time (see Table 3.4). The apparent lower levels of reproducibility for GCF IL-6, TNF-α and IL-1β following storage for 6 months in a -80 ^{o}C freezer should, however, be interpreted with caution. The levels for IL-6 were below the level of detection in sample A, explaining the larger inter-plate variations seen for this sample. Additionally, at month 6 increased variations between duplicates in sample B for all cytokines also contributed to higher inter-plate variations. Also, for all cytokines, the month 6 analysis produced higher levels for all 4 cytokines in comparison to the analysis at month 0. This suggests protein degradation is not a significant issue. These data indicate that storage of GCF samples in a -80 ^{o}C freezer for 6 months may lead to an increase in inter-plate variation but the effect that such storage has on the level of cytokines present in samples is not clear. Therefore, to minimise such variations the time between sampling and analysis was minimised by analysing samples through-out the study time-frame.

Table 3.5 Inter-plate variation of multi-spot cytokine assay for GCF samples stored for 6 months in a -80 ^{o}C freezer

Sample	Time (months)	IL-6 (pg/ml)	IL-1β (pg/ml)	TNF-α (pg/ml)	IFN-γ (pg/ml)
A	**0**	0.16	167	2.11	0.99
		0.29	161	1.73	1.03
A	**6**	0.46	203	2.43	2.9
		0.51	279	1.82	2.0
	Mean	0.35	202.5	2.02	1.73
	SD	0.16	54.27	0.32	0.91
	Inter-plate variation	44.8	26.8	15.6	52.5
B	**0**	2.51	512	2.77	3.2
		2.55	560	3	2.81
B	**6**	2.84	843	3.94	3.48
		3.86	936	5.61	5.39
	Mean	2.94	712.75	3.83	3.72
	SD	0.63	208.52	1.29	1.15
	Inter-plate variation	21.5	29.255	33.68	30.83
Mean inter-plate variation		33.1	28.0	24.7	41.7
SD inter-plate variation		16.5	1.7	12.8	15.3

Chapter 4 Investigation of the general and oral health status of patients with T2DM

4.1 Introduction

The epidemiological evidence from association studies confirms that diabetes is a risk factor for periodontal disease. The risk for developing periodontitis is greater if diabetes control is poor and those with good diabetic control do not have a greater risk of periodontal disease than non-diabetic patients (Nelson *et al.*, 1990; Emrich *et al.*, 1991; Taylor *et al.*, 1998a; Tsai *et al.*, 2002). Furthermore, evidence suggests that those with moderate levels of periodontal disease have a 2-fold increased risk for developing T2DM (Demmer et al., 2008) and diabetics with severe periodontal disease are six times more likely to have poor glycaemic control (Taylor et al., 1996). This highlights a bi-directional relationship between these two diseases. Furthermore, a recent meta-analysis of cross-sectional data has demonstrated a positive association between periodontal disease and obesity, a known risk factor for T2DM (Chaffee and Weston, 2010). This highlights the importance of considering oral health within a wider perspective of maintaining systemic health, however, previous research has demonstrated that the attitudes towards oral health of patients with diabetes are poor in comparison to the general population (Allen *et al.*, 2008).

Recently, a small number of interventional studies have used data from full-mouth clinical periodontal measurements to describe the periodontal status of patients with T2DM and periodontal disease prior to the provision of periodontal management (Correa *et al.*, 2008; Dag *et al.*, 2009; Correa *et al.*, 2010; Kardesler *et al.*, 2010; Santos *et al.*, 2010). Some studies demonstrated no significant differences in the clinical periodontal status at baseline (Dag *et al.*, 2009; Kardesler *et al.*, 2010), whilst another

demonstrated that the diabetic group had higher levels of periodontal disease compared to non-diabetic subjects (Correa et al., 2008).

4.2 Results

4.2.1 Demographic data

Demographic findings for the diabetic and non-diabetic subjects are summarised in Table 4.1. Of note, no significant differences between the T2DM and non-diabetic groups were found for gender, age, ethnicity, smoking status and pack years. This demonstrates that the non-diabetic and T2DM groups were appropriately matched for gender, age, and ethnicity and were balanced for smoking status. There was a 9% prevalence of current smokers in the present study in both the T2DM and non-diabetic groups.

The demographic findings did, however, highlight some important differences between diabetic and non-diabetic patients. The IMD rank differed significantly between the T2DM subjects [9198 (3384-21930)] and the non-diabetic subjects [18213 (7658-25035)], demonstrating that diabetic subjects lived in more deprived areas. The subjects with T2DM had significantly higher systolic blood pressure [146.9 (±21.2)] compared to the non-diabetic group [136.6 (±18.9)]. Similarly, patients with T2DM had significantly higher BMI [32.6 (29.8-36.0)] compared to the non-diabetic patients [27.3 (25.0-29.5)]. Furthermore, Table 4.1 and Figure 4.1 show that proportions of T2DM and non-diabetic patients in each BMI category were significantly different, with the T2DM group containing a higher proportion of obese (42.6%) and morbidly obese (30.7%) subjects compared to 10.8% and 13.3%, respectively, in the non-diabetic group (Table 4.1 and Figure 4.1).

Table 4.2 summarises subject demographic data following further categorisation of subjects based on their periodontal diagnosis. When demographic data for these six

categories (diabetic subjects with healthy periodontal tissues; diabetic subjects with gingivitis; diabetic subjects with periodontitis; non-diabetic subjects with healthy periodontal tissues; non-diabetic subjects with gingivitis and non-diabetic subjects with periodontitis) were analysed, there were no significant differences found between the groups for gender or age. However, there were significant differences in the proportions in the ethnicity categories with a higher proportion of Black and Asian subjects within the T2DM groups. The clinical significance of this, however, is likely to be limited, given to the very low numbers of Black and Asian subjects recruited (5 out of a total of 184 subjects). The IMD rank differed significantly between T2DM subjects with gingivitis [7046 (2320-17948)] and non-diabetic subjects with gingivitis [21195 (16093-26175)], showing subjects with T2DM living in more deprived areas.

For subjects with healthy periodontal tissues and gingivitis, the systolic blood pressure was significantly higher in subjects with T2DM [146.1 (±20.6) mmHg and 150.1 (±21.2) mmHg] compared to non-diabetic subjects [122.2 (±11.0) mmHg and 135 (±18.3) mmHg]. Additionally, within the non-diabetic group, systolic blood pressure was significantly lower in those with healthy periodontal tissues [122.2 (±11.0) mmHg] compared to those with either gingivitis [135 (±18.3) mmHg] or periodontitis [141.0 (±19.1) mmHg].

When considering BMI, subjects with healthy periodontal tissues, gingivitis or periodontitis, the BMI was significantly higher in subjects with T2DM [31.2 (24.5-34.6) kg/m^2, 32.9 (30.7-36.7) kg/m^2 and 33.0 (29.9-36.5) kg/m^2] compared to non-diabetic subjects [24.7 (23.1-27.7) kg/m^2, 27.3 (26.3-30.9) kg/m^2 and 28.1 (25.2-32.0) kg/m^2]. Furthermore, Figure 4.2 highlights there were significant differences in the proportions of subjects in each of the BMI categories with a higher proportion of obese and morbidly obese subjects in the diabetic group compared to the non-diabetic group. Interestingly, BMI was significantly higher in non-diabetic subjects with periodontitis

[28.1 (25.2-32.0) kg/m^2] compared to non-diabetic subjects with healthy periodontal tissues [24.7 (23.1-27.7) kg/m^2]. Similarly, in subjects with T2DM, the BMI appeared higher in the periodontitis group [33.0 (29.9-36.5) kg/m^2] compared to the group with healthy periodontal tissues [31.2 (24.5-34.6) kg/m^2], although the difference failed to reach significance (Table 4.3 and Figure 4.2).

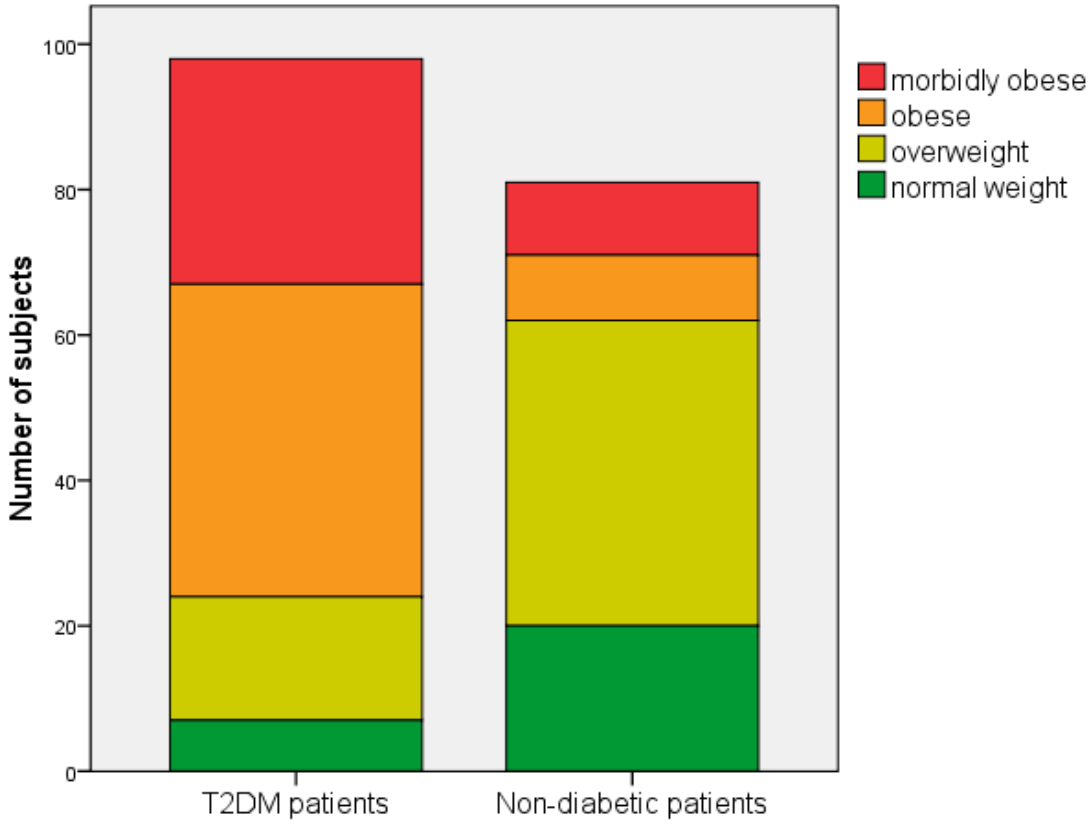
When considering smoking status, Figure 4.3 shows, that for both diabetic and non-diabetic subjects, there was a tendency towards a higher proportion of current or ex-smokers in those with periodontitis compared to those with gingivitis or healthy periodontal tissue, although, this difference failed to reach significance. Interestingly, pack years was lowest in subjects with healthy periodontal tissues [5.0 (4.0-15.0) in T2DM subjects and 10.9 (4.4-23.0) in non-diabetic subjects] and highest in subjects with periodontitis [30 (10.6-40.0) in T2DM subjects and 15.0 (5.2-30.0) in non-diabetic subjects], although the difference failed to reach significance (Table 4.3 and Figure 4.3).

Table 4.1 Demographic data from the T2DM and non-diabetic groups

	T2DM patients (n=101)	Non-diabetic patients (n=83)	p-value
Gender (n (%))			
Male	67 (66.3)	50 (60.2)	NS
Age (years)	49 (45-53)	48 (43-54)	NS
Ethnicity (n (%))			
Caucasian	96 (95.1)	83(100.0)	
Black	1 (1.0)		NS
Asian	4(4.0)		
IMD rank	9198 (3384-21930)	18213 (7658-25035)	< **0.001**
Smoking			
Status (n (%))			
Current	9 (8.9)	9 (10.8)	
Ex	37 (36.6)	25 (30.1)	NS
Never	55 (54.5)	49 (59.0)	
Pack years *	16 (10.0-38.8)	15 (4.9-23.8)	NS
Systolic blood pressure (mmHg)	146.9 (±21.2)	136.6 (±18.9)	< **0.001**
Diastolic blood pressure (mmHg)	81.0 (74.0-90.0)	81.0 (74.0-88.0)	NS
BMI (kg/m^2)	32.6 (29.8-36.0)	27.3 (25.0-29.5)	< **0.001**
Status (n(%))			
normal weight	7 (6.9)	20 (24.1)	
overweight	19 (18.8)	43 (51.8)	< **0.001**
obese	43 (42.6)	9 (10.8)	
morbidly obese	31(30.7)	11 (13.3)	

P-values determined using chi-squared test for discrete variables, Mann-Whitney U tests for continuous non-parametric variables (age, pack years, IMD rank, diastolic blood pressure and BMI) and independent t-test for continuous parametric variables (systolic blood pressure). * Applicable only to current and ex-smokers (n=46 diabetic subjects, n=34 non-diabetics subjects). Means (±SD) are presented for parametric data and medians (IQR) are presented for non-parametric data.

Figure 4.1 Categorisation of T2DM and non-diabetic patients based on BMI



Stacked bar chart of subjects categorised based on BMI for T2DM patients (normal weight n=7, overweight n=17, obese n=43, morbidly obese n=31) and non-diabetic patients (normal weight n=20, overweight n=42, obese n=9, morbidly obese n=10). normal weight 18.5-24.9 kg/m^2, overweight 25.0-29.9 kg/m^2, obese 30-34.9 kg/m^2, morbidly obese >35 kg/m^2.

Table 4.2 Subject demographic data comparing groups based on diabetic status and periodontal diagnosis

	Diabetic subjects (n=101)			Non-diabetic subjects (n=83)			p-value
	Health (n=14)	**Gingivitis (n=39)**	**Periodontitis (n=48)**	**Health (n=16)**	**Gingivitis (n=19)**	**Periodontitis (n=48)**	
Male (n(%))	5 (35.7)	26 (66.7)	36 (75.0)	4 (25.0)	11 (57.9)	35 (72.9)	NS
Age (years)	49.0 (±7.8)	47.3 (±6.8)	50.1 (±5.8)	48.0 (±7.5)	46.6 (±8.1)	48.8 (±6.7)	NS
Ethnicity (n(%))							
Caucasian	13 (92.9)	38 (97.4)	45 (93.8)	16 (100)	19 (100)	48 (100)	
Black	1 (7.1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**< 0.05**
Asian	0 (0)	1 (7.1)	3 (6.3)	0 (0)	0 (0)	0 (0)	
IMD rank	10718 (2133-20143)	7046 (2320-17948)[$]	15579 (3865-24013)	19357 (11702-26868)	21195 (16093-26175)	16024 (5090-23060)	[$]**< 0.001**
Smoking status (n(%))							
Current	0 (0)	3 (7.7)	6 (12.5)	0 (0)	2 (10.5)	7 (14.6)	
Ex	3 (21.4)	10 (25.6)	24 (50.0)	4 (25.0)	4 (21.1)	17 (35.4)	NS
Never	11 (78.6)	26 (66.7)	18 (37.5)	12 (75.0)	13 (68.4)	24 (50.0)	
Pack years [*]	5.0 (4.0-15.0)	15.0 (10.0-24.0)	30.0 (10.6-40.0)	10.9 (4.4-23.0)	11.1 (2.51-26.3)	15.0 (5.2-30.0)	NS

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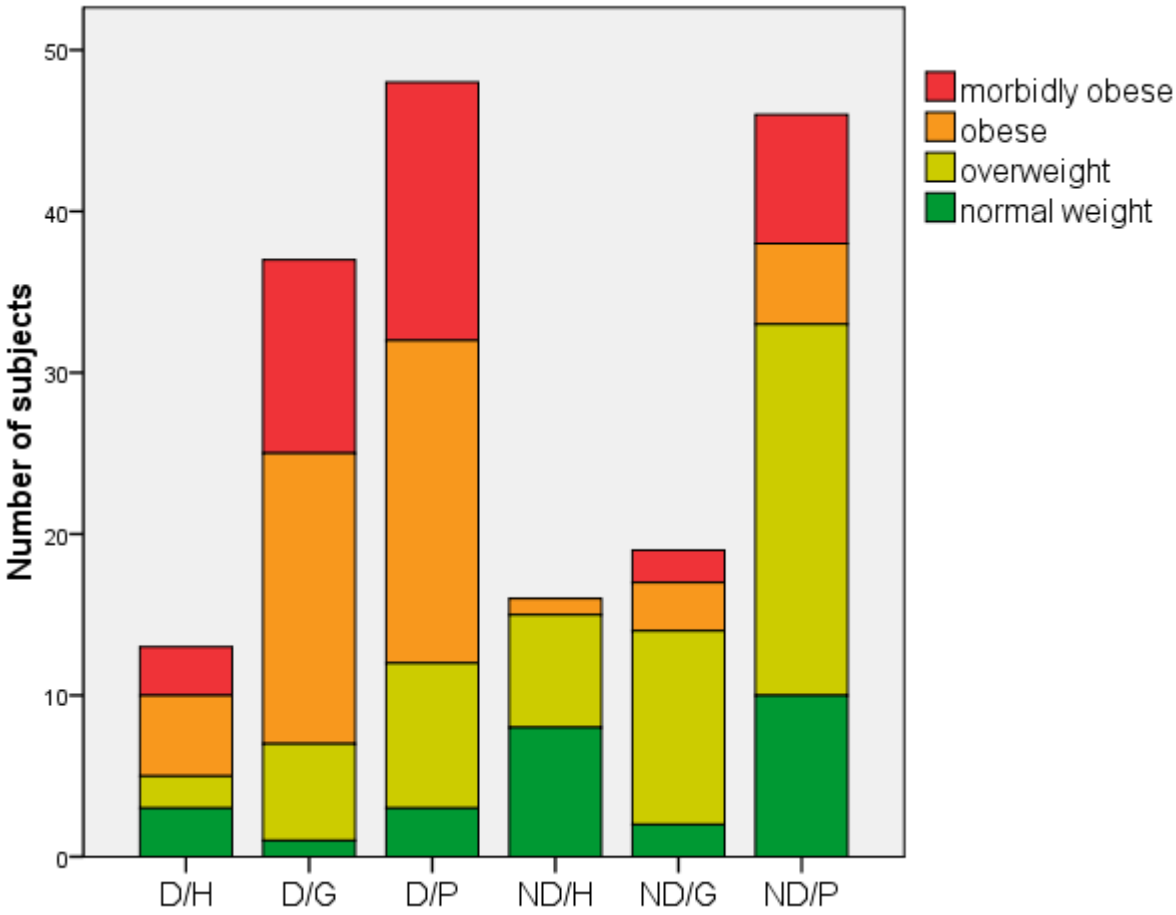
	Diabetic subjects (n=101)			Non-diabetic subjects (n=83)			p-value
	Health (n=14)	**Gingivitis (n=39)**	**Periodontitis (n=48)**	**Health (n=16)**	**Gingivitis (n=19)**	**Periodontitis (n=48)**	
Systolic BP (mmHg)	146.1 (±20.6)$	150.1 (±21.2)$	144.8 (±21.5)	122.2 (±11.0)¶,#	135 (±18.3)	141.0 (±19.1)	$,¶,# **< 0.05**
Diastolic BP (mmHg)	80.0 (71.8-90.0)	87.4 (76.0-92.0)	79.5 (74.0-88.8)	79.0 (69.8-82.5)	80.0 (73.0-88.0)	83.0 (76.0-93.5)	NS
BMI (kg/m^2)	31.2 (24.5-34.6)$	32.9 (30.7-36.7)$	33.0 (29.9-36.5)$	24.7 (23.1-27.7)¶	27.3 (26.3-30.9)	28.1 (25.2-32.0)	$,¶ **< 0.05**
BMI status (n(%))**							
Normal weight	3 (23.1)	1 (2.7)	3 (6.3)	8 (50.0)	2 (10.5)	10 (21.7)	**< 0.001**
overweight	2 (15.4)	6 (16.2)	9 (18.8)	7 (43.8)	12 (63.2)	23 (50.0)	
obese	5 (38.5)	18 (48.6)	20 (41.7)	1 (6.3)	3 (15.8)	5 (10.9)	
morbidly obese	3 (14.3)	12 (32.4)	16 (33.3)	0 (0)	2 (10.5)	8 (17.4)	

P-values were determined using chi-squared test for discrete variables, Kruskal-Wallis test with Mann-Whitney U post hoc tests for continuous non-parametric variables (pack years, IMD rank, diastolic blood pressure and BMI) and one way ANOVA test with post-hoc independent t-test for continuous parametric variables (age and systolic blood pressure). * Applicable only to current and ex-smokers (n=3 diabetic with healthy perio, n=13 diabetic with gingivitis, n=29 diabetic with periodontitis, n=4 non-diabetic with healthy perio, n=6 non-diabetic with gingivitis, n=23 non-diabetic with periodontitis). Mean (±SD) is presented for parametric data and median (IQR) is presented for non-parametric data. ** Missing data for 2 non-diabetic subjects with periodontitis.

$ indicates a comparison within rows between diabetic and non-diabetic groups with the same periodontal diagnosis
# indicates a comparison within rows between periodontally healthy and gingivitis groups within either diabetes or non-diabetes groups
¶ indicates a comparison within rows between periodontally healthy and periodontitis groups within either diabetes or non-diabetes groups
† indicates a comparison within rows between gingivitis and periodontitis groups within either diabetes or non-diabetes groups

Figure 4.2 Categorisation using BMI of subjects within groups based on periodontal and diabetes status

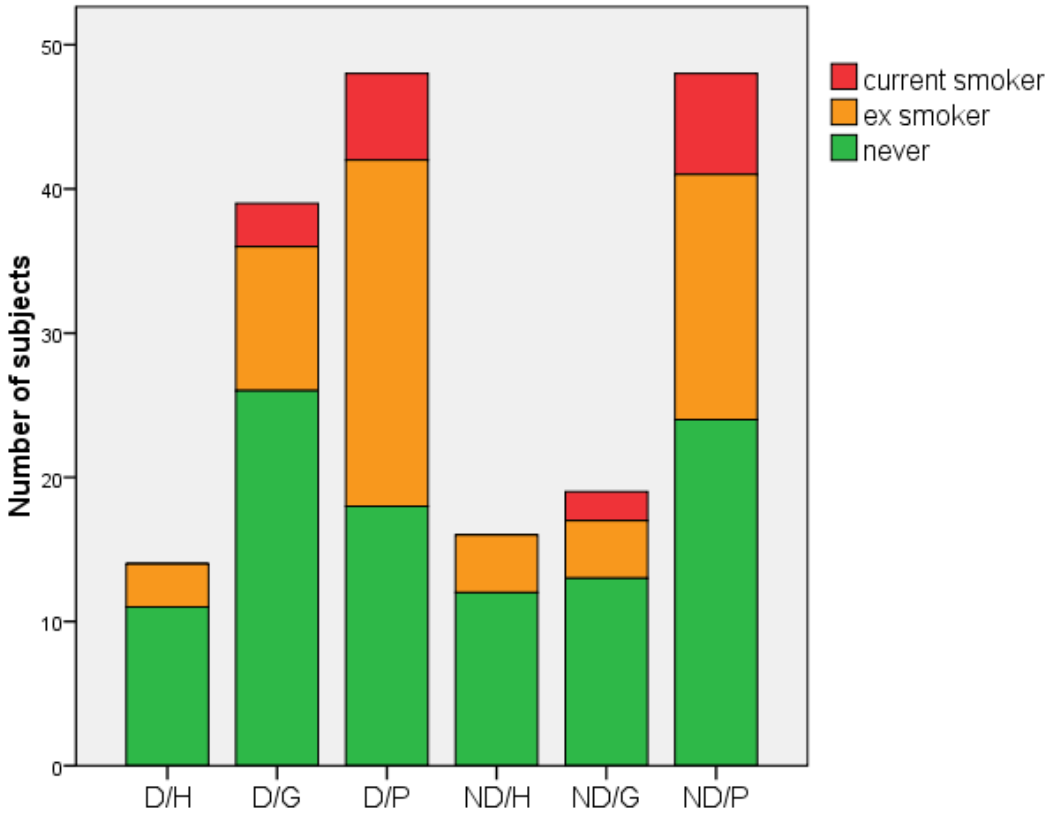


Stacked bar chart of subjects categorised based on BMI for D/H patients (normal weight n=3, overweight n=2, obese n=5, morbidly obese n=3), D/G patients (normal weight n=1, overweight n=6, obese n=18, morbidly obese n=12), D/P patients (normal weight n=3, overweight n=9, obese n=20, morbidly obese n=16), ND/H patients (normal weight n=8, overweight n=7, obese n=1), ND/G patients (normal weight n=2, overweight n=12, obese n=3, morbidly obese n=2), ND/P patients (normal weight n=10, overweight n=23, obese n=5, morbidly obese n=8)

normal weight 18.5-24.9 kg/m^2, overweight 25.0-29.9 kg/m^2, obese 30-34.9 kg/m^2, morbidly obese >35 kg/m^2.

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

Figure 4.3 Categorisation using smoking status of subjects within groups based on periodontal and diabetes status



Stacked bar chart of subjects categorised based on smoking status for D/H patients (never smoked n=11, ex-smoker n=3), D/G patients (current smoker n=3, never smoked n=26, ex-smoker n=10), D/P patients (current smoker n=6, never smoked n=18, ex-smoker n=24), ND/H patients (never smoked n=12, ex-smoker n=4), ND/G patients (current smoker n=2, never smoked n=13, ex-smoker n=4) ND/P patients (current smoker n=7, never smoked n=24, ex-smoker n=17).

current smoker, ex-smoker, never smoked

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

4.2.2 Diabetes Care data

Table 4.3 summarises data regarding diabetes care for the subjects with T2DM and demonstrates that of the 101 subjects with T2DM that were assessed at baseline, 60.4% gave a family history of diabetes and 32.7% presented with at least one diabetic complication, not including periodontitis. 94.9% of subjects with T2DM had received examinations of their feet and eyes within the past 12 months. Based on HbA1c values, 46.5% of subjects with diabetes could be categorised as having good glycaemic control (HbA1c <7.0%) (WHO, 1999). However, only 26.3% of subjects met the more stringent HbA1c target of 6.5% set out in more recent national T2DM management guidelines for the UK (NICE, 2008) (Table 4.3).

Table 4.4 presents data following categorisation of subjects based on their periodontal diagnosis and demonstrates that subjects with gingivitis [6.0 (3.0-13.0 years] or periodontitis [7.0 (3.0-10.0) years] presented with a longer history of diabetes compared to those with healthy periodontal tissues [2.5 (1.0-5.0) years]. Furthermore, Table 4.5 and Figure 4.4 highlight significant differences in the proportions of subjects meeting the UK target for glycaemic control of HbA1c $\leq$ 6.5 %, with a higher proportion of subjects with healthy periodontal tissues meeting the target (64.3%) and progressively fewer subjects reaching this target in the gingivitis (26.3%) and periodontitis groups (14.9%) respectively (Table 4.4 and Figure 4.4). Table 4.5 also shows there were significant differences in the proportions of subjects with specific management regimes with a higher proportion of subjects with healthy periodontal tissues being managed by dietary intervention alone (42.9%) and progressively fewer subjects being managed by diet alone in the gingivitis (12.8%) and periodontitis (8.5%) groups, respectively. Conversely, a higher proportion of subjects with periodontitis (29.8%) required insulin and there were progressively fewer subjects requiring insulin in the gingivitis (17.9%) and healthy periodontal tissues (0.0%) groups respectively.

Table 4.4 also shows a higher proportion of subjects reporting a diabetic complication was seen in subjects with periodontitis [35.4%] and gingivitis [35.9%] compared to subjects with healthy periodontal tissues [14.3%], although the difference failed to reach statistical significance (Table 4.4).

Table 4.3 Diabetes care data for patients with T2DM

	Diabetic patients (n=101)
Years since diabetes diagnosis (years)	5 (2-10)
Family history (n (%))	
Positive finding	61 (60.4)
Diabetes Management (n (%)) *	
Diet only	15 (15.0)
Oral hypoglycaemic drugs	64 (64.0)
With insulin	21 (21.0)
Glycaemic control categories (n (%)) **	
Good (<7.0%)	46 (46.5)
Moderate (7.0-8.5%)	25 (25.3)
Poor (>8.5%)	28 (28.3)
Glycaemic control target met (≤6.5%) (n (%))	26 (26.3)
Patient perception of glycaemic control (n (%)) *	
Good	44 (44.0)
Moderate	36 (36.0)
Poor	20 (20.0)
Diabetic complications (n (%))	
Positive finding	33 (32.7)
Screening for complications (n(%))**	
Eye examination	93 (94.9)
Foot examination	93 (94.9)
Taking lipid lowering medication (n(%))	73 (72.3)

Means (±SD) are presented for parametric data and medians (IQR) are presented for non-parametric data. * Applicable to n=100. ** Applicable to n=99. *** Applicable to n=98.

Table 4.4 Diabetes care data for patients with T2DM within groups based on periodontal diagnosis

	Diabetic subjects (n=101)			p-value
	Healthy (n=14)	Gingivitis (n=39)	Periodontitis (n=48)	
Years since diabetes diagnosis (years)	2.5 (1.0-5.0) [#,¶]	6.0 (3.0-13.0)	7.0 (3.0-10.0)	[#,¶] < 0.05
Family history – positive finding (n(%))	10 (71.4)	26 (66.7)	25 (52.1)	NS
Diabetes Management (n(%)) [*]				
Diet only	5 (42.9)	5 (12.8)	5 (10.4)	
Oral hypoglycaemic drugs	8 (57.1)	27 (69.2)	29 (60.4)	< 0.01
With insulin	0 (0)	7 (17.9)	14 (29.2)	
Glycaemic control target met (≤6.5%) (n(%))	9 (64.3)	10 (26.3)	7 (14.9)	< 0.001
Glycaemic control categories (n(%)) [**]				
Good (<7.0%)	9 (64.3)	19 (50.0)	18 (38.3)	
Moderate (7.0-8.5%)	1 (7.1)	12 (31.6)	12 (25.5)	NS
Poor (>8.5%)	4 (28.6)	7 (18.4)	17 (36.2)	
Glycaemic control target met (≤6.5%) (n (%))	9 (64.3)	10 (26.3)	7 (14.9)	

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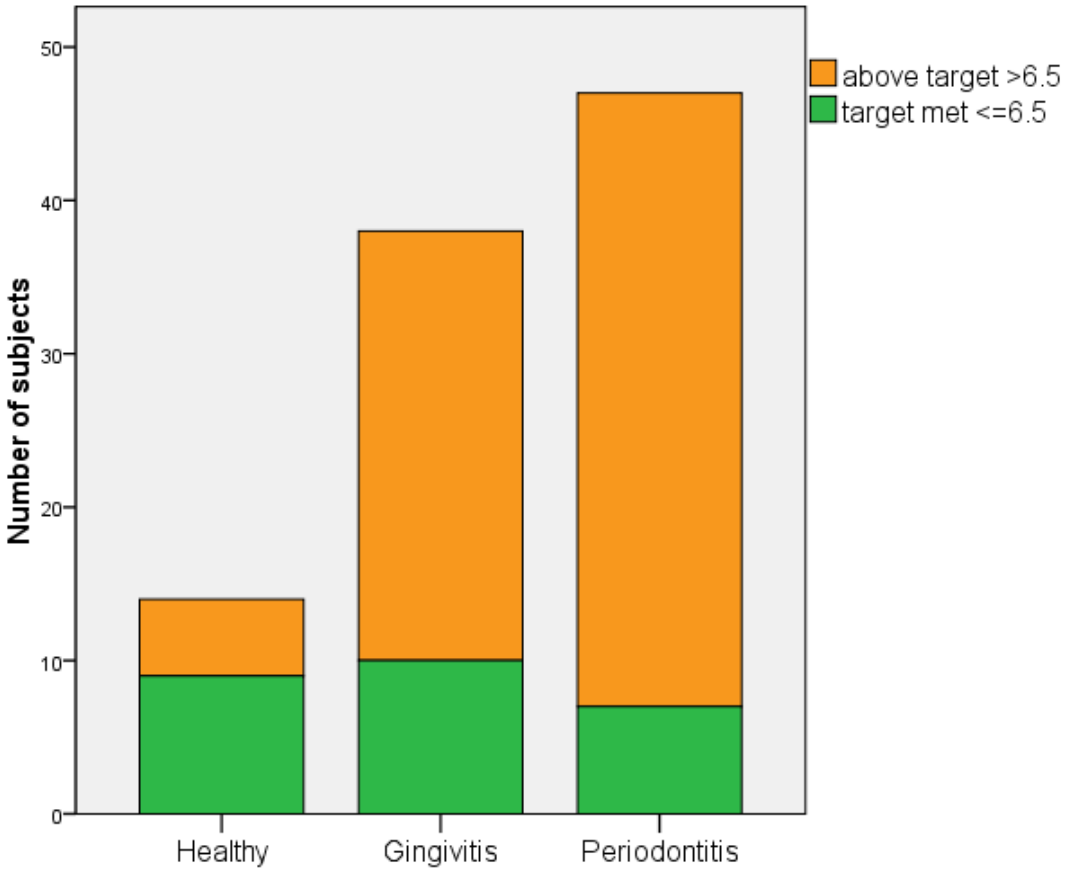
	Diabetic subjects (n=101)			p-value
	Healthy (n=14)	Gingivitis (n=39)	Periodontitis (n=48)	
Patient perception of glycaemic control (n(%)) ***				
Good	9 (64.3)	16 (41.0)	19 (40.4)	
Moderate	4 (28.6)	15 (38.5)	17 (36.2)	NS
Poor	1 (7.1)	8 (20.5)	11 (23.4)	
Diabetic complications (n(%))				
Positive finding	2 (14.3)	14 (35.9)	17 (35.4)	NS
Eye screening for complications (n(%))****	13 (92.9)	38 (97.4)	41 (93.2)	NS
Foot screening for complications (n(%))****	13 (92.9)	37 (94.9)	42 (95.5)	NS
Taking lipid lowering medication (n(%))	10 (71.4)	26 (66.7)	37 (77.1)	NS

P-values were determined using chi-squared test for discrete variables, Kruskal-Wallis test with Mann-Whitney U post hoc tests for continuous non-parametric variables (years since diabetes diagnosed) and one way ANOVA test with post-hoc independent t-test for continuous parametric variables (age at diagnosis). Mean (±SD) is presented for parametric data and median (IQR) is presented for non-parametric data. * Applicable to n=13 diabetic with healthy perio, n=39 diabetic with gingivitis, n=48 diabetic with periodontitis. ** Applicable to n=14 diabetic with healthy perio, n=38 diabetic with gingivitis, n=47 diabetic with periodontitis. *** Applicable to n=14 diabetic with healthy perio, n=39 diabetic with gingivitis, n=47 diabetic with periodontitis. **** Applicable to n=14 diabetic with healthy perio, n=39 diabetic with gingivitis, n=44 diabetic with periodontitis.

# indicates a comparison within rows between periodontally healthy and gingivitis groups within diabetic subjects
¶ indicates a comparison within rows between periodontally healthy and periodontitis groups within diabetic subjects
† indicates a comparison within rows between gingivitis and periodontitis groups within diabetic subjects

Figure 4.4 Categorisation of T2DM subjects achieving national UK target of HbA1c ≤ 6.5% within groups based on periodontal



Stacked bar chart of T2DM subjects categorised based achievement of national UK target of HbA1c ≤6.5% for subjects with healthy periodontal tissues (>6.5% n=7, ≤HbA1c n=17), gingivitis (>6.5% n=7, ≤HbA1c n=17) and periodontitis (>6.5% n=7, ≤HbA1c n=17).

■ >6.5% HbA1c (target not achieved), ■ ≤6.5% HbA1c target (target achieved)

4.2.3 Oral and dental data

Oral and dental findings are summarised in Table 4.5. Of note when comparing the T2DM and the non-diabetic groups, significant differences were found relating to dry mouth and caries into dentine. Dry mouth was reported more frequently by subjects with T2DM [14.9%] compared to non-diabetics [2.4%]. Furthermore, dry mouth was clinically present more often in subjects with T2DM [7.9%] compared to non-diabetic subjects [1.2%]. Caries into dentine was clinically present more often in subjects with T2DM [20.8%] compared to subjects without diabetes [4.9%] (Table 4.5).

Table 4.6 presents data following further categorisation of subjects based on their periodontal diagnosis and demonstrates a lack of significant differences between groups apart from teeth restored involving 1-3 surfaces in which subjects with T2DM and healthy periodontal tissues [9.9±3.9] had significantly more restored teeth than diabetic subjects with either gingivitis [6.5±3.6] or periodontitis [7.0±3.6]. Subjects with T2DM and gingivitis [6.5±3.6] had significantly less restored teeth than non-diabetic subjects with gingivitis [9.0±4.2]. There were also differences in the proportions of subjects with at least one tooth with caries into dentine, with subjects with T2DM and either gingivitis or periodontitis being more likely to have at least one carious tooth, although this difference failed to reach statistical significance and was detected as a trend ($p=0.075$) (Table 4.6).

Table 4.5 Oral and dental data comparing subjects with and without T2DM

	Diabetic subjects (n=101)	Non-diabetic subjects (n=83)	p-value
Abnormal Soft Tissue findings (n(%))			
Positive finding	7 (6.8)	3 (3.6)	NS
Clinician assessed dry mouth (n(%))			
Positive finding	8(7.9)	1(1.2)	**< 0.05**
Patient reported dry mouth (n(%))			
Positive finding	15 (14.9)	2 (2.4)	**< 0.01**
Pain in previous month			
Positive finding	13 (12.9)	6 (7.2)	NS
Removable Prosthesis (n(%))			
None	92 (91.1)	79 (95.2)	
Acrylic	6 (5.9)	4 (4.8)	NS
Chrome	3 (3.0)		
Teeth present	25.0 (22.0-28.0)	26.0 (24.0-27.0)	NS
Sound & unrestored teeth	16.0 (6.2)	16.1 (4.8)	NS
Restored teeth (1-3 surfaces)	8.0 (5.0-10.0)	7.0 (5.0-11.5)	NS
n (%) with at least one	96.0 (93.2)	82 (98.8)	NS
Restored teeth (+4 surfaces)	0.0 (0.0-2.0)	0.0 (0.0-2.0)	NS
n (%) with at least one	46 (45.5)	32 (38.5)	NS
Teeth with caries into dentine	0.0 (0.0-0.0)	0.0 (0.0-0.0)	**< 0.001**
n (%) with at least one	21 (20.8)	4 (4.9)	**< 0.05**
Broken down teeth	0.0 (0.0-0.0)	0.0 (0.0-0.0)	NS
n (%) with at least one	7 (6.9)	2 (2.4)	NS
Endodontically treated teeth*	0.0 (0.0-0.0)	0.0 (0.0-1.0)	NS
n (%) with at least one	9 (20.9)	12 (33.3)	NS
Periapical radiolucencies*	0.0 (0.0-0.0)	0.0 (0.0-0.8)	NS
n (%) with at least one	7 (16.3)	8 (22.2)	NS

P-values determined using chi-squared test for discrete variables, Mann-Whitney U tests for continuous non-parametric variables and independent t-test for continuous parametric variables. *(n=43 diabetic subjects, n=36 non-diabetics subjects).

Table 4.6 Oral and Dental data comparing groups based on diabetic status and periodontal diagnosis

	Diabetic subjects (n=101)			Non-diabetic subjects (n=83)			p-value
	Healthy (n=14)	Gingivitis (n=39)	Periodontitis (n=48)	Healthy (n=16)	Gingivitis (n=19)	Periodontitis (n=48)	
Abnormal soft tissue -positive finding (n(%))	1 (7.1)	3 (7.7)	3 (6.35)	0 (0)	1 (5.2)	2 (4.2)	NS
Clinically dry mouth -positive finding (n(%))	2 (14.3)	3 (7.7)	3 (6.3)	0 (0)	1 (5.2)	0 (0)	NS
Reported dry mouth - positive finding (n(%))	3 (21.4)	5 (12.8)	7 (14.6)	0 (0)	1 (5.2)	1 (2.1)	NS
Pain – positive finding (n(%))	1 (7.1)	5 (12.8)	7 (14.6)	0 (0)	3 (15.7)	3 (6.3)	NS
Removable Prosthesis (n(%))							
None	14 (100)	35 (89.7)	43 (89.6)	15 (93.8)	18 (94.7)	46 (95.8)	
Acrylic	0 (0)	2 (5.1)	4 (8.3)	1 (6.3)	1 (5.2)	2 (4.2)	NS
Chrome	0 (0)	2 (5.1)	1 (2.1)	0 (0)	0 (0)	0 (0)	
Teeth present	25.0 (23.5-28.0)	26.0 (22.0-27.0)	25.0 (22.0-29.0)	27.0 (24.5-28.0)	25.0 (23.0-27)	26.0 (23.0-27.0)	NS
Sound & unrestored teeth	13.2 (±5.6)	16.3 (±5.8)	16.6 (±6.5)	17.1 (±5.0)	14.8 (±5.2)	17.0 (±4.5)	NS
Restored teeth (1-3 surfaces)	9.9 (±3.9)[#,¶]	6.5 (±3.6)[$]	7.0 (±3.6)	8.4 (±4.4)	9.0 (±4.2)	7.5 (±3.8)	[#,¶,$]**< 0.05**
n (%) with at least one	14 (100.0)	36 (92.3)	43 (89.6)	14 (87.5)	19 (100.0)	46 (95.8)	NS
Restored teeth (+4 surfaces)	1.0 (0.0-3.0)	0.0 (0.0-2.0)	0.0 (0.0-2.0)	0.0 (0.0-2.3)	0.0 (0.0-1.0)	0.0 (0.0-2.0)	NS
n (%) with at least one	7 (50.0)	18 (46.2)	21(43.8)	5 (31.3)	6 (31.6)	21 (43.8)	NS

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	Diabetic subjects (n=101)			Non-diabetic subjects (n=83)			p-value
	Healthy (n=14)	Gingivitis (n=39)	Periodontitis (n=48)	Healthy (n=16)	Gingivitis (n=19)	Periodontitis (n=48)	
Teeth with caries into dentine	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)$	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	$< **0.05**
n (%) with at least one	1(7.1)	9 (23.1)	11 (22.9)	2 (12.5)	2 (10.5)	2 (4.2)	**0.075**
Broken down teeth	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	NS
n (%) with at least one	0 (0.0)	3 (7.7)	4 (8.3)	0 (0.0)	1 (5.3)	1 (2.0)	NS
Endodontically treated teeth*	0	0.0 (0.0-0.0)	0.0 (0.0-0.25)	0	2.0 (1.0-3.0)	0.0 (0.0-1.0)	NS
Periapical radiolucencies *	0	0.0 (0.0-0.75)	0.0 (0.0-0.0)	0	0.0 (0.0-0.0)	0.0 (0.0-1.0)	NS

P-values were determined using chi-squared test for discrete variables, Kruskal-Wallis test with Mann-Whitney U post hoc tests for continuous non-parametric variables (years since diabetes diagnosed) and one way ANOVA test with post-hoc independent t-test for continuous parametric variables (age at diagnosis). Means (±SD) are presented for parametric data and medians (IQR) are presented for non-parametric data. *(n=5 diabetic subjects with gingivitis, n=38 diabetic subjects with periodontitis, n=2 non-diabetic subjects with gingivitis, n=34 non-diabetic subjects with periodontitis).

$ indicates a comparison within rows between diabetics and non-diabetic group with the same periodontal diagnosis
# indicates a comparison within rows between periodontally healthy and gingivitis groups within either diabetes or non-diabetes groups
¶ indicates a comparison within rows between periodontally healthy and periodontitis groups within either diabetes or non-diabetes groups
† indicates a comparison within rows between gingivitis and periodontitis groups within either diabetes or non-diabetes group

4.2.4 Oral health behaviour data

Table 4.7 summarises oral health behaviour data for diabetic patients and non-diabetic patients. Of note, when comparing the T2DM and the non-diabetic groups, there were significant differences in the proportions of subjects for each of the oral health behaviours, with a higher proportion of non-diabetic subjects reporting that they attend a GDP regularly, or reporting that they had attended at GDP in the last 12 months. Additionally, there were significant differences in the reason for the last visit to a GDP, with a higher proportion of non-diabetics attending for a check-up and a higher proportion of diabetics making emergency attendances. There were also significant differences in the frequency of tooth-brushing and interproximal cleaning, with a higher proportion of non-diabetics tooth-brushing at least twice a day and performing interproximal cleaning at least 3 times weekly (Table 4.7).

Table 4.8 presents data following further categorisation of subjects based on their periodontal diagnosis and demonstrates that there were significant differences in the proportions of subjects for each of the oral health behaviours. Figure 4.5 and 4.6 highlight that non-diabetic groups appeared to have a higher proportion of subjects tooth-brushing at least twice daily and attending a GDP regularly compared to diabetic subjects with the same periodontal condition (Table 4.8 and Figures 4.5 and 4.6).

Table 4.7 Oral health behaviour data comparing subjects with and without T2DM

	Diabetic subjects (n=101)	Non-diabetic subjects (n=83)	p-value
Attends GDP regularly			
positive finding (n(%))	62 (62.0)	62 (76.5)	**< 0.05**
Attended GDP within 12 months			
positive finding (n(%))	68 (68.0)	70 (86.4)	**< 0.01**
Reason for last visit (n(%)) *			
N/A	35 (35.0)	7 (9.1)	
Check-up	38 (38.0)	55 (71.4)	
Emergency	14 (14.0)	6 (7.8)	**< 0.001**
Restoration	3 (3.0)	5 (6.5)	
Periodontal therapy	1 (1.0)	3 (3.9)	
other	9 (9.0)	1 (1.3)	
Frequency of tooth-brushing (n(%))**			
<1/day	7 (7.0)	2 (2.4)	
1/day	29 (29.9)	5 (6.0)	**< 0.001**
2/day	60 (60.0)	68 (81.9)	
>2/day	4 (4.0)	8 (9.6)	
Frequency of interproximal (n(%))**			
never	65 (65.0)	30 (36.1)	**< 0.001**
1/wk	10 (10.0)	14 (16.9)	
>3/wk	25 (25.0)	39 (47.0)	

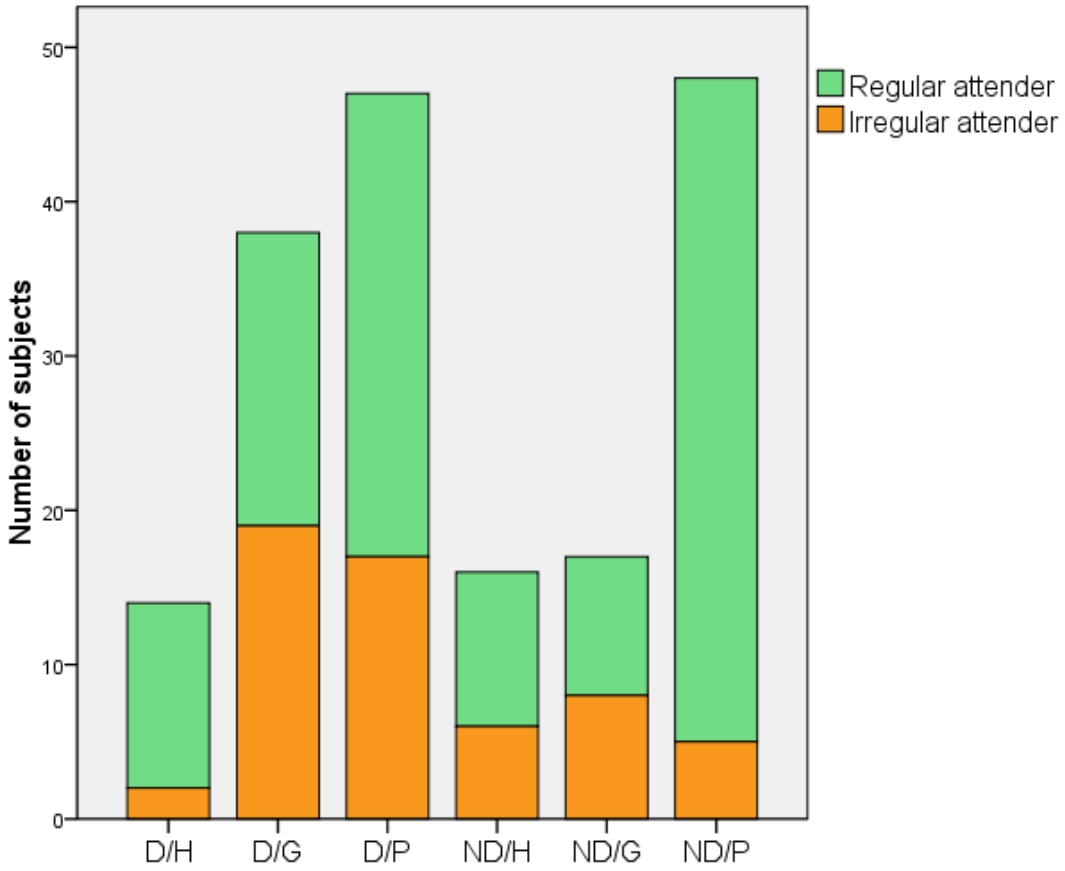
P-values were determined using chi-squared test for discrete variables. * n=100 diabetic subjects, n=77 non-diabetics subjects. ** n=100 diabetic subjects, n=83 non-diabetics subjects.

Table 4.8 Oral health behaviour data comparing groups based on diabetic status and periodontal diagnosis

	Diabetic (n=101)			Non-diabetic (n=83)			p-value
	Healthy (n=14)	**Gingivitis (n=39)**	**Periodontitis (n=48)**	**Healthy (n=16)**	**Gingivitis (n=19)**	**Periodontitis (n=48)**	
Attends GDP regularly–positive finding(n(%))	12 (85.7)	19 (48.7)	31 (64.6)	10 (62.5)	9 (47.4)	43 (89.6)	**< 0.001**
Attended GDP within 12 months-positive finding (n(%))	11 (78.6)	23 (59.0)	34 (70.8)	13 (81.3)	10 (52.6)	47 (97.9)	**< 0.001**
Reason for last visit (n(%)) *							
N/A	3 (21.4)	16 (41.0)	16 (33.3)	1 (6.3)	5 (26.3)	1 (2.1)	
Check-up	8 (57.1)	11 (28.2)	19(39.6)	10 (62.5)	5 (26.3)	40 (83.3)	
Emergency	2 (14.3)	7 (17.9)	5 (10.4)	2 (12.5)	1 (5.2)	3 (6.25)	**< 0.001**
Restoration	1 (7.1)	1 (2.6)	1 (2.1)	1 (6.3)	3 (15.8)	1 (2.1)	
Periodontal	0 (0)	0 (0)	1 (2.1)	0 (0)	0 (0)	3(6.25)	
Other	0 (0%)	3 (7.7)	6 (12.5)	0 (0)	1 (5.2)	0 (0)	
Frequency of tooth-brushing (n(%)) **							
<1/day	0 (0)	3 (7.7)	4 (8.3)	1 (6.3)	0 (0)	1 (2.1)	
1/day	2 (14.3)	15 (38.5)	12 (25.0)	0 (0)	1 (5.2)	4 (8.3)	**< 0.01**
2/day	11 (78.6)	19 (48.7)	30 (62.5)	14 (87.5)	18 (90.5)	36 (76.1)	
>2/day	1 (7.1)	1 (2.6)	2 (4.2)	1(6.3)	0 (0)	7 (14.6)	
Frequency of inter-proximal cleaning (n(%)) **							
never	5 (35.7)	30 (76.9)	30 (62.5)	2 (12.5)	9 (47.4)	19 (39.6)	
1/wk	6 (42.9)	1 (2.6)	3 (6.3)	3 (18.8)	6 (31.6)	5 (10.4)	**< 0.001**
>3/wk	3 (21.4)	7 (17.9)	15 (31.3)	11 (68.8)	4 (21.1)	24 (50.0)	

P-values were determined using chi-squared test for discrete variables. * n= 14 diabetic subjects with healthy periodontal tissues, n=38 diabetic subjects with gingivitis, n=48 diabetic subjects with periodontitis, n=14 non-diabetic subjects with healthy periodontal tissues, n=15 non-diabetic subjects with gingivitis, n=48 non-diabetic subjects with periodontitis. ** n= 14 diabetic subjects with healthy periodontal tissues, n=38 diabetic subjects with gingivitis, n=48 diabetic subjects with periodontitis, n=16 non-diabetic subjects with healthy periodontal tissues, n=19 non-diabetic subjects with gingivitis, n=48 non-diabetic subjects with periodontitis.

Figure 4.5 Categorisation using attendance at a GDP of subjects within groups based on periodontal and diabetes status

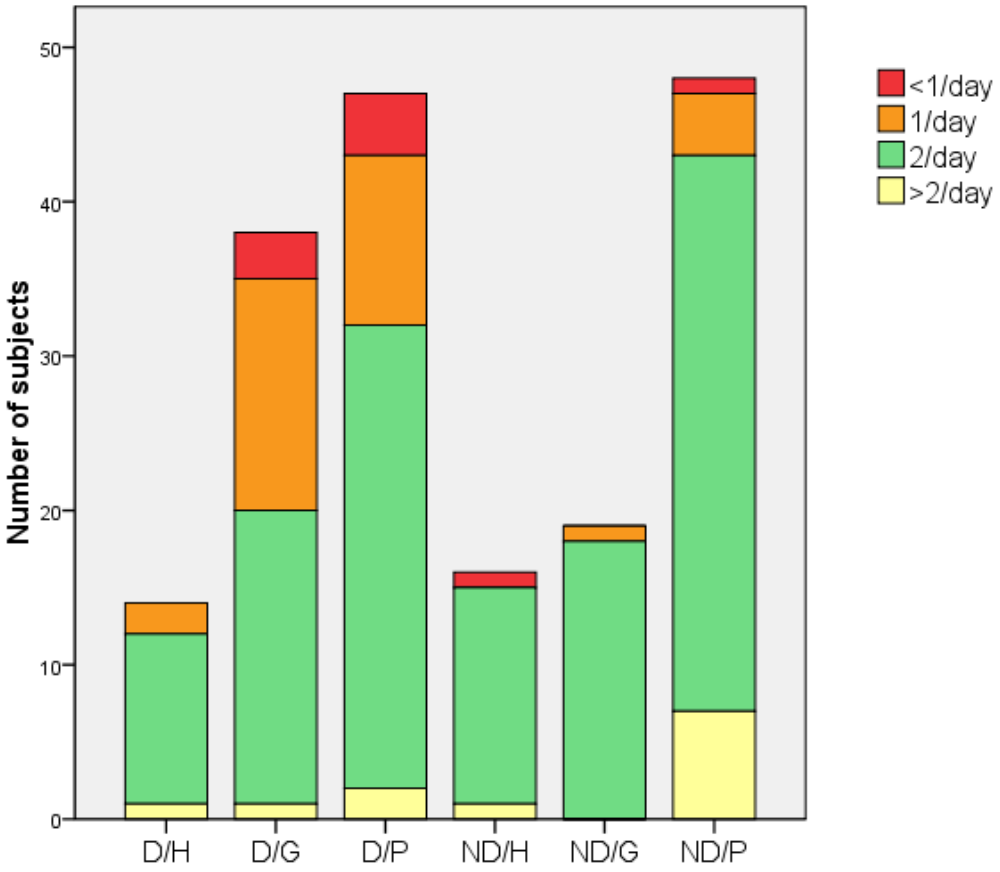


Stacked bar chart of subjects categorised based on regular attendance at a GDP for D/H patients (regular attender at GDP n=12, irregular attender at GDP n= 2), D/G patients (regular attender at GDP n=19, irregular attender at GDP n= 19), D/P patients (regular attender at GDP n=31, irregular attender at GDP n=17), ND/H patients (regular attender at GDP n=10, irregular attender at GDP n=6), ND/G patients (regular attender at GDP n=9, irregular attender at GDP n=8) ND/P patients (regular attender at GDP n=43, irregular attender at GDP n=5).

■ Attends GDP regularly, ■ Irregular attender at GDP

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

Figure 4.6 Categorisation using frequency of tooth-brushing of subjects within groups based on periodontal and diabetes status



Stacked bar chart of subjects categorised based on frequency of tooth brushing for D/H patients (once per day n= 2, twice per day n= 11, > twice per day n= 1), D/G patients (< once per day n=3, once per day n= 15, twice per day n=19 , > twice per day n=1), D/P patients (< once per day n=4, once per day n=12 , twice per day n= 30, > twice per day n=2), ND/H patients (< once per day n=1, twice per day n=14 , > twice per day n=1), ND/G patients (once per day n=1 , twice per day n=18) ND/P patients (< once per day n=1, once per day n=4 , twice per day n= 36, >twice per day n=7).

Tooth-brushing < once per day Tooth-brushing once per day
Tooth-brushing twice per day Tooth-brushing > twice per day

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

4.2.5 Pre-treatment clinical biochemistry data

Table 4.9 summarises the pre-treatment clinical biochemistry data for diabetic patients and non-diabetic patients. Of note, when comparing the T2DM and the non-diabetic groups there were significant differences for HbA1c, triglycerides, HDL, non-HDL and cholesterol. Subjects with diabetes had significantly lower levels of HDL, non-HDL [3.2 (2.7-3.9) mmol/L] and total cholesterol [4.4 (3.9-5.3) mmol/L] compared to non-diabetics [4.1(3.5-4.6) mmol/L and 5.5 (5.0-6.1) mmol/L]. As would be expected, HbA1c was significantly higher subjects with diabetes [7.2 (6.5-8.9)%] compared to the non-diabetic group subjects [5.5 (5.3-5.7)%]. Levels of high sensitive CRP also appeared higher in subjects with T2DM [2.3 (0.9-4.5) mg/L] compared to non-diabetic subjects [1.9 (0.8-3.9) mg/L], however the difference failed to reach statistical significance (Table 4.9 and Figure 4.7).

Table 4.10 presents pre-treatment clinical biochemistry data following further categorisation of subjects based on their periodontal diagnosis and demonstrates that HbA1c, as expected, was significantly higher in diabetic subjects compared to the non-diabetic subjects regardless of periodontal condition. Interestingly, when comparing groups in subjects with T2DM, HbA1c appeared higher in subjects with periodontitis [7.5 (6.7-9.2)%] compared to subjects with healthy periodontal tissues [6.4 (5.9-9.0)%]. However, the Bonferroni-Holm correction of p-values for multiple comparison placed the critical p-value at 0.017, therefore, this difference was detected as trend (p=0.036) (Table 4.10).

When comparing groups with different periodontal conditions, triglyceride levels were significantly higher in subjects with T2DM with healthy periodontal tissues [3.1 (2.2-3.9) mmol/L] and periodontitis [2.5 (1.7-4.2) mmol/L] compared to non-diabetic subjects with healthy periodontal tissues [1.0 (0.8-2.7) mmol/L] and periodontitis [1.7 (1.2-2.7) mmol/L]. When considering subjects with T2DM,

triglyceride levels were significantly higher in those with healthy periodontal tissues [3.1 (2.2-3.9) mmol/L] compared to those with periodontitis [2.5 (1.7-4.2) mmol/L] and lower in those with gingivitis [1.8 (1.3-2.6) mmol/L] compared to those with periodontitis [2.5 (1.7-4.2) mmol/L] (Table 4.10).

Following categorisation of data based on periodontal diagnosis, HDL levels were significantly lower in subjects with T2DM with healthy periodontal tissues [1.2 (1.2-1.6) mmol/L], gingivitis [1.2 (1.3-2.6) mmol/L] and periodontitis [1.1 (1.0-1.3) mmol/L] compared to non-diabetic subjects with healthy periodontal tissues [1.7 (1.5-2.0) mmol/L], gingivitis [1.5 (1.3-1.7) mmol/L] and periodontitis [1.4 (1.1-1.6) mmol/L]. In subjects with T2DM, HDL levels in subjects with healthy periodontal tissues [1.2 (1.2-1.6) mmol/L] appeared higher compared to subjects with periodontitis [1.1 (1.0-1.3) mmol/L]. However, the Bonferroni-Holm correction of p-values for multiple comparison placed the critical p-value at 0.017, therefore, the difference was detected as trend (p=0.04). Similarly, in non-diabetic subjects, HDL levels in subjects with healthy periodontal tissues [1.7 (1.5-2.0) mmol/L] appeared higher compared to subjects with periodontitis [1.4 (1.1-1.6) mmol/L]. However, the Bonferroni-Holm correction of p-values for multiple comparison placed the critical p-value at 0.017, therefore, the difference was detected as trend (p=0.021) (Table 4.10).

Following categorisation of data based on periodontal diagnosis, non-HDL levels were significantly lower in T2DM subjects with gingivitis [3.0 (2.3-3.7) mmol/L] and periodontitis [3.3 (2.8-3.9) mmol/L] compared to the non-diabetic subjects with gingivitis [4.4 (3.8-4.7) mmol/L] and periodontitis [4.2 (3.6-4.6) mmol/L]. When considering subjects with T2DM, non-HDL levels were significantly higher in those with healthy periodontal tissues [4.3 (3.0-5.1) mmol/L] compared to those with gingivitis [3.0 (2.3-3.7) mmol/L]. Similarly, in subjects with T2DM, non-HDL levels in those with healthy periodontal tissues [4.3 (3.0-5.1) mmol/L] appeared higher compared

to subjects with periodontitis [3.3 (2.8-3.9) mmol/L]. However, the Bonferroni-Holm correction of p-values for multiple comparison placed the critical p-value at 0.025, therefore, this difference was detected as trend (p=0.04) (Tables 4.9 and 4.10).

Following categorisation of data based on periodontal diagnosis, total-cholesterol levels were significantly higher in T2DM subjects with healthy periodontal tissues [5.5 (4.3-6.4) mmol/L] compared to T2DM subjects with gingivitis [4.2 (3.7-5.9) mmol/L]. Similarly, in subjects with T2DM, total cholesterol levels appeared higher in subjects with healthy periodontal tissues [5.5 (4.3-6.4) mmol/L] compared to those with periodontitis [4.4 (4.0-5.3) mmol/L]. However, the Bonferroni-Holm correction of p-values for multiple comparison placed the critical p-value at 0.025, therefore, this difference was detected as trend (p=0.026) (Tables 4.9 and 4.10).

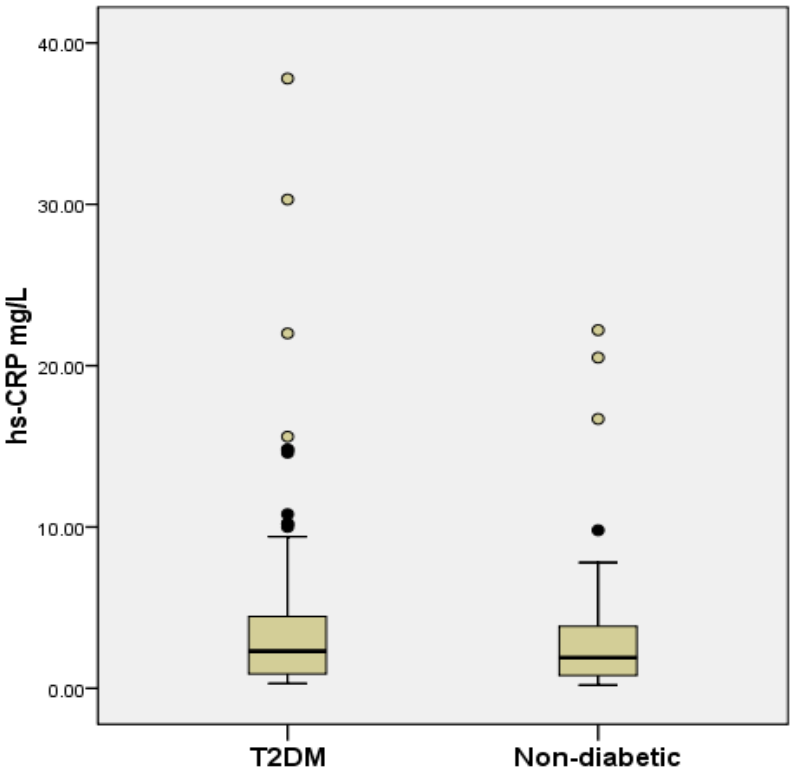
When considering hsCRP levels, subjects with T2DM and healthy periodontal tissues [2.4 (0.8-5.5) mg/L] had significantly higher hsCRP levels than non-diabetic subjects with healthy periodontal tissues [0.6 (0.2-1.5) mg/L]. Also, in non-diabetic subjects, those with healthy periodontal tissues [0.6 (0.2-1.5) mg/L] had significantly lower hsCRP levels compared to subjects with gingivitis [2.3 (0.8-4.2) mg/L] or periodontitis [2.3 (1.1-4.3) mg/L]. However, in subjects with T2DM similar differences were not found when comparing subjects with healthy periodontal tissues [2.4 (0.8-5.5) mg/L], gingivitis [2.4 (1.0-4.4) mg/L] and periodontitis [2.1 (0.9-4.6) mg/L] (Table 4.10).

Table 4.9 Pre-treatment clinical biochemistry data comparing subjects with and without T2DM

	Diabetics (n=99)	Non-diabetics (n=79)	p-value
HbA1c (%)	7.2 (6.5-8.9)	5.5 (5.3-5.7)	< 0.001
Triglycerides (mmol/L)	2.3 (1.5-3.6)	1.6 (1.1-2.4)	< 0.001
HDL (mmol/L)	1.2 (1-1.4)	1.5 (1.2-1.8)	< 0.001
Non-HDL (mmol/L)	3.2 (2.7-3.9)	4.1 (3.5-4.6)	< 0.001
Total cholesterol (mmol/L)	4.4 (3.9-5.3)	5.5 (5.0-6.1)	< 0.001
High sensitive CRP (mg/L)*	2.3 (0.9-4.5)	1.9 (0.8-3.9)	0.25

P-values determined using Mann Whitney-U tests as all variables are continuous non-parametric. Median and IQR presented for this non-parametric data. *(92 T2DM and 71 non-diabetic subjects)

Figure 4.7 Pre-treatment serum levels of high sensitive CRP in subjects with T2DM and in non-diabetic subjects



Boxplot of pre-treatment serum levels of high sensitive CRP in 92 T2DM and 71 non-diabetic subjects**.** Statistics: Mann Whitney-U test (differences were non-significant). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Table 4.10 Pre-treatment clinical biochemistry data comparing groups based on diabetic status and periodontal diagnosis

	Diabetic subjects (n=99)			Non-diabetic subjects (n=79)			p-value
	Healthy (n=14)	Gingivitis (n=38)	Periodontitis (n=47)	Healthy (n=15)	Gingivitis (n=17)	Periodontitis (n=47)	
HbA1c (%)	6.4 (5.9-9.0) [$]	7.0 (6.5-8.3) [$]	7.5 (6.7-9.2) [$]	5.5 (5.3-5.6)	5.7 (5.3-5.9)	5.5 (5.3-5.7)	[$]< 0.001
Triglycerides (mmol/L)	3.1 (2.2-3.9) [#, $]	1.8 (1.3-2.6) [†]	2.5 (1.7-4.2) [$]	1.0 (0.8-2.7)	1.7 (1.4-2.1)	1.7 (1.2-2.7)	[†,#,$]< 0.05
HDL (mmol/L)	1.2 (1.2-1.6) [$]	1.2 (1.1-1.5) [$]	1.1 (1.0-1.3) [$]	1.7 (1.5-2.0)	1.5 (1.3-1.7)	1.4 (1.1-1.6)	[$]< 0.05
Non-HDL (mmol/L)	4.3 (3.0-5.1) [#]	3.0 (2.3-3.7) [$]	3.3 (2.8-3.9) [$]	3.5 (2.9-4.3)	4.4 (3.8-4.7)	4.2 (3.6-4.6)	[$,#]< 0.05
Total cholesterol (mmol/L)	5.5 (4.3-6.4) [#]	4.2 (3.7-5.0) [$]	4.4 (4.0-5.3) [$]	5.1 (4.7-6.0)	5.9 (5.1-6.3)	5.5 (5.0-6.1)	[#, $]< 0.01
High sensitive CRP (mg/L)[*]	2.4 (0.8-5.5) [$]	2.4 (1.0-4.4)	2.1 (0.9-4.6)	0.6 (0.2-1.5) [#,¶]	2.3 (0.8-4.2)	2.3 (1.1-4.3)	[$,#,¶]< 0.05

P-values were determined using Kruskal-Wallis test with Mann-Whitney U post hoc tests for continuous non-parametric variables (all data). Medians (IQR) are presented for this non-parametric data. [*](n=13 diabetic subjects with healthy periodontal tissues, n=36 diabetic subjects with gingivitis, n=43 diabetic subjects with periodontitis, n=15 non-diabetic subjects with healthy periodontal tissues, n=14 non-diabetic subjects with gingivitis, n=42 non-diabetic subjects with periodontitis).

[$] indicates a comparison within rows between diabetics and non-diabetic group with the same periodontal diagnosis
[#] indicates a comparison within rows between periodontally healthy and gingivitis groups within either diabetes or non-diabetes groups
[¶] indicates a comparison within rows between periodontally healthy and periodontitis groups within either diabetes or non-diabetes groups
[†] indicates a comparison within rows between gingivitis and periodontitis groups within either diabetes or non-diabetes group

4.2.6 Pre-treatment clinical periodontal data

Table 4.11 summarises the clinical periodontal data for diabetic patients and non-diabetic patients before periodontal management. Of note, when comparing the T2DM and the non-diabetic groups, there were significant differences in mean PI, PESA, % sites with PD ≥6mm and % of sites with LOA ≥6mm. The subjects with T2DM had significantly higher PI [0.8 (0.6-1.0)mmol/L] compared to the non-diabetic subjects [0.6 (0.3-0.8)mmol/L]. Conversely, in subjects with T2DM, PESA [1038.9 (817.9-1327.6) mm^2] was significantly lower compared to non-diabetic subjects [1372.7 (849.2-1801.0) mm^2]. Furthermore, T2DM subjects had significantly fewer sites with PD ≥ 6 mm [0.0 (0.0-2.0)] and fewer sites with LOA ≥ 6 mm [0.7 (0.0-4.4)] compared to non-diabetic subjects [0.7 (0.0-11.3) and 3.2 (0.0-15.1) respectively] (Table 4.11).

Table 4.12 presents pre-treatment clinical periodontal data following further categorisation of subjects based on their periodontal diagnosis. When considering levels of plaque control, PI was significantly higher in diabetic subjects with healthy periodontal tissues [0.4 (0.3-0.8)], gingivitis [0.9 (0.7-1.0)] and periodontitis [0.8 (0.6-1.1)] compared to the non-diabetic subjects with healthy periodontal tissues [0.2 (0.0-0.4)], gingivitis [0.6 (0.4-0.7)] and periodontitis [0.6 (0.4-0.8)]. When comparing the subjects with T2DM, PI was significantly higher in those with gingivitis [0.9 (0.7-1.0)] compared to those with healthy periodontal tissues [0.4 (0.3-0.8)]. Similarly, in subjects with T2DM, PI was significantly higher in those with periodontitis [0.8 (0.6-1.1)] compared to those with healthy periodontal tissues [0.4 (0.3-0.8)]. For non-diabetic subjects, PI was significantly higher in those with gingivitis [0.6 (0.4-0.7)] compared to those with healthy periodontal tissues [0.2 (0.0-0.4)]. Similarly, in non-diabetic subjects, PI was significantly higher in those with periodontitis [0.6 (0.4-0.8)]. compared to those with healthy periodontal tissues [0.2 (0.0-0.4)] (Table 4.12 and Figure 4.8).

Table 4.12 presents pre-treatment clinical periodontal data following further categorisation of subjects based on their periodontal diagnosis. When considering levels of gingival inflammation, mGI was significantly higher in diabetic subjects with healthy periodontal tissues [0.8 (0.5-1.6)] and gingivitis [1.9 (1.3-2.5)] compared to the non-diabetic subjects with healthy periodontal tissues [0.5(0.3-0.8)] and gingivitis [1.3 (0.8-1.7)]. When comparing the subjects with T2DM, mGI was significantly higher in those with gingivitis [1.9 (1.3-2.5)] compared to those with healthy periodontal tissues [0.8 (0.5-1.6)]. Similarly, in subjects with T2DM, mGI was significantly higher in those with periodontitis [2.0 (1.5-2.7)] compared to those with healthy periodontal tissues [0.8 (0.5-1.6)]. For non-diabetic subjects, mGI was significantly higher in those with gingivitis [1.3 (0.8-1.7)] compared to those with healthy periodontal tissues [0.5 (0.3-0.8)]. Similarly, in non-diabetic subjects, mGI was significantly higher in those with periodontitis [2.4 (2.0-2.7)] compared to those with healthy periodontal tissues [0.5 (0.3-0.8)]. Additionally, in non-diabetic subjects mGI was significantly higher in the periodontitis group [2.4 (2.0-2.7)] compared to those with gingivitis [1.3 (0.8-1.7)] (Table 4.12 and Figure 4.9).

When considering bleeding on probing, % BOP was significantly higher in diabetic subjects with healthy periodontal tissues [4.5 (0.7-13.1)%] and gingivitis [35.1 (25.0-44.9)%] compared to the non-diabetic subjects with healthy periodontal tissues [0.7(0.0-2.6)%] and gingivitis [22.0 (17.3-32.6)%]. When comparing the subjects with T2DM, % BOP was significantly higher in those with gingivitis [35.1 (25.0-44.9)%] compared to those with healthy periodontal tissues [4.5 (0.7-13.1)%]. Similarly, in subjects with T2DM, % BOP was significantly higher in those with periodontitis [46.0 (30.0-60.7)%] compared to those with healthy periodontal tissues [4.5 (0.7-13.1)%]. For non-diabetic subjects, % BOP was significantly higher in those with gingivitis [22.0 (17.3-32.6)%] compared to those with healthy periodontal tissues [0.7 (0.0-2.6)%].

Similarly, in non-diabetic subjects, % BOP was significantly higher in those with periodontitis [43.0 (29.4-56.7)%] compared to those with healthy periodontal tissues [0.7 (0.0-2.6)%]. Additionally, in non-diabetic subjects % BOP was significantly higher in the periodontitis group [43.0 (29.4-56.7)%] compared to those with gingivitis [22.0 (17.3-32.6)%] (Table 4.12 and Figure 4.10).

When considering probing depths, mean PD was significantly higher in diabetic subjects with healthy periodontal tissues [1.7 (1.6-1.8) mm] and gingivitis [2.1 (1.2-2.2) mm] compared to the non-diabetic subjects with healthy periodontal tissues [1.6 (1.5-1.7) mm] and gingivitis [1.9 (1.8-2.1) mm]. When comparing the subjects with T2DM, mean PD was significantly higher in those with gingivitis [2.1 (1.2-2.2) mm] compared to those with healthy periodontal tissues [1.7 (1.6-1.8) mm]. Similarly, in subjects with T2DM, mean PD was significantly higher in those with periodontitis [2.8 (2.4-3.2) mm] compared to those with healthy periodontal tissues [1.7 (1.6-1.8) mm]. Additionally, in subjects with T2DM, mean PD was significantly higher in the periodontitis group [2.8 (2.4-3.2) mm] compared to those with gingivitis [2.1 (1.2-2.2) mm]. For non-diabetic subjects, mean PD was significantly higher in those with gingivitis [1.9 (1.8-2.1) mm] compared to those with healthy periodontal tissues [1.6 (1.5-1.7) mm]. Similarly, in non-diabetic subjects, mean PD was significantly higher in those with periodontitis [2.9(2.5-3.5) mm] compared to those with healthy periodontal tissues [1.6(1.5-1.7) mm]. Additionally, in non-diabetic subjects mean PD was significantly higher in the periodontitis group [2.9(2.5-3.5) mm] compared to those with gingivitis [1.9(1.8-2.1) mm] (Table 4.12 and Figure 4.11).

When considering the percentage of sites with PD $\geq$ 4 mm, there was a significantly greater % in diabetic subjects with healthy periodontal tissues [0.7 (0.0-1.7) %] compared to the non-diabetic subjects with healthy periodontal tissues [0.0 (0.0-0.0) %]. When comparing the subjects with T2DM, % of sites with PD $\geq$ 4 mm was

significantly higher in those with gingivitis [3.6 (1.4-7.0) %] compared to those with healthy periodontal tissues [0.7 (0.0-2.7) %]. Similarly, in subjects with T2DM, % of sites with PD ≥ 4 mm was significantly higher in those with periodontitis [20.2 (12.7-35.2) %] compared to those with healthy periodontal tissues [0.7 (0.0-1.7) %]. Additionally, in subjects with T2DM, the % of sites with PD ≥ 4 mm was significantly higher in the periodontitis group [20.2 (12.7-35.2) %] compared to those with gingivitis [3.6 (1.4-7.0) %]. For non-diabetic subjects, % of sites with PD ≥ 4 mm was significantly higher in those with gingivitis [2.0 (0.6-5.1) %] compared to those with healthy periodontal tissues [0.0 (0.0-0.0) %]. Similarly, in non-diabetic subjects, % of sites with PD ≥ 4 mm was significantly higher in those with periodontitis [24.2 (18.0-40.0) %] compared to those with healthy periodontal tissues [0.0 (0.0-0.0) %]. Additionally, in non-diabetic subjects % of sites with PD ≥ 4 mm was significantly higher in the periodontitis group [24.2 (18.0-40.0) %] compared to those with gingivitis [2.0 (0.6-5.1) %] (Table 4.12 and Figure 4.12).

When considering the percentage of PD sites ≥5mm, there were no significant differences found in any periodontal category between T2DM and non-diabetic subjects. When comparing the subjects with T2DM, % of sites with PD ≥ 5 mm was significantly higher in those with gingivitis [0.0 (0.0-1.2) %] compared to those with healthy periodontal tissues [0.0 (0.0-0.0) %], although the clinical significance of this difference is likely to be minimal. Similarly, in subjects with T2DM, % of sites with PD ≥ 5 mm was significantly higher in those with periodontitis [10.9 (8.1-18.2) %] compared to those with healthy periodontal tissues [0.0 (0.0-0.0) %]. Additionally, insubjects with T2DM, the % of sites with PD ≥ 5 mm was significantly higher in the periodontitis group [10.9 (8.1-18.2) %] compared to those with gingivitis [0.0 (0.0-1.2) %]. For non-diabetic subjects, % of sites with PD ≥ 5 mm was significantly higher in those with gingivitis [0.0 (0.0-0.0) %] compared to those with healthy periodontal

tissues [0.0 (0.0-0.0) %], although the clinical significance of this difference is likely to be minimal. Similarly, in non-diabetic subjects, % of sites with PD ≥ 5 mm was significantly higher in those with periodontitis [16.0 (8.0-30.8) %] compared to those with healthy periodontal tissues [0.0 (0.0-0.0) %]. Additionally, in non-diabetic subjects % of sites with PD ≥ 5 mm was significantly higher in the periodontitis group [16.0 (8.0-30.8) %] compared to those with gingivitis [0.0 (0.0-0.0) %] (Table 4.12 and Figure 4.13).

The percentage of PD sites ≥ 6 mm, was significantly lower in diabetic subjects with periodontitis [2.7 (0.7-7.1) %] compared to the non-diabetic subjects with periodontitis [8.7 (2.6-16.7) %]. When comparing the subjects with T2DM, % of sites with PD ≥ 6 mm was significantly higher in those with periodontitis [2.7 (0.7-7.1) %] compared to those with healthy periodontal tissues [0.0 (0.0-0.0) %]. Additionally, in subjects with T2DM, the % of sites with PD ≥ 6 mm was significantly higher in the periodontitis group [2.7 (0.7-7.1) %] compared to those with gingivitis [0.0 (0.0-0.0) %]. For non-diabetic subjects, % of sites with PD ≥ 6 mm was significantly higher in those with periodontitis [8.7 (2.6-16.7) %] compared to those with healthy periodontal tissues [0.0 (0.0-0.0) %]. Additionally, in non-diabetic subjects % of sites with PD ≥ 6 mm was significantly higher in the periodontitis group [8.7 (2.6-16.7) %] compared to those with gingivitis [0.0 (0.0-0.0) %] (Table 4.12 and Figure 4.14).

When considering loss of attachment, mean LOA was significantly higher in diabetic subjects with gingivitis [2.3 (2.1-2.4) mm] compared to the non-diabetic subjects with gingivitis [2.1 (2.0-2.3) mm]. When comparing the subjects with T2DM, mean LOA was significantly higher in those with gingivitis [2.3 (2.1-2.4)] compared to those with healthy periodontal tissues [1.9 (1.8-2.1)]. Similarly, in subjects with T2DM, mean LOA was significantly higher in those with periodontitis [3.1 (2.7-3.9)] compared to those with healthy periodontal tissues [1.9 (1.8-2.1)]. Additionally, in subjects with

T2DM, the mean LOA was significantly higher in the periodontitis group [3.1 (2.7-3.9)] compared to those with gingivitis [2.3 (2.1-2.4)]. For non-diabetic subjects, the mean LOA significantly higher in those with gingivitis [2.1 (2.0-2.3)] compared to those with healthy periodontal tissues [1.8 (1.6-2.0)]. Similarly, in non-diabetic subjects, mean LOA was significantly higher in those with periodontitis [3.4 (2.9-4.2)] compared to those with healthy periodontal tissues [1.8 (1.6-2.0)]. Additionally, in non-diabetic subjects mean LOA was significantly higher in the periodontitis group [3.4 (2.9-4.2)] compared to those with gingivitis [2.1 (2.0-2.3)] (Table 4.12).

When considering the percentage of sites with LOA ≥ 4mm, there were no significant differences found in any periodontal category between T2DM and non-diabetic subjects. When comparing the subjects with T2DM, the % of sites with LOA ≥ 4 mm was significantly higher in those with gingivitis [5.8 (1.8-10.1) %] compared to those with healthy periodontal tissues [2.3 (0.5-6.1) %]. Similarly, in subjects with T2DM, the % of sites with LOA ≥ 4 mm was significantly higher in those with periodontitis [32.0 (19.5-51.2) %] compared to those with healthy periodontal tissues [2.3 (0.5-6.1) %]. Additionally, in subjects with T2DM, the % of sites with LOA ≥ 4 mm was significantly higher in the periodontitis group [32.0 (19.5-51.2) %] compared to those with gingivitis [5.8 (1.8-10.1) %]. For non-diabetic subjects, the % of sites with LOA ≥ 4 mm significantly higher in those with gingivitis [4.2 (0.7-8.2) %] compared to those with healthy periodontal tissues [1.0 (0.0-4.0) %]. Similarly, in non-diabetic subjects, the % of sites with LOA ≥ 4 mm was significantly higher in those with periodontitis [39.9(25.6-58.0) %] compared to those with healthy periodontal tissues [1.0(0.0-4.0) %]. Additionally, in non-diabetic subjects the % of sites with LOA ≥ 4 mm was significantly higher in the periodontitis group [39.9(25.6-58.0) %] compared to those with gingivitis [4.2(0.7-8.2) %] (Table 4.12).

When considering the percentage of LOA sites ≥ 5 mm, there were no significant differences found in any periodontal category between T2DM and non-diabetic subjects. When comparing the subjects with T2DM, the % of sites with LOA ≥ 5 mm was significantly higher in those with periodontitis [17.3 (10.6-31.7) %] compared to those with healthy periodontal tissues [0.5 (0.0-1.7) %]. Additionally, in subjects with T2DM, the % of sites with LOA ≥ 5 mm was significantly higher in the periodontitis group 17.3 (10.6-31.7) %] compared to those with gingivitis [0.8 (0.0-2.6) %]. For non-diabetic subjects, % of sites with LOA ≥ 5 mm was significantly higher in those with gingivitis [0.0 (0.0-1.6) %] compared to those with healthy periodontal tissues [0.0 (0.0-0.0) %], although the clinical significance of this difference is likely to be minimal. Similarly, in non-diabetic subjects, % of sites with LOA ≥ 5 mm was significantly higher in those with periodontitis [24.7 (14.5-37.2) %] compared to those with healthy periodontal tissues [0.0 (0.0-0.0) %]. Additionally, in non-diabetic subjects % of sites with LOA ≥ 5 mm was significantly higher in the periodontitis group [24.7 (14.5-37.2) %] compared to those with gingivitis [0.0 (0.0-1.6) %] (Table 4.12).

When considering the percentage of sites with LOA ≥ 6 mm, there was a significantly greater % in non-diabetic subjects with periodontitis [12.7 (6.0-25.6) %] compared to the diabetic subjects with and periodontitis [4.6 (1.2-13.3) %]. Conversely, there was a significantly greater % in diabetic subjects with gingivitis [0.0 (0.0-0.7) %] compared to the non-diabetic subjects with and gingivitis [0.0 (0.0-0.0) %]. When comparing the subjects with T2DM, the % of sites with LOA ≥ 6 mm was significantly higher in those with periodontitis [4.6 (1.2-13.3) %] compared to those with healthy periodontal tissues [0.0 (0.0-0.2) %]. Additionally, in subjects with T2DM, the % of sites with LOA ≥ 6 mm was significantly higher in the periodontitis group [4.6 (1.2-13.3) %] compared to those with gingivitis [0.0 (0.0-0.7) %]. For non-diabetic subjects, % of sites with LOA ≥ 6 mm was significantly higher in those with periodontitis [12.7

(6.0-25.6) %] compared to those with healthy periodontal tissues [0.0 (0.0-0.0) %]. Additionally, in non-diabetic subjects % of sites with LOA ≥ 6 mm was significantly higher in the periodontitis group [12.7 (6.0-25.6) %] compared to those with gingivitis [0.0 (0.0-0.0) %] (Table 4.12).

When considering periodontal epithelial surface area, PESA was a significantly greater in non-diabetic patients with periodontitis [1744.9±519.9 mm^2] compared to diabetic subjects with periodontitis [1444.7±495.1 mm^2]. When comparing the subjects with T2DM, the PESA was significantly higher in those with gingivitis [921.5±184.3 mm^2] compared to those with healthy periodontal tissues [698.5±197.3 mm^2]. Similarly, in subjects with T2DM, the PESA was significantly higher in those with periodontitis [1444.7±495.1 mm^2] compared to those with healthy periodontal tissues [698.5±197.3 mm^2]. Additionally, in subjects with T2DM, the PESA was significantly higher in the periodontitis group [1444.7±495.1 mm^2] compared to those with gingivitis [921.5±184.3 mm^2]. For non-diabetic subjects, the PESA was significantly higher in those with gingivitis [921.4±154.4 mm^2] compared to those with healthy periodontal tissues [787.2±82.1 mm^2]. Similarly, in non-diabetic subjects, the PESA was significantly higher in those with periodontitis [1744.9±519.9 mm^2] compared to those with healthy periodontal tissues [787.2±82.1 mm^2]. Additionally, in non-diabetic subjects the PESA was significantly higher in the periodontitis group [1744.9±519.9 mm^2] compared to those with gingivitis [921.4±154.4 mm^2] (Table 4.12, Figure 4.15)

When considering the periodontal surface area which was considered inflamed due to the presence of bleeding on probing, there was no significant differences in PISA were found in any periodontal category between T2DM and non-diabetic subjects. When comparing the subjects with T2DM, the PISA was significantly higher in those with gingivitis [342.4 (239.4-439.1) mm^2] compared to those with healthy periodontal tissues [24.7 (0.0-89.8) mm^2]. Similarly, in subjects with T2DM, the PISA was

significantly higher in those with periodontitis [683.0 (439.1-1085.5) mm^2] compared to those with healthy periodontal tissues [24.7 (0.0-89.8) mm^2]. Additionally, subjects with T2DM, the PISA was significantly higher in the periodontitis group [683.0 (439.1-1085.5) mm^2] compared to those with gingivitis [342.4 (239.4-439.1) mm^2]. For non-diabetic subjects, the PISA significantly higher in those with gingivitis [242.6 (195.6-353.3) mm^2] compared to those with healthy periodontal tissues [4.48 (0.0-23.2) mm^2]. Similarly, in non-diabetic subjects, the PISA was significantly higher in those with periodontitis [897.3 (683.6-1232.9) mm^2] compared to those with healthy periodontal tissues [4.48(0.0-23.2) mm^2]. Additionally, in non-diabetic subjects the PISA was significantly higher in the periodontitis group [897.3(683.6-1232.9) mm^2] compared to those with gingivitis [242.6(195.6-353.3) mm^2] (Table 4.12 and Figure 4.16)

Table 4.11 Pre-treatment clinical periodontal data comparing subjects with and without T2DM

	Diabetic subjects (n=101)	Non-diabetic subjects (n=83)	p-value
Month 0 mean mGI	1.9 (1.3-2.5)	1.8 (0.8-2.5)	NS
Month 0 mean PI	0.8 (0.6-1.0)	0.6 (0.3-0.8)	< 0.001
Month 0 BOP (%)	36.3 ±22.4	31.6 ±22.7	NS
Month 0 mean PD (mm)	2.4 (2.0-2.8)	2.5 (1.8-3.1)	NS
Month 0 mean recession (mm)	0.3 (0.1-0.5)	0.3 (0.2-0.6)	NS
Month 0 mean LOA (mm)	2.7 (2.2-3.1)	2.9 (2.0-3.5)	NS
Month 0 PESA (mm^2)	1038.9 (817.9-1327.6)	1372.7 (849.2-1801.0)	< 0.05
Month 0 PISA (mm^2)	526.7 (201.1-746.8)	634.1 (145.3-963.0)	NS
Month 0 % PD sites ≥4mm	7.8 (2.2-18.5)	14.7 (0.6-31.3)	NS
Month 0 total assessed sites	150 (132.0-162.0)	156.0 (144.0-162.0)	NS
Month 0 % PD sites ≥5mm	2.9 (0.0-10.5)	5.6 (0.0-21.8)	NS
Month 0 % PD sites ≥6mm	0.0 (0.0-2.0)	0.7 (0.0-11.3)	< 0.01
Month 0 % LOA sites ≥4mm	12. (4.3-31.1)	17.9 (3.3-41.7)	NS
Month 0 % LOA sites ≥5mm	4.0 (0.68-16.2)	8.3 (0.0-27.9)	NS
Month 0 % LOA sites ≥6mm	0.7(0.0-4.4)	3.2 (0.0-15.1)	< 0.05

P-values determined using independent t-test for continuous parametric variables (% BOP) and Mann-Whitney U tests for the remaining continuous non-parametric variables. Means (±SD) are presented for parametric data and medians (IQR) are presented for non-parametric data.

Table 4.12 Pre-treatment clinical periodontal data comparing groups based on diabetic status and periodontal diagnosis

	Diabetic subjects (n=101)			Non-diabetic subjects (n=83)			p-value
	Healthy (n=14)	Gingivitis (n=39)	Periodontitis (n=48)	Healthy (n=16)	Gingivitis (n=19)	Periodontitis (n=48)	
Month 0 mean PI	0.4 (0.3-0.8) [#,¶,$]	0.9 (0.7-1.0) [$]	0.8 (0.6-1.1) [$]	0.2 (0.0-0.4) [#,¶]	0.6 (0.4-0.7)	0.6 (0.4-0.8)	[#,¶]< 0.001 [$]< 0.05
Month 0 mean mGI	0.8 (0.5-1.6) [#,¶,$]	1.9 (1.3-2.5) [$]	2.0 (1.5-2.7)	0.5 (0.3-0.8) [#,¶]	1.3 (0.8-1.7) [†]	2.4 (2.0-2.7)	[#,¶,†]< 0.001 [$]< 0.05
Month 0 BOP (%)	4.5 (0.7-13.1) [#,¶,$]	35.1(25.0-44.9) [†,$]	46.0 (30.0-60.7)	0.7 (0.0-2.6) [#,¶]	22.0 (17.3-32.6) [†]	43.0 (29.4-56.7)	[#,¶]< 0.001 [†,$]< 0.05
Month 0 mean PD (mm)	1.7 (1.6-1.8) [#,¶,$]	2.1(1.2-2.2) [†,$]	2.8 (2.4-3.2)	1.6 (1.5-1.7) [#,¶]	1.9 (1.8-2.1) [†]	2.9 (2.5-3.5)	[#,¶,†]< 0.001 [$]< 0.05
Month 0 mean recession (mm)	0.3 (0.1-0.4)	0.2 (0.1-0.3) [†]	0.4 (0.3-0.7)	0.2 (0.1 0.4) [¶]	0.2 (0.1-0.3) [†]	0.4 (0.2-0.9)	[†,¶]< 0.01
Month 0 mean LOA (mm)	1.9 (1.8-2.1) [#,¶]	2.3 (2.1-2.4) [†,$]	3.1 (2.7-3.9)	1.8 (1.6-2.0) [#,¶]	2.1 (2.0-2.3) [†]	3.4 (2.9-4.2)	[#,¶,†]< 0.001 [$]< 0.05
Month 0 PESA (mm^2)	698.5±197.3 [#,¶]	921.5±184.3 [†]	1444.7±495.1 [$]	787.2±82.1 [#,¶]	921.4±154.4 [†]	1744.9±519.9	[¶,†]< 0.001 [#,$]< 0.01
Month 0 PISA (mm^2)	24.7 (0.0-89.8) [#,¶]	342.4 (239.4-439.1) [†]	683.0 (439.1-1085.5)	4.48 (0.0-23.2) [#,¶]	242.6 (195.6-353.3) [†]	897.3 (683.6-1232.9)	[†,¶]< 0.001
Month 0 total sites assessed	150.0 (141.0-159.0)	150.0 (132.0-162.0)	150.0 (132.0-162.0)	156.0 (144.0-156.0)	150 (144.0-156.0)	156.0 (138.0-162.0)	NS
Month 0 % PD sites ≥4mm	0.7 (0.0-1.7) [#,¶,$]	3.6(1.4-7.0) [†]	20.2 (12.7-35.2)	0.0 (0.0-0.0) [#,¶]	2.0 (0.6-5.1) [†]	24.2 (18.0-40.0)	[#,¶,$,†]< 0.001
Month 0 % PD sites ≥5mm	0.0 (0.0-0.0) [#,¶]	0.0 (0.0-1.2) [†]	10.9 (8.1-18.2)	0.0 (0.0-0.0) [#,¶]	0.0 (0.0-0.0) [†]	16.0 (8.0-30.8)	[#,¶] < 0.001 [†]< 0.05
Month 0 % PD sites ≥6mm	0.0 (0.0-0.0) [¶]	0.0 (0.0-0.0) [†]	2.7 (0.7-7.1) [$]	0.0 (0.0-0.0) [¶]	0.0 (0.0-0.0) [†]	8.7 (2.6-16.7)	[¶,†]< 0.001 [$]< 0.01

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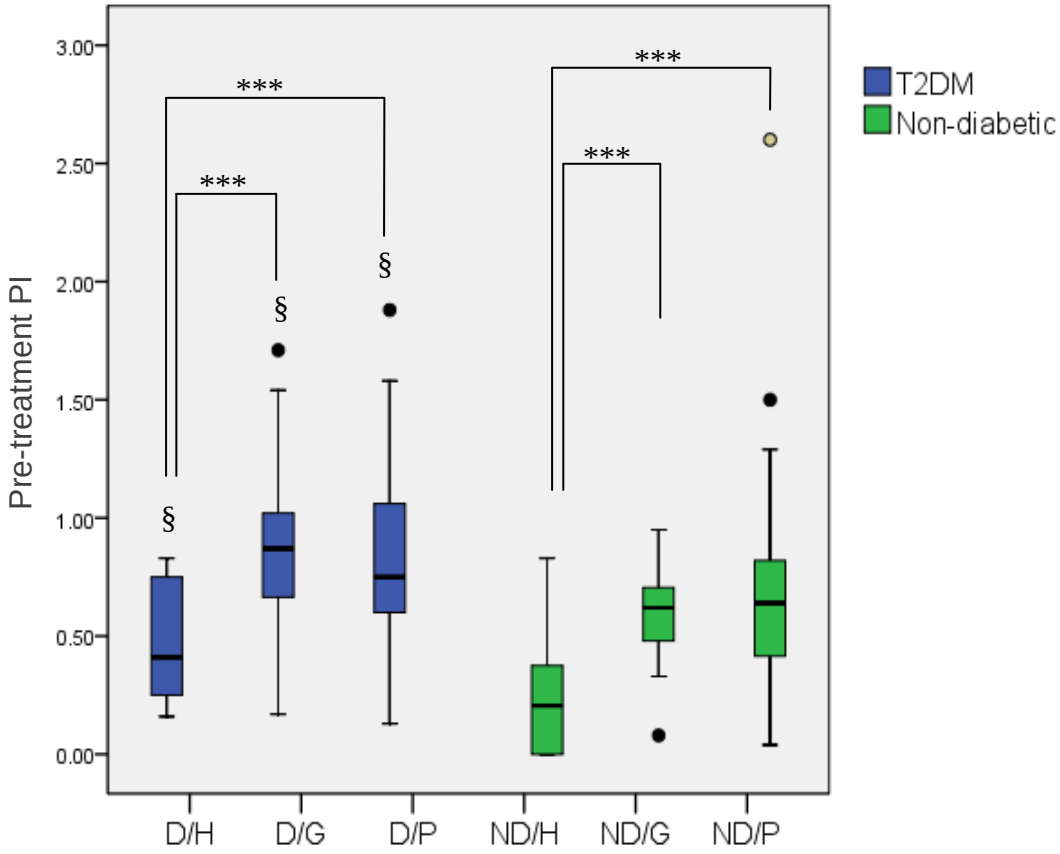
	Diabetic subjects (n=101)			Non-diabetic subjects (n=83)			p-value
	Healthy (n=14)	**Gingivitis (n=39)**	**Periodontitis (n=48)**	**Healthy (n=16)**	**Gingivitis (n=19)**	**Periodontitis (n=48)**	
Month 0 % LOA sites ≥4mm	2.3 (0.5-6.1) [#,¶]	5.8 (1.8-10.1) [†]	32.0 (19.5-51.2)	1.0 (0.0-4.0) [#,¶]	4.2 (0.7-8.2) [†]	39.9 (25.6-58.0)	[#]< 0.05 [¶,†]< 0.001
Month 0 % LOA sites ≥5mm	0.5 (0.0-1.7) [¶]	0.8 (0.0-2.6) [†]	17.3 (10.6-31.7)	0.0 (0.0-0.0) [#,¶]	0.0 (0.0-1.6) [†]	24.7 (14.5-37.2)	[#,¶,†]< 0.05
Month 0 % LOA sites ≥6mm	0.0 (0.0-0.2) [¶]	0.0 (0.0-0.7) [†,$]	4.6 (1.2-13.3) [$]	0.0 (0.0-0.0) [¶]	0.0 (0.0-0.0) [†]	12.7 (6.0-25.6)	[¶,†]< 0.001 [$]< 0.05

P-values were determined using chi-squared test for discrete variables, Kruskal-Wallis test with Mann-Whitney U post hoc tests for continuous non-parametric variables and one way ANOVA test with post-hoc independent t-test for continuous parametric variables (PESA). Mean (±SD) is presented for parametric data and median (IQR) is presented for non-parametric data.

[$] indicates a comparison within rows between diabetics and non-diabetic group with the same periodontal diagnosis
[#] indicates a comparison within rows between periodontally healthy and gingivitis groups within either diabetes or non-diabetes groups
[¶] indicates a comparison within rows between periodontally healthy and periodontitis groups within either diabetes or non-diabetes groups
[†] indicates a comparison within rows between gingivitis and periodontitis groups within either diabetes or non-diabetes groups

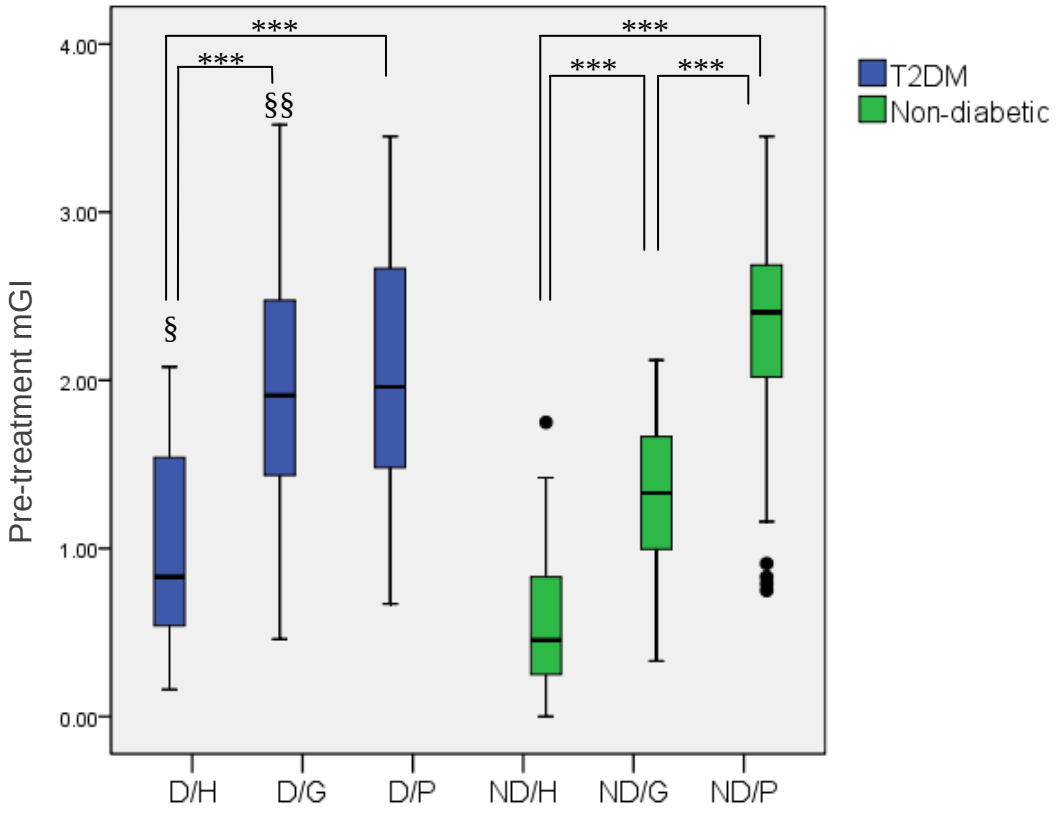
Figure 4.8 Pre-treatment mean PI data comparing groups based on diabetic status and periodontal diagnosis



Boxplots of pre-treatment mean PI data in 100 T2DM subjects (healthy periodontal tissues n=14, gingivitis n=39, periodontitis n=47) and 83 non-diabetic subjects (healthy periodontal tissues n=16, gingivitis n=19, periodontitis n=48)**.** Statistics: Kruskal-Wallis with Mann Whitney-U post hoc test *<0.05, **p<0.01, ***p<0.001(according to periodontal status within T2DM or non-diabetic group); § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups within the corresponding periodontal status). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

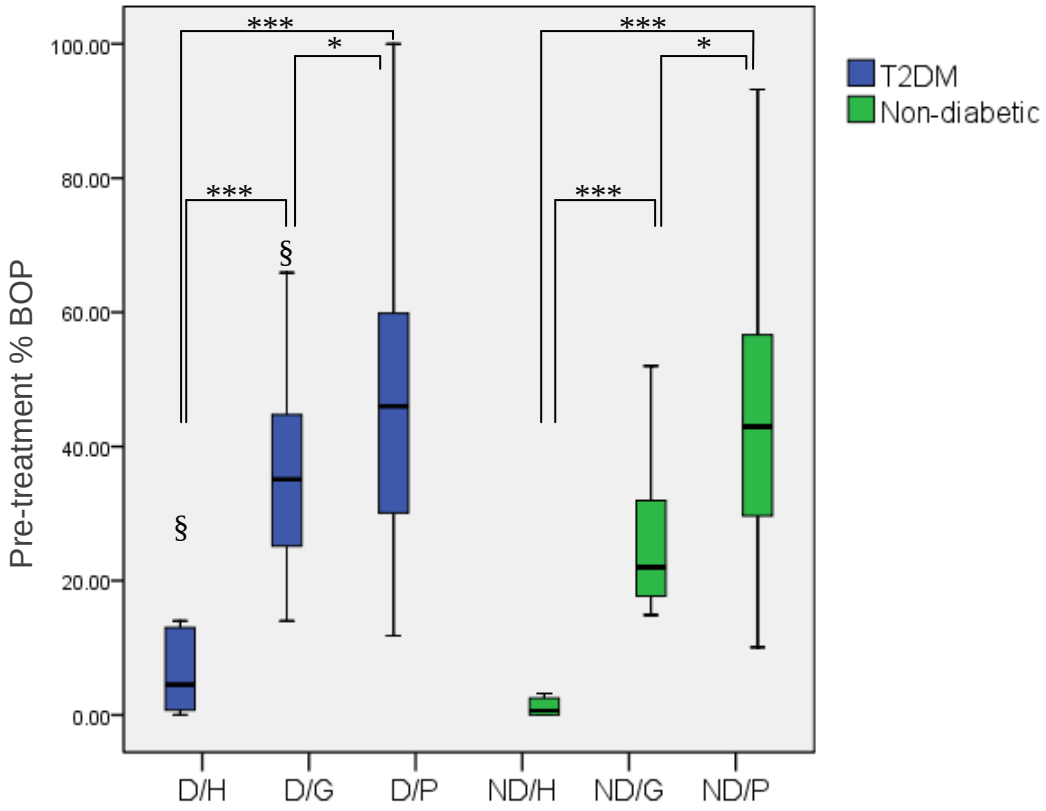
Figure 4.9 Pre-treatment mean mGI data comparing groups based on diabetic status and periodontal diagnosis



Boxplots of pre-treatment mean mGI data in 100 T2DM subjects (healthy periodontal tissues n=14, gingivitis n=39, periodontitis n=47) and 83 non-diabetic subjects (healthy periodontal tissues n=16, gingivitis n=19, periodontitis n=48)**.** Statistics: Kruskal-Wallis with Mann Whitney-U post hoc test *<0.05, **p<0.01, ***p<0.001(according to periodontal status within T2DM or non-diabetic group); § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups within the corresponding periodontal status). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

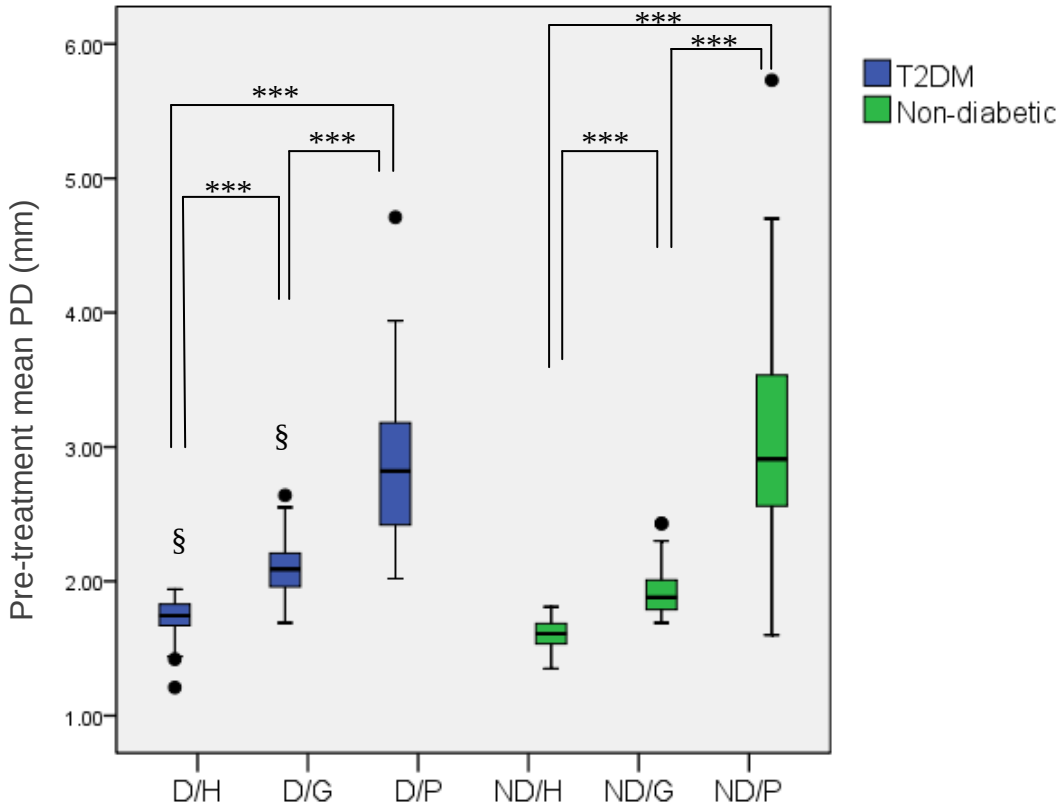
Figure 4.10 Pre-treatment mean % BOP data comparing groups based on diabetic status and periodontal diagnosis



Boxplots of pre-treatment % BOP data in 100 T2DM subjects (healthy periodontal tissues n=14, gingivitis n=39, periodontitis n=47) and 83 non-diabetic subjects (healthy periodontal tissues n=16, gingivitis n=19, periodontitis n=48)**.** Statistics: Kruskal-Wallis with Mann Whitney-U post hoc test *<0.05, **p<0.01, ***p<0.001(according to periodontal status within T2DM or non-diabetic group); § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups within the corresponding periodontal status). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

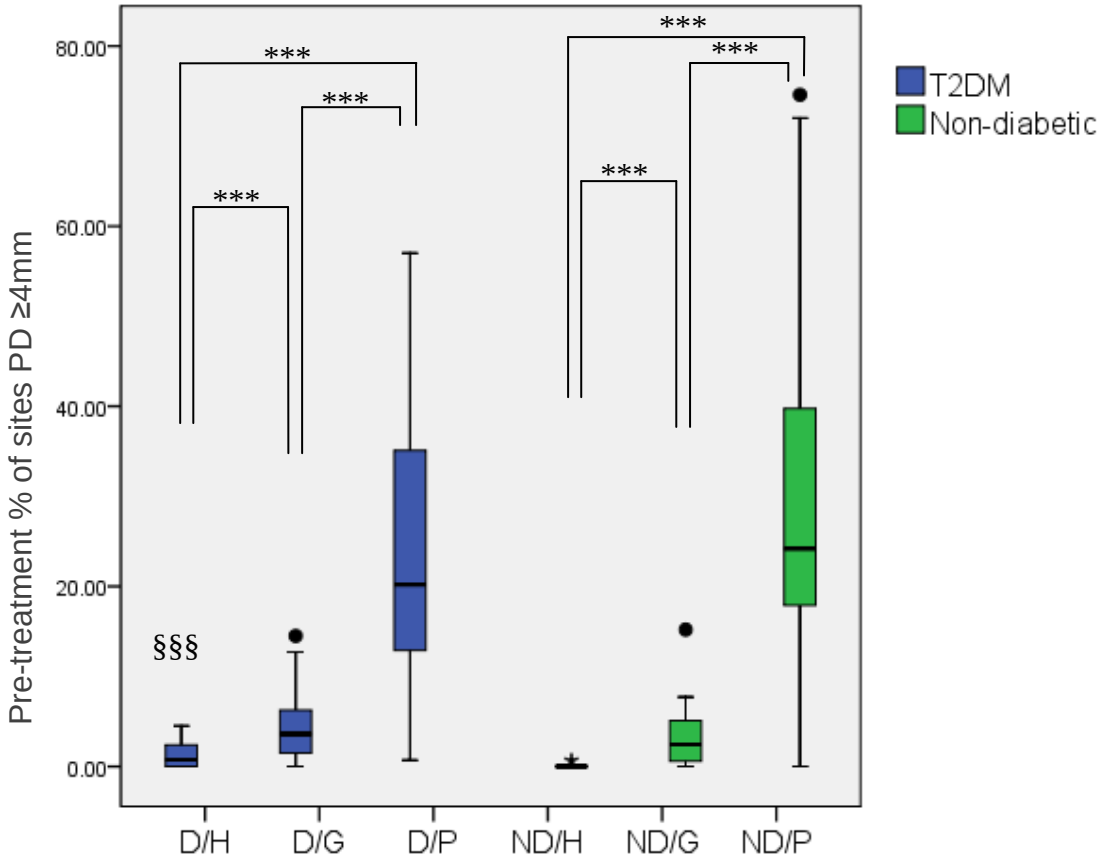
Figure 4.11 Pre-treatment mean PD data comparing groups based on diabetic status and periodontal diagnosis



Boxplots of pre-treatment mean PD data in 100 T2DM subjects (healthy periodontal tissues n=14, gingivitis n=39, periodontitis n=45) and 83 non-diabetic subjects (healthy periodontal tissues n=16, gingivitis n=18, periodontitis n=47)**.** Statistics: Kruskal-Wallis with Mann Whitney-U post hoc test *<0.05, **p<0.01, ***p<0.001(according to periodontal status within T2DM or non-diabetic group); § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups within the corresponding periodontal status). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

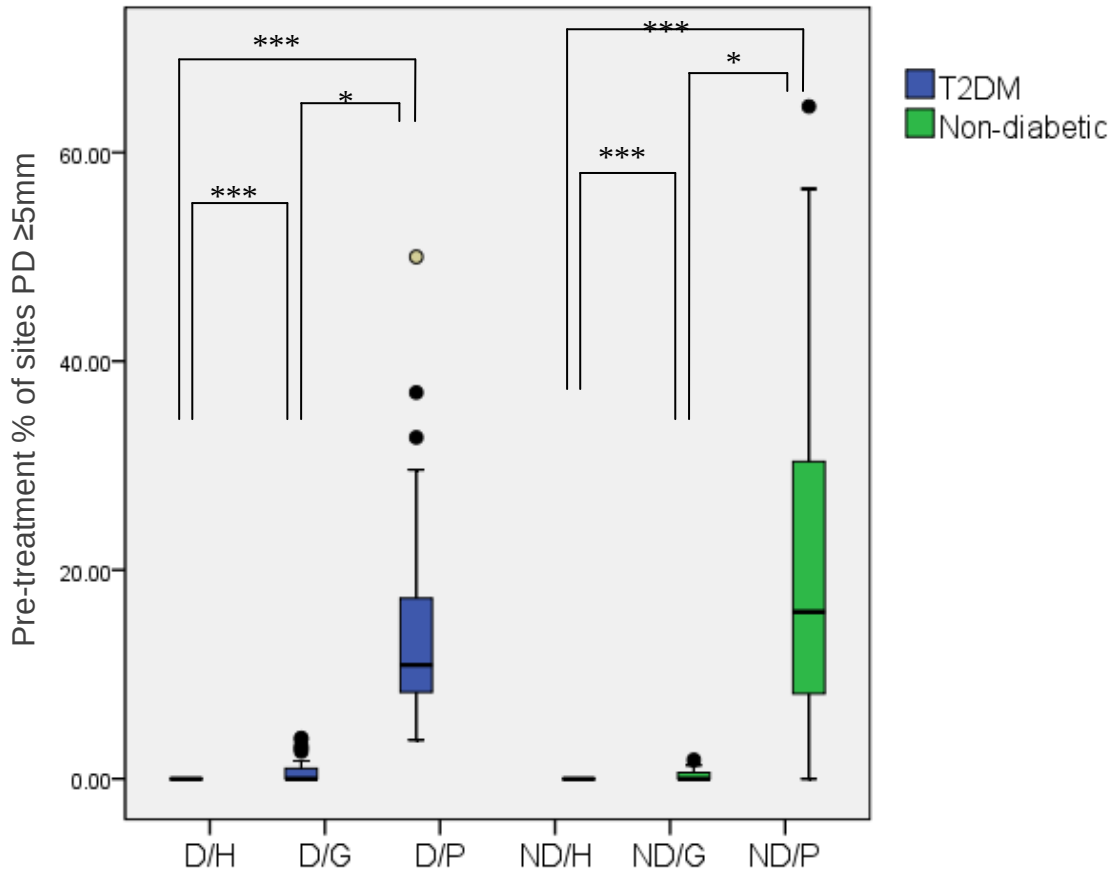
Figure 4.12 Pre-treatment data for the % of sites with PD ≥4mm comparing groups based on diabetic status and periodontal diagnosis



Boxplots of pre-treatment data for the % of sites with PD ≥4mm in 98 T2DM subjects (healthy periodontal tissues n=14, gingivitis n=39, periodontitis n=45) and 81 non-diabetic subjects (healthy periodontal tissues n=16, gingivitis n=18, periodontitis n=47)**.** Statistics: Kruskal-Wallis with Mann Whitney-U post hoc test *<0.05, **p<0.01, ***p<0.001(according to periodontal status within T2DM or non-diabetic group); § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups within the corresponding periodontal status). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

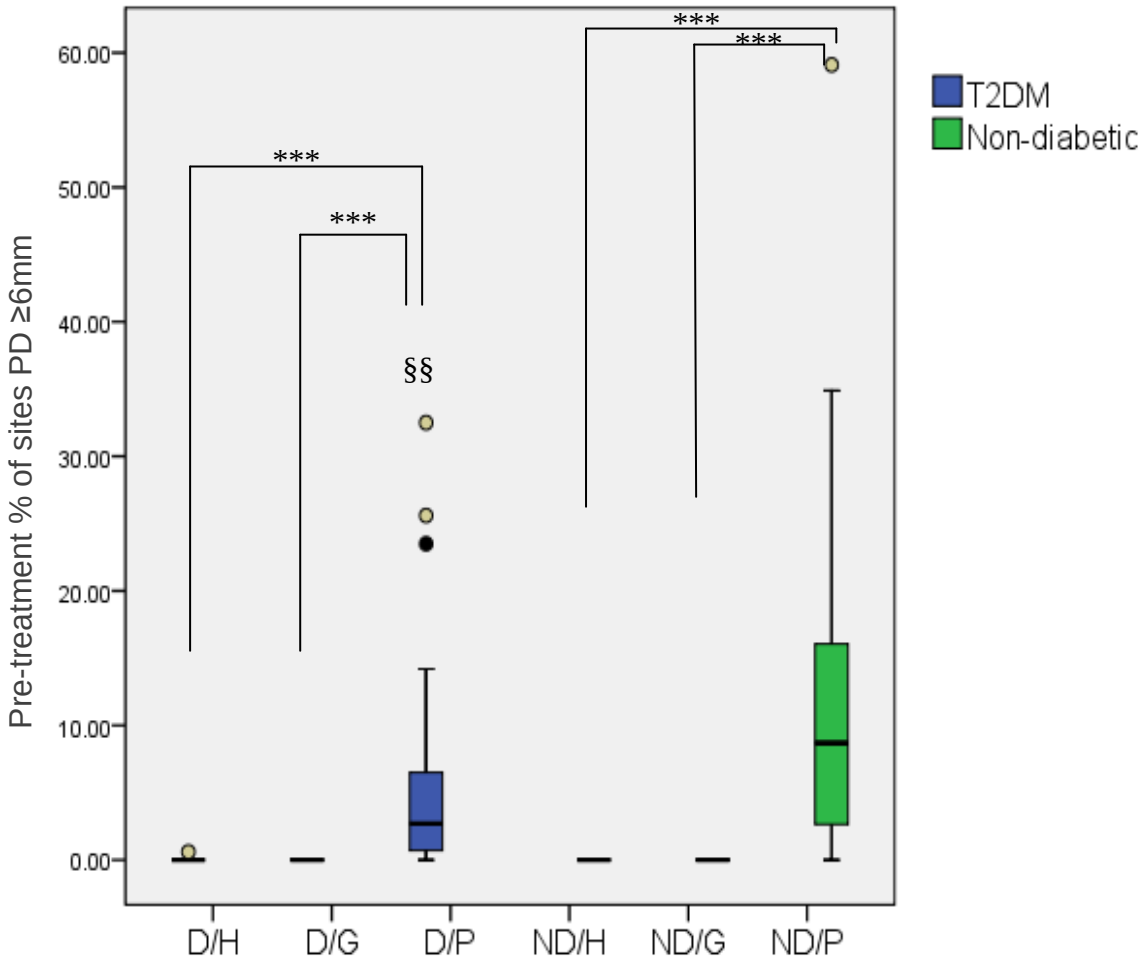
Graph 4.13 Pre-treatment data for the % of sites with PD ≥5mm comparing groups based on diabetic status and periodontal diagnosis



Boxplots of pre-treatment data for the % of sites with PD ≥5mm in 98 T2DM subjects (healthy periodontal tissues n=14, gingivitis n=39, periodontitis n=45) and 81 non-diabetic subjects (healthy periodontal tissues n=16, gingivitis n=18, periodontitis n=47)**.** Statistics: Kruskal-Wallis with Mann Whitney-U post hoc test *<0.05, **p<0.01, ***p<0.001(according to periodontal status within T2DM or non-diabetic group); § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups within the corresponding periodontal status). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

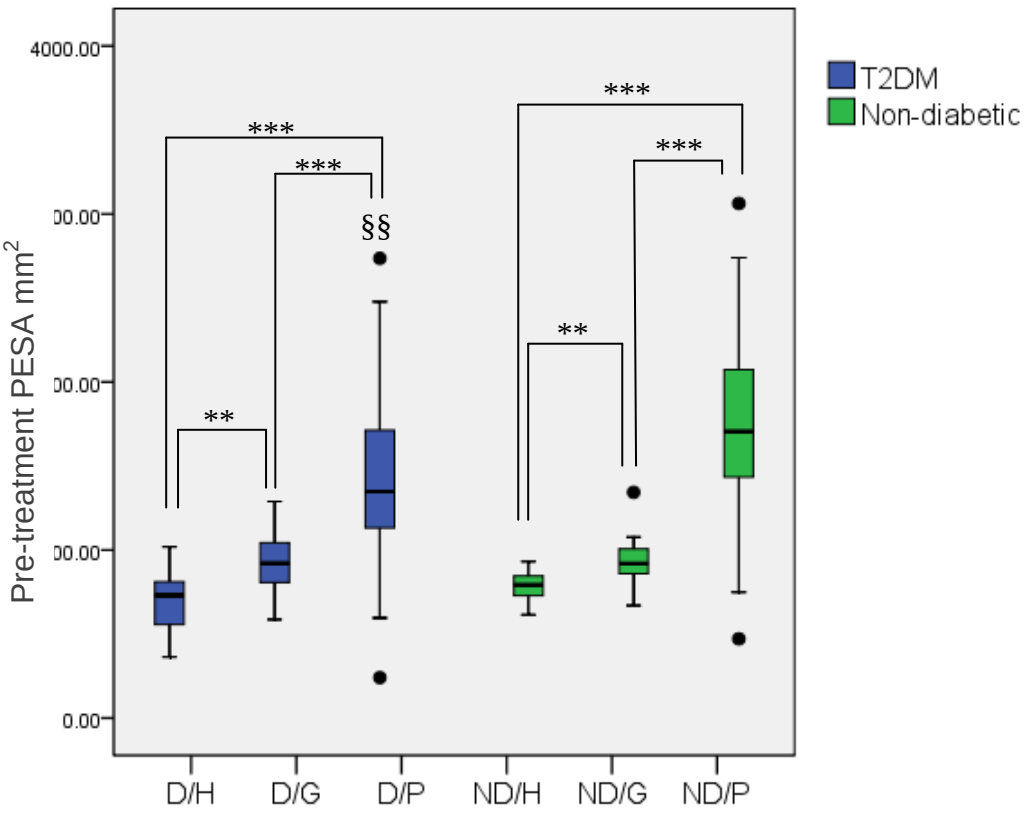
Figure 4.14 Pre-treatment data for the % of sites with PD ≥6mm comparing groups based on diabetic status and periodontal diagnosis



Boxplots of pre-treatment data for the % of sites with PD ≥6mm in 98 T2DM subjects (healthy periodontal tissues n=14, gingivitis n=39, periodontitis n=45) and 81 non-diabetic subjects (healthy periodontal tissues n=16, gingivitis n=18, periodontitis n=47)**.** Statistics: Kruskal-Wallis with Mann Whitney-U post hoc test *<0.05, **p<0.01, ***p<0.001(according to periodontal status within T2DM or non-diabetic group); § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups within the corresponding periodontal status). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

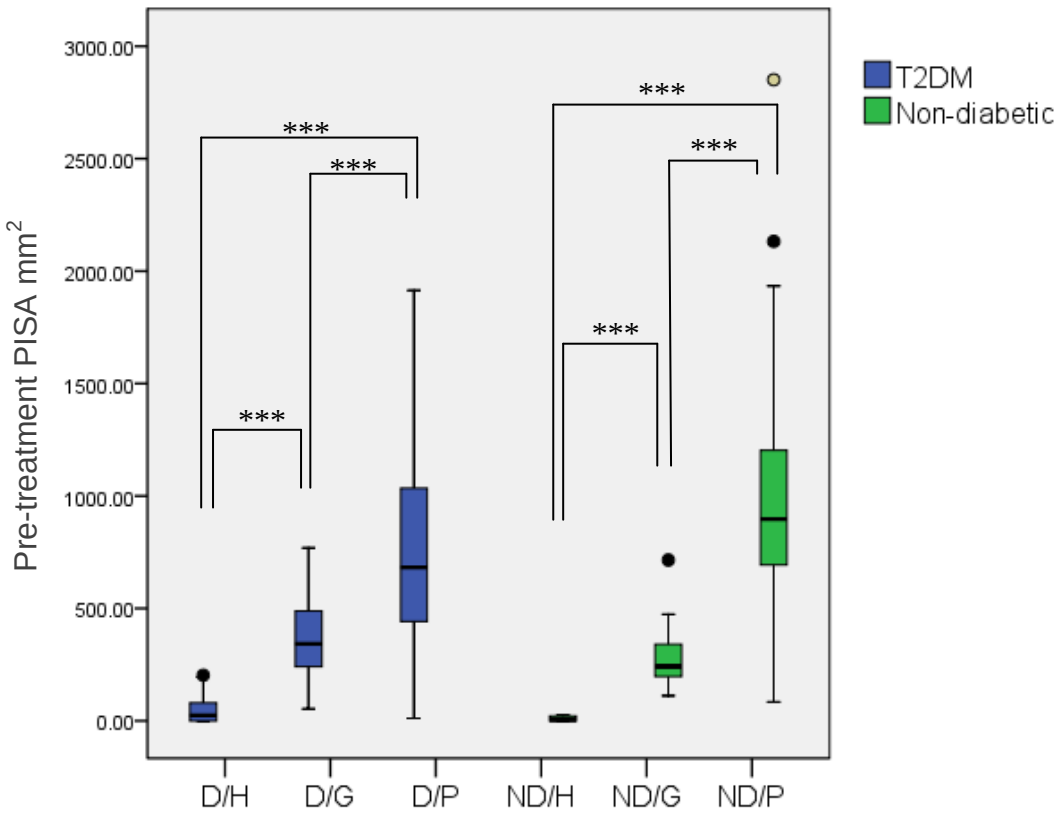
Figure 4.15 Pre-treatment PESA data comparing groups based on diabetic status and periodontal diagnosis



Boxplots of pre-treatment PESA data in 97 T2DM subjects (healthy periodontal tissues n=14, gingivitis n=39, periodontitis n=44) and 81 non-diabetic subjects (healthy periodontal tissues n=16, gingivitis n=18, periodontitis n=47)**.** Statistics: ANOVA with independent sample t-test post hoc test *<0.05, **p<0.01, ***p<0.001(according to periodontal status within T2DM or non-diabetic group); § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups within the corresponding periodontal status). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

Figure 4.16 Pre-treatment PISA data comparing groups based on diabetic status and periodontal diagnosis



Boxplots of pre-treatment PISA data in 97 T2DM subjects (healthy periodontal tissues n=14, gingivitis n=39, periodontitis n=44) and 81 non-diabetic subjects (healthy periodontal tissues n=16, gingivitis n=18, periodontitis n=47)**.** Statistics: Kruskal-Wallis with Mann Whitney-U post hoc test *<0.05, **p<0.01, ***p<0.001(according to periodontal status within T2DM or non-diabetic group); § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups within the corresponding periodontal status). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

4.3 Discussion

In Western developed countries, the prevalence of T2DM is closely linked to socio-economic status, with diabetic individuals more likely to live in deprived areas of the UK (Ismail *et al.*, 1999; Evans *et al.*, 2000). Within the present study, subjects' socio-economic status was assessed using IMD. A number of indicators, covering a range of economic, social and housing issues are combined to produce a single IMD in each small area in England. Each area can then be ranked relative to one another according to their level of deprivation. In the current study the IMD rank differed significantly between the T2DM subjects [9198 (3384-21930)] and the non-diabetic subjects [18213 (7658-25035)] ($p < 0.001$), with diabetic subjects living in areas ranked higher for IMD. This corroborates previous research (Ismail *et al.*, 1999; Evans *et al.*, 2000), and shows that within the North East of England, individuals with T2DM live in more deprived areas compared to non-diabetic subjects.

Smoking is a recognised risk factor for periodontal disease (Tomar and Asma, 2000). Interestingly, there was a 9% prevalence of current smokers in the present study in both the T2DM and non-diabetic groups. This is markedly lower than the 21% prevalence rate for smoking in England (Robinson and Harris, 2011). Subjects' past experience of healthcare could impact on smoking habits. Smoking cessation is an integral part of the management of T2DM (NICE, 2008) and a key part of the oral health advice provided to patients by the oral health profession. The lower prevalence of current smoking in the present study compared to that found in the general population could reflect the impact of smoking cessation advice previously received as part of either their diabetes management and/or their oral healthcare. Furthermore, the subjects participating in this study have actively opted in, indicating the presence of a healthcare seeking behaviour that may not be reflected in the general population of England.

Previous research has also shown that smokers are up to 4 times more likely to have periodontal disease compared with non-smokers with length of smoking history and number of cigarettes smoked per day being important factors to consider (Tomar and Asma, 2000; Calsina *et al.*, 2002). The importance of smoking history in the development of periodontitis is highlighted in the current study with pack years, regardless of diabetes status, being lowest in subjects with healthy periodontal tissues and highest in subjects with periodontitis, although the differences failed to reach statistical significance.

Previous research has clearly demonstrated that obesity is a powerful risk factor for T2DM (Chan et al., 1994), with general obesity, as measured by BMI strongly predicting the risk of T2DM (Wang et al., 2005). It has also been said that the leading cause for developing insulin resistance is obesity (Kahn et al., 2006). Patients with T2DM had significantly higher BMI [32.6 (29.8-36.0) kg/m^2] compared to the non-diabetic patients [27.3 (25.0-29.5) kg/m^2] ($p < 0.001$). Furthermore, the T2DM group contained a higher proportion of obese (43%) and morbidly obese (31%) subjects compared to 11% and 13% in the non-diabetic group, respectively.

Cross-sectional studies have demonstrated a positive association between periodontal disease and obesity (Pischon *et al.*, 2007). Interestingly, in the current study, BMI was significantly higher in non-diabetic subjects with periodontitis [28.1 (25.2-32.0)] compared to non-diabetic subjects with healthy periodontal tissues [24.7 (23.1-27.7)] ($p < 0.05$). Similarly, in subjects with T2DM, with the BMI appeared higher in the periodontitis group [33.0 (29.9-36.5)] compared to the group with healthy periodontal tissues [31.2 (24.5-34.6)], although the difference failed to reach significance. Therefore, the current study would appear to support data from a recent meta-analysis that, notwithstanding the limitations stated by the authors, provided evidence of a greater

mean CAL among obese individuals and a higher BMI among periodontal patients (Chaffee and Weston, 2010).

T2DM and hypertension are commonly associated conditions, both of which carry an increased risk of cardiovascular and renal disease (Garcia *et al.*, 1974; Turner *et al.*, 1998b). The prevalence of hypertension in T2DM is higher than that in the general population, especially in younger patients (HDS, 1993). Tight control of BP in hypertensive patients with T2DM has been shown to provide a 24% reduction in diabetic complications and a 32% reduction in deaths related to diabetes (Turner et al., 1998a) and this is now reflected within national UK guidelines for the management of T2DM (NICE, 2008). However, despite the clear guidance for the intensive management of hypertension in subjects with T2DM, the present study demonstrates that systolic BP is significantly higher in subjects with T2DM [146.9±21.2 mmHg] compared to non-diabetic subjects [136.6±18.9 mmHg] ($p<0.001$). Despite clinical trials having convincingly demonstrated that intensive treatment of hypertension among patients with diabetes reduces diabetic complications and deaths related to diabetes (Turner *et al.*, 1998a) the mean BP for subjects with T2DM included in this study was 147±21 mmHg /81(74-90) mmHg which is above the target (140/80 mmHg) set out within UK management guidelines for T2DM (NICE, 2008). This supports previous research that consistently found that most diabetic patients do not achieve recommended levels of blood pressure control (Martin *et al.*, 1995; Harris, 2000). A lack of medication intensification by clinicians in hypertensive subjects with T2DM is recognised as a major factor in patients not achieving the recommended BP targets (Berlowitz *et al.*, 2003) although, the reasons why clinicians are not more aggressively managing hypertension in patients with T2DM remains unclear. However, the implications for patients with T2DM not achieving adequate blood pressure control

remain significant, placing them at an increased risk of life limiting diabetic complications, such as CVD (Turner *et al.*, 1998b).

In patients with T2DM, the risk of diabetic complications is strongly associated with hyperglycaemia, with reductions in HbA1c leading to a reduced the risk of developing diabetic complications (UKPDS, 1998a). Each 1% reduction in mean HbA1c has been shown to be associated with reductions in risk of 21% for diabetic complications (Stratton *et al.*, 2000). Such data have been influential in the development of management guidelines for T2DM, which advocate using dietary and lifestyle interventions with medication, as required, to achieve and maintain the ideal target HbA1c level of 6.5% or less (NICE, 2008). Interestingly, in the current study, only 26.3% of subjects met the HbA1c target of 6.5% set out in the UK T2DM management guidelines (NICE, 2008). The most recent National Diabetes Audit for 2009-2010 showed that 66.5% of people with T2DM were achieving a HbA1c of 7.5% or less (National Diabetes Audit, 2011), however, disappointingly, the % of subjects achieving the optimal target of 6.5% for T2DM set by NICE was not published , although it is reasonable to propose the % of subjects would be less than 66.5%. Although the benefits of more intensive management in preventing or delaying the development and progression of diabetic complications are well documented, achieving an ideal HbA1c has to be balanced against the risk of hypoglycaemic episodes. Previous research has shown that subjects who receive intensive management of hyperglycemia, such as through the use of insulin, have more hypoglycaemic episodes than subjects not receiving intensive management of blood glucose (UKPDS, 1998a). Risk of hypoglycaemic episodes may be one reason why subjects are not intensively managed to achieve an ideal HbA1c target of 6.5%.

Research shows that a deterioration of insulin secretion over time is the usual course in most patients with T2DM, and many patients will have deficient insulin

secretion after 10 years of diabetes (Wallace and Matthews, 2002). The rate of deterioration in diabetes control is around 1.5% HbA1c per 10 years and it thought to be a result of a decline in β-cell function (Home, 2008b). Patients with a longer history of T2DM may therefore find it more difficult to achieve adequate blood glucose control and potentially they are exposed for a greater length of time to diabetes related risk factors. In this study, subjects with gingivitis [6.0 (3.0-13.0 years] or periodontitis [7.0 (3.0-10.0) years] presented with a significantly longer history of diabetes compared to those with healthy periodontal tissues [2.5 (1.0-5.0) years] ($p < 0.05$) (Table 4.4). Furthermore, there were significant differences in the proportions of subjects meeting the UK target for glycaemic control of HbA1c ≤ 6.5 %, with a higher proportion of subjects with healthy periodontal tissues meeting the target (64.3%) and progressively fewer subjects reaching this target in the gingivitis (26.3%) and periodontitis groups (14.9%) respectively (Table 4.4 and Figure 4.4). There were also significant differences in the proportions of subjects with specific management regimes with a higher proportion of subjects with healthy periodontal tissues being managed by dietary intervention alone (42.9%) and progressively fewer subjects being managed by diet alone in the gingivitis (12.8%) and periodontitis (8.5%) groups, respectively. Conversely, a higher proportion of subjects with periodontitis (29.8%) required insulin and there were progressively fewer subjects requiring insulin in the gingivitis (17.9%) and healthy periodontal tissues (0.0%) groups respectively (Table 4.4).

Taken collectively, this highlights that the deterioration in glycaemic control appears to be mirrored by worsening periodontal health. Thus, as with other diabetes complications, the duration of diabetes and the degree of glycaemic control would appear to be important factors in the deterioration of periodontal health. The 2 way relationship between T2DM and periodontal disease could provide an explanation for this. T2DM or specifically hyperglycaemia may modify inflammatory processes

contributing to local dysregulated immune-inflammatory responses, causing increased periodontal destruction and likewise, inflammatory changes within the periodontal tissues may impact on glycaemic control and the systemic health of those with T2DM. However, the influence of health behaviours on both T2DM and periodontal disease should also not be overlooked. For example, the levels of compliance with health care behaviours required to achieve good glycaemic control are likely to be mirrored in the quality of oral hygiene practices achieved. Thus the relationship between T2DM and periodontal disease could be a reflection of healthcare behaviours.

In the current study, 1/3 of the T2DM subjects had at least one diabetic complication. Previous research demonstrates more cardiovascular complications in T2DM subjects with periodontitis compared to T2DM subjects without periodontitis (Jansson et al., 2006). In the current study, a higher proportion of subjects reporting a diabetic complication was seen in subjects with periodontitis [35.4%] and gingivitis [35.9%] compared to subjects with healthy periodontal tissues [14.3%], although the difference failed to reach statistical significance. Within the literature, epidemiological data suggest a link between periodontitis and an increased risk of cardiovascular events (Humphrey *et al.*, 2008). Similarly, the association between T2DM and CVD is well known (Stratton *et al.*, 2000) as is the association between T2DM and periodontal diseases (Taylor, 2001). However, the nature of the associations between these three diseases remains unclear, with periodontitis, CVD and T2DM all sharing a number of risk factors. Common pathological mechanisms could result in increased susceptibility to these three diseases, however interventional trials are required to clarify the relationship between periodontal disease, cardiovascular disease and T2DM.

When considering patient care pathways within diabetes management, it is interesting to note that in the current study, 95% of subjects with T2DM had received examinations of their feet and eyes within the past 12 months. This clearly demonstrates

the robust patient pathway that exists for screening for diabetic complications. However, the same is not true of screening for oral complications of T2DM, with as many as 1/3 of subjects with T2DM in this study reporting not seeing a dentist in the past 12 months. Thus, a regular opportunity for the screening of oral complications in this disease susceptible population is clearly being lost.

The current study demonstrates that, compared to the non-diabetic group, the T2DM group had a higher number of subjects who presented with clinically detectable dry mouth. This supports previous research demonstrating a higher prevalence of xerostomia in patients with T2DM compared to non-diabetic subjects, with polyuria and medications being cited as the potential causes of dry mouth in subjects with T2DM (Sandberg *et al.*, 2000; Hintao *et al.*, 2007; Borges *et al.*, 2010).

Furthermore, in agreement with previous research, in the T2DM group in this current study, the percentage of subjects with at least one carious tooth was over 4 times greater in subjects with T2DM (21%) compared to non-diabetic subjects (4.9%) ($p < 0.05$) (Sandberg *et al.*, 2000; Hintao *et al.*, 2007). The most recent UK Adult Dental Health Survey showed that in the general population just under 1/3 of dentate adults (31%) had obvious tooth decay (Steele and O' Sullivan, 2011), which is higher than for subjects within the current study. However, given the cumulative nature of dental disease and the potential of caries to cause pain and sepsis, it is disappointing that, within the current study, subjects with T2DM appear to be 4 times more likely to be experiencing dental caries. This is particularly relevant given the importance of diet and nutrition for subjects with T2DM. Possible reasons for this difference could include differences in diet, although as part of the management of T2DM patients are actively advised to limit their intake of carbohydrates, which is also an important message for caries prevention. The differences may also be a reflection of the recruitment strategy for the current study in which subjects with T2DM were recruited from databases of

T2DM patients held in both primary and secondary care settings whereas non-diabetic patients were recruited from either those referred from general dental practice into the restorative department within the School of Dental Sciences or patients seen on student treatment clinics within the School of Dental Sciences.

Previous research has demonstrated that the attitudes towards oral health of patients with diabetes are poor in comparison with the findings from surveys of the general population (Allen et al., 2008). Other studies have reported on the dental care habits of subjects with T2DM both with and without periodontal disease, but providing no comparison with non-diabetic subjects (Jansson et al., 2006). In this current study, the T2DM group reported poorer oral health behaviours (including attendance at a dentist and oral hygiene practices) compared to the non-diabetic group. T2DM subjects reported a less favourable pattern of attendance at the GDP, with 62% of T2DM subjects regularly attending and 68% attending in the past 12 months compared to 77% and 86%, respectively, for non-diabetic subjects. This supports previous reports that 70% T2DM subjects have attended a dentist in the past 12 months (Jansson et al., 2006). The current study showed that T2DM subjects reported less favourable oral hygiene practices, with 64% of T2DM subjects toothbrushing at least twice a day, 30% once a day, 7% less than once a day and 25% cleaning interproximally more than 3 times a week compared to 92%, 5%, 2% and 47% respectively for non-diabetic subjects. This supports reports that approximately 90% of subjects with T2DM toothbrush at least once a day but is not in agreement with reports that 51% of subjects with T2DM clean interproximally more than 3 times a week (Jansson et al., 2006). Interestingly, attendance pattern and tooth brushing frequency in the T2DM subjects from this study were comparable with findings from the general UK population, with the UK Adult Dental Health Survey (Steele and O' Sullivan, 2011) reporting that 61% of the general population attend a dentist regularly and 75% clean at least twice a day. In contrast, the

attendance patterns and tooth brushing frequency in the non-diabetic subjects included in the current study appear to be more favourable than that of the general UK population.

Possible reasons for more favourable oral health behaviours in the non-diabetic subjects compared to either the T2DM subjects or data from the general population could reflect the recruitment strategy used in the current study, with the non-diabetic subjects being recruited from patients referred from general dental practice or from patients seen on student treatment clinics within the School of Dental Sciences and therefore likely to be motivated, oral health seeking individuals, that have previously received oral hygiene advice. On the other hand, when considering the reasons for the poorer oral health behaviours in the T2DM group compared to the non-diabetic group seen in this current study, it is important to recognise that given the potential severe and life threatening complications of diabetes, oral problems may not be a major priority for this group of patients. Furthermore, in the current study, patients were not matched based on regular attendance at a dentist or deprivation. This could introduce a potential bias in oral health behaviours, in that 77% of non-diabetics as compared to 62% of diabetics reported attending a dentist regularly and the T2DM subjects lived in more deprived areas than the non-diabetic subjects. It is not unreasonable to think that regular attenders at a dentist would have better oral health knowledge and thus more favourable oral health behaviours. Furthermore, answers to questions may be subject to recall bias due to participants giving socially acceptable responses to questions pertaining to oral health behaviours (Allen *et al.*, 2008).

Interestingly, the current study demonstrates that, compared to the non-diabetic group, subjects with T2DM had lower total cholesterol and lower non-HDL, which are both considered indicators of risk for CVD. Potentially, this reflects the UK management guidelines for T2DM which recommends dietary advice to optimise blood

lipid profile and the use of lipid lowering therapy to reduce CVD risk in T2DM subjects (NICE, 2008). Therefore, it is feasible that the T2DM subjects in the current study are receiving more aggressive management of CVD risks factors than the non-diabetic subjects. In this current study, 72% of subjects with T2DM were taking lipid lowering medication; however, comparable data for non-diabetic subjects were not collected in this study. Within the published literature, the few studies that present blood lipid profiles in patients with diabetes and periodontal disease use fasting samples (Cutler *et al.*, 1999a; Kardesler *et al.*, 2010), thus preventing a direct comparison with values obtained in this current study (which used non-fasting samples). One study found no significant differences in total cholesterol, triglycerides and low density lipoprotein levels between subjects with T2DM and non-diabetic subjects (Kardesler *et al.*, 2010). This was not supported by data from the current study that found T2DM subjects had significantly higher triglyceride levels and significantly lower levels of HDL, non-HDL and total cholesterol compared to non-diabetic subjects. Another study demonstrated a trend for increased levels of triglyceride in subjects with T2DM compared to non-diabetic subjects (Cutler *et al.*, 1999a), thus supporting data from the current study.

Currently the data regarding levels of hsCRP in patients with T2DM and periodontitis is limited (Correa *et al.*, 2010; Kardesler *et al.*, 2010). One study reported no significant difference in hsCRP levels between subjects with T2DM and non-diabetic subjects (Kardesler *et al.*, 2010) although a lack of a non-diabetic group in a different study prevents a similar comparison (Correa *et al.*, 2010). In the current study, levels of hsCRP appeared higher in subjects with T2DM [2.3(0.9-4.5) mg/L] compared to non-diabetic subjects [1.9(0.8-3.9) mg/L], however the difference failed to reach statistical significance (Table 4.10 and Figure 4.6). Due to the wide variation seen in hsCRP levels, further studies with larger sample sizes would be required to clarify whether CRP levels are actually higher in subjects with T2DM compared to non-diabetic

subjects. Interestingly, when subjects were further categorised based on periodontal status, subjects with T2DM and healthy periodontal tissues [2.4(0.8-5.5) mg/L] had significantly higher hsCRP levels than non-diabetic subjects with the same periodontal status [0.6(0.2-1.5) mg/L] ($p < 0.05$), potentially supporting published evidence that demonstrates the association between elevated systemic inflammation and the development of T2DM (Bertoni et al., 2010). Evidence from a meta-analysis also that demonstrated that CRP is consistently elevated in patients with periodontitis compared to patient with healthy periodontal tissues (Paraskevas et al., 2008). This was confirmed in the current study, which showed that in non-diabetic subjects, those with healthy periodontal tissues [0.6(0.2-1.5) mg/L] had significantly lower hsCRP levels compared to subjects with gingivitis [2.3(0.9-4.6) mg/L] or periodontitis [2.4(0.8-5.5) mg/L] ($p < 0.05$). However, in subjects with T2DM, similar differences were not found when comparing subjects with healthy periodontal tissues [2.4(0.8-5.5) mg/L], gingivitis [2.4(1.0-4.4) mg/L] and periodontitis [2.1(0.9-4.6) mg/L]. Perhaps the raised background levels of CRP seen in subjects with T2DM masks any differences in CRP levels caused by inflammation in the periodontal tissues. The assessment of systemic inflammation in the current study, did not however, take into account the potential impact of medication on systemic inflammation. For example, as part of the management of CVD risk, statins have been shown to lower LDL and also reduce CRP levels, with both mechanisms contributing independently to CVD risk reduction (Devaraj *et al.*, 2007; Ridker and Silvertown, 2008).

This study showed that, as expected, HbA1c was higher in the T2DM group compared to non-diabetic group (Table 4.10). Previous research demonstrated higher HbA1c levels were found in subjects with poorer periodontal health (Jansson *et al.*, 2006; Chen *et al.*, 2010). This current study found a trend for a higher HbA1c level in T2DM subjects with periodontitis [7.5 (6.7-9.2)%] compared to T2DM subjects with

healthy periodontal tissues [6.4 (5.9-9.0)%]. Given both the variability of HbA1c seen in subjects with T2DM and the statistical requirement to correct the p-values to compensate for multiple comparisons, the numbers of subjects included in this study were too small to clarify the relationship of HbA1c and periodontal status.

When the HbA1c level for T2DM subjects with periodontitis was compared to that reported in previous research, it would appear comparable to some studies (Kiran *et al.*, 2005; Chen *et al.*, 2010) but lower than other studies (Correa et al., 2010). Interestingly, some studies categorised T2DM subjects based on glycaemic control with the T2DM subjects with periodontitis in our study having a HbA1c level that was lower than the 'poorly controlled group' and higher than the 'well controlled group' used in previous studies (Dag *et al.*, 2009; Kardesler *et al.*, 2010). Given the proposed interplay between hyperglycaemia and inflammatory mediators (Stumvoll *et al.*, 2005), it could be viewed as important to ensure that HbA1c levels are consistent in all studies. However, limiting inclusion of T2DM subjects into studies based on their HbA1c value would reduce how accurately results can be generalised to the T2DM population as a whole. Stratification of subjects based on their HbA1c levels could be used; however adequate numbers of subjects would be required in each category of HbA1c. Stratification of subjects based on HbA1c was not built into the recruitment strategy for the current study and to prevent low n values in groups, data were not subsequently stratified when the data was analysed.

Previous epidemiological evidence has reported an increased prevalence and severity of periodontal disease in subjects with T2DM compared to non-diabetic subjects (Shlossman *et al.*, 1990; Sandberg *et al.*, 2000; Mattout *et al.*, 2006; Moles, 2006). In more recent interventional studies evaluating the response to periodontal therapy, the pre-treatment periodontal condition of subjects with and without diabetes was well matched (Dag *et al.*, 2009; Kardesler *et al.*, 2010) or the T2DM subjects had

higher levels of BOP and mean PD (Correa et al., 2008). In the current study, when exploring the pre-treatment periodontal status of subjects with T2DM in comparison to those without diabetes, no significant difference was found in the mean PD for subjects with T2DM and periodontitis [2.8 (2.4-3.2) mm] compared to non-diabetic subjects with periodontitis [2.9 (2.5-3.5) mm]. Interestingly, the mean PD data from this current study are comparable to data from some previous studies (Kiran *et al.*, 2005; Dag *et al.*, 2009) but are lower than data from other studies (Correa *et al.*, 2008; Kardesler *et al.*, 2010), indicating potential variations in extent of periodontal disease between different studies. However, only one previous study supplemented the mean PD data with data on the % of sites with advanced periodontal disease, showing that when considering the % of PD sites $\geq$ 7 mm, the differences between subjects with T2DM [9.0 (1.0-22.0)%] and non-diabetic subjects [5.5 (3.0-14.0)%] were found not be significant (Correa et al., 2008). Periodontal disease rarely affects all parts of the periodontium equally and mean PD provides only a crude description of the PD found in each subject. Therefore, the use of mean PD, without additional data on the % of sites with advanced periodontal disease, is a limitation of all other studies in this field of work. In the current study, the % of PD sites $\geq$ 6 mm was significantly lower in subjects with T2DM and periodontitis [2.7 (0.7-7.1)%] compared to non-diabetic subjects with periodontitis [8.7 (2.6-16.7)%] ($p < 0.01$), indicating more severe periodontal disease was present in non-diabetic subjects compared to subjects with T2DM (Table 4.14 and Figure 4.13). This may reflect the differences in the recruitment pools used for diabetic and non-diabetic subjects in the current study. As previously described, T2DM subjects were recruited from medical databases of T2DM patients held in both primary and secondary care settings whereas the non-diabetic subjects were recruited from patients referred from general dental practice or from patients seen on student treatment clinics within the School of Dental Sciences. Although, diabetic and non-diabetic subjects were matched

based on their periodontal diagnosis, the extent of periodontal disease was not however considered in this process. This is a limitation of the current study and highlights a need to stratify periodontal case selection based on extent and severity of disease to ensure more robust matching of groups with regards to periodontal status in future studies.

In the current study, exploration of the data relating to gingival inflammation indicates different patterns for subjects with T2DM compared to non-diabetic subjects, with significantly higher levels of mGI and % BOP in subjects with T2DM and healthy tissues [0.8 (0.5-1.6) and 4.5 (0.7-13.1)] or gingivitis [1.9 (1.3-2.5) and 35.1 (25.0-44.9)] compared to non-diabetic subjects with healthy periodontal tissues [0.5 (0.3-0.8) and 0.7 (0.0-2.6)] and gingivitis [1.3 (0.8-1.7) and 22.0 (17.3-32.6)] ($p < 0.05$) (Table 4.12 and Figure 4.8 and 4.9). This supports data from previous studies that demonstrate significantly higher levels of gingival inflammation in subjects with T2DM compared to non-diabetic subjects (Lu and Yang, 2004; Campus *et al.*, 2005; Mattout *et al.*, 2006; Correa *et al.*, 2008). A potential explanation for the higher levels of gingival inflammation seen in subjects with T2DM is that the increased gingival inflammation is a manifestation of the upregulated systemic inflammation seen in diabetes. In the current study, the same pattern of increased gingival inflammation in subjects with T2DM compared to non-diabetic subjects is not replicated in subjects with periodontitis. There were no significant differences in % BOP and mGI in subjects with T2DM and periodontitis [46.0 (30.0-60.7) and 2.0 (1.5-2.7)] compared to non-diabetic subjects with periodontitis [43.0 (29.4-56.7) and 2.4 (2.0-2.7)]. It is not unreasonable to presume that the more severe periodontal disease present in non-diabetic subjects compared to subjects with T2DM likely masked the presence of a higher background level of gingival inflammation in subjects with T2DM with periodontitis compared to non-diabetic subjects who had periodontitis.

In previously published research, both PESA and PISA have been used to investigate the condition of the periodontal tissues (Nesse *et al.*, 2008; Nesse *et al.*, 2009). Using measurements for LOA and recession at 6 sites per tooth, the surface area of the periodontium is calculated, taking into account variations in the root surface area of each tooth, and PESA (mm^2) is the sum value for the periodontal surface area for the whole mouth. To calculate the surface area of inflamed periodontal tissue, the periodontal surface area for each tooth is multiplied by the proportion of sites around the tooth affected by BOP, and PISA (mm^2) is the sum value for the periodontal inflamed surface area for the whole mouth.

In the current study, PESA is able to represent key aspects of the PD data, highlighting differences in PD between subjects with difference periodontal status. For example, when comparing the subjects with T2DM, the PESA was significantly higher in those with gingivitis [921.5±184.3 mm^2] compared to those with healthy periodontal tissues [698.5±197.3 mm^2] ($p < 0.01$), the PESA was significantly higher in the periodontitis group [1444.7±495.1 mm^2] compared to those with gingivitis [921.5±184.3 mm^2] ($p < 0.001$) and the PESA was significantly higher in those with periodontitis [1444.7±495.1 mm^2] compared to those with healthy periodontal tissues [698.5±197.3 mm^2] ($p < 0.001$). Similarly, for non-diabetic subjects, the PESA significantly higher in those with gingivitis [921.4±154.4 mm^2] compared to those with healthy periodontal tissues [787.2±82.1] mm^2 ($p < 0.01$), the PESA was significantly higher in the periodontitis group [1744.9±519.9 mm^2] compared to those with gingivitis [921.4±154.4 mm^2] ($p < 0.001$) and the PESA was significantly higher in those with periodontitis [1744.9±519.9 mm^2] compared to those with healthy periodontal tissues [787.2±82.1 mm^2] ($p < 0.001$) (Table 4.14, Figure 4.14). Furthermore, the present study demonstrated that PESA was more sensitive than the mean PD data in detecting the presence of advanced periodontal disease. PESA was significantly greater in non-

diabetic subjects with periodontitis [1744.9±519.9 mm^2] compared to subjects with T2DM and periodontitis [1444.7±495.1 mm^2] ($p < 0.01$). This supports the significantly greater % of PD sites ≥6mm in non-diabetic subjects [8.7 (2.6-16.7)%] compared to subjects with T2DM [2.7 (0.7-7.1)%] ($p < 0.01$). Thus, PESA was able to detect the more advanced periodontal disease present in the non-diabetic subjects compared to subjects with T2DM. Conversely, when considering mean PD data, no significant differences were demonstrated between non-diabetic subjects [2.9 (2.4-3.2) mm] and non-diabetic subjects [2.9 (2.5-3.5) mm], highlighting that mean PD fails to detect the differences in PD that were present. In the current study, PESA not only supports the conventional PD data, but is also able to better indicate the extent and severity of the periodontal disease present (Table 4.12).

PISA incorporates both periodontal surface area and BOP data into a single variable. In the current study, PISA was able to represent key differences between subjects with difference periodontal status. For example, when comparing the subjects with T2DM, the PISA was significantly higher in those with gingivitis [342.4 (239.4-439.1) mm^2] compared to those with healthy periodontal tissues [24.7 (0.0-89.8) mm^2] ($p < 0.001$), the PISA was significantly higher in the periodontitis group [683.0 (439.1-1085.5) mm^2] compared to those with gingivitis [342.4 (239.4-439.1) mm^2] ($p < 0.001$) and the PISA was significantly higher in those with periodontitis [683.0 (439.1-1085.5) mm^2] compared to those with healthy periodontal tissues [24.7 (0.0-89.8) mm^2] ($p < 0.001$). Similarly, for non-diabetic subjects, the PISA was significantly higher in those with gingivitis [242.6 (195.6-353.3) mm^2] compared to those with healthy periodontal tissues [4.48 (0.0-23.2) mm^2] ($p < 0.001$), the PISA was significantly higher in the periodontitis group [897.3 (683.6-1232.9) mm^2] compared to those with gingivitis [242.6 (195.6-353.3) mm^2] ($p < 0.001$) and the PISA was significantly higher in those with periodontitis [897.3 (683.6-1232.9) mm^2] compared to those with healthy

periodontal tissues [4.48(0.0-23.2) mm^2] ($p < 0.001$) (Table 4.14, Figure 4.14). PISA was greater in non-diabetic subjects with periodontitis [897.3 (683.6-1232.9) mm^2] compared to subjects with T2DM and periodontitis [683.0 (439.1-1085.5) mm^2], however, the differences failed to reach significance. Thus, although PISA showed a similar pattern, it was not able to confirm (with statistical significance) the significantly greater % of PD sites ≥ 6 mm in non-diabetic subjects [8.7 (2.6-16.7)%] compared to subjects with T2DM [2.7 (0.7-7.1) %] ($p < 0.01$). This indicates that the incorporation of BOP into PISA makes it less sensitive than PESA in detecting differences in the extent and severity of periodontal disease as defined by PD. However, given that BOP data remains key in the clinical assessment of inflammation in the periodontal tissues, it would be appropriate to present both PESA and PISA when evaluating the clinical periodontal status.

Summary of key findings from chapter 4

- Subjects with T2DM had significantly higher BMI compared to the non-diabetic subjects and systolic BP was significantly higher in subjects with T2DM compared to non-diabetic subjects.
- Subjects with gingivitis or periodontitis presented with a significantly longer history of diabetes compared to those with healthy periodontal tissues and fewer reached the UK target for HbA1c. Also, insulin was required in a higher proportion of subjects with periodontitis compared to those with healthy periodontal tissues.
- 95% of subjects with T2DM had received examinations of their feet and eyes within the past 12 months. However, 1/3 of subjects with T2DM had not seen a dentist in the past 12 months.

- Subjects with T2DM had a significantly higher % of subjects with dry mouth compared to those without diabetes. Also, the percentage of subjects with at least one carious tooth was over 4 times greater in subjects with T2DM compared to non-diabetic subjects.
- The T2DM group reported poorer oral health behaviours (including attendance at a dentist and oral hygiene practices) compared to the non-diabetic group.
- Levels of cholesterol and non-HDL were significantly lower in subjects with T2DM compared to non-diabetic subjects.
- Levels of hsCRP appeared higher in subjects with T2DM compared to non-diabetic subjects, however the difference failed to reach statistical significance. Also, in non-diabetic subjects hsCRP was significantly lower in those with healthy periodontal tissues compared to subjects with gingivitis or periodontitis.
- A trend for higher HbA1c in T2DM subjects with periodontitis compared to T2DM subjects with healthy periodontal tissues was seen.
- No significant difference was found in the mean PD for subjects with T2DM and periodontitis compared to non-diabetic subjects with periodontitis. However, the % of PD sites ≥ 6 mm was significantly lower in subjects with T2DM and periodontitis compared to non-diabetic subjects with periodontitis.
- Higher levels of gingival inflammation were seen in subjects with T2DM and gingivitis compared to non-diabetic subjects with gingivitis, although this difference is not replicated in subjects with periodontitis.
- PESA is able to represent key aspects of the PD data, highlighting differences in PD between subjects with different periodontal status and detecting differences in the extent and severity of periodontal disease between T2DM and non-diabetic subjects.

Chapter 5 Analysis of pre-treatment local and systemic cytokine levels in patients with T2DM

5.1 Introduction

A number of prospective studies have demonstrated an association between higher circulating levels of inflammatory mediators, such as IL-6 and CRP, and the development of T2DM (Pradhan *et al.*, 2001; Spranger *et al.*, 2003b; Bertoni *et al.*, 2010). This relationship between inflammation and T2DM is mediated through adiposity, insulin resistance and β-cell dysfunction (Donath *et al.*, 2003; Pickup, 2004).

In the pathogenesis of periodontal disease, the importance of the host response and the production of inflammatory cytokines is well recognised. Research has demonstrated elevated inflammatory cytokine levels in tissue and GCF samples in disease compared to health (Stashenko *et al.*, 1991b; Irwin and Myrillas, 1998; Engebretson *et al.*, 2002; Lin *et al.*, 2005; Zhong *et al.*, 2007) with reductions following successful periodontal management (Engebretson et al., 2002). Published data on circulating levels of inflammatory cytokines in periodontitis are currently very limited (Marcaccini *et al.*, 2009; Sun *et al.*, 2009), as are data for salivary levels of inflammatory cytokines in periodontitis (Miller *et al.*, 2006; Gursoy *et al.*, 2009; Teles *et al.*, 2009; Costa *et al.*, 2010).

Clearly, inflammatory cytokines contribute to the pathogenesis of both T2DM and periodontitis. Alterations in immunologically active molecules as a result of T2DM have the potential to influence the cytokine network within the periodontium and thus contribute to local periodontal tissue destruction. Furthermore, inflammation within the periodontium has the potential to contribute to the systemic inflammatory burden and thus influence the pathogenic mechanisms present in T2DM. It is therefore surprising that, until very recently, relatively few studies had investigated the role of inflammatory

cytokines in patients with T2DM and periodontal disease (Cutler *et al.*, 1999a; Engebretson *et al.*, 2007; Correa *et al.*, 2008; Dag *et al.*, 2009; Correa *et al.*, 2010; Kardesler *et al.*, 2010; Santos *et al.*, 2010).

5.2 Results

5.2.1 Analysis of local and systemic cytokine levels before periodontal management

5.2.1.1 Analysis of pre-treatment serum cytokine levels in T2DM and non-diabetic patients with or without periodontal disease

Table 5.1 and Figure 5.1 show that before periodontal treatment, the levels of TNF-α, IL-1β and INF-γ in serum were significantly higher in subjects with T2DM [7.45 (5.07-9.23) pg/ml, 0.18 (0.01-0.37) pg/ml and 1.35 (0.66-2.50) pg/ml] compared to non-diabetic subjects [5.25 (3.15-7.69) pg/ml, 0.04 (0.00-0.10) pg/ml and 0.78 (0.43-1.88) pg/ml] ($p<0.01$). However, caution must be used when interpreting these results given that serum levels of IL-1β and INF-γ for both groups are around the lower limits of detection for the assay used (Table 5.1 and Figure 5.1).

Table 5.2 presents serum cytokine concentrations before periodontal treatment following further categorisation of subjects based on their periodontal diagnosis. When considering IL-6 levels in serum, there were no significant differences found in any periodontal category between T2DM and non-diabetic subjects. When comparing the subjects with T2DM, serum IL-6 appeared higher in those with gingivitis [1.33 (0.42-2.65) pg/ml] compared to those with healthy periodontal tissues [0.50 (0.33-0.81) pg/ml]. However, the Bonferroni-Holm correction of p-values for multiple comparisons placed the critical p-value at 0.017, therefore, this difference was detected as trend ($p=0.018$). Likewise, when comparing the subjects with T2DM, serum IL-6 appeared higher in those with gingivitis [1.33 (0.42-2.65) pg/ml] compared to those with

periodontitis [0.51 (0.34-1.50) pg/ml]. However, the Bonferroni-Holm correction of p-values for multiple comparisons placed the critical p-value at 0.025, therefore, this difference was detected as trend (p=0.041). Furthermore, in subjects with T2DM, serum IL-6 levels were not significantly different in those with healthy periodontal tissues [0.50 (0.33-0.81) pg/ml] compared to those with periodontitis [0.51 (0.34-1.50) pg/ml]. In non-diabetic subjects, serum IL-6 levels were not significantly different in those with healthy periodontal tissues [0.69 (0.43-1.11) pg/ml], gingivitis [0.75 (0.41-1.42) pg/ml] and periodontitis [0.59 (0.32-0.93) pg/ml] (Table 5.2).

When considering serum TNF-α, the levels were significantly higher in diabetic subjects with periodontitis [7.10 (3.25-9.30) pg/ml] compared to the non-diabetic subjects with periodontitis [3.44 (2.34-7.24) pg/ml] ($p < 0.05$). However, there were no significant differences in serum TNF-α levels in those with gingivitis or healthy periodontal tissues when comparing T2DM and non-diabetic subjects. In subjects with T2DM, serum TNF-α levels were not significantly different in those with healthy periodontal tissues [8.02 (6.34-9.24) pg/ml], gingivitis [7.69 (5.62-9.26) pg/ml] and periodontitis [7.10 (3.25-9.30) pg/ml]. For non-diabetic subjects, serum TNF-α was significantly higher in those with periodontally healthy tissues [6.28 (4.4-8.81) pg/ml] compared to those with periodontitis [3.44 (2.34-7.24) pg/ml] ($p < 0.05$). Also, in non-diabetic subjects, serum TNF-α was significantly higher in those with gingivitis [6.39 (5.21-8.03) pg/ml] compared to those with periodontitis [3.44 (2.34-7.24) pg/ml] ($p < 0.05$). However, in non-diabetic subjects, there were no significant differences in serum TNF-α between those with healthy periodontal tissues [6.28 (4.4-8.81) pg/ml] and those with gingivitis [6.39 (5.21-8.03) pg/ml] (Table 5.2).

When considering serum IL-1β, the level was significantly higher in diabetic subjects with healthy periodontal tissues, gingivitis and periodontitis [0.28 (0.09-0.40) pg/ml, 0.24 (0.04-0.40) pg/ml] and 0.08 (0.00-0.34) pg/ml] compared to non-diabetic

subjects with healthy periodontal tissues, gingivitis and periodontitis respectively [0.09 (0.06-0.14) pg/ml, 0.09 (0.02-0.20) pg/ml and 0.00 (0.00-0.48) pg/ml] ($p < 0.05$). In subjects with T2DM, serum IL-1β levels were not significantly different in those with healthy periodontal tissues [0.28 (0.09-0.40) pg/ml], gingivitis [0.24 (0.04-0.40) pg/ml] and periodontitis [0.08 (0.00-0.34) pg/ml]. For non-diabetic subjects, serum IL-1β was significantly higher in those with periodontally healthy tissues [0.09 (0.06-0.14) pg/ml] compared to those with periodontitis [0.00 (0.00-0.05) pg/ml] ($p <0.001$). Also, in non-diabetic subjects, serum IL-1β was significantly higher in those with gingivitis [0.09 (0.02-0.20) pg/ml] compared to those with periodontitis [0.00 (0.00-0.05) pg/ml] ($p < 0.001$). However, in non-diabetic subjects, there were no significant differences in serum IL-1β between those with healthy periodontal tissues [0.09 (0.06-0.14) pg/ml] and those with gingivitis [0.09 (0.02-0.20) pg/ml]. Caution must however, be used when interpreting these results given that serum levels of IL-1β for all subjects were around the lower limits of detection for the assay used (Table 5.2).

When considering serum IFN-γ, the level was significantly higher in diabetic subjects with gingivitis and periodontitis [1.74 (0.77-5.80) pg/ml and 1.09 (0.56-2.50) pg/ml] compared to the non-diabetic subjects with gingivitis and periodontitis [0.79 (0.58-2.67) pg/ml and 0.57 (0.26-1.33) pg/ml] ($p < 0.05$). In subjects with T2DM, serum IFN-γ levels were not significantly different in those with healthy periodontal tissues [1.19 (0.71-1.62) pg/ml], gingivitis [1.74 (0.77-5.80) pg/ml] and periodontitis [1.09 (0.56-2.50) pg/ml]. For non-diabetic subjects, serum IFN-γ was significantly higher in those with periodontally healthy tissues [1.78 (0.67-3.64) pg/ml] compared to those with periodontitis [0.57 (0.26-1.33) pg/ml] ($p < 0.01$). However, in non-diabetic subjects, there were no significant differences in serum IFN-γ between those with healthy periodontal tissues [1.78 (0.67-3.64) pg/ml] and gingivitis [0.79 (0.58-2.67) pg/ml]. Likewise, in non-diabetic subjects, there were no significant differences in

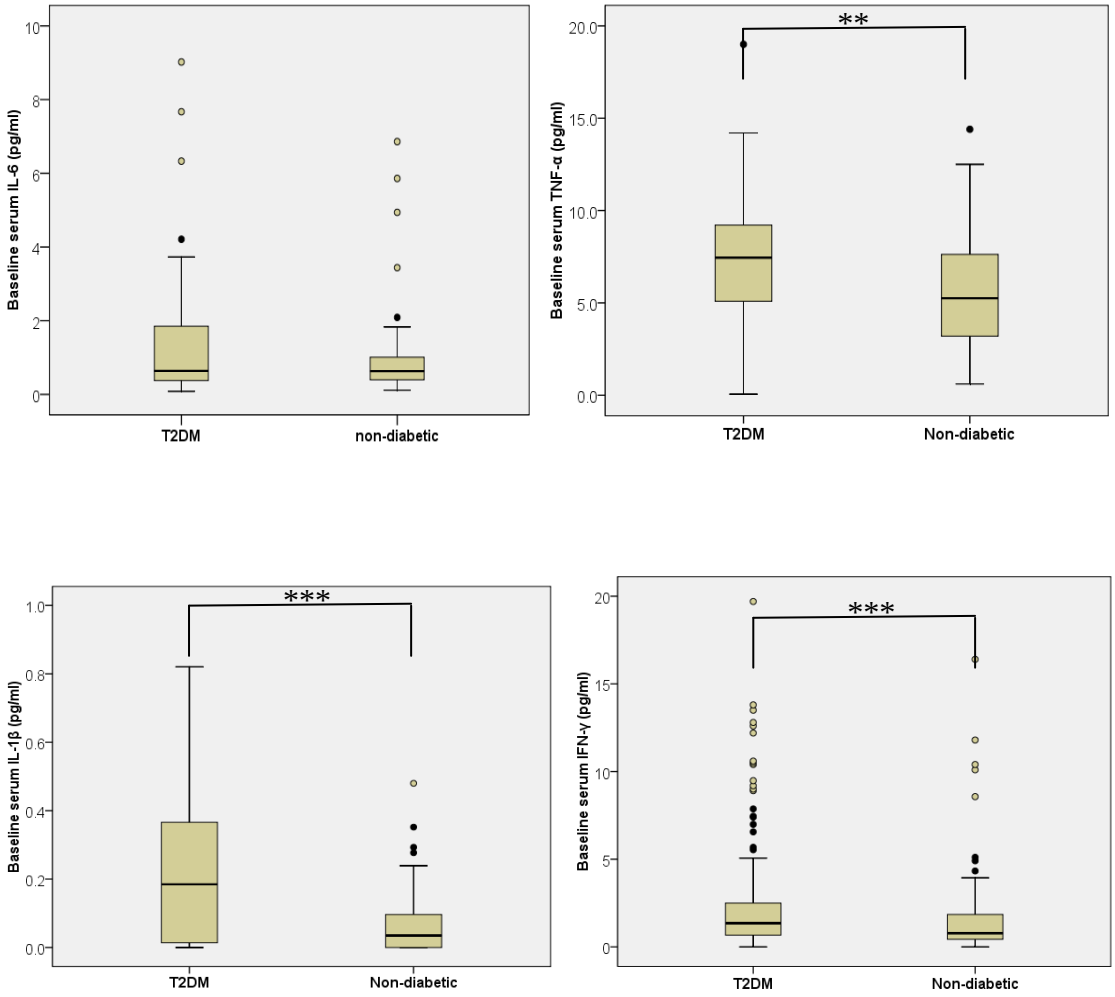
serum IFN-γ between those with gingivitis [0.79 (0.58-2.67) pg/ml] and periodontitis [0.57 (0.26-1.33) pg/ml]. Caution must however be used when interpreting these results given that serum levels of INF-γ for some subjects are around the lower limits of detection for the assay used (Table 5.2).

Table 5.1: Serum cytokine concentrations before periodontal treatment in T2DM patients and non-diabetic patients

	Diabetic subjects (n=99)	Non-diabetic subjects (n=79)	p-value
IL-6 (pg/ml)	0.64 (0.37-1.86)	0.63 (0.39-1.03)	NS
TNF-α (pg/ml)	7.45 (5.07-9.23)	5.25 (3.15-7.69)	**<0.01**
IL-1β (pg/ml)	0.18 (0.01-0.37)	0.04 (0.00-0.10)	**<0.001**
IFN-γ (pg/ml)	1.35 (0.66-2.50)	0.78 (0.43-1.88)	**<0.01**

P-values determined using Mann-Whitney U tests for continuous non-parametric variables and median (IQR) is presented for this non-parametric data.

Figure 5.1 Pre-treatment serum levels of IL-6, TNF-α, IL-1β and IFN-γ in subjects with T2DM and in non-diabetic subjects



Boxplots of pre-treatment serum levels of IL-6, TNF-α, IL-1β and IFN-γ in 99 T2DM and 79 non-diabetic subjects. Statistics: Mann Whitney-U test * $p<0.05$, **$p<0.01$, ***$p<0.001$. ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the boundaries.

Table 5.2 Pre-treatment serum cytokine data comparing groups based on diabetic status and periodontal diagnosis

	Diabetic subjects (n=98)			Non-diabetic subjects (n=79)			p-value
	Healthy (n=14)	Gingivitis (n=38)	Periodontitis (n=46)	Healthy (n=15)	Gingivitis (n=19)	Periodontitis (n=45)	
IL-6 (pg/ml)	0.50 (0.33-0.81)	1.33 (0.42-2.65)	0.51 (0.34-1.50)	0.69 (0.43-1.11)	0.75 (0.41-1.42)	0.59 (0.32-0.93)	NS
TNF-α (pg/ml)	8.02 (6.34-9.24)	7.69 (5.62-9.26)	7.10 (3.25-9.30)$^{\$}$	6.28 (4.4-8.81)¶	6.39 (5.21-8.03)†	3.44 (2.34-7.24)	$^{¶,†,\$}\leq 0.05$
IL-1β (pg/ml)	0.28 (0.09-0.40)$^{\$}$	0.24 (0.04-0.40)$^{\$}$	0.08 (0.00-0.34)$^{\$}$	0.09 (0.06-0.14)¶	0.09 (0.02-0.20)†	0.00 (0.00-0.05)	$^{¶,†}\leq 0.001$ $^{\$}\leq 0.05$
IFN-γ (pg/ml)	1.19 (0.71-1.62)	1.74 (0.77-5.80)$^{\$}$	1.09 (0.56-2.50)$^{\$}$	1.78 (0.67-3.64)¶	0.79 (0.58-2.67)	0.57 (0.26-1.33)	$^{¶}\leq 0.01$ $^{\$}\leq 0.05$

P-values were determined using Kruskal-Wallis test with Mann-Whitney U post hoc tests for continuous non-parametric variables (all data). Median (IQR) is presented for this non-parametric data.

$^{\$}$ indicates a comparison within row between diabetics and non-diabetic group with the same periodontal diagnosis
$^{\#}$ indicates a comparison within rows between periodontally healthy and gingivitis groups within either diabetes or non-diabetes groups
¶ indicates a comparison within rows between periodontally healthy and periodontitis groups within either diabetes or non-diabetes groups
† indicates a comparison within rows between gingivitis and periodontitis groups within either diabetes or non-diabetes group

5.2.1.2 Analysis of pre-treatment saliva cytokine levels in T2DM and non-diabetic patients with or without periodontal disease

Table 5.3 and Figure 5.2 show that before periodontal treatment, the levels of TNF-α, IL-1β and INF-γ in saliva were significantly higher in non-diabetic subjects [2.59 (1.40-3.98) pg/ml, 46.00 (22.00-90.43) pg/ml and 1.48 (0.64-2.12) pg/ml] compared to subjects with T2DM [1.38 (0.53-2.70) pg/ml, 26.95 (12.08-59.90) pg/ml and 0.81 (0.35-1.34) pg/ml] ($p < 0.01$). Also, the levels of salivary IL-6 were higher in the non-diabetic subjects [2.18 (0.89-5.72) pg/ml] compared to the subjects with T2DM [1.74 (0.72-4.18) pg/ml], although this difference was detected as a trend ($p=0.065$). Caution must, however, be used when interpreting these results for salivary levels of IL-6 and IFN-γ as some data are around the lower limits of detection for the assays used (Table 5.3 and Figure 5.2).

Table 5.4 presents saliva cytokine levels before periodontal treatment following further categorisation of subjects based on their periodontal diagnosis. When considering IL-6 levels in saliva, there were no significant differences found in any periodontal category between T2DM and non-diabetic subjects. In subjects with T2DM, levels of IL-6 in saliva were significantly higher in those with periodontitis [2.29 (1.27-4.83) pg/ml] compared to those with healthy periodontal tissues [0.80 (0.25-3.25) pg/ml] ($p < 0.05$). In subjects with T2DM, levels of IL-6 in saliva showed no significant differences in those with gingivitis [1.37 (0.65-3.84) pg/ml] compared to healthy periodontal tissues. Likewise in subjects with T2DM, levels of IL-6 in saliva showed no significant differences those with gingivitis [1.37 (0.65-3.84) pg/ml] compared to those with periodontitis [2.29 (1.27-4.83) pg/ml]. In non-diabetic subjects, saliva IL-6 levels were not significantly different in those with healthy periodontal tissues [2.13 (1.27-4.83) pg/ml], gingivitis [1.85 (0.85-4.07) pg/ml] and periodontitis [2.41 (0.95-7.26) pg/ml]. Caution must be used when interpreting the results for salivary levels of IL-6 as

data for some subjects are around the lower limits of detection for the assay used (Table 5.4).

When considering saliva TNF-α, the level was significantly higher in non-diabetic subjects with gingivitis and periodontitis [2.30 (1.55-4.08) pg/ml and 2.59 (1.18-3.97) pg/ml] compared to subjects with T2DM and gingivitis and periodontitis [1.17 (0.52-2.04) pg/ml and 1.58 (0.61-3.08) pg/ml] ($p < 0.05$). However, no significant differences were found in levels of TNF-α in saliva between non-diabetic subjects with healthy periodontal tissues [2.79 (1.63-4.54) pg/ml] and diabetic subjects with healthy periodontal tissue [2.26 (0.42-4.29) pg/ml]. In subjects with T2DM, there were no significant differences in saliva TNF-α levels between those with healthy periodontal tissues [2.26 (0.42-4.29) pg/ml], gingivitis [1.17 (0.52-2.04) pg/ml] and periodontitis [1.58 (0.61-3.08) pg/ml]. For non-diabetic subjects, there were no significant differences in saliva TNF-α levels between those with healthy periodontal tissues [2.79 (1.63-4.54) pg/ml], gingivitis [2.30 (1.55-4.08) pg/ml] and periodontitis [2.59 (1.18-3.97) pg/ml] (Table 5.2).

When considering IL-1β in saliva, the level was significantly higher in non-diabetic subjects with gingivitis and periodontitis [43.00 (24.70-93.70) pg/ml and 62.60 (39.20-97.10) pg/ml] compared to subjects with T2DM and gingivitis and periodontitis [16.70 (7.99-49.60) pg/ml and 38.65 (20.45-68.28) pg/ml] ($p < 0.05$). However, no significant differences were found in levels of IL-1β between non-diabetic subjects with healthy periodontal tissues [17.60 (14.20-31.95) pg/ml] and diabetic subjects with healthy periodontal tissue [14.20 (3.26-43.50) pg/ml]. In subjects with T2DM, levels of IL-1β in saliva was significantly higher in those with periodontitis [38.65 (20.45-68.28) pg/ml] compared to those with healthy periodontal tissues [14.20 (3.26-43.50) pg/ml] ($p< 0.05$). Similarly, in subjects with T2DM, levels of IL-1β in saliva were significantly higher in those with periodontitis [38.65 (20.45-68.28) pg/ml] compared to those with

gingivitis [16.70 (7.99-49.60) pg/ml] ($p < 0.05$). However, in subjects with T2DM, no significant differences in the levels of IL-1β in saliva were seen in those with gingivitis [16.70 (7.99-49.60) pg/ml] compared to healthy periodontal tissues [14.20 (3.26-43.50) pg/ml]. In non-diabetic subjects, levels of IL-1β in saliva were significantly higher in those with periodontitis [62.60 (39.20-97.10) pg/ml] compared to those with healthy periodontal tissues [17.60 (14.20-31.95) pg/ml] ($p < 0.05$). Similarly, in non-diabetic subjects, levels of IL-1β in saliva were significantly higher in those with gingivitis [43.00 (24.70-93.70) pg/ml] compared to those with healthy periodontal tissues [17.60 (14.20-31.95) pg/ml] ($p < 0.01$). In non-diabetic subjects, no significant differences in the levels of IL-1β in saliva were seen in those with gingivitis [43.00 (24.70-93.70) pg/ml] compared to those with periodontitis [62.60 (39.20-97.10) pg/ml] Therefore, for both T2DM and non-diabetic subjects, IL-1β levels in saliva increased as the periodontal status worsened (Table 5.4).

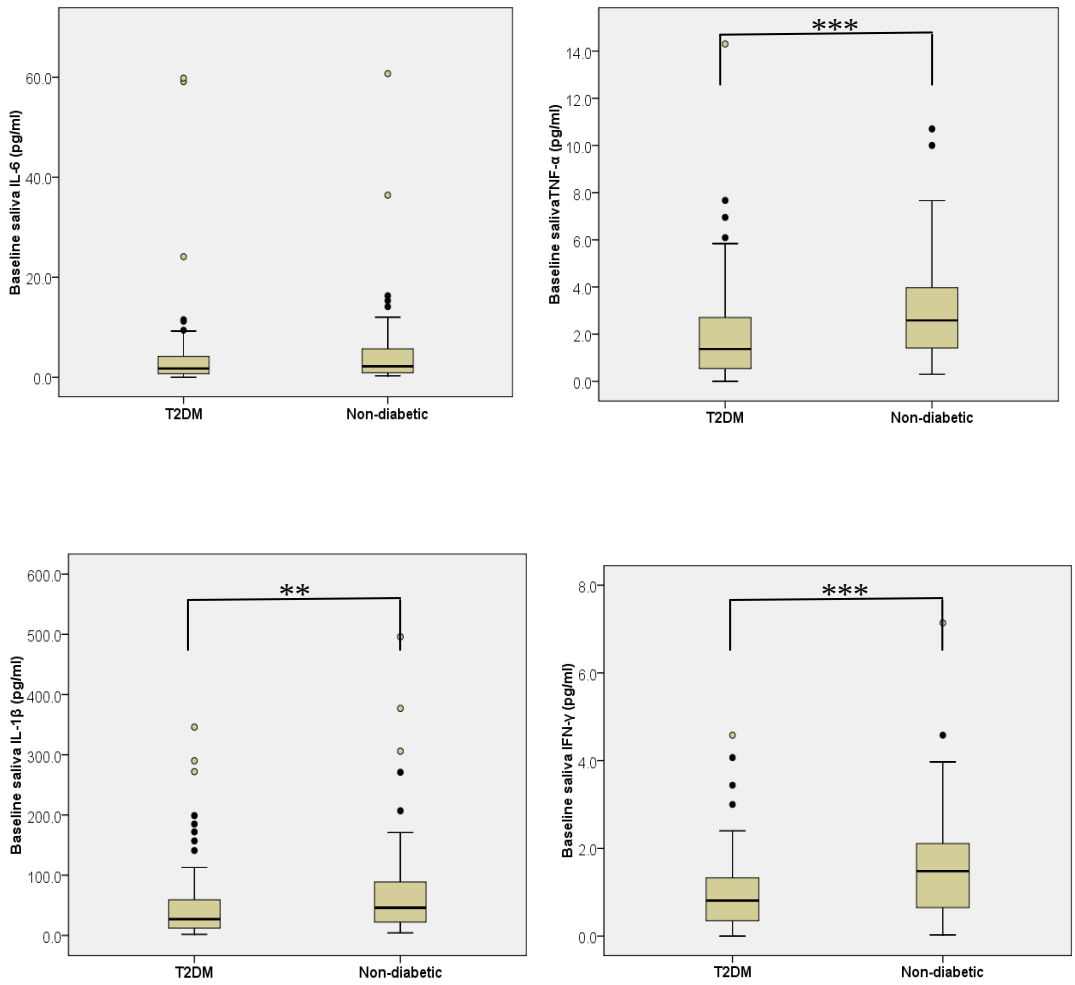
When considering IFN-γ in saliva, the levels were significantly higher in non-diabetic subjects with healthy periodontal tissues, gingivitis and periodontitis [1.74 (0.46-2.09) pg/ml, 1.07 (0.53-1.65) pg/ml and 1.55 (0.86-2.24) pg/ml] compared to diabetic subjects with healthy periodontal tissues, gingivitis and periodontitis respectively [0.58 (0.17-1.51) pg/ml, 0.64 (0.34-0.98) pg/ml and 0.86 (0.47-1.60) pg/ml] ($p < 0.05$). In subjects with T2DM, there were no significant differences in saliva IFN-γ levels between those with healthy periodontal tissues [0.58 (0.17-1.51) pg/ml], gingivitis [0.64 (0.34-0.98) pg/ml] and periodontitis [0.86 (0.47-1.60) pg/ml]. For non-diabetic subjects, there were no significant differences in saliva IFN-γ levels between those with healthy periodontal tissues [1.74 (0.46-2.09) pg/ml], gingivitis [1.07 (0.53-1.65) pg/ml] and periodontitis [1.55 (0.86-2.24) pg/ml]. However, caution must be used when interpreting the results for salivary levels of IFN-γ as data for some subjects are around the lower limits of detection for the assay used (Table 5.4).

Table 5.3 Saliva cytokine concentrations before periodontal treatment in T2DM patients and non-diabetic patients

	Diabetic subjects (n=101)	Non-diabetic subjects (n=83)	p-value
IL-6 (pg/ml)	1.74 (0.72-4.18)	2.18 (0.89-5.72)	0.065
TNF-α (pg/ml)	1.38 (0.53-2.70)	2.59 (1.40-3.98)	**<0.001**
IL-1β (pg/ml)	26.95 (12.08-59.90)	46.00 (22.00-90.43)	**<0.01**
IFN- (pg/ml)	0.81 (0.35-1.34)	1.48 (0.64-2.12)	**<0.001**

P-values determined using Mann-Whitney U tests for continuous non-parametric variables and median (IQR) is presented for this non-parametric data.

Figure 5.2 Pre-treatment saliva levels of IL-6, TNF-α, IL-1β and IFN-γ in subjects with T2DM and in non-diabetic subjects



Boxplots of pre-treatment saliva levels of IL-6, TNF-α, IL-1β and IFN-γ in 100 T2DM and 80 non-diabetic subjects. Statistics: Mann Whitney-U test * $p<0.05$, **$p<0.01$, ***$p<0.001$. ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the boundaries.

Table 5.4 Pre-treatment saliva cytokine data comparing groups based on diabetic status and periodontal diagnosis

	Diabetic subjects (n=100)			Non-diabetic subjects (n=80)			p-value
	Healthy (n=13)	Gingivitis (n=39)	Periodontitis (n=48)	Healthy (n=16)	Gingivitis (n=19)	Periodontitis (n=45)	
IL-6 (pg/ml)	0.80 (0.25-3.25) [¶]	1.37 (0.65-3.84)	2.29 (1.27-4.83)	2.13 (1.27-4.83)	1.85 (0.85-4.07)	2.41 (0.95-7.26)	[¶]<0.05
TNF-α (pg/ml)	2.26 (0.42-4.29)	1.17 (0.52-2.04)[$]	1.58 (0.61-3.08)[$]	2.79 (1.63-4.54)	2.30 (1.55-4.08)	2.59 (1.18-3.97)	[$]<0.05
IL-1β (pg/ml)	14.20 (3.26-43.50) [¶]	16.70 (7.99-49.60)[†,$]	38.65 (20.45-68.28) [$]	17.60 (14.20-31.95)[#,¶]	43.00 (24.70-93.70)	62.60 (39.20-97.10)	[¶,†,$]<0.05 [#]<0.01
IFN-γ (pg/ml)	0.58 (0.17-1.51)[$]	0.64 (0.34-0.98)[$]	0.86 (0.47-1.60)[$]	1.74 (0.46-2.09)	1.07 (0.53-1.65)	1.55 (0.86-2.24)	[$]<0.05

P-values were determined using Kruskal-Wallis test with Mann-Whitney U post hoc tests for continuous non-parametric variables (all data). Median (IQR) is presented for this non-parametric data.

[$] indicates a comparison within row between diabetics and non-diabetic group with the same periodontal diagnosis
[#] indicates a comparison within rows between periodontally healthy and gingivitis groups within either diabetes or non-diabetes groups
[¶] indicates a comparison within rows between periodontally healthy and periodontitis groups within either diabetes or non-diabetes groups
[†] indicates a comparison within rows between gingivitis and periodontitis groups within either diabetes or non-diabetes group

5.2.1.3 Analysis of pre-treatment GCF cytokine levels in T2DM and non-diabetic patients with or without periodontal disease

Table 5.5 shows that before periodontal treatment, no significant differences in the levels of IL-6, TNF-α and IL-1β in GCF were found in subjects with T2DM [1.50 (0.65-3.88) pg/ml, 3.03 (1.66-6.04) pg/ml and 202.80 (90.76-420.09) pg/ml] compared to non-diabetic subjects [1.74 (0.76-3.08) pg/ml, 3.09 (1.66-5.88) pg/ml and 205.25 (83.78-537.00) pg/ml]. A trend for higher GCF levels of IFN-γ in the non-diabetic group [2.62 (1.01-5.66) pg/ml] compared to the T2DM group [1.66 (0.71-4.58) pg/ml] was demonstrated (p=0.08). However, caution must be used when interpreting the results for GCF levels of IFN-γ as data were around the lower limits of detection for the assay used (Table 5.5).

Table 5.6 presents GCF cytokine levels before periodontal treatment following further categorisation of subjects based on their periodontal diagnosis. When considering IL-6 levels in GCF, there were no significant differences found in any periodontal category between T2DM and non-diabetic subjects. In subjects with T2DM, the levels of IL-6 in GCF appeared to be higher in those with periodontitis [1.97 (0.98-5.17) pg/ml] compared to those with healthy periodontal tissues [0.99 (0.48-2.25) pg/ml]. However, the Bonferroni-Holm correction of p-values for multiple comparisons placed the critical p-value at 0.017, therefore, this difference was detected as trend (p=0.037). Furthermore, apparent differences in GCF IL-6 levels between those with healthy periodontal tissues [0.99 (0.48-2.25) pg/ml] and gingivitis [1.44 (0.53-3.61) pg/ml] and between those with gingivitis [1.44 (0.53-3.61) pg/ml] and periodontitis [1.97 (0.98-5.17) pg/ml] failed to reach statistical significance. In non-diabetic subjects, levels of IL-6 in GCF was significantly higher in those with periodontitis [2.25 (1.12-3.37) pg/ml] compared to those with healthy periodontal tissues [1.00 (0.26-2.69) pg/ml] ($p < 0.05$). Similarly, in non-diabetic subjects, levels of IL-6 in GCF were significantly higher in those with

periodontitis [2.25 (1.12-3.37) pg/ml] compared to those with gingivitis [0.90 (0.39-2.12) pg/ml] ($p < 0.05$). In non-diabetic subjects, no significant difference was detected between those with gingivitis and those with healthy periodontal tissues (Table 5.6 and Figure 5.3).

When considering TNF-α levels in GCF, there were no significant differences found in any periodontal category between T2DM and non-diabetic subjects. In subjects with T2DM, the levels of TNF-α in GCF were significantly higher in those with periodontitis [4.16 (2.7-6.69) pg/ml] compared to those with gingivitis [2.28 (1.30-3.06) pg/ml]. However, in subjects with T2DM, the apparent differences in GCF TNF-α levels between those with healthy periodontal tissues [1.32 (0.05-7.20) pg/ml] and periodontitis [4.16 (2.7-6.69) pg/ml] and between those with gingivitis [2.28 (1.30-3.06) pg/ml] and healthy periodontal tissues [1.32 (0.05-7.20) pg/ml] failed to reach statistical significance. In non-diabetic subjects, levels of TNF-α in GCF were significantly higher in those with periodontitis [4.49 (2.55-7.04) pg/ml] compared to those with healthy periodontal tissues [1.83 (1.23-2.90) pg/ml] ($p < 0.01$). Similarly, in non-diabetic subjects, levels of IL-6 in GCF were significantly higher in those with periodontitis [2.25 (1.12-3.37) pg/ml] compared to those with gingivitis [0.90 (0.39-2.12) pg/ml] ($p < 0.01$) (Table 5.6 and Figure 5.4).

When considering IL-1β levels in GCF, there were no significant differences found in any periodontal category between T2DM and non-diabetic subjects. In subjects with T2DM, the levels of IL-1β in GCF were significantly higher in those with gingivitis [173.41 (77.60-268.19) pg/ml] compared to those with healthy periodontal tissue [84.95 (40.66-130.61) pg/ml] ($p < 0.05$). Likewise, in subjects with T2DM, the levels of IL-1β in GCF were significantly higher in those with periodontitis [344.33 (156.16-572.50) pg/ml] compared to those with periodontal healthy tissues [84.95 (40.66-130.61) pg/ml] ($p < 0.001$). Furthermore, in subjects with T2DM, the levels of IL-1β in GCF were

significantly higher in those with periodontitis [344.33 (156.16-572.50) pg/ml] compared to those with gingivitis [173.41 (77.60-268.19) pg/ml] ($p < 0.001$). In non-diabetic subjects, the levels of IL-1β in GCF were significantly higher in those with gingivitis [116.25 (56.50-176.53) pg/ml] compared to those with healthy periodontal tissue [54.26 (25.79-100.65) pg/ml] ($p< 0.05$). Likewise, in non-diabetic subjects, the levels of IL-1β in GCF were significantly higher in those with periodontitis [413.38 (213.84-770.56) pg/ml] compared to those with periodontal healthy tissues [54.26 (25.79-100.65) pg/ml] ($p < 0.001$). Furthermore, in non-diabetic subjects, the levels of IL-1β in GCF were significantly higher in those with periodontitis [413.38 (213.84-770.56) pg/ml] compared to those with gingivitis [116.25 (56.50-176.53) pg/ml] ($p < 0.001$). Therefore, for both T2DM and non-diabetic subjects, IL-1β levels in GCF increased as the periodontal status worsened (Table 5.6 & Figure 5.5).

When considering IFN-γ in GCF, the levels were significantly higher in non-diabetic subjects with periodontitis [4.40 (2.18-7.09) pg/ml] compared to diabetic subjects with periodontitis [2.51 (1.11-5.24) pg/ml] ($p < 0.05$). However, no significant differences were found in IFN-γ levels in GCF between T2DM and non-diabetic subjects for those with healthy periodontal tissues or gingivitis. In subjects with T2DM, levels of IFN-γ in GCF appeared higher in those with periodontitis [2.51 (1.11-5.24) pg/ml] compared to those with healthy periodontal tissues [0.53 (0.34-5.76) pg/ml]. However, the Bonferroni-Holm correction of p-values for multiple comparisons placed the critical p-value at 0.025, therefore, this difference was detected as trend ($p=0.032$). In subjects with T2DM, levels of IFN-γ in GCF appeared higher in those with periodontitis [2.51 (1.11-5.24) pg/ml] compared to those with gingivitis [2.17 (0.65-2.50) pg/ml]. However, the Bonferroni-Holm correction of p-values for multiple comparisons placed the critical p-value at 0.017, therefore, this difference was detected as trend ($p=0.018$). In non-diabetic subjects, levels of IFN-γ were significantly higher in those with periodontitis

[4.40 (2.18-7.09) pg/ml] compared those with healthy periodontal tissues [0.72 (0.58-2.16) pg/ml] ($p < 0.001$). Likewise, in non-diabetic subjects, levels of IFN-γ were significantly higher in those with periodontitis [4.40 (2.18-7.09) pg/ml] compared to those with gingivitis [1.85 (1.01-2.44) pg/ml] ($p < 0.001$). However, caution must be used when interpreting the results for GCF levels of IFN-γ as some data for all groups were around the lower limits of detection for the assay used (Table 5.6 and Figure 5.6).

When considering GCF volume, the volume was significantly higher in diabetic subjects with gingivitis [0.44±0.23 μl] compared to non-diabetic subjects with gingivitis [0.28(±0.15) μl] ($p < 0.01$). However, no significant differences were found in GCF volume between T2DM and non-diabetic subjects for those with healthy periodontal tissues or periodontitis. In subjects with T2DM, the GCF volumes were significantly higher in those with gingivitis [0.44±0.23 μl] compared to those with healthy periodontal tissue [0.27±0.11 μl] ($p < 0.01$). Likewise, in subjects with T2DM, GCF volume was significantly higher in those with periodontitis [0.53±0.23 μl] compared to those with healthy periodontal tissues [0.27±0.11 μl] ($p < 0.001$). However, in subjects with T2DM the apparent difference in GCF volume between those with gingivitis [0.44±0.23 μl] and periodontitis [0.53±0.23 μl] failed to reach statistical significance. In non-diabetic subjects, the GCF volumes were significantly higher in those with periodontitis [0.53±0.25 μl] compared to those with healthy periodontal tissue [0.21±0.10 μl] ($p < 0.01$). Likewise, in subjects with T2DM, GCF volume was significantly higher in those with periodontitis [0.53±0.25 μl] compared to those with gingivitis [0.28±0.15 μl] ($p < 0.001$). However, in non-diabetic subjects, the difference in GCF volume between those with healthy periodontal tissues [0.21±0.10 μl] and gingivitis [0.28±0.15 μl] failed to reach statistical significance. Overall, for both T2DM and non-diabetic subjects, GCF volume increased as the periodontal status worsens (Table 5.6).

Table 5.5 Pre-treatment GCF cytokine concentrations and volume in T2DM patients and non-diabetic patients

	Diabetics (n=100)	Non-diabetics (n=83)	p-value
GCF Vol (µl)	0.43 (0.29-0.58)	0.35 (0.21-0.60)	NS
IL-6 (pg/ml)	1.50 (0.65-3.88)	1.74 (0.76-3.08)	NS
TNF-α (pg/ml)	3.03 (1.66-6.04)	3.09 (1.66-5.88)	NS
IL-1β (pg/ml)	202.80 (90.76-420.09)	205.25 (83.78-537.00)	NS
IFN-γ (pg/ml)	1.66 (0.71-4.58)	2.62 (1.01-5.66)	NS

P-values determined using Mann-Whitney U tests for continuous non-parametric variables and median (IQR) is presented for this non-parametric data.

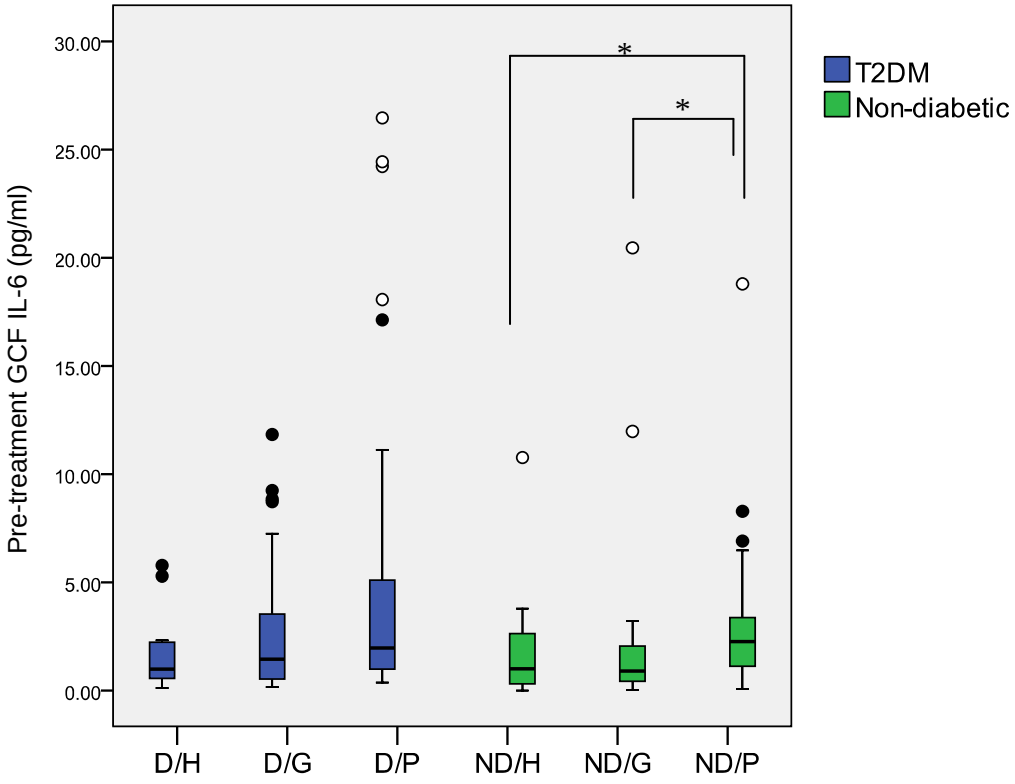
Table 5.6 Pre-treatment GCF volume and GCF cytokine data comparing groups based on diabetic status and periodontal diagnosis

	Diabetic subjects (n=100)			Non-diabetic subjects (n=83)			p-value
	Healthy (n=14)	Gingivitis (n=38)	Periodontitis (n=48)	Healthy (n=16)	Gingivitis (n=19)	Periodontitis (n=48)	
GCF Vol (µl)	0.27 (±0.11)[#,¶]	0.44 (±0.23)[$]	0.53 (±0.23)	0.21 (±0.10)[¶]	0.28 (±0.15)[†]	0.53 (±0.25)	[#,$]<0.01 [¶,†]<0.001
IL-6 (pg/ml)	0.99 (0.48-2.25)	1.44 (0.53-3.61)	1.97 (0.98-5.17)	1.00 (0.26-2.69)[¶]	0.90 (0.39-2.12)[†]	2.25 (1.12-3.37)	[¶,†]<0.05
TNF-α (pg/ml)	1.32 (0.05-7.20)	2.28 (1.30-3.06)[†]	4.16 (2.7-6.69)	1.83 (1.23-2.90)[¶]	2.23 (1.66-4.30)[†]	4.49 (2.55-7.04)	[¶,†]<0.01
IL-1β (pg/ml)	84.95 (40.66-130.61)[#,¶]	173.41 (77.60-268.19)[†]	344.33 (156.16-572.50)	54.26 (25.79-100.65)[#,¶]	116.25 (56.50-176.53)[†]	413.38 (213.84-770.56)	[#]<0.05 [¶,†]<0.001
IFN-γ (pg/ml)	0.53 (0.34-5.76)	2.17 (0.65-2.50)	2.51 (1.11-5.24)[$]	0.72 (0.58-2.16)[¶]	1.85 (1.01-2.44)[†]	4.40 (2.18-7.09)	[¶,†]<0.001 [$]<0.05

P-values were determined using chi-squared test for discrete variables, Kruskal-Wallis test with Mann-Whitney U post hoc tests for continuous non-parametric variables and one way ANOVA test with post-hoc independent t-test for continuous parametric variables (GCF vol). Mean (±SD) is presented for parametric data and median (IQR) is presented for non-parametric data.

[$] indicates a comparison within row between diabetics and non-diabetic group with the same periodontal diagnosis
[#] indicates a comparison within rows between periodontally healthy and gingivitis groups within either diabetes or non-diabetes groups
[¶] indicates a comparison within rows between periodontally healthy and periodontitis groups within either diabetes or non-diabetes groups
[†] indicates a comparison within rows between gingivitis and periodontitis groups within either diabetes or non-diabetes group

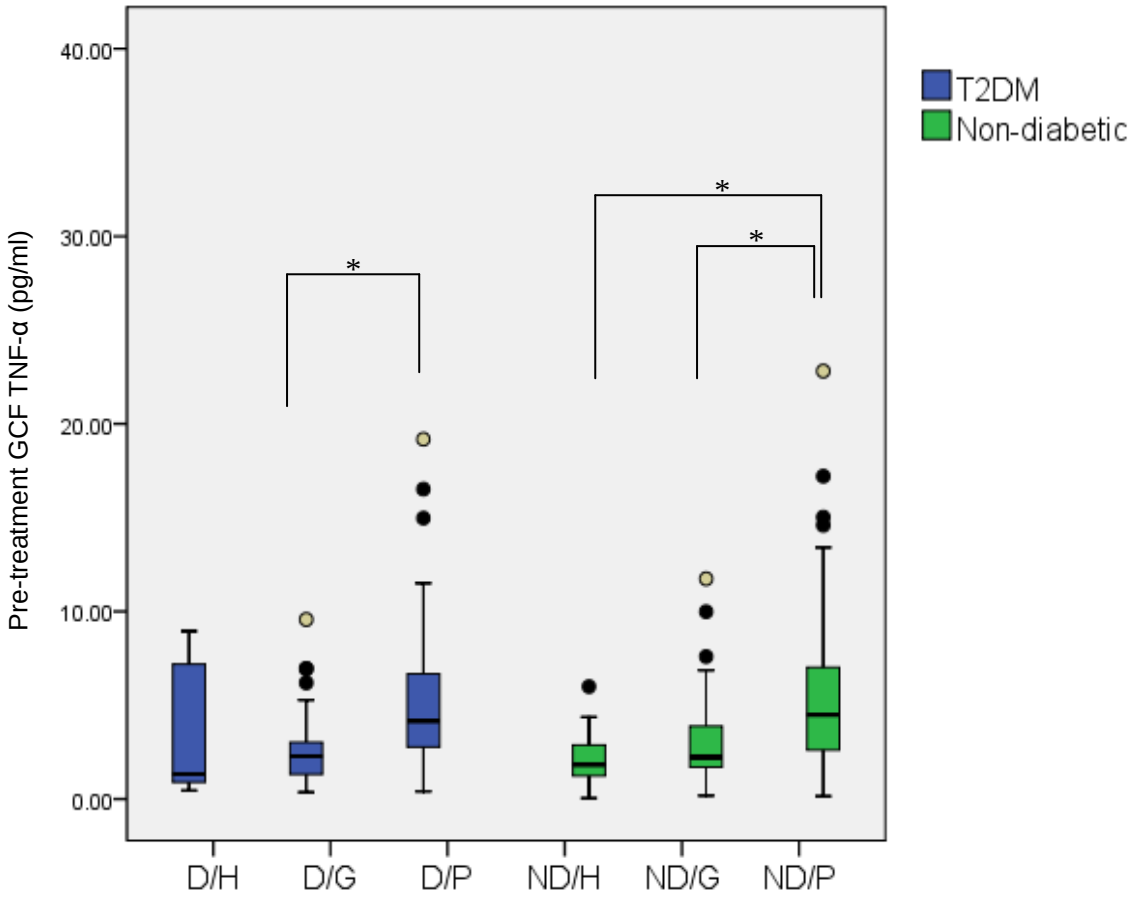
Figure 5.3 Pre-treatment GCF levels of IL-6 comparing groups based on diabetic status and periodontal diagnosis



Boxplots of pre-treatment GCF IL-6 data in 100 T2DM subjects (healthy periodontal tissues n=14, gingivitis n=38, periodontitis n=48) and 83 non-diabetic subjects (healthy periodontal tissues n=16, gingivitis n=19, periodontitis n=48)**.** Statistics: Kruskal-Wallis with Mann Whitney-U post hoc test *<0.05, **p<0.01, ***p<0.001(according to periodontal status within T2DM or non-diabetic group); § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups within the corresponding periodontal status). ○ outlier more that 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

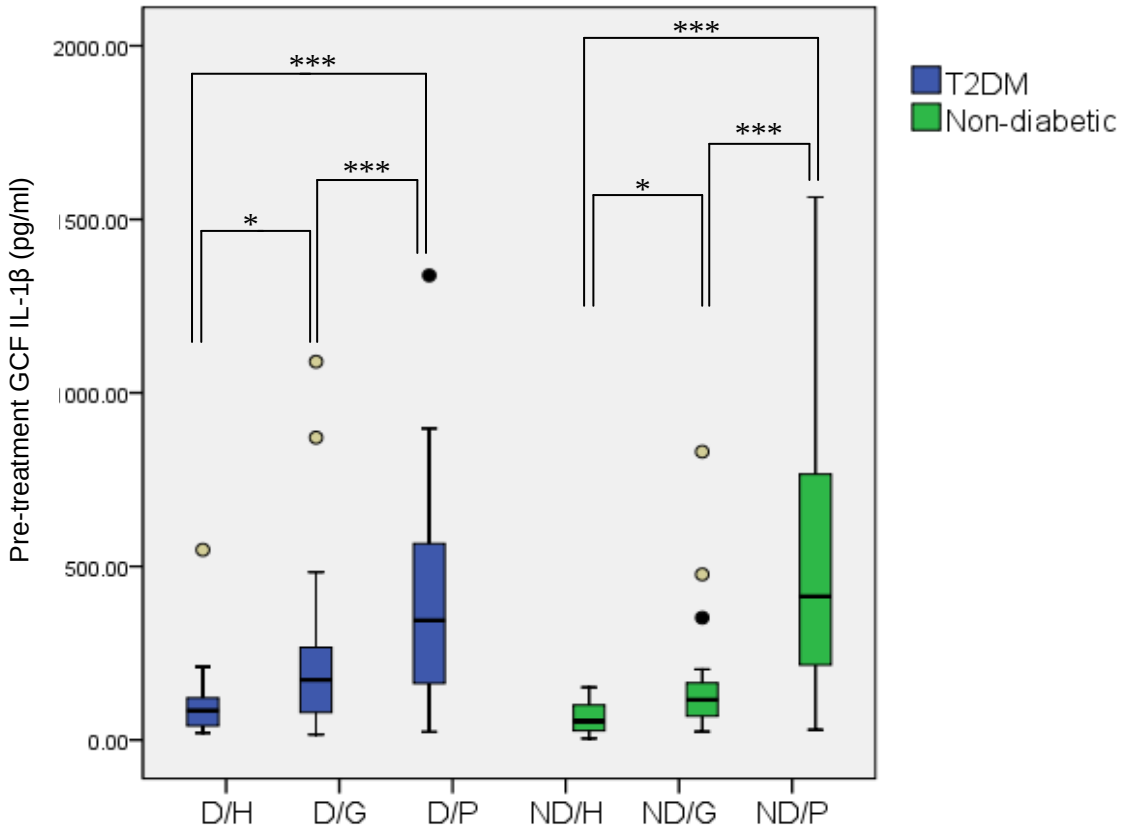
Figure 5.4 Pre-treatment GCF levels of TNF-α comparing groups based on diabetic status and periodontal diagnosis



Boxplots of pre-treatment GCF TNF-α data in 100 T2DM subjects (healthy periodontal tissues n=14, gingivitis n=38, periodontitis n=48) and 83 non-diabetic subjects (healthy periodontal tissues n=16, gingivitis n=19, periodontitis n=48)**.** Statistics: Kruskal-Wallis with Mann Whitney-U post hoc test *<0.05, **p<0.01, ***p<0.001(according to periodontal status within T2DM or non-diabetic group); § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups within the corresponding periodontal status). ○ outlier more that 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

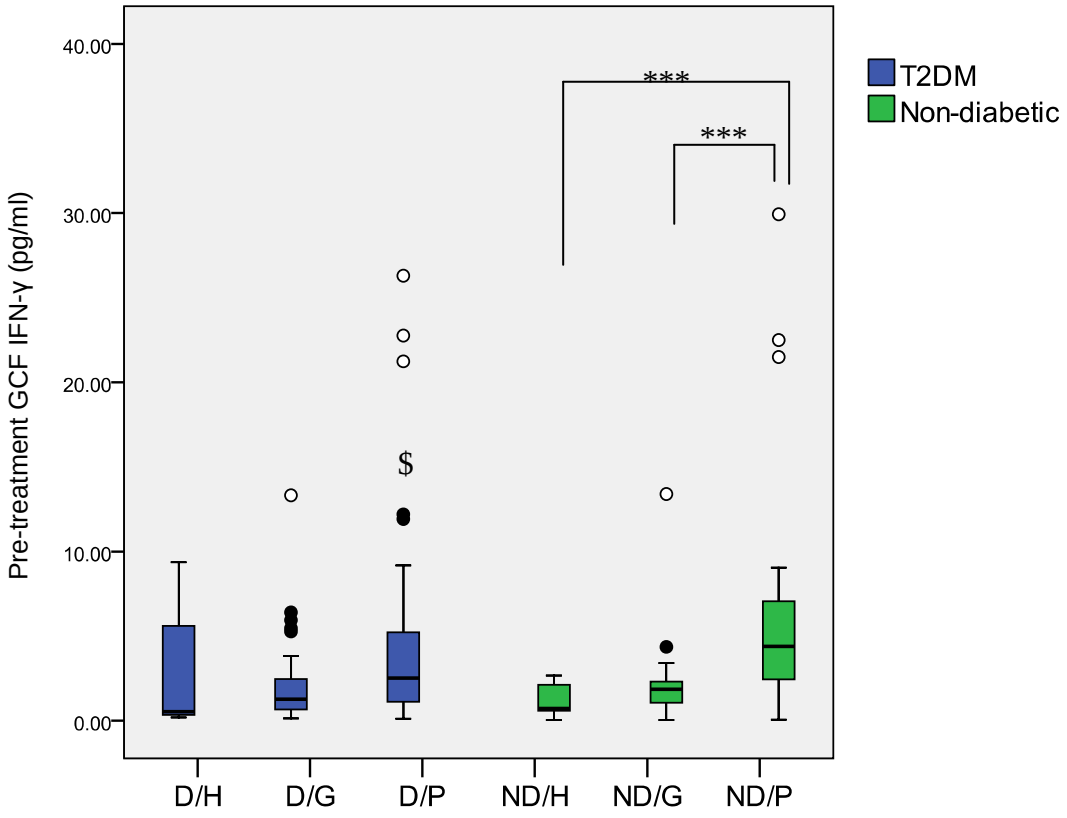
Figure 5.5 Pre-treatment GCF levels of IL-1β comparing groups based on diabetic status and periodontal diagnosis



Boxplots of pre-treatment GCF IL-1β data in 100 T2DM subjects (healthy periodontal tissues n=14, gingivitis n=38, periodontitis n=48) and 83 non-diabetic subjects (healthy periodontal tissues n=16, gingivitis n=19, periodontitis n=48)**.** Statistics: Kruskal-Wallis with Mann Whitney-U post hoc test *<0.05, **p<0.01, ***p<0.001(according to periodontal status within T2DM or non-diabetic group); § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups within the corresponding periodontal status). ○ outlier more that 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

Figure 5.6 Pre-treatment GCF levels of IFN-γ comparing groups based on diabetic status and periodontal diagnosis



Boxplots of pre-treatment GCF IFN-γ data in 100 T2DM subjects (healthy periodontal tissues n=14, gingivitis n=38, periodontitis n=48) and 83 non-diabetic subjects (healthy periodontal tissues n=16, gingivitis n=19, periodontitis n=48)**.** Statistics: Kruskal-Wallis with Mann Whitney-U post hoc test *<0.05, **p<0.01, ***p<0.001(according to periodontal status within T2DM or non-diabetic group); § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups within the corresponding periodontal status). ○ outlier more that 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

D/H: T2DM and healthy periodontal tissues
D/G: T2DM and gingivitis
D/P: T2DM and periodontitis
ND/H: Non-diabetic subjects with healthy periodontal tissues
ND/G: Non-diabetic subjects with gingivitis
ND/P: Non-diabetic subjects with periodontitis

5.2.2 Exploration of the associations between clinical periodontal parameters, cytokine levels and clinical markers of diabetes control and inflammation

5.2.2.1 The relationship of HbA1c and hsCRP with clinical periodontal parameters

Pre-treatment levels of HbA1c and hs-CRP were investigated for correlations with mGI, BOP, mean PD, PESA and PISA. Correlations were first undertaken for all subjects and then repeated taking diabetes status into account (Tables 5.7, 5.8 and 5.9).

When considering all subjects (diabetic and non-diabetic), a significant negative correlation was demonstrated between HbA1c levels and PESA (Spearman's ρ =-0.17, $p<0.05$). All other correlations between HbA1c levels and mGI, BOP, mean PD and PISA were non-significant (Table 5.7). In subjects with T2DM and for non-diabetic subjects, no significant correlations were found between levels of HbA1c and mGI, BOP, mean PD, PESA and PISA (Tables 5.8 and 5.9).

When considering all subjects (diabetic and non-diabetic), significant positive correlations were demonstrated between hsCRP levels and mGI (Spearman's ρ =0.24, $p<0.05$) and mean PD (Spearman's ρ =0.16, $p<0.05$). All other correlations between hsCRP levels and BOP, PESA and PISA were non-significant (Table 5.7). When the data were split according to diabetes status, no significant correlations were found between levels of hsCRP and mGI, BOP, mean PD, PESA and PISA in subjects with T2DM (Table 5.8). However, in non-diabetic subjects, significant positive correlations were shown between hsCRP levels and mGI (Spearman's ρ= 0.23, $p<0.05$), BOP (Spearman's ρ= 0.26, $p<0.05$), mean PD (Spearman's ρ= 0.26, $p<0.01$) and PISA (Spearman's ρ= 0.27, $p<0.05$) (Table 5.9).

Table 5.7 Correlations between HbA1c and hsCRP levels and clinical periodontal parameters for all subjects

Clinical parameters	Spearman rank Correlation Co-efficient	
	HbA1c (%)	Hs CRP (mg/L)
mGI	0.05	0.24†
BOP (%)	0.04	0.15
Mean PD (mm)	-0.05	0.16‡
PESA (mm^2)	-0.17‡	-0.01
PISA (mm^2)	0.04	-0.09

Table 5.8 Correlations between HbA1c and hsCRP levels and clinical periodontal parameters for T2DM subjects

Clinical parameters	Spearman rank Correlation Co-efficient	
	HbA1c (%)	Hs CRP (mg/L)
mGI	0.03	-0.07
BOP (%)	-0.02	-0.14
Mean PD (mm)	-0.21	-0.05
PESA (mm^2)	0.06	-0.17
PISA (mm^2)	0.10	-0.11

Table 5.9 Correlations between HbA1c and hsCRP levels and clinical periodontal parameters for non-diabetic subjects

Clinical parameters	Spearman rank Correlation Co-efficient	
	HbA1c (%)	Hs CRP (mg/L)
mGI	0.03	0.23‡
BOP (%)	-0.06	0.26‡
Mean PD (mm)	-0.07	0.26‡
PESA (mm^2)	-0.05	0.17
PISA (mm^2)	-0.04	0.27‡

Tables show Spearman rank correlation coefficients with colour indicates strength of correlation: small (r=0.10 to 0.29), medium (r=0.30 to 0.49) and large (r=0.50 to 1.00) (Cohen, 1988). *p<0.001, †p<0.01, ‡ p<0.05.

5.2.2.2 The relationship of HbA1c and hsCRP levels with cytokine levels in serum, saliva and GCF

Pre-treatment levels of HbA1c and hs-CRP were investigated for correlations with levels of IL-6, TNF-α, IL-1β and IFN-γ in serum, saliva and GCF. Correlations were first undertaken for all subjects and then repeated taking diabetes status into account (Tables 5.10, 5.11 and 5.12).

When considering all subjects (diabetic and non-diabetic), significant positive correlations were demonstrated between HbA1c levels and serum levels of IL-6 (Spearman's $\rho = 0.16$, $p<0.05$), TNF-α (Spearman's $\rho = 0.18$, $p<0.05$) and IL-1β (Spearman's $\rho = 0.21$, $p<0.01$) and all other correlations between HbA1c levels and serum cytokines were non-significant (Table 5.10). In subjects with T2DM only, a significant negative correlation was demonstrated between HbA1c levels and serum IL-1β levels (Spearman's $\rho = -0.26$, $p<0.05$) and all other correlations between HbA1c levels and serum cytokines were non-significant (Table 5.11). In non-diabetic subjects, no significant correlations between levels of HbA1c and serum levels of IL-6, TNF-α, IL-1β and IFN-γ were found (Table 5.12).

Additionally, in all subjects, significant negative correlations were demonstrated between HbA1c levels and saliva levels of TNF-α (Spearman's $\rho = -0.18$, $p<0.05$) and IFN-γ (Spearman's $\rho = -0.15$, $p<0.05$) and all other correlations between HbA1c levels and saliva cytokines were non-significant (Table 5.10). In subjects with T2DM only, a significant positive correlation was demonstrated between HbA1c levels and saliva levels of IL-1β (Spearman's $\rho = -0.27$, $p<0.05$) and IFN-γ (Spearman's $\rho = 0.20$, $p<0.01$) and all other correlations between HbA1c levels and saliva cytokines were non-significant (Table 5.11). In non-diabetic subjects, no significant correlations between levels of HbA1c and saliva levels of IL-6, TNF-α, IL-1β and IFN-γ were found (Table 5.12).

Furthermore, in all subjects and when the data were split according to diabetes status, no significant correlations between levels of HbA1c and GCF levels of IL-6, TNF-α, IL-1β and IFN-γ were found (Tables 5.10, 5.11 and 5.12).

When considering all subjects, and following categorisation according to diabetes status, no significant correlations between levels of hsCRP and serum or saliva levels of IL-6, TNF-α, IL-1β and IFN-γ were found (Tables 5.10, 5.11 and 5.12). When considering cytokine levels in GCF, no significant correlations between levels of hsCRP and GCF levels of IL-6, TNF-α, IL-1β and IFN-γ were found when all subjects or subjects with T2DM alone were considered. In non-diabetic subjects, a significant positive correlation was demonstrated between hsCRP levels and GCF levels of IL-1β (Spearman's $\rho = -0.32$, $p < 0.01$) (Tables 5.10, 5.11 and 5.12).

Table 5.10 Correlations between HbA1c and hsCRP levels and cytokine levels in serum, saliva and GCF for all subjects

Clinical Parameters	Spearman Rank Correlation Co-efficient											
	Serum cytokines				Saliva cytokines				GCF cytokines			
	IL-6	TNF-α	IL-1β	IFN-γ	IL-6	TNF-α	IL-1β	IFN-γ	IL-6	TNF-α	IL-1β	IFN-γ
HbA1c (%)	0.16‡	0.18‡	0.21†	0.15	-0.72	-0.18‡	-0.10	-0.15‡	0.05	0.001	0.00	-0.14
hsCRP (mg/L)	0.10	-0.09	0.04	0.13	0.58	-0.01	0.06	0.07	0.02	0.05	0.15	0.10

Table 5.11 Correlations between HbA1c and hsCRP levels and cytokine levels in serum, saliva and GCF for T2DM subjects

Clinical Parameters	Spearman Rank Correlation Co-efficient											
	Serum cytokines				Saliva cytokines				GCF cytokines			
	IL-6	TNF-α	IL-1β	IFN-γ	IL-6	TNF-α	IL-1β	IFN-γ	IL-6	TNF-α	IL-1β	IFN-γ
HbA1c (%)	0.09	-0.08	-0.26‡	-0.15	0.14	0.18	0.27†	0.20†	0.16	0.19	0.11	0.06
hsCRP (mg/L)	0.09	-0.15	0.78	0.04	0.07	0.03	0.06	0.10	-0.08	-0.02	-0.01	-0.03

Table 5.12 Correlations between HbA1c and hsCRP levels and cytokine levels in serum, saliva and GCF for non-diabetic subjects

Clinical Parameters	Spearman Rank Correlation Co-efficient											
	Serum cytokines				Saliva cytokines				GCF cytokines			
	IL-6	TNF-α	IL-1β	IFN-γ	IL-6	TNF-α	IL-1β	IFN-γ	IL-6	TNF-α	IL-1β	IFN-γ
HbA1c (%)	0.18	0.03	0.10	0.09	-0.06	0.06	0.02	0.13	-0.15	-0.17	-0.03	-0.10
hsCRP (mg/L)	0.15	-0.12	-0.15	0.18	0.06	-0.02	-0.10	0.07	0.16	0.16	0.32†	0.21

Tables show correlation coefficients: small (r=0.10 to 0.29), medium (r=0.30 to 0.49) and large (r=0.50 to 1.00) (Cohen, 1988) *p<0.001, †p<0.01, ‡ p<0.05

5.2.2.3 The relationship of cytokine levels in serum, saliva and GCF with clinical periodontal parameters

Pre-treatment levels of inflammatory cytokines in serum, saliva and GCF were investigated for correlations with mGI, BOP, mean PD, PESA and PISA. Correlations were first undertaken for all subjects (Table 5.13) and then repeated taking diabetes status into account (Tables 5.14 and 5.15). A series of scatter plots were used to graphically present correlations of BOP, mean PD, PESA and PISA with IL-6, TNF-α, IL-1β and IFN-γ in serum, saliva and GCF (Figure 5.7 to 5.18)

When considering all subjects, a significant negative correlations was determined between TNF-α levels in serum and mean PD (Spearman's ρ= -0.15, $p<0.05$) and PESA (Spearman's ρ= -0.18, $p<0.05$). A significant negative correlation was also determined between IL-1β levels in serum and mean PD (Spearman's ρ= -0.26, $p<0.001$), PESA (Spearman's ρ= -0.30, $p<0.001$) and PISA (Spearman's ρ= -0.26, $p<0.001$) (Table 5.13 and Figure 5.8). When the data were split according to diabetes status, similar negative correlations between clinical periodontal parameters and serum TNF-α and IL-1β were demonstrated in non-diabetic subjects but no such correlations were demonstrated in T2DM subjects (Tables 5.14 and 5.15). Caution must however be used when interpreting these results given that serum levels of the cytokines measured are around the lower limits of detection for the assay used.

When considering all subjects, significant positive correlations were determined between IL-6 levels in saliva and mGI (Spearman's ρ= 0.35, $p<0.001$), BOP (Spearman's ρ= 0.25, $p<0.01$), mean PD (Spearman's ρ= 0.20, $p<0.01$) and PESA (Spearman's ρ= 0.24, $p<0.001$) and PISA (Spearman's ρ= 0.27, $p<0.001$) (Table 5.13 and Figure 5.11). Similarly, significant positive correlations were determined between IL-1β levels in saliva and mGI (Spearman's ρ= 0.35, $p<0.001$), mean PD (Spearman's

ρ= 0.40, p<0.001) and PESA (Spearman's ρ= 0.46, p<0.001) and PISA (Spearman's ρ= 0.45, p<0.001) (Table 5.13 and Figure 5.13). When the data were split according to diabetes status, similar positive correlations between clinical periodontal parameters and saliva IL-6 and IL-1β levels were also demonstrated in non-diabetic and T2DM subjects (Tables 5.14 and 5.15).

When considering all subjects, significant positive correlations were demonstrated between IL-6 levels in GCF and mGI (Spearman's ρ= 0.30, p<0.001), BOP (Spearman's ρ= 0.37, p<0.001), mean PD (Spearman's ρ= 0.35, p<0.001) and PESA (Spearman's ρ= 0.32, p<0.001) and PISA (Spearman's ρ= 0.39, p<0.001) (Table 5.13 and Figure 5.15). Similarly, significant positive correlations were also demonstrated between TNF-α levels in GCF and mGI (Spearman's ρ= 0.23, p<0.01), BOP (Spearman's ρ= 0.30, p<0.001), mean PD (Spearman's ρ= 0.40, p<0.001) and PESA (Spearman's ρ= 0.35, p<0.001) and PISA (Spearman's ρ= 0.38, p<0.001) (Table 5.13 and Figure 5.16). Additionally, significant positive correlations were also between IL-1β levels in GCF and mGI (Spearman's ρ= 0.51, p<0.001), BOP (Spearman's ρ= 0.58, p<0.001), mean PD (Spearman's ρ= 0.66, p<0.001) and PESA (Spearman's ρ= 0.59, p<0.001) and PISA (Spearman's ρ= 0.66, p<0.001) (Table 5.10 and Figure 5.17). Furthermore, significant positive correlations were shown between IFN-γ levels in GCF and mGI (Spearman's ρ= 0.33, p<0.001), BOP (Spearman's ρ= 0.31, p<0.001), mean PD (Spearman's ρ= 0.46, p<0.001) and PESA (Spearman's ρ= 0.42, p<0.001) and PISA (Spearman's ρ= 0.47, p<0.001) (Table 5.13 and Figure 5.18). When the data were split according to diabetes status, similar positive correlations between clinical periodontal parameters and GCF IL-6, TNF-α, IL-1β and IFN-γ levels were also demonstrated in non-diabetic and T2DM subjects, although the correlations were smaller in magnitude in the T2DM subjects (Tables 5.14 and 5.15).

Table 5.13 Relationships among clinical periodontal parameters and cytokine levels in serum, saliva and GCF for all subjects

Clinical Parameters	Spearman Rank Correlation Co-efficient											
	Serum cytokines				Saliva cytokines				GCF cytokines			
	IL-6	TNF-α	IL-1β	IFN-γ	IL-6	TNF-α	IL-1β	IFN-γ	IL-6	TNF-α	IL-1β	IFN-γ
mGI	-0.04	-0.08	-0.02	-0.10	0.35*	0.13	0.35*	0.10	0.30*	0.23†	0.51*	0.33*
BOP (%)	-0.04	-0.09	-0.09	0.05	0.25†	-0.07	0.35	0.04	0.37*	0.30*	0.58*	0.31*
Mean PD (mm)	-0.08	-0.15‡	-0.26*	-0.13	0.20†	-0.05	0.40*	0.13	0.35*	0.40*	0.66*	0.46*
PESA (mm2)	-0.11	-0.18‡	-0.30*	-0.17‡	0.24*	0.07	0.46*	0.20†	0.32*	0.35*	0.59*	0.42*
PISA (mm2)	-0.03	-0.13	-0.26*	-0.10	0.27*	0.01	0.45*	0.12	0.39*	0.38*	0.66*	0.47*

Table 5.14 Relationships among periodontal clinical parameters and cytokine levels in serum, saliva and GCF for T2DM subjects

Clinical Parameters	Spearman Rank Correlation Co-efficient											
	Serum cytokines				Saliva cytokines				GCF cytokines			
	IL-6	TNF-α	IL-1β	IFN-γ	IL-6	TNF-α	IL-1β	IFN-γ	IL-6	TNF-α	IL-1β	IFN-γ
mGI	0.02	0.09	0.17	0.06	0.42*	0.25‡	0.32†	0.17	0.24‡	0.18	0.38*	0.24‡
BOP (%)	0.17	0.09	-0.05	0.22‡	0.28†	-0.29	0.30†	0.10	0.32†	0.19	0.48*	0.31†
Mean PD (mm)	0.06	0.07	-0.18	0.08	0.24‡	0.01	0.38*	0.24‡	0.27†	0.28†	0.56*	0.35†
PESA (mm2)	-0.00	0.01	-0.19	0.02	0.26‡	0.11	0.40*	0.24‡	0.26‡	0.24‡	0.49*	0.27†
PISA (mm2)	0.10	0.06	-0.18	0.81	0.30†	0.04	0.40*	0.14	0.34†	0.25‡	0.55*	0.34†

Table 5.15 Relationships among periodontal biochemistry parameters and cytokine levels in serum, saliva and GCF for non-diabetic subjects

Clinical Parameters	Spearman Rank Correlation Co-efficient											
	Serum cytokines				Saliva cytokines				GCF cytokines			
	IL-6	TNF-α	IL-1β	IFN-γ	IL-6	TNF-α	IL-1β	IFN-γ	IL-6	TNF-α	IL-1β	IFN-γ
mGI	-0.00	-0.42*	-0.31†	-0.34†	0.31†	0.12	0.47*	0.10	0.34†	0.29†	0.61*	0.47*
BOP (%)	-0.18	-0.43*	-0.37†-	-0.21	0.26‡	-0.13	0.50*	0.06	0.44*	0.45*	0.69*	0.53*
Mean PD (mm)	-0.22	-0.41*	-0.47*	-0.31†	0.14	-0.09	0.48*	0.06	0.42*	0.53*	0.72*	0.60*
PESA (mm2)	-0.19	-0.36†	-0.41*	-0.27‡	0.17	-0.06	0.50*	0.09	0.41*	0.48*	0.67*	0.59*
PISA (mm2)	-0.19	-0.38†	-0.44*	-0.25‡	0.25‡	0.00	0.56*	0.09	0.56*	0.51*	0.72*	0.59*

Tables show correlation coefficients: small (r=0.10 to 0.29), medium (r=0.30 to 0.49) and large (r=0.50 to 1.00) (Cohen, 1988) *p<0.001, †p<0.01, ‡ p<0.05,

Figure 5.7 The relationship of serum IL6 with clinical periodontal parameters in all subjects

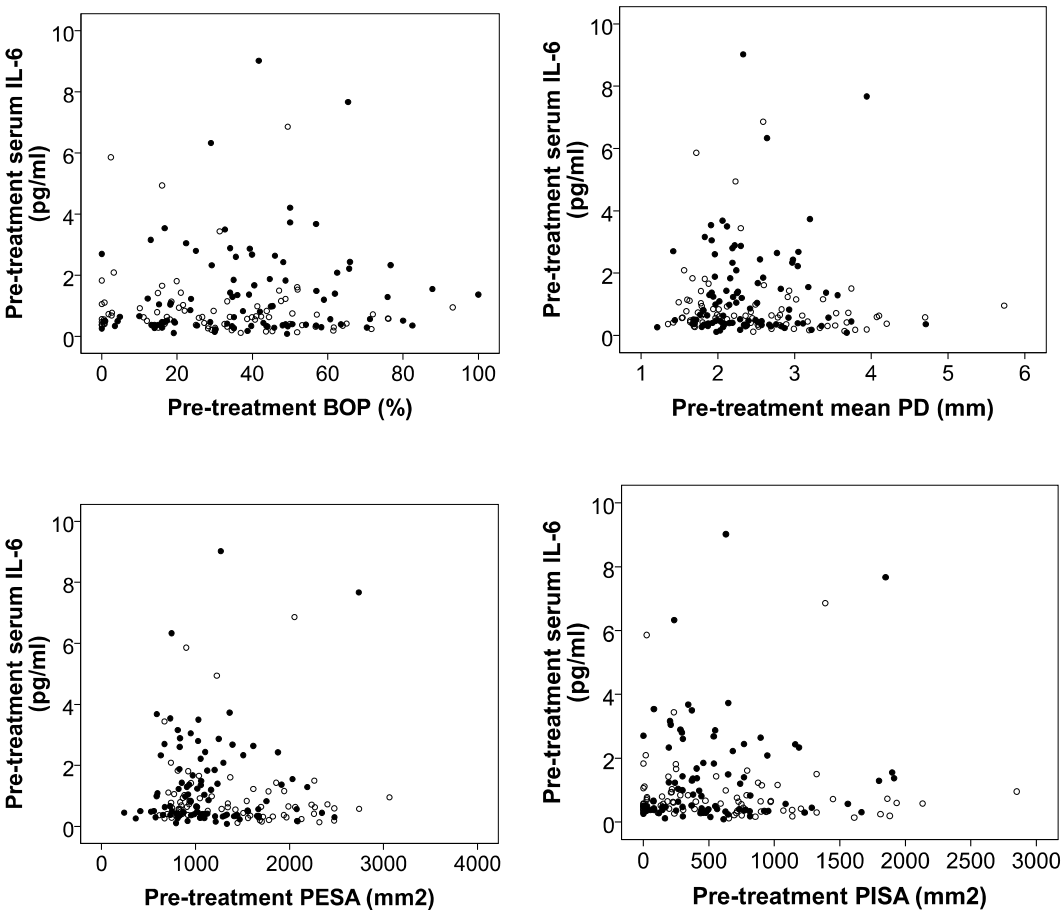


Figure shows Spearman correlation of serum IL-6 concentrations with BOP (%), mean PD (mm), PESA (mm2) and PISA (mm^2) in all subjects ● T2DM subjects and ○ non-diabetic subjects (n=182). The addition of a trend-line demonstrates the presence of a significant correlation.

Figure 5.8 The relationship of serum TNF-α with clinical periodontal parameters in all subjects

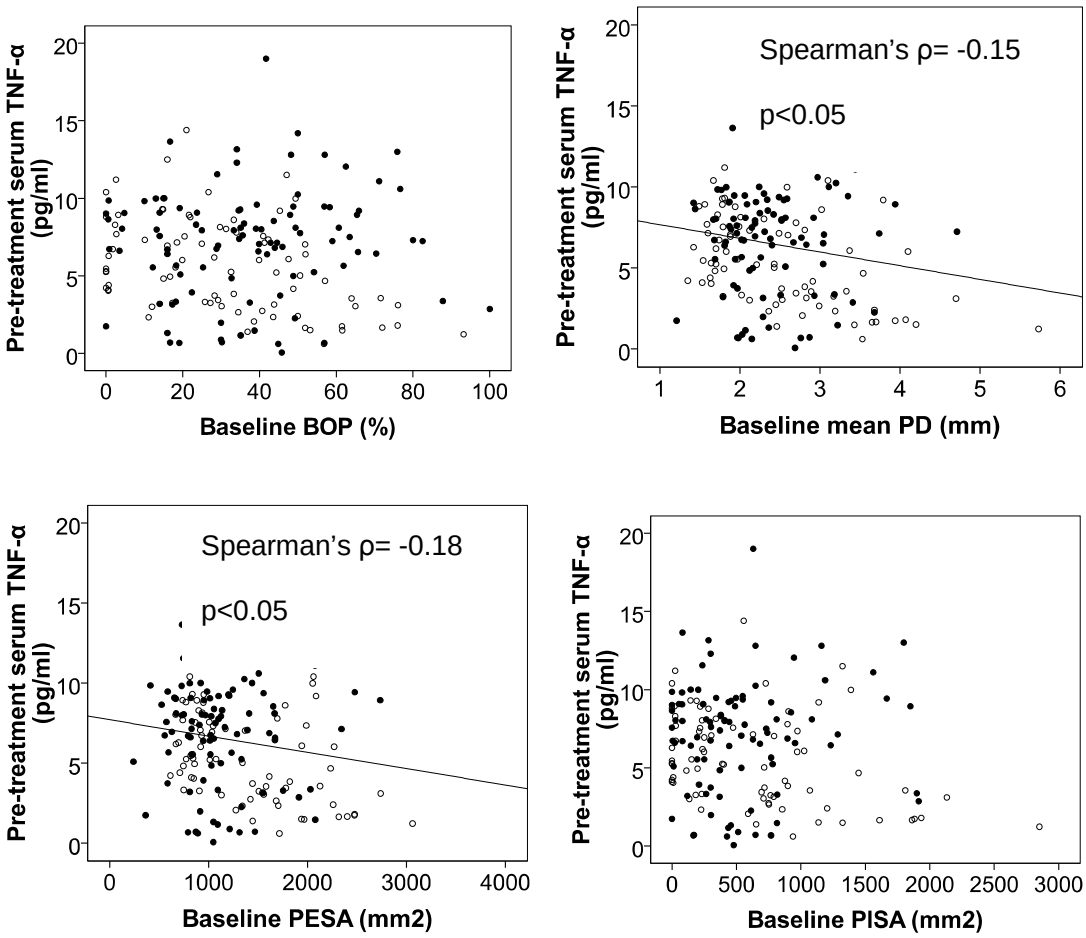


Figure shows Spearman correlation of serum TNF-α concentrations with BOP (%), mean PD (MM), PESA (mm2) and PISA (mm^2) in all subjects ● T2DM subjects and ○ non-diabetic subjects (n=182). The addition of a trend-line demonstrates the presence of a significant correlation.

Figure 5.9 The relationship of serum IL-1β with clinical periodontal parameters in all subjects

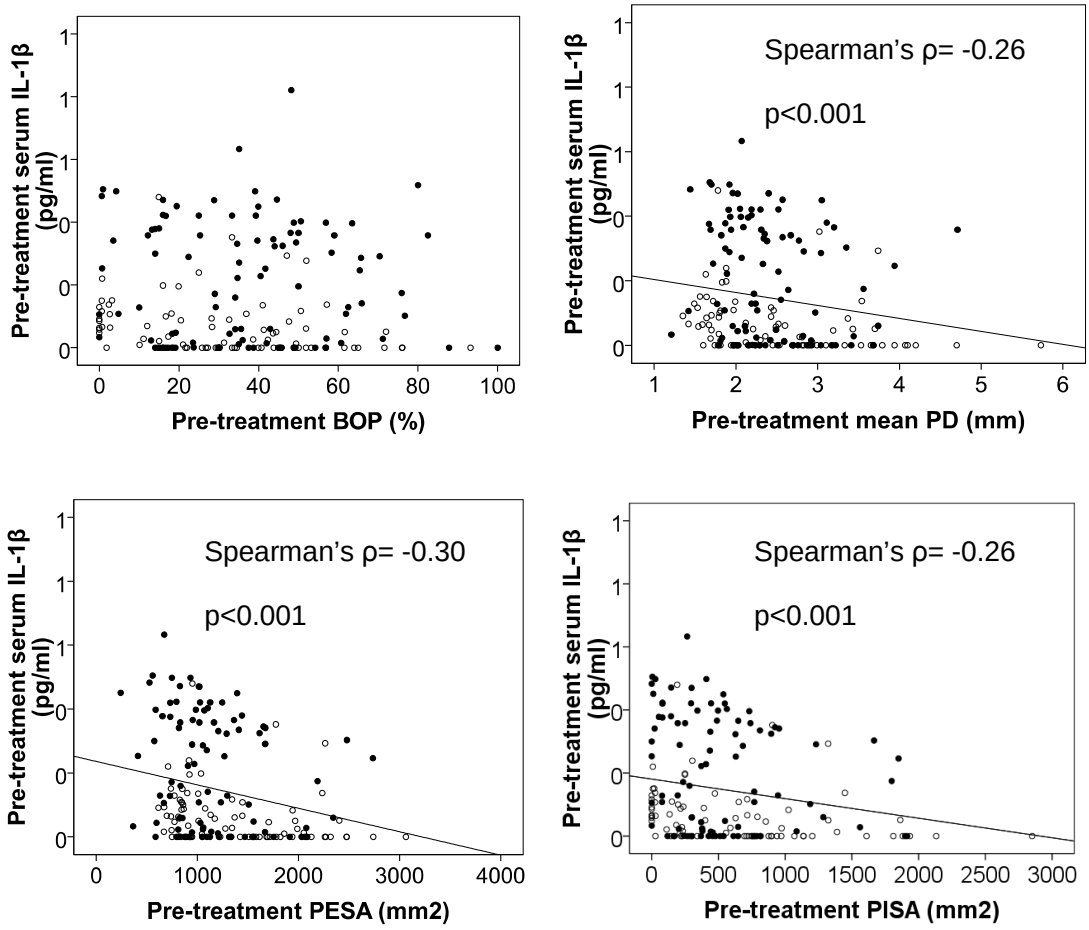


Figure shows Spearman correlation of serum IL-1β concentrations with BOP (%), mean PD (mm), PESA (mm2) and PISA (mm^2) in all subjects ● T2DM subjects and ○ non-diabetic subjects (n=182). The addition of a trend-line demonstrates the presence of a significant correlation.

Figure 5.10 The relationship of serum IFN-γ with clinical periodontal parameters in all subjects

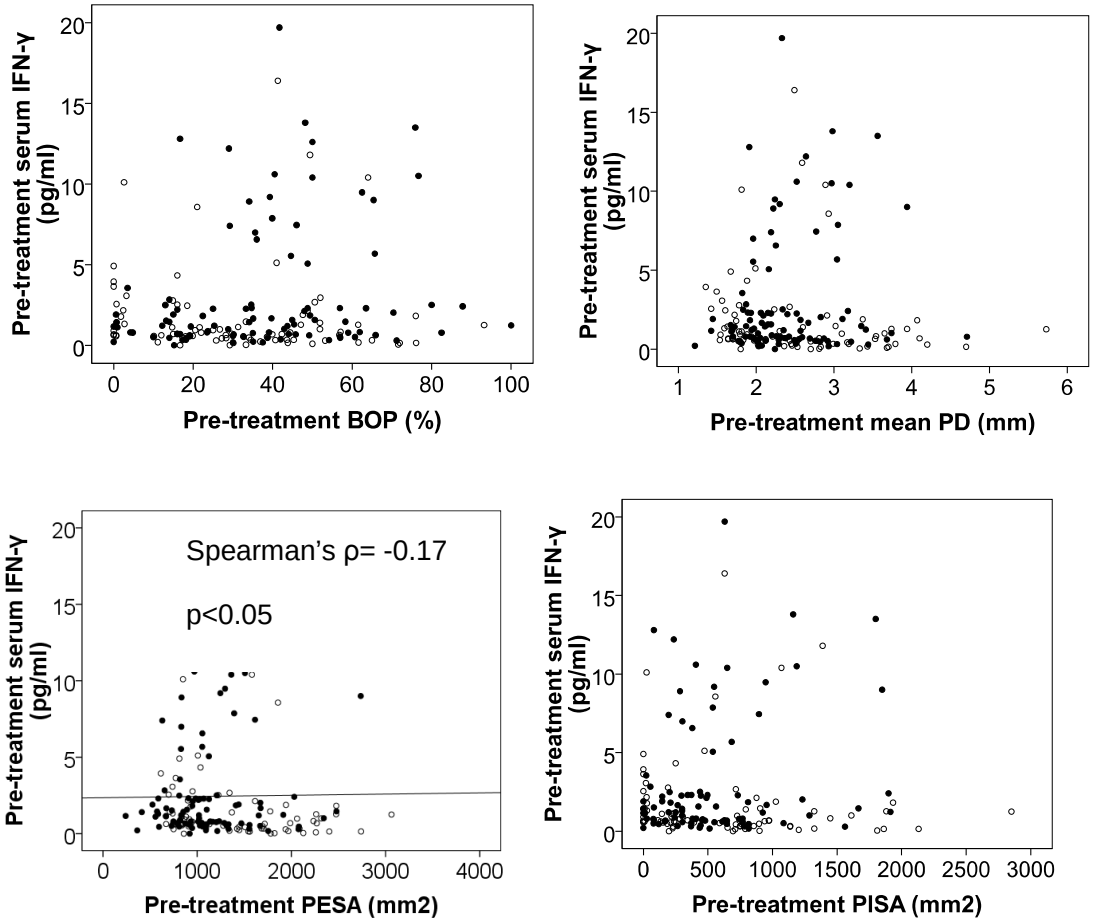


Figure shows Spearman correlation of serum IFN-γ concentrations with BOP (%), mean PD (mm), PESA (mm2) and PISA (mm^2) in all subjects ● T2DM subjects and ○ non-diabetic subjects (n=182). The addition of a trend-line demonstrates the presence of a significant correlation.

Figure 5.11 The relationship of saliva IL-6 with clinical periodontal parameters in all subjects

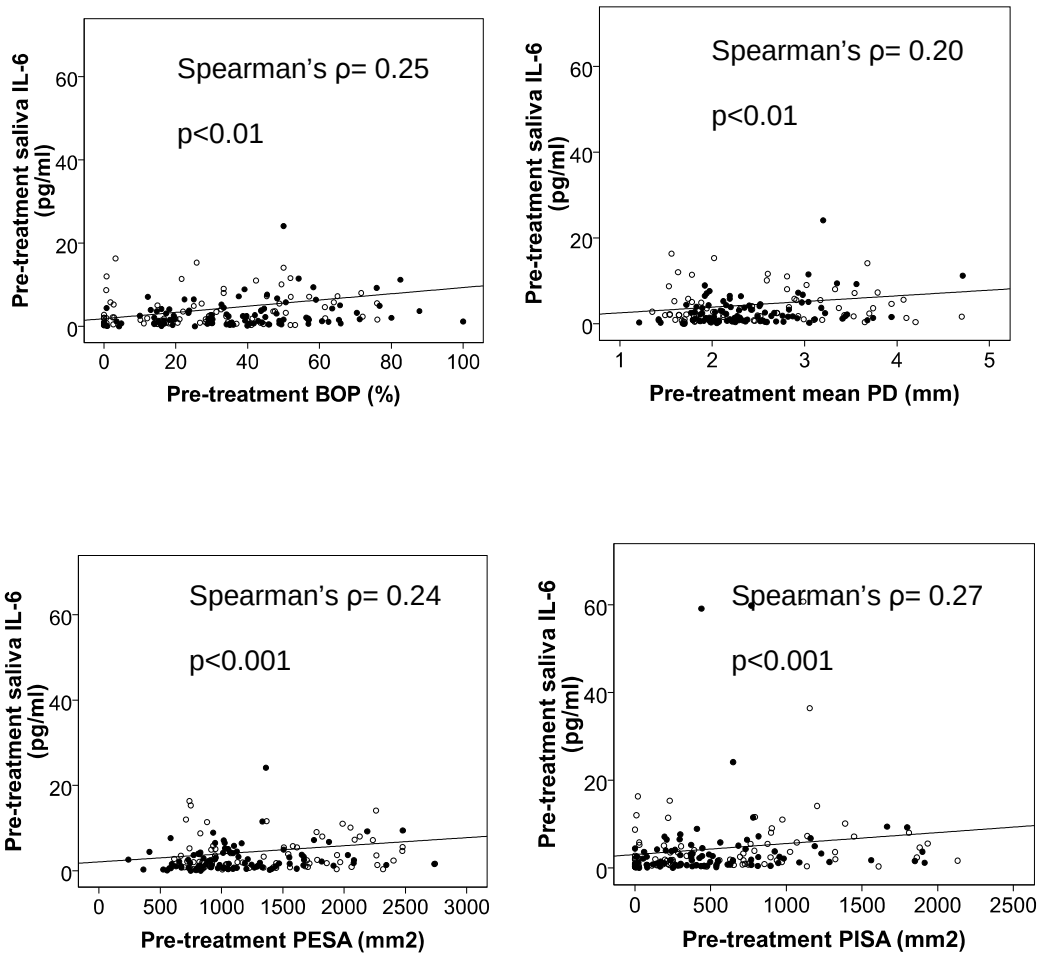


Figure shows Spearman correlation of saliva IL-6 concentrations with BOP (%), mean PD (mm), PESA (mm2) and PISA (mm^2) in all subjects ● T2DM subjects and ○ non-diabetic subjects (n=182). The addition of a trend-line demonstrates the presence of a significant correlation.

Figure 5.12 The relationship of saliva TNF-α with clinical periodontal parameters in all subjects

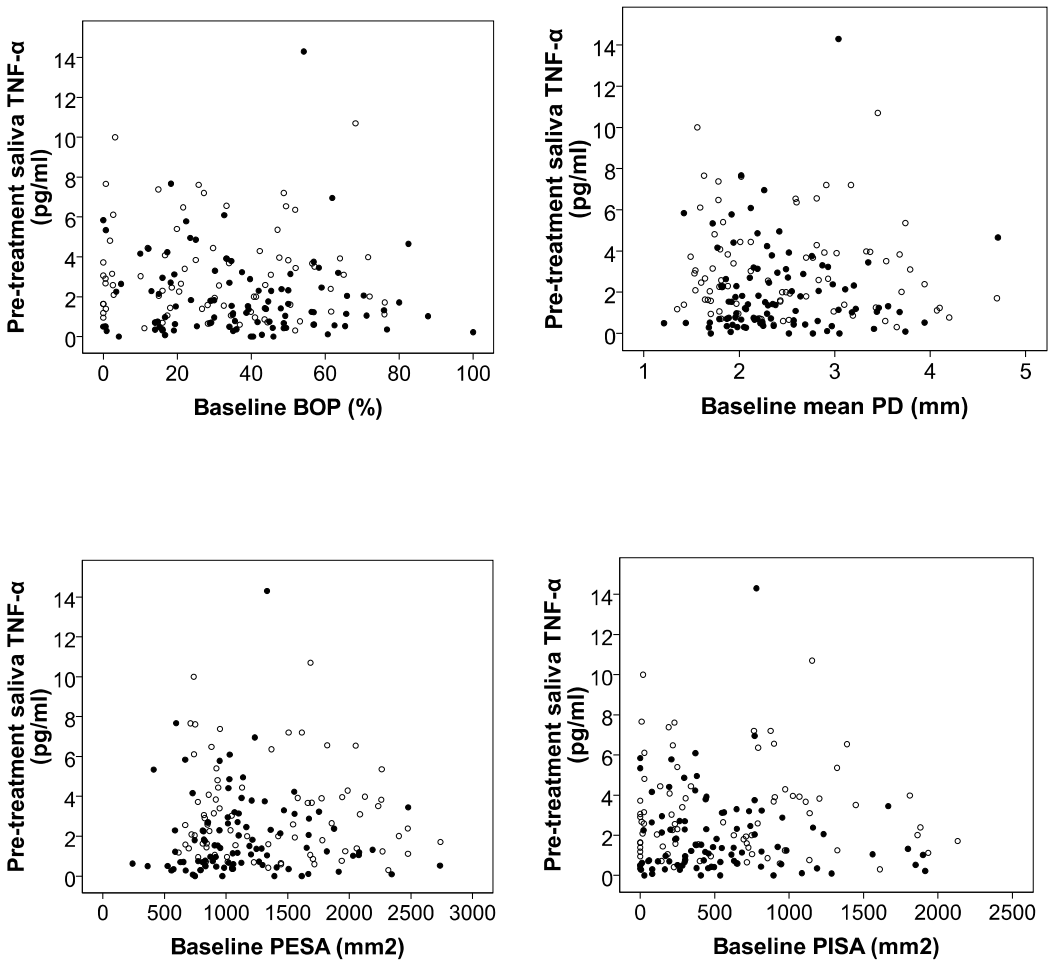


Figure shows Spearman correlation of saliva TNF-α concentrations with BOP (%), mean PD (mm), PESA (mm2) and PISA (mm^2) in all subjects ● T2DM subjects and ○ non-diabetic subjects (n=182). The addition of a trend-line demonstrates the presence of a significant correlation.

Figure 5.13 The relationship of saliva IL-1β with clinical periodontal parameters in all subjects

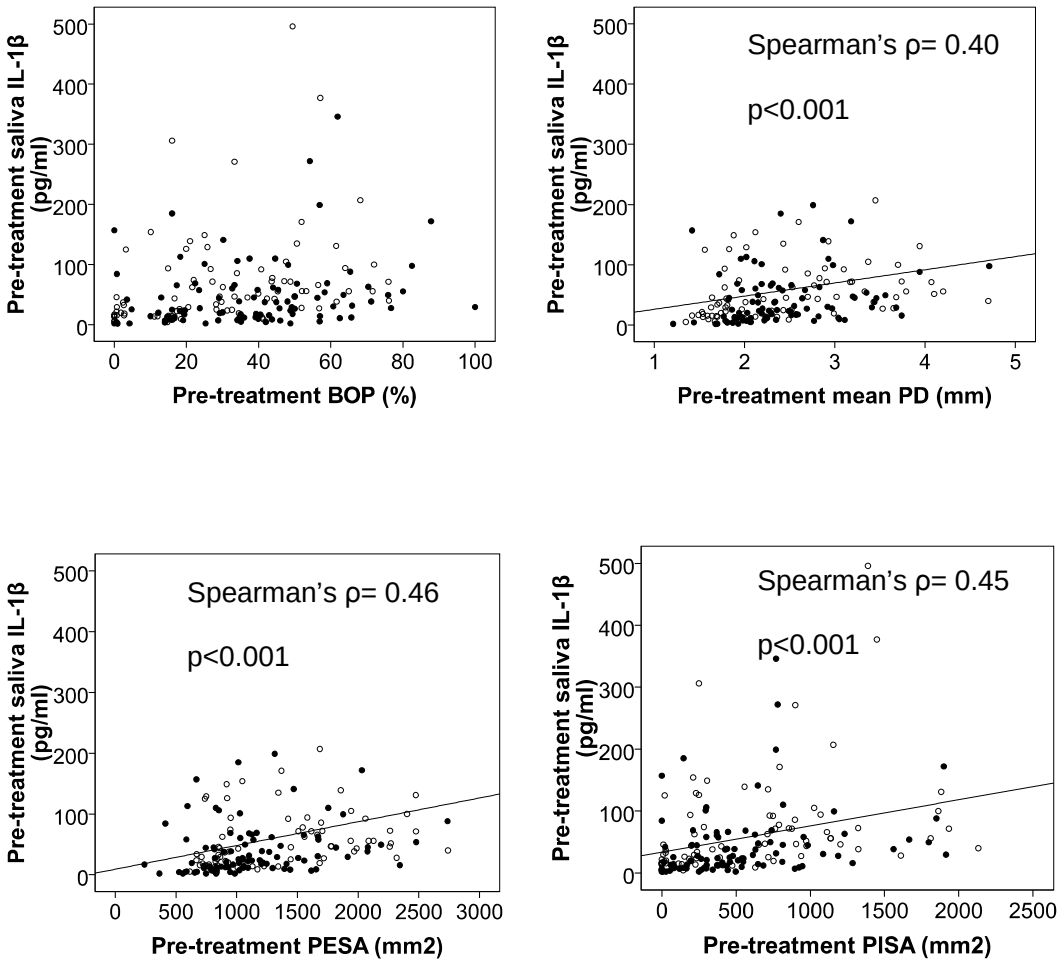


Figure shows Spearman correlation of saliva IL-1β concentrations with BOP (%), mean PD (mm), PESA (mm2) and PISA (mm^2) in all subjects ● T2DM subjects and ○ non-diabetic subjects (n=182). The addition of a trend-line demonstrates the presence of a significant correlation.

Figure 5.14 The relationship of saliva INF-γ with clinical periodontal parameters in all subjects

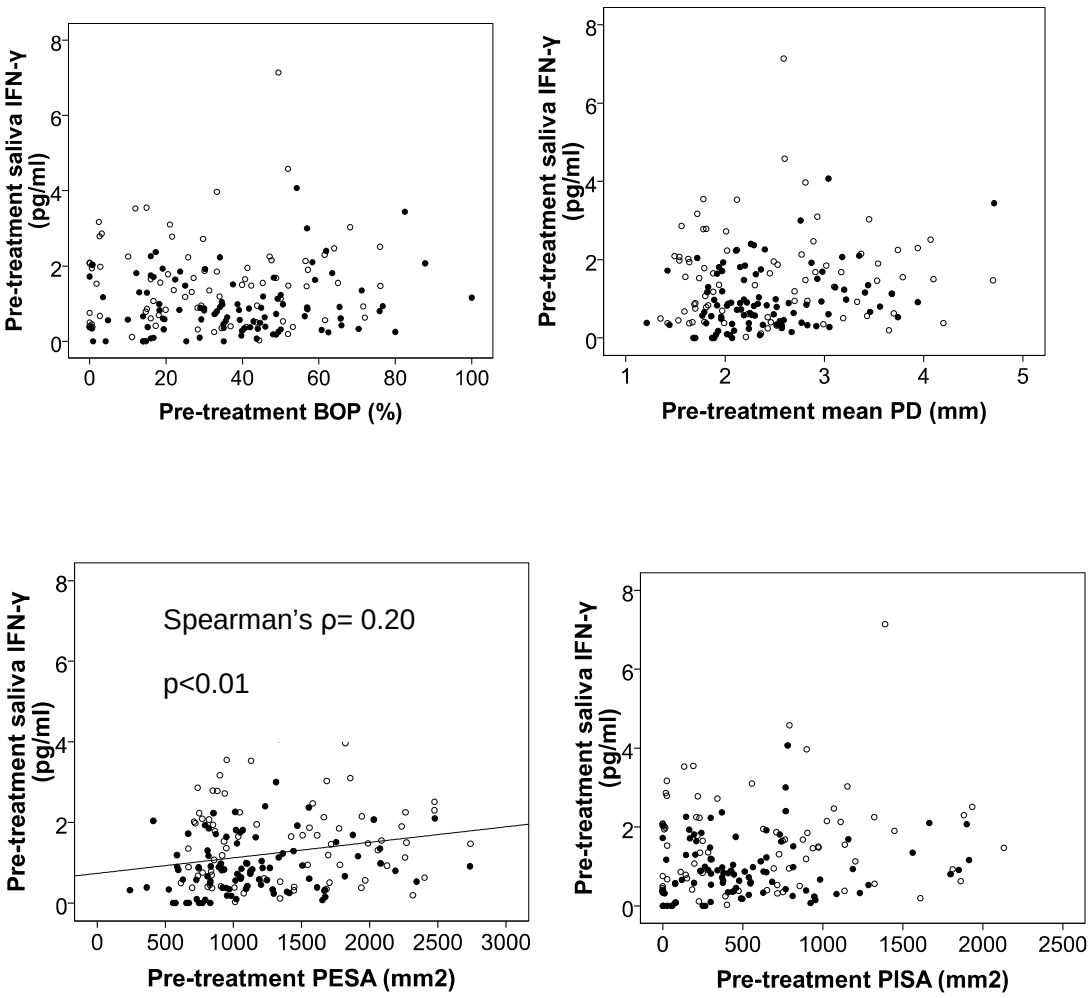


Figure shows Spearman correlation of saliva IFN-γ concentrations with BOP (%), mean PD (mm), PESA (mm2) and PISA (mm^2) in all subjects ● T2DM subjects and ○ non-diabetic subjects (n=182). The addition of a trend-line demonstrates the presence of a significant correlation.

Figure 5.15 The relationship of GCF IL-6 with clinical periodontal parameters in all subjects

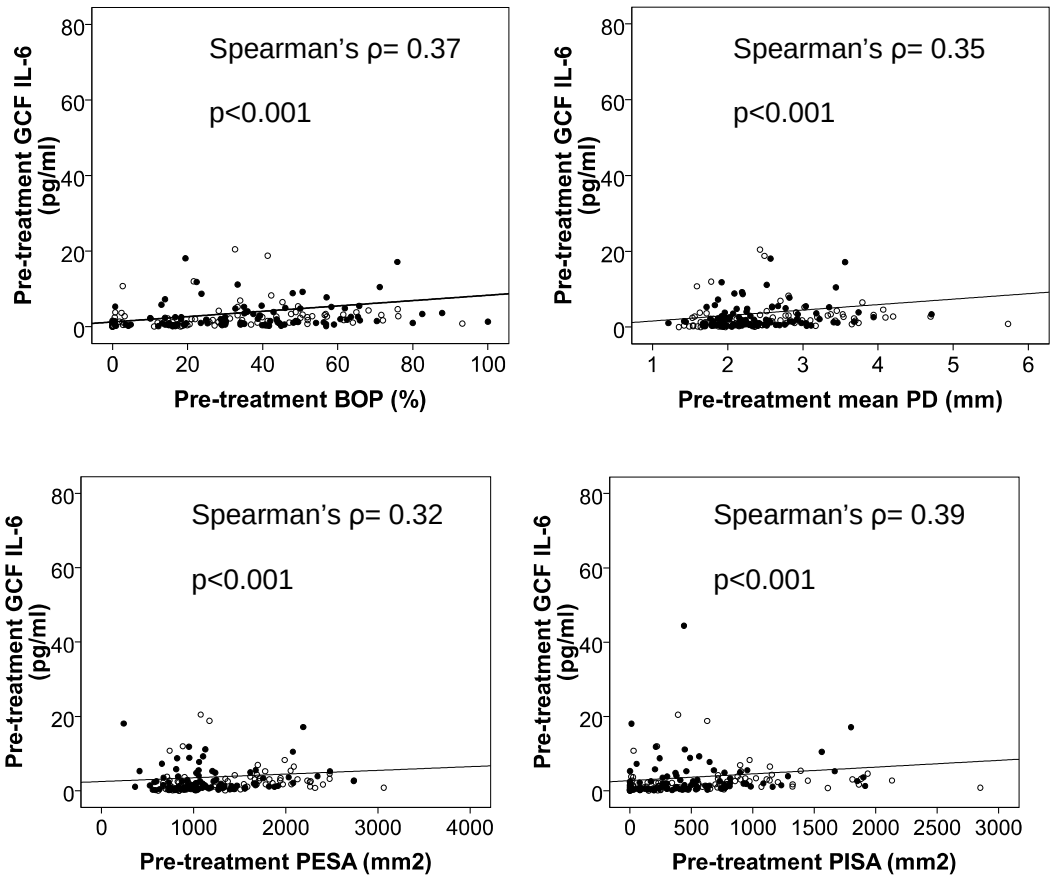


Figure shows Spearman correlation of GCF IL-6 concentrations with BOP (%), mean PD (mm), PESA (mm2) and PISA (mm^2) in all subjects ● T2DM subjects and ○ non-diabetic subjects (n=182). The addition of a trend-line demonstrates the presence of a significant correlation.

Figure 5.16 The relationship of GCF TNF-α with clinical periodontal parameters in all subjects

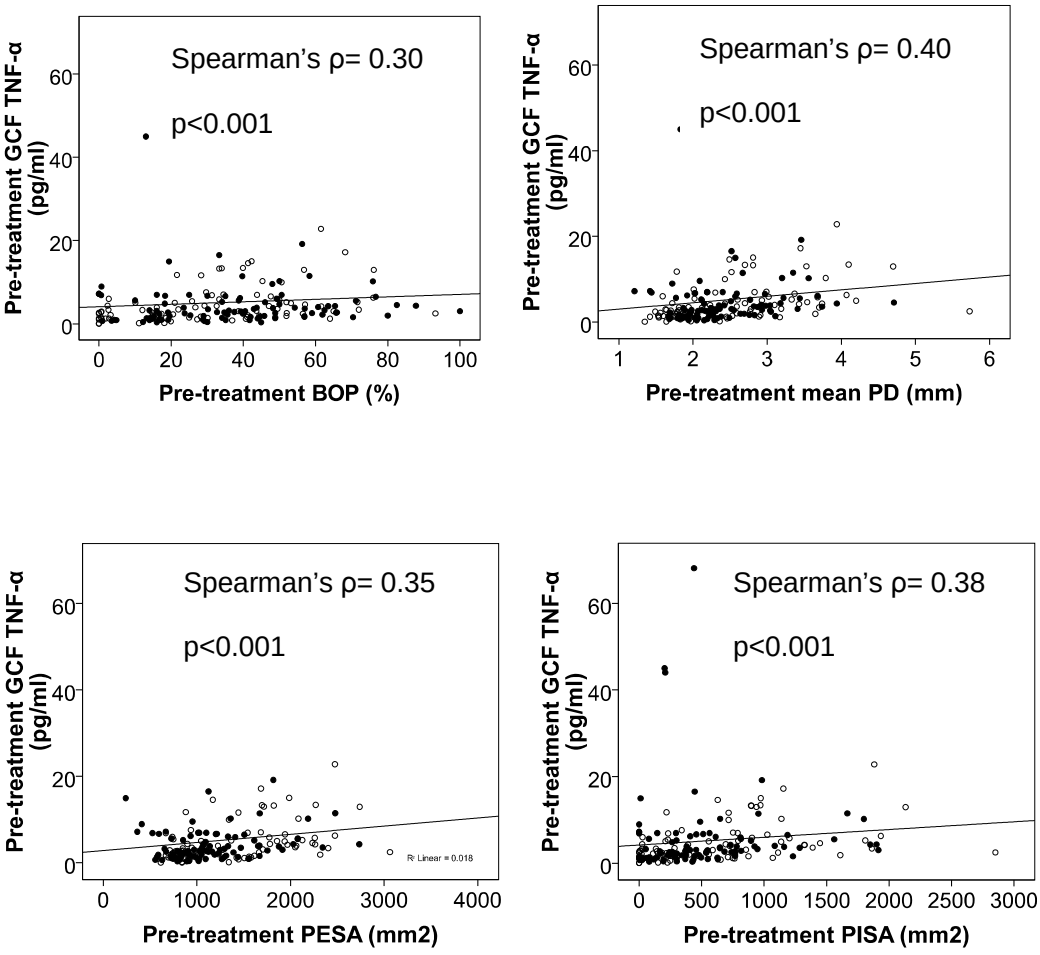


Figure shows Spearman correlation of GCF TNF-α concentrations with BOP (%), mean PD (mm), PESA (mm2) and PISA (mm^2) in all subjects ● T2DM subjects and ○ non-diabetic subjects (n=182). The addition of a trend-line demonstrates the presence of a significant correlation.

Figure 5.17 The relationship of GCF IL-1β with clinical periodontal parameters in all subjects

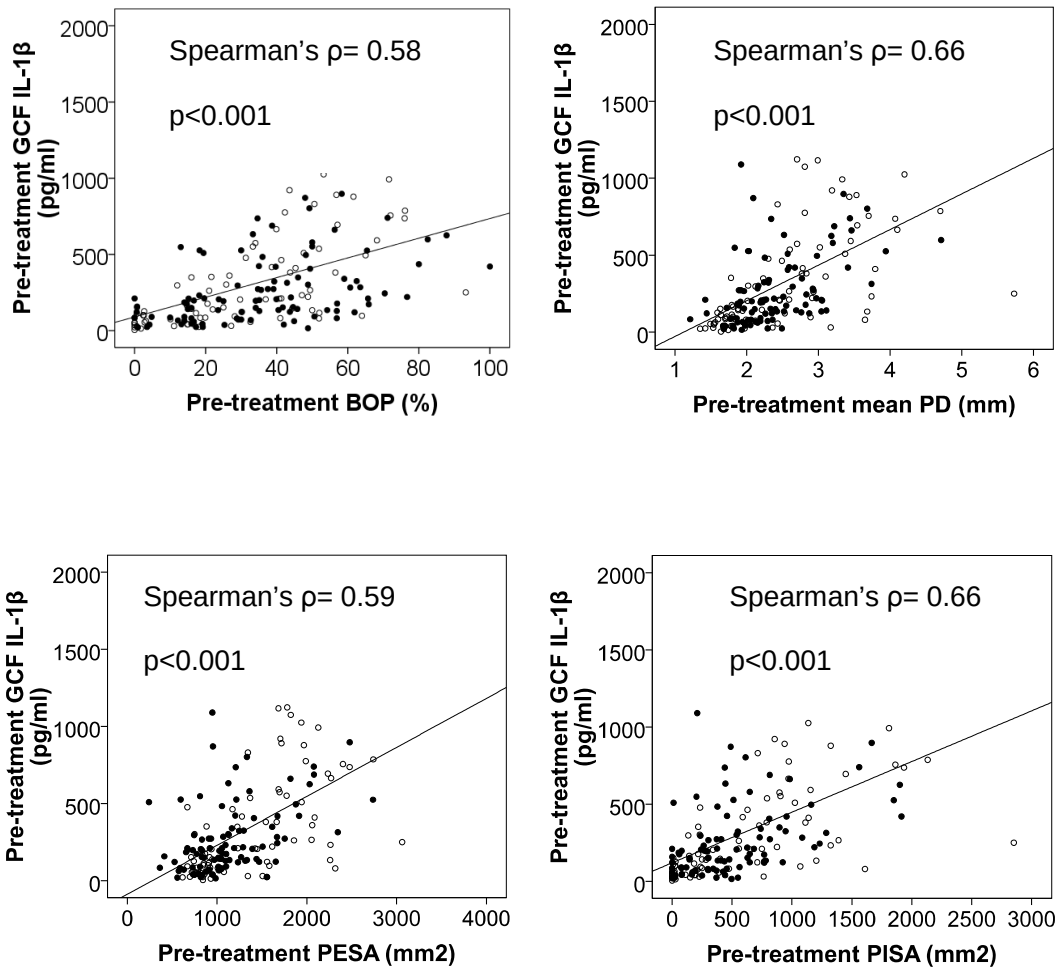


Figure shows Spearman correlation of GCF IL-1β concentrations with BOP (%), mean PD (mm), PESA (mm2) and PISA (mm^2) in all subjects ● T2DM subjects and ○ non-diabetic subjects (n=182). The addition of a trend-line demonstrates the presence of a significant correlation.

Figure 5.18 The relationship of GCF IFN-γ with clinical periodontal parameters in all subjects

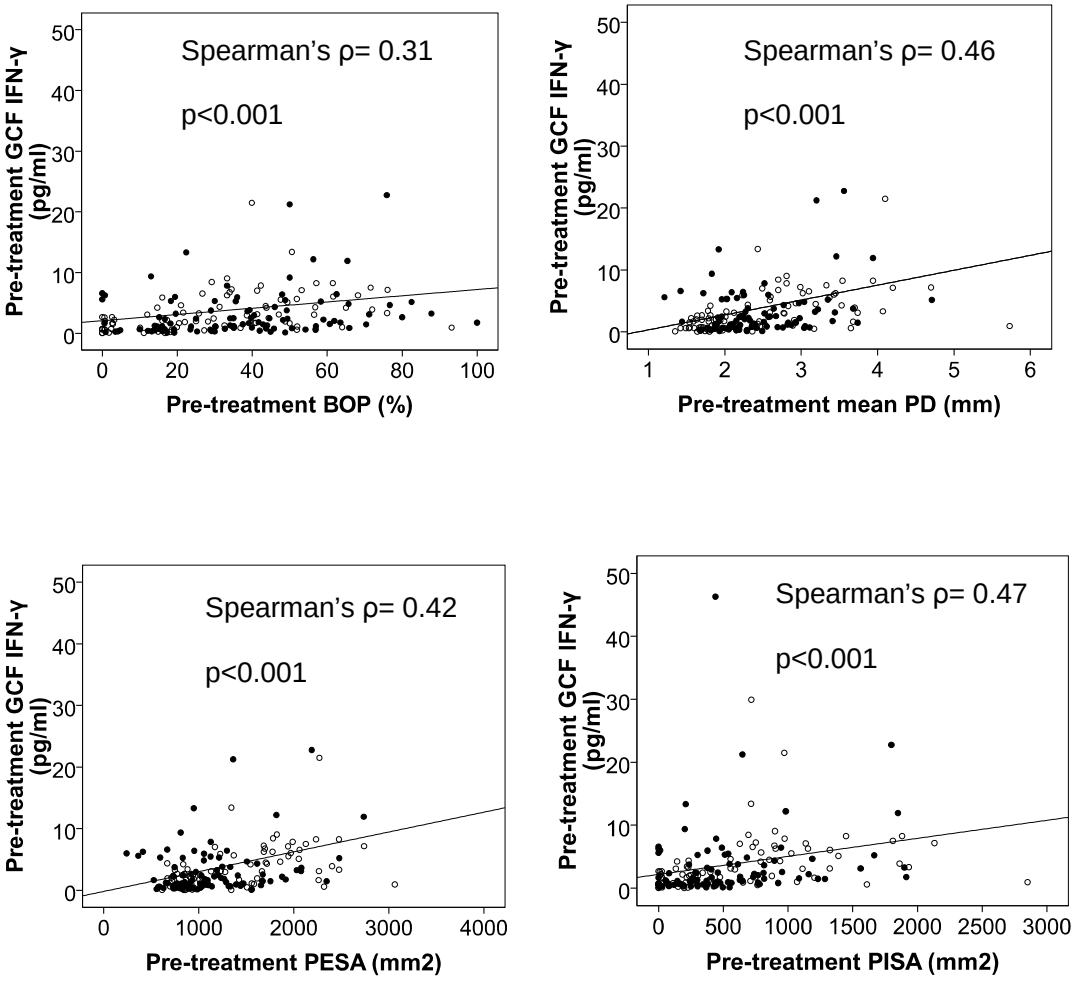


Figure shows Spearman correlation of GCF IFN-γ concentrations with BOP (%), mean PD (MM), PESA (mm2) and PISA (mm^2) in all subjects ● T2DM subjects and ○ non-diabetic subjects (n=182). The addition of a trend-line demonstrates the presence of a significant correlation.

5.2.3 PISA as a predictor of periodontal status

The potential of PISA in predicating gingivitis or periodontitis was assessed in multinomial logistic regression models. Periodontal status (periodontal health, gingivitis or periodontitis) was assigned as the dependent variable, using periodontal health as the reference category. All tests were conducted on measurements taken before periodontal treatment and groups were not divided according to diabetic status. In a multinomial logistic regression model, PISA was identified as a predicator of the periodontal status with PISA being a significant predicator of gingivitis ($p < 0.01$) and periodontitis ($p < 0.01$).

5.2.4 Inflammatory cytokine levels in GCF as predictors of PISA

The potential of GCF IL-6, TNF-α, IL-1β and IFN-γ as predictors of PISA was assessed in linear regression models. PISA was assigned as the dependent variable and GCF IL-6, TNF-α, IL-1β and IFN-γ were assigned as the independent variables. All tests were conducted on measurements taken before periodontal treatment and groups were not divided according to diabetic status. In linear regression models, GCF IL-1β and IFN-γ concentrations were determined as significant predicators of PISA ($p < 0.001$). A trend for GCF TNF-α concentration as a predicator of PISA was noted ($p = 0.089$), however, GCF IL-6 concentration was not a predictor for PISA ($p = 0.125$).

5.2.5 Inflammatory cytokine levels in saliva as predictors of PISA

The potential of saliva IL-6 and IL-1β as predictors of PISA was assessed in linear regression models. PISA was assigned as the dependent variable and saliva IL-6 and IL-1β were assigned as the independent variables. All tests were conducted on measurements taken before periodontal treatment and groups were not divided according to diabetic status. No significant correlations had been demonstrated of saliva

TNF-α and IFN-γ with PISA, thus the potential of saliva TNF-α and IFN-γ as predictors of PISA was not assessed. In linear regression models, saliva IL-1β ($p < 0.001$) and IL-6 ($p < 0.05$) concentrations were determined as significant predicators of PISA.

5.3 Discussion

Cytokine levels in serum

Previous research has demonstrated an association between higher circulating levels of inflammatory mediators and the development of T2DM, with significantly higher levels of CRP, IL-6 and TNF-α found in subjects with T2DM compared to non-diabetic subjects (Pradhan *et al.*, 2001; Spranger *et al.*, 2003b; Bertoni *et al.*, 2010). The current study supports this, with levels of TNF-α, IL-1β and INF-γ in serum being significantly higher in subjects with T2DM [7.45 (5.07-9.23) pg/ml, 0.18 (0.01-0.37) pg/ml and 1.35 (0.66-2.50) pg/ml] compared to non-diabetic subjects [5.25 (3.15-7.69) pg/ml, 0.04 (0.00-0.10) pg/ml and 0.78 (0.43-1.88) pg/ml] ($p < 0.01$) (Table 5.1). Similarly, following further categorisation of the serum cytokine data based on periodontal status, serum TNF-α, IL-1β and IFN-γ levels were significantly higher in diabetic subjects with periodontitis [7.10 (3.25-9.30) pg/ml, 0.08 (0.00-0.34) pg/ml and 1.09 (0.56-2.50) pg/ml] compared to non-diabetic subjects with periodontitis [3.44 (2.34-7.24) pg/ml, 0.00 (0.00-0.48) pg/ml and 0.57 (0.26-1.33) pg/ml] ($p < 0.05$). Again, this corroborates previous research showing significantly higher circulating levels of inflammatory mediators, such as CRP, IL-6 and TNF-α in subjects with T2DM compared to non-diabetic controls (Pradhan *et al.*, 2001; Spranger *et al.*, 2003b; Bertoni *et al.*, 2010).

Currently, data on serum cytokine levels in subjects with T2DM and periodontitis are limited. One study showed no significant difference in pre-treatment serum TNF-α

levels between subjects with poorly controlled T2DM [12.44±5.27 pg/ml], well controlled T2DM [9.92±3.33 pg/ml] and non-diabetic subjects [10.42±4.80 pg/ml] (Dag *et al.*, 2009). Similarly, another study showed no significant difference in serum TNF-α or IL-6 levels between subjects with T2DM and non-diabetic subjects. Unfortunately, however, detailed evaluation of the data from this study is precluded due to a lack of numerical values within the publication, with the authors presenting serum cytokine levels in graphs alone (Kardesler *et al.*, 2010). Data from the present study, with levels of TNF-α, IL-1β and INF-γ in serum being significantly higher in subjects with T2DM [7.45 (5.07-9.23) pg/ml, 0.18 (0.01-0.37) pg/ml and 1.35 (0.66-2.50) pg/ml] compared to non-diabetic subjects [5.25 (3.15-7.69) pg/ml, 0.04 (0.00-0.10) pg/ml and 0.78 (0.43-1.88) pg/ml] ($p < 0.01$), do not support the data from these two previously published studies. Another previously published study presents plasma levels of IL-6, TNF-α, IL-1β and IFN-γ in subjects with T2DM and periodontitis [2.1±0.3 pg/ml, 0.3±0.3 pg/ml, 0.3±0.3 pg/ml and 2.8±1.3 pg/ml] (O'Connell *et al.*, 2008). However, a lack of a non-diabetic group prevents a comparison of levels in subjects with and without T2DM from being made. Furthermore, the use of plasma (not serum) prevents a direct comparison to data from the current study. Similarly, a further study, also presents plasma levels of IL-6 and TNF-α in subjects with T2DM and periodontitis [3.1 (2.1-4.2) pg/ml and 5.6 (4.3-7.4) pg/ml], although again a lack of a non-diabetic group prevents a comparison of levels in subjects with and without T2DM from being made and again the use of plasma (not serum) prevents a direct comparison to data from the current study (Correa *et al.*, 2010). Taken collectively, data from the current study along with previously published data suggest that subjects with T2DM have higher circulating levels of some inflammatory cytokines. A further study, within the general medical literature, evaluated hsCRP levels and serum levels of IL-1β, IL-6 and TNF-α in 89 patients with

T2DM (periodontal status unknown) finding low levels of IL-1β [0.5 (0.3-0.8) pg/ml] and IL-6 [7.0 (3.5-12.1) pg/ml] and undetectable levels of TNF-α in serum (Castoldi *et al.*, 2007).

Published data demonstrated that in non-diabetic subjects, serum IL-6 levels were significantly higher in the periodontitis group compared to the control group without periodontitis, although the authors only present this difference graphically and do not report the numerical values (Marcaccini et al., 2009). In another study, the level of plasma IL-6 in patients with aggressive periodontitis (1.20 pg/ml) was significantly higher than that in subjects without periodontitis (0.08 pg/ml) (Sun *et al.*, 2009) . In a further small study, serum levels of IL-1β were evaluated, but in many of the subjects with periodontitis and the majority of subjects without periodontitis, no IL-1β was measureable in serum (Mengel *et al.*, 2002). Interestingly, in this current study it would appear the reverse is true, with serum TNF-α levels, IL-1β and IFN-γ in non-diabetic subjects being significantly lower in those with periodontitis [3.44 (2.34-7.24) pg/ml, 0.00 (0.00-0.48) pg/ml and 0.57 (0.26-1.33) pg/ml] compared to those with healthy periodontal tissues [6.28 (4.4-8.81) pg/ml, 0.09 (0.06-0.14) pg/ml and 1.78 (0.67-3.64) pg/ml] and no significant difference in serum IL-6 levels in non-diabetic subjects with periodontitis [0.59 (0.32-0.93) pg/ml] compared to non-diabetic subjects with healthy periodontal tissues [0.69 (0.43-1.11) pg/ml]. Interestingly, in the current study, differences in serum cytokine levels between subjects with and without periodontitis were not seen in subjects with T2DM. For example, in subjects with T2DM, serum IL-6, TNF-α levels, IL-1β and IFN-γ were not significantly different in subjects with periodontitis [0.51 (0.34-1.50) pg/ml, 7.10 (3.25-9.300 pg/ml, 0.08 (0.00-0.34) pg/ml and 1.09 (0.56-2.50) pg/ml] compared to those with healthy periodontal tissues [0.50 (0.33-0.81) pg/ml, 8.02 (6.34-9.24) pg/ml, 0.28 (0.09-0.40) pg/ml and 1.19 (0.71-1.62)

pg/ml]. Published studies, investigating the circulating levels of inflammatory cytokines in subjects with T2DM have not included subjects without periodontitis (O'Connell *et al.*, 2008; Dag *et al.*, 2009; Correa *et al.*, 2010; Kardesler *et al.*, 2010), therefore, no comparison can be made with the data from the current study which assessed serum cytokine levels in diabetic subjects both with and without periodontitis. Overall it would however appear that data from the current study doesn't support the hypothesis that subjects with periodontitis have higher levels of systemic inflammation compared to subjects without periodontitis. The variations seen in the presented serum cytokine levels and the lack of agreement between the data from the current study and published research could be due to heterogeneity of methodology. Differences in analytical techniques, storage of samples and case definition of periodontitis may all impact on the data that are presented. Additionally, the low levels of cytokines present in serum which are around the lower limit of detection for assays used, lead to larger intra- and inter-study variations. Large variations, coupled with small sample sizes have the potential to produce a greater number of chance findings. Therefore, to clarify the role of circulating inflammatory cytokines in the relationship between T2DM and periodontitis, further, well designed, studies of adequate power are required.

Cytokine levels in Saliva

Recently, a small number of studies have investigated inflammatory cytokine levels in saliva in subjects with and without periodontitis (Miller *et al.*, 2006; Gursoy *et al.*, 2009; Teles *et al.*, 2009; Costa *et al.*, 2010). One previous study demonstrated significantly higher levels of IL-1β in saliva from subjects with periodontitis [665.7±267.5 pg/ml] compared to those without periodontitis [467.8±279.8 pg/ml] (Gursoy *et al.*, 2009). Another study also found significantly higher levels of IL-1β in saliva from subjects with periodontitis [753.7±1022.4 pg/ml] compared to those without

periodontitis [212.8±167.4 pg/ml] (Miller *et al.*, 2006). This increase in IL-1β levels in saliva with the development of periodontitis is supported by data from the current study, which show in non-diabetic subjects, levels of IL-1β in saliva were significantly higher in those with periodontitis [62.60 (39.20-97.10) pg/ml] compared to those with healthy periodontal tissues [17.60 (14.20-31.95) pg/ml] ($p < 0.01$) and similarly in subjects with T2DM, levels of IL-1β in saliva were significantly higher in those with periodontitis [38.65 (20.45-68.28) pg/ml] compared to those with healthy periodontal tissues [14.20 (3.26-43.50) pg/ml] ($p < 0.05$) (Table 5.4). Previous studies have also demonstrated elevated levels in saliva of IL-6 in subjects with periodontitis compared to those without periodontitis (Costa et al., 2010), although numerical values were not published, with differences presented graphically. A different study however, found no significant differences in IL-6 and TNF-α levels in saliva in subjects with periodontitis [3.6±5.9 pg/ml and 2.9±4.0 pg/ml] compared to control subjects without periodontitis [3.1±3.6 pg/ml and 2.7±2.8 pg/ml] (Gursoy *et al.*, 2009). In the current study subjects with T2DM and periodontitis have significantly higher levels of IL-6 in saliva [2.29 (1.27-4.83) pg/ml] compared to those with healthy periodontal tissues [0.80 (0.25-3.25) pg/ml] ($p < 0.05$), although the pattern was not replicated in non-diabetic subjects, with no significant differences found in saliva IL-6 levels between those with healthy periodontal tissues [2.13 (1.27-4.83) pg/ml] and periodontitis [2.41 (0.95-7.26) pg/ml]. In a further published study, a number of inflammatory cytokines in saliva were quantified using a multiplex bead immunoassay, demonstrating no significant differences between periodontitis and periodontally healthy groups with regards to levels of IL-6, TNF-α, IL-1β and IFN-γ (Teles et al., 2009). Unfortunately, however, detailed evaluation of the data from this study is precluded due to a lack of numerical

values within the publication, with the authors presenting saliva cytokine levels in graphs alone.

Currently, there is very little published research investigating the role of inflammatory cytokines in saliva in subjects with T2DM and periodontitis. One small study found no significant differences in IL-6 levels in saliva when subjects with T2DM and periodontitis (n=24) were compared to non-diabetic subjects with periodontitis (n=24) (Costa et al., 2010), although again numerical values were not presented. This lack of difference in IL-6 levels in saliva between subjects with and without T2DM was not supported in the current study. Comparing all non-diabetic subjects (n=101) with all subjects with T2DM (n=83), data from the current study showed the levels of salivary IL-6 were higher in the non-diabetic subjects [2.18 (0.89-5.72) pg/ml] compared to the subjects with T2DM [1.74 (0.72-4.18) pg/ml], although this difference was detected as a trend (p=0.065) (Table 5.3). Furthermore, in the current study, the levels of TNF-α, IL-1β and INF-γ in saliva were significantly higher in non-diabetic subjects [2.59 (1.40-3.98) pg/ml, 46.00 (22.00-90.43) pg/ml and 1.48 (0.64-2.12) pg/ml] compared to subjects with T2DM [1.38 (0.53-2.70) pg/ml, 26.95 (12.08-59.90) pg/ml and 0.81 (0.35-1.34) pg/ml] (Table 5.3). One explanation for the higher levels of salivary cytokine in non-diabetic subjects compared to subjects with T2DM seen in the current study is the greater severity and extent of periodontitis seen in non-diabetic subjects compare to subjects with T2DM, indicated by significantly greater PESA and % of sites with PD $\geq$ 6 mm [1372.7 (849.2-1801.0) mm^2 and 0.7(0.0-11.3)%] compared to subjects with T2DM [1038.9(817.9-1327.6) mm^2 and 0.0(0.0-0.2)%] (Table 4.12). This clinical difference between subjects with and without diabetes most probably reflect the differences in the recruitment pools used for diabetic and non-diabetic subjects in the current study and although, diabetics and non-diabetic subjects were matched based on

their periodontal diagnosis, the extent of periodontal disease was not however considered in this process.

Following further categorisation of subjects based on their periodontal diagnosis, the current study demonstrated that TNF-α, IL-1β and IFN-γ in saliva, were significantly higher in non-diabetic subjects with periodontitis [2.59 (1.18-3.97) pg/ml, 62.60 (39.20-97.10) pg/ml and 1.55 (0.86-2.24) pg/ml] compared to subjects with T2DM and periodontitis [1.58 (0.61-3.08) pg/ml, 38.65 (20.45-68.28) pg/ml and 0.86(0.47-1.60) pg/ml] ($p < 0.05$) (Table 5.4). Similarly, TNF-α, IL-1β and IFN-γ in saliva, were significantly higher in non-diabetic subjects with gingivitis [2.30 (1.55-4.08) pg/ml, 43.00 (24.70-93.70) pg/ml and 1.07 (0.53-1.65) pg/ml] compared to subjects with T2DM and gingivitis [1.17 (0.52-2.04) pg/ml, 16.70 (7.99-49.60) pg/ml and 0.64 (0.34-0.98) pg/ml] ($p < 0.05$) (Table 5.4). For subjects with periodontitis, a possible explanation for this is that the non-diabetic group had a significantly greater PESA and % of sites with PD ≥6mm [1744.9± and 8.7(2.6-16.7)] and compared to subjects with T2DM [1444.7±495.1 and 2.7(0.7-7.1)]. This indicates that more severe periodontal disease was present in non-diabetic subjects compared to subjects with T2DM and most likely reflects the differences in the recruitment pools used for diabetic and non-diabetic subjects in the current study and a lack of stratified periodontal case selection based on extent and severity of disease when matching T2DM and non-diabetic subjects (Table 4.13 and Figures 4.13 & 4.14). However, no such differences in these clinical PD parameters were demonstrated between the T2DM and non-diabetic groups with gingivitis (Table 4.13 and Figures 4.13 & 4.14). Additionally, the higher levels of salivary cytokines seen in non-diabetic subjects with gingivitis compared to subjects with T2DM with gingivitis do not appear to reflect the levels of local inflammation, as assessed by mGI and % BOP, which show significantly higher levels

in T2DM with gingivitis [1.9 (1.3-2.5) and 35.1 (25.0-44.9)] compared to non-diabetic subjects with gingivitis [1.3 (0.8-1.7) and 22.0 (17.3-32.6)] (Table 4.13 and Figures 4.8 and 4.9).

Overall, data from the current study and published research suggest that salivary levels of IL-1β and possibly IL-6 increase with the development of periodontitis. This would suggest that select salivary biomarkers may reflect periodontal status. However, this pattern is not universally demonstrated and a lack of clarity remains regarding the effect of periodontitis on levels of other inflammatory cytokines in saliva, with further studies required to clarify this.

There are numerous possible explanations for the variations seen in the salivary cytokine levels within currently available data. It is wise to remember that the periodontium will not be the sole source of inflammatory cytokines found in saliva, with the salivary glands and oral mucosa possible contributors to the levels of cytokines found in saliva. Heterogeneity of methodology between studies is another possible explanation for variations in cytokine levels, with differences in analytical techniques, sampling techniques, storage of samples and case definition of periodontitis all potentially impacting on the data that are presented. Additionally, some inflammatory cytokines were present in saliva at low levels which are around the lower limit of detection for assays used and thus leading to larger intra- and inter- study variations. This coupled with the small sample sizes, used in previous studies, have the potential to produce a greater number of chance findings. Therefore, to clarify the role of circulating inflammatory cytokines in the relationship between T2DM and periodontitis, further, well designed, studies of adequate power are required. One specific limitation in the current study is the technique used to sample saliva. This involved an oral rinse using 10ml of saline and this consequently diluted the saliva sample. This dilution has the

potential to take already low levels of inflammatory cytokine nearer to or below the lower level of detection for the assay used and thus increasing the variability in the data. Additionally, because the actual volume of whole saliva in each sample is not known, it is not possible to clarify the levels of dilution each sample underwent, causing greater and unpredictable variation in salivary cytokine data.

Cytokine levels in GCF

The current study showed no significant differences in GCF levels of the IL-6, TNF-α, IL-1β and IFN-γ, between T2DM subjects [1.50 (0.65-3.88 pg/ml, 3.03 (1.66-6.04) pg/ml, 202.80 (90.76-420.09) pg/ml and 1.66 (0.71-4.58) pg/ml] and non-diabetic subjects [1.74 (0.76-3.08 pg/ml, 3.09 (1.66-5.88) pg/ml, 205.25 (83.78-537.00) pg/ml and 2.62(1.01-5.66) pg/ml]. Furthermore, following categorisation of subjects based on periodontal status, the apparent higher levels of in GCF levels of IL-6, TNF-α and IL-1β in non-diabetic subjects with periodontitis [2.25 (1.12-3.37) pg/ml, 4.49 (2.55-7.04) pg/ml and 413.38 (213.84-770.56) pg/ml] compared to subjects with T2DM and periodontitis [1.97 (0.98-5.17) pg/ml, 4.16 (2.7-6.69) pg/ml and 344.33 (156.16-572.50) pg/ml] did not reach significance. Only, GCF IFN-γ levels were significantly higher in non-diabetic subjects with periodontitis [4.40 (2.18-7.09) pg/ml] compared to T2DM subjects with periodontitis [2.51 (1.11-5.24) pg/ml] ($p < 0.05$). The higher levels, albeit non-significant, of GCF cytokines in non-diabetic subjects with periodontitis compared to subjects with T2DM and periodontitis, may well be a reflection of the more severe periodontal disease that was present in non-diabetic subjects compared to subjects with T2DM (Table 4.13). However, the large variations in GCF cytokine levels seen in the present study provide a possible reason for the lack of significant differences in GCF cytokine levels between diabetic and non-diabetic subjects with periodontitis. An additional reason for a lack of difference may be due to a higher levels of background

inflammation present in the periodontium of subjects with T2DM, as possibly indicated by the higher, albeit non significant, levels of IL-6, TNF-α, IL-1β and IFN-γ in GCF in subjects with T2DM and gingivitis [1.44 (0.53-3.61) pg/ml, 2.28 (1.30-3.06) pg/ml, 173.41 (77.60) pg/ml and 2.17 (0.65-2.50) pg/ml] compared to non-diabetic subjects with gingivitis [0.90 (0.39-2.12) pg/ml, 2.23 (1.66-4.30) pg/ml, 116.25 (56.50-176.53) pg/ml and 1.85 (1.01-2.44) pg/ml] (Table 5.6).

To date, there are a limited number of published studies that investigated GCF cytokine levels in subjects with T2DM and periodontitis compared to non-diabetic subjects with periodontitis. One study demonstrated higher levels of GCF IL-6 in T2DM subjects with periodontitis compared to non-diabetic subjects with periodontitis (Kardesler et al., 2011). Unfortunately, however, detailed evaluation of the data from this study is precluded due to a lack of numerical values within the publication, with the authors presenting serum cytokine levels in graphs alone. This is not replicated in the current study, which found no significant difference in GCF levels of IL-6 in subjects with T2DM and periodontitis [1.97 (0.98-5.17) pg/ml] compared to non-diabetic subjects with periodontitis [2.25 (1.12-3.37) pg/ml]. Another published study showed no differences in pre-treatment GCF IL-1β levels between T2DM subjects with periodontitis [140.9 (106.0-177.7) pg/ml] and non-diabetic subjects with periodontitis [159.6 (145.71-197.1) pg/ml] (Correa et al., 2008), supporting data in the current study which found no significant difference in the pre-treatment GCF levels of IL-1β in subjects with T2DM and periodontitis [344.33 (156.16-572.50) pg/ml] compared to non-diabetic subjects with periodontitis [413.38 (213.84-770.56) pg/ml].

There are additional studies that have investigated GCF cytokine levels in subjects with well and poorly controlled T2DM; however a lack of a non-diabetic group for comparison is a limitation to these studies (Engebretson *et al.*, 2007; Santos *et al.*,

2010). One study showed higher GCF IL-1β levels in subjects with poorly controlled T2DM [89.0 (61.7-116.3) pg/ml] compared to those with well controlled T2DM [49.1 (18.3-80) pg/ml] (Engebretson *et al.*, 2004). Another study demonstrated higher levels of GCF IFN-γ in well controlled compared to poorly controlled T2DM subjects, but no differences in GCF TNF-α in well controlled compared to poorly controlled T2DM subjects (Santos *et al.*, 2010), although numerical values were not published and the data were presented graphically. In the current study, T2DM subjects were not sub-divided based on level of control thus preventing direct comparison to these previous studies (Engebretson *et al.*, 2004; Santos *et al.*, 2010).

Heterogeneity of methodology between studies is a possible explanation for the variations in GCF cytokine levels seen both within the published literature and also compared to the current study. Differences in analytical techniques, GCF sampling techniques, methods for elution of GCF cytokines from Periopapers, storage of samples and case definition of periodontitis could all potentially impact on the data that is presented. Specifically, when considering the method used for eluting the cytokines in GCF from the Periopapers, a wide variety of protocols are used within studies. In one study, for example, 5 or 6 GCF Periopaper samples were pooled into 1 tube containing 1ml of PBS, followed by 40 minutes of standing and then centrifugation at 3000 g for 10 minutes before storing within a freezer at -70^{o}C (Correa *et al.*, 2008). Another study placed each GCF Periopaper sample into a tube containing 250 µl of PBS containing protease inhibitor, followed by 'vortexing' for 30 seconds and then centrifugation for 5 minutes before storing within a freezer at -20^{o}C (Santos *et al.*, 2010). By comparison, a different study placed each GCF Periopaper sample into 500 µl of PBS followed by shaking for 45 minutes before storing within a freezer at ¯40^{o}C (Kardesler *et al.*, 2011). As demonstrated in the verification studies conducted within the current study (Table

3.2), both the methodology for elution and the volumes of solution into which the GCF cytokines are eluted has an impact on the recoverability of cytokines from Periopapers (Table 3.2). Consequently, a lack of consistency within the literature for the elution of GCF cytokines from Periopapers will increase inter-study variations in GCF cytokine levels and thus prevent clear conclusions regarding the role of GCF cytokines in subjects with T2DM and periodontitis from being made.

Currently, there are no published data comparing GCF cytokine levels in T2DM subjects with and without periodontitis. However, previously research has investigated GCF levels of key inflammatory mediators in periodontitis in systemically healthy subjects, showing increased levels of IL-1β in GCF from subjects with periodontitis (Preiss and Meyle, 1994; Figueredo *et al.*, 1999; Engebretson *et al.*, 2002; Zhong *et al.*, 2007) and elevated IL-6 levels in GCF from diseased sites compared with control samples (Mogi *et al.*, 1999; Lin *et al.*, 2005). Data from another study, in subjects without systemic conditions such as T2DM, demonstrated that subjects with periodontitis had significantly higher levels of GCF IL-1β compared to periodontally healthy subjects (Teles *et al.*, 2010). Furthermore, data from a further small study using a site based analysis of multiple cytokines, demonstrated that only IL-1β and IL-1α differed when healthy and diseased sites were compared, with higher levels for both mediators in diseased sites (Thunell *et al.*, 2010). The current study supports previous data, with elevated levels of pro-inflammatory cytokines in GCF in subjects with periodontitis compared to subjects with healthy periodontal tissues. For example, in non-diabetic subjects, levels of IL-6, TNF-α, IL-1β and IFN-γ in GCF were significantly higher in those with periodontitis [2.25 (1.12-3.37) pg/ml, 4.49 (2.55-7.04) pg/ml, 413.38 (213.84-770.56) pg/ml and 4.40 (2.18-7.09) pg/ml] compared to those with healthy periodontal tissues [1.00 (0.26-2.69) pg/ml, 1.83 (1.23-2.90) pg/ml, 54.26

(25.79-100.65) pg/ml and 0.72 (0.58-2.16) pg/ml]. In subjects with T2DM, the pattern is still present but less clear, with significantly higher levels of IL-1β in GCF being significantly higher in those with periodontitis [344.33 (156.16-572.50) pg/ml] compared to those with healthy periodontal tissue [84.95 (40.66-130.61) pg/ml]. In subjects with T2DM, the higher levels of IL-6, TNF-α and IFN-γ in GCF in those with periodontitis [1.97 (0.98-5.17) pg/ml, 4.16 (2.7-6.69) pg/ml and 2.51 (1.11-5.24) pg/ml] compared to those with healthy periodontal tissues [0.99 (0.48-2.25) pg/ml, 1.32 (0.05-7.20) pg/ml] and 0.53 (0.34-5.76) pg/ml] were detected as trends (Table 5.6 and Figures 5.3 to 5.6).

Furthermore, data from the current study demonstrates that GCF IL-1β is a promising indicator of periodontal disease, reflecting not only difference between subjects with healthy periodontal tissues and periodontitis, but also differences between subjects with healthy periodontal tissues and gingivitis and likewise between subjects with gingivitis and periodontitis. This corroborates data from previous studies which demonstrates elevated GCF IL-1β and IL-6 from diseased periodontal sites compared to healthy sites (Mogi *et al.*, 1999; Lin *et al.*, 2005; Thunell *et al.*, 2010). However, these studies evaluating GCF cytokine levels in periodontitis, all presented data in a site specific manner. Interestingly, one additional study shows clinically healthy sites from subjects with periodontitis had higher levels of IL-1β than clinically healthy sites from periodontally healthy subjects (Teles et al., 2010), highlighting that the case definition on a subject levels may affect local site specific cytokine levels. To date, there remains a lack of published data of GCF cytokine levels for subjects with or without periodontitis.

Relationship between HbA1c, hsCRP levels and periodontal parameters

In one previously published study, a dose-response relationship was shown between PISA and HbA1c in subjects with T2DM, indicating that PISA was a predictor significantly associated with HbA1c levels (Nesse *et al.*, 2009). However, a number of other studies, collecting full mouth periodontal data in subjects with T2DM and periodontitis and undertaking correlation tests, did not report significant correlations between HbA1c levels and clinical periodontal parameters (Engebretson *et al.*, 2007; Dag *et al.*, 2009; Costa *et al.*, 2010; Santos *et al.*, 2010). In line with this, the current study found no significant correlations between HbA1c and mGI, %BOP, mean PD and PISA. A significant negative correlation was demonstrated between HbA1c and PESA (Spearman's ρ=-0.17, $p<0.05$). However, given that the correlation was small and is not supported by other correlations, it is likely this was simply a chance finding. One potential explanation of the lack of correlation found within the current study and previous studies is that, in addition to the inflammatory burden from periodontal disease, many conditions potentially impact on glycaemic control, including obesity and duration of diabetes. Therefore, variations in HbA1c, unrelated to the periodontal condition, prevent the correlation tests in these relatively small studies from detecting possible associations between worsening glycaemic control and increased periodontal severity that have been previously demonstrated in epidemiological studies (Taylor, 2001).

Given that CRP has been shown to be elevated in subjects with T2DM (Bertoni et al., 2010) and in subjects with periodontitis (Paraskevas et al., 2008) and considering the importance of inflammation in the proposed mechanism linking T2DM and periodontitis, it is surprising that data evaluating hsCRP in patients with T2DM and periodontitis are currently very limited (Correa *et al.*, 2010; Kardesler *et al.*, 2010) .

Therefore, in subjects with T2DM, correlations between hsCRP levels and clinical periodontal parameters have yet to be explored within the published literature. In a recent study of systemically healthy patients with and without periodontitis, a significant positive correlation was demonstrated between log serum IL-6 levels and mean PD (r=0.39, p=0.000) and mean LOA (r=0.42, p=0.000) (Sun *et al.*, 2009). In the current study, significant positive correlations were demonstrated between hsCRP, mGI (Spearman's ρ =0.24, $p<0.05$) and mean PD (Spearman's ρ =0.16, $p<0.05$), when all subjects were considered (Table 5.7). Interestingly, when the data was split according to diabetes status, no significant correlations between hsCRP and the clinical periodontal parameters were found in subjects with T2DM (Table 5.8), whereas, in non-diabetic subjects, significant positive correlations were shown between hsCRP and mGI (Spearman's ρ= 0.23, $p<0.05$), BOP (Spearman's ρ= 0.26, $p<0.05$), mean PD (Spearman's ρ= 0.26, $p<0.01$) and PISA (Spearman's ρ= 0.27, $p<0.05$) (Tables 5.9). Overall, these data would suggest that increasing severity and extent of periodontitis are associated with increasing levels of systemic levels of inflammation, as indicated by hsCRP. One possible explanation for this is that as the inflammatory burden from the periodontium increases, so do systemic levels of inflammation. This is supported by previous estimates that the cumulative size of all periodontal lesions in patients with untreated severe periodontitis may amount to $20cm^2$ (Loos, 2005). A potential explanation for the lack of significant correlations in T2DM subjects is that perhaps the systemic condition of T2DM subjects, such as obesity, concurrent CVD or the routine use of statin medication, may mask the relationship between the periodontal condition and hs-CRP levels in subjects with T2DM. It must also be remembered that although the correlations demonstrated between hsCRP and clinical periodontal parameters were significant, they were small in magnitude.

Relationship between HbA1c, hsCRP and inflammatory cytokines

Currently, there are limited published data exploring the relationship between HbA1c and levels of inflammatory cytokines in GCF in subjects with T2DM. In one study evaluating GCF levels of TNF-α, INF-γ, IL-4, IL-17 and IL-23 in subjects with T2DM, a significant positive correlation was demonstrated between GCF TNF-α, IL-4 and IL-17 levels and HbA1c ($r=0.25$; $p<0.05$, $r=0.41$; $p<0.001$ and $r=0.50$; $p<0.001$ respectively) and significant negative correlations between GCF levels of IFN-γ and HbA1c ($r=-0.06$; $p<0.001$) (Santos *et al.*, 2010). In a further study of subjects with T2DM and periodontitis, HbA1c was significantly correlated with GCF IL-1β levels ($r=0.371$, $p=0.01$) (Engebretson et al., 2004). Furthermore, this study identified HbA1c as an independent predictor of high GCF IL-1β and demonstrated that patients with HbA1c > 8% had significantly higher mean GCF IL-1β levels than patients with HbA1c < 8% (Engebretson et al., 2004). In the current study, similar correlations between levels of inflammatory cytokines in GCF and HbA1c levels were not replicated, such that no significant correlations between levels of HbA1c and GCF levels of IL-6, TNF-α, IL-1β and IFN-γ were found, either when all subjects were considered or when subjects were split based on diabetes status (Table 5.7, 5.8 and 5.9). However, the significant correlations demonstrated in previous research between HbA1c and GCF cytokines are small in magnitude (Engebretson *et al.*, 2004; Santos *et al.*, 2010) and so it is not necessarily surprising that data from the current study failed to corroborate these findings. Other studies evaluating both GCF cytokine levels and HbA1c do not present correlation data and this could either due correlations not being undertaken or, due to publication bias, non-significant correlations were not reported within the publications (Correa *et al.*, 2008; Kardesler *et al.*, 2011).

Currently, there is one published study exploring the relationship between HbA1c and levels of inflammatory cytokines in saliva in subjects with T2DM, demonstrating a positive correlation between salivary IL-6 levels and HbA1c levels in subjects with T2DM ($r=0.60$; $p<0.01$) (Costa et al., 2010). This suggests that increasing levels of salivary IL-6 are associated with poorer glycaemic control, as indicated by increasing HbA1c levels. This was not, however, supported by data from the current study, which failed to demonstrate a correlation between levels of IL-6 in saliva and HbA1c. In fact, significant negative correlations between HbA1c levels and saliva levels of TNF-α ($r=-0.18$, $p<0.05$) and IFN-γ ($r=-0.15$, $p<0.05$) were demonstrated in the present study in all subjects. The current study did, however, demonstrate a significant positive correlation between HbA1c levels and saliva levels of IL-1β in subjects with T2DM ($r=0.27$, $p<0.05$) and IFN-γ ($r=0.20$, $p<0.010$). The inconsistency between previous research and the current study could be due to the variation in HbA1c levels and levels of cytokines in saliva coupled with the relatively small sample size used, both of which would increase the likelihood of chance findings.

Within the current literature, there is one published study exploring the correlations between circulating cytokine levels and HbA1c levels in subjects with T2DM and periodontitis, demonstrating there was no significant positive correlation found between plasma TNF-α levels and HbA1c ($r=0.10$, $p=0.50$) (Engebretson *et al.*, 2007). Within the general medical literature however, a previous study evaluating the relationship between circulating levels of IL-6 and HbA1c demonstrated that a significant positive correlation was demonstrated between serum Il-6 levels and HbA1c levels ($\beta=0.58$, $p=0.04$) (Kado *et al.*, 1999). This is supported by data from the current study that demonstrates significant positive correlations between HbA1c and serum

levels of IL-6 (Spearman's $\rho = 0.16$, $p<0.05$), TNF-α (Spearman's $\rho = 0.18$, $p<0.05$) and IL-1β (Spearman's $\rho = 0.21$, $p<0.01$) when all subjects are considered.

In subjects with T2DM and periodontitis, the relationship between hsCRP levels and inflammatory cytokines has yet to be explored within the published literature. In a recent study, of systemically healthy patients with and without periodontitis, no significant correlations were demonstrated between serum IL-6 and hsCRP in patients with and without periodontitis (Marcaccini *et al.*, 2009), which is surprising given that circulating IL-6 is reported as a major regulator of CRP production (Yudkin *et al.*, 2000). A further study, within the general medical literature, evaluated correlations between hsCRP levels and serum levels of IL-1β, IL-6 and TNF-α and reported no significant correlations between hsCRP and serum levels of IL-1β, IL-6 and TNF-α (Castoldi *et al.*, 2007). This is supported in the current study that shows no significant correlations between levels of hsCRP and serum levels of IL-6, TNF-α, IL-1β and IFN-γ. Overall, in the current study, therefore, systemic or local level inflammatory cytokines do not appear related to either HbA1c (a marker of glycaemic control) or hsCRP (a biomarker of systemic inflammation).

Correlations between periodontal parameters and cytokine levels in saliva & GCF

Previous published research has begun to explore the relationship between clinical periodontal parameters and levels of inflammatory cytokines in GCF in subjects with T2DM. One study demonstrates that levels of GCF IL-1β were associated with increasing CAL in subjects with T2DM (Engebretson et al., 2007). In another study, levels of IL-1β in GCF showed significant correlations between PD, CAL and BOP in subjects with T2DM (Engebretson et al., 2004). However, one further study in subjects with T2DM failed to show a significant correlation between levels of TNF-α, INF-γ, IL-4, IL-17 and IL-23 in GCF and mean PD or mean LOA (Santos *et al.*, 2010). Recently,

an additional study, evaluating GCF levels of IL-1β in systemically healthy subjects with and without periodontitis, demonstrated significant positive correlations between levels of IL-1β in GCF and mean PD ($r=0.75$, $p<0.001$), % BOP ($r=0.75$, $p<0.001$) (Teles *et al.*, 2010). The previously demonstrated correlations between GCF levels of inflammatory cytokines and clinical periodontal parameters are corroborated in the current study, which found that significant positive correlations were consistently demonstrated between mGI, BOP, mean PD, PESA and PISA and GCF levels of IL-6 (Table 5.13 and Figure 5.13), TNF-α (Table 5.13 and Figure 5.16), IL-1β (Table 5.10 and Figure 5.17) and IFN-γ (Table 5.13 and Figure 5.18). This suggests that as the clinical periodontal measurements increase, the levels IL-6, TNF-α, IL1β and IFN-γ in GCF also increase.

One study evaluating levels of IL-1β in saliva in subjects with and without periodontitis demonstrated significant positive correlations between levels of Il-1β in saliva and BOP ($r=0.41$, $p=0.001$), the % of sites with PD ≥4mm ($r=0.53$, $p=0.0001$) and the % of sites with PD ≥5mm ($r=0.53$, $p=0.0001$) (Miller *et al.*, 2006). This was supported in another study that reported significant correlations with salivary IL-1β levels and clinical periodontal measurements, although a failure to report the correlation coefficients limits interpretation. (Tobon-Arroyave *et al.*, 2008). Furthermore, logistic regression analysis showed that salivary IL-1β was associated in a dose-response manner with advanced periodontitis (Gursoy *et al.*, 2009). A further study however, used correlations to explore associations between the levels of salivary cytokines and clinical periodontal parameters in subjects with and without periodontitis, but failed to demonstrate a significant correlation between salivary IL-1β and BOP, mean PD or mean LOA (Teles *et al.*, 2009). In the current study, significant positive correlations were determined between mGI, %BOP, PESA and PISA and levels in saliva of IL-6

(Table 5.13 and Figure 5.11) and IL-1β (Table 5.13 and Figure 5.13). This suggests that as the clinical periodontal measurements increase, the levels IL-6 and IL1β in saliva also increase.

Overall, it would appear that increases in the severity and extent of periodontitis are associated with increasing levels of IL-1β, α-TNF- and IL-6 in GCF (Table 5.13), supporting the recognised key role of these pro-inflammatory cytokines within the pathogenesis of periodontitis (Kinane *et al.*, 2011; Preshaw and Taylor, 2011). Furthermore, this situation is mirrored in saliva, with increasing levels of IL-1β and IL-6 being associated with increasing severity and extent of periodontitis (Table 5.13), suggest that cytokines produced within the periodontal tissues are also present in saliva. On the whole, the correlations were smaller in magnitude for T2DM subjects compared to non-diabetic subjects (Tables 5.14 and 5.15) perhaps highlighting that within this current study, non-diabetic subjects with periodontitis had a greater extent of disease compared to T2DM subjects with periodontitis.

Correlations between periodontal parameters and cytokine levels in serum

A previous study, evaluating levels of TNF-α, IFN-γ (along with other cytokines) in subjects with T2DM and non-diabetic subjects, demonstrated a significant positive correlation between TNF-α levels in serum and PD (r=0.55, p=0.033) and GI (r=0.70, p=0.003) (Dag *et al.*, 2009). However, in a further study of subjects with T2DM, the picture is less clear, with log transformed data for TNF-α levels in plasma demonstrating a positive correlation with CAL (r=0.40, p=0.009) but not mean PD (r=0.28, p=0.07) or BOP (r=0.30, p=0.053) (Engebretson *et al.*, 2007). In a different study of 84 patients with periodontitis and 65 patients without periodontitis, significant positive correlations were demonstrated between log transformed data for serum IL-6 levels and mean PD (r=0.39, p=0.000) and mean LOA (r=0.42, p=0.000) (Sun *et al.*,

2009). In a further study of 25 patients with periodontitis and 20 patients without periodontitis, a significant positive correlation was see between serum IL-6 and mean PD ($r=0.31$, $p<0.025$) (Marcaccini *et al.*, 2009). However, this was not supported by data from the current study, which failed to demonstrate significant correlations between IL-6 levels in serum and clinical periodontal parameters, and found significant negative correlations between serum levels of TNF-α, IL-1β and clinical periodontal parameters when all subjects are considered. It is difficult to provide an explanation as to why the increases in the severity and extent of periodontitis appeared to be associated with decreasing circulating levels of TNF-α, IL-1β. However, given that it is not a consistent finding, it could potentially be a chance finding. To clarify the relationship between circulating inflammatory cytokines and periodontitis in subjects with T2DM, further studies of adequate power are required.

Inflammatory cytokines in GCF and saliva as predictors of periodontal status

Currently, large variations exist within the literature regarding the definition and classification of periodontal disease within research (Preshaw, 2009). Current classifications of periodontitis fail to quantify the amount of inflamed periodontal tissue which is required to accurately describe the inflammatory burden posed by periodontitis. PISA has recently been developed to describe the quantity of inflamed periodontal tissue (Nesse et al., 2008). PISA, calculated using LOA, recession and BOP, is reported to reflect the surface area of bleeding pocket epithelium (mm^2). Whilst theoretically PISA appears to be offer a better method of classifying periodontitis, there remains a need to assess the validity of PISA to ensure it correlates with conventional measures of periodontal disease. This was confirmed in the current study, in which a multinomial logistic regression model identified PISA as a predictor of periodontal status, with PISA being a significant predictor of gingivitis and periodontitis.

The identification of biomarkers that would effectively identify subjects with periodontitis would facilitate better patient management. In the current study, linear regression models demonstrated GCF IL-1β and GCF IFN-γ levels were significant predictors of PISA and a trend for GCF TNF-α levels as a predictor of PISA was noted. Likewise, salivary IL-6 and IL-1β levels were determined as significant predictors of PISA. Therefore, these findings confirm that increased levels of these local inflammatory cytokines predict higher PISA and more advanced disease, and conversely lower levels of these cytokines predict lower PISA and less advanced disease.

Interestingly, in a recent study, a dose-response relationship was identified between PISA and HbA1c in T2DM subjects, with multiple linear regression analyses demonstrating PISA was a predictor significantly associated with HbA1c (Nesse et al., 2009). In contrast, the current study found no correlation between PISA and HbA1c for T2DM subjects (Table 5.7) and thus a regression analysis was not performed. Interestingly, the median (IQR) for PISA in the published study was 151 (39-307) mm^2 in comparison to 417 (195-733) mm^2 for T2DM subjects in the current study, indicating a greater severity of periodontal disease in the current study.

Summary of key findings from chapter 5

- Serum levels of TNF-α, IL-1β and IFN-γ were significantly higher in subjects with T2DM compared to non-diabetic subjects. Furthermore, serum levels of TNF-α, IL-1β and IFN-γ were significantly higher in T2DM subjects with periodontitis compared to non-diabetic subjects with periodontitis.
- In non-diabetic subjects, serum TNF-α, IL-1β and IFN-γ were significantly higher in those with healthy periodontal tissues compared to subjects with periodontitis.

- In both diabetic and non-diabetic subjects, IL-1β in saliva was significantly higher in subjects with periodontitis compared to those with healthy periodontal tissues. Also, in subjects with T2DM, IL-6 in saliva was significantly higher in subjects with periodontitis compared to those with healthy periodontal tissues.
- TNF-α, IL-1β and IFN-γ in saliva was significantly higher in non-diabetic subjects compared to subjects with T2DM. Furthermore, compared to subjects with T2DM and periodontitis, non-diabetic subjects with periodontitis had significantly higher levels of TNF-α, IL-1β and IFN-γ in saliva.
- No significant differences in GCF IL-6, TNF-α, IL-1β and IFN-γ were found between subjects with T2DM and non-diabetic subjects.
- Non-diabetic subjects with periodontitis had apparently higher levels of GCF IL-6, TNF-α and IL-1β compared to T2DM subjects with periodontitis, although the differences failed to reach statistical significance.
- T2DM subjects with gingivitis had apparently higher levels of GCF IL-6, IL-1β and IFN-γ compared to non-diabetic subjects with gingivitis, although the differences failed to achieve statistical significance.
- In both diabetic and non-diabetic subjects, IL-1β in GCF was significantly higher in subjects with periodontitis compared to those with healthy periodontal tissues.
- Levels of IL-6, TNF-α and IFN-γ in GCF were significantly higher in non-diabetic subjects with periodontitis compared to non-diabetics with healthy periodontal tissues. Similarly, in subjects with T2DM the same differences in GCF levels of IL-6, TNF-α and IFN-γ were detected as trends.
- When all subjects were considered, significant positive correlations were found between hsCRP and mGI and mean PD. Furthermore, in non-diabetic subjects,

significant positive correlations were found between hsCRP and mGI, % BOP, mean PD and PISA. However, no significant correlations were demonstrated between hsCRP and serum levels of IL-6, IL-1β, TNF-α and IFN-γ.

- Significant positive correlations were consistently demonstrated between mGI, mean PD, PESA and PISA and GCF levels of IL-6, TNF-α and IL-1β. Furthermore, GCF levels of IL-1β and IFN-γ were significant predictors of PISA and a trend for GCF TNF-α levels as a predictor of PISA was noted.
- Significant positive correlations were found between mGI, %BOP, PESA and PISA and saliva levels of IL-6 and IL-1β. Furthermore, salivary levels of IL-6 and IL-1β were significant predictors of PISA.

Chapter 6 Response to non-surgical management in subjects with T2DM and the impact on markers of diabetes control and local and systemic cytokine levels

6.1 Introduction

The role of inflammatory cytokines in the pathogenesis of periodontal disease is well recognised (Preshaw and Taylor, 2011). Likewise, inflammatory cytokines are key players in the relationship between inflammation and T2DM. Cytokines, such as TNF-α, IL-1β and IL-6, contribute to the development of T2DM through obesity, insulin resistance and β-cell dysfunction (Donath *et al.*, 2003; Pickup, 2004; Wellen and Hotamisligil, 2005).

In recent years, a number of controlled interventional studies have confirmed positive clinical periodontal treatment outcomes following NSM in patients with T2DM (Stewart *et al.*, 2001; Kiran *et al.*, 2005; Promsudthi *et al.*, 2005; Jones *et al.*, 2007; Correa *et al.*, 2008; O'Connell *et al.*, 2008; Dag *et al.*, 2009; Kardesler *et al.*, 2010; Kardesler *et al.*, 2011). Overall, clinical treatment outcomes assessed in these studies include reduction in probing depths, gain in clinical attachment and reduction in bleeding on probing (Cobb, 2002). More recently, composite assessments have also been used to evaluate the periodontal condition of patients, incorporating within a numerical score, both periodontal epithelial surface area and BOP (Nesse et al., 2008) and thus providing an evaluation of the inflammatory burden produced from the periodontium.

In addition to the observational evidence highlighting the relationship between periodontal disease and T2DM, increasing numbers of interventional studies have been

conducted to explore the effects of periodontal disease on glycaemic control in patients with diabetes. However, consideration of intervention studies both controlled (Stewart *et al.*, 2001; Kiran *et al.*, 2005; Promsudthi *et al.*, 2005; Jones *et al.*, 2007; O'Connell *et al.*, 2008) and non-controlled (Grossi *et al.*, 1997; Iwamoto *et al.*, 2001) demonstrated inconsistent results regarding the impact of periodontal management on HbA1c. Furthermore, 2 meta-analyses have also been conducted in an attempt to clarify whether periodontal treatment has an effect on glycaemic control (Janket *et al.*, 2005; Darre *et al.*, 2008). However, the situation remains unclear, with one previous meta-analysis producing a significant reduction in HbA1c (Darre et al., 2008) whilst another gave a non-significant reduction in HbA1c (Janket et al., 2005). In the most recent meta-analysis, when compared against no treatment or usual treatment, NSM gave a statistically significant 0.40% HbA1c reduction (p=0.04) (Simpson *et al.*, 2010).

Research demonstrates an association between raised serum lipids and periodontitis (Cutler *et al.*, 1999b; Losche *et al.*, 2000; Noack *et al.*, 2000; Fentoglu *et al.*, 2009; Fentoglu *et al.*, 2010). Given that hyperlipidemia has been described as one of the factors associated with diabetes-induced immune cell alterations (Iacopino and Cutler, 2000), it is surprising that only a few studies have investigated the effect of improved periodontal health on serum lipid profiles in subjects with T2DM (Christgau *et al.*, 1998; Kiran *et al.*, 2005; Kardesler *et al.*, 2010) .

It is increasingly recognised that inflammation plays a role in the development of T2DM. CRP production is part of the non-specific acute-phase response to inflammation, infection and tissue damage (Pepys and Hirschfield, 2003). A recent systematic review concluded that there was strong evidence from cross-sectional studies that plasma CRP was elevated in periodontitis affected subjects compared with controls (Paraskevas et al., 2008). Furthermore, studies have begun to describe the

effect of reducing periodontal inflammation on CRP and circulating inflammatory cytokine levels (Ide *et al.*, 2003; D'Aiuto *et al.*, 2004; Marcaccini *et al.*, 2009). In subjects with T2DM, CRP has been proposed as a cardiovascular risk marker (Pfutzner and Forst, 2006) as well as a useful marker in predicting the risk of developing T2DM (Freeman *et al.*, 2002; Duncan *et al.*, 2003). Despite the importance of inflammation in both T2DM and periodontitis, few studies have investigated the effect of improved periodontal health on CRP levels in subjects with T2DM (Christgau *et al.*, 1998; Correa *et al.*, 2010; Kardesler *et al.*, 2010).

Recently, studies have begun to investigate the role of inflammatory cytokines in patients with T2DM and periodontal disease (Cutler *et al.*, 1999a; Engebretson *et al.*, 2007; Correa *et al.*, 2008; Dag *et al.*, 2009; Correa *et al.*, 2010; Kardesler *et al.*, 2010; Santos *et al.*, 2010) and more specifically the impact of periodontal management on cytokine levels in patients with T2DM and periodontitis (Correa *et al.*, 2008; Dag *et al.*, 2009; Correa *et al.*, 2010; Kardesler *et al.*, 2010; Santos *et al.*, 2010; Kardesler *et al.*, 2011). Although such studies have begun to elucidate the role of inflammatory cytokines within patients with T2DM and periodontitis, differences in sampling and analytical techniques, inconsistencies in the selection of cytokines investigated, small sample sizes and lack of non-diabetic control groups currently prevents clear conclusions from being made.

6.2 Results

6.2.1 Changes in BMI, blood pressure and medical history following non-surgical periodontal management (NSM)

Table 6.1 and Figure 6.1 summarise the BMI data following non-surgical periodontal management for patients with T2DM and periodontitis and non-diabetic patients with periodontitis. Of note, BMI was significantly higher in patients with

T2DM at each time point. Also, within the subjects with T2DM, BMI showed a significant reduction from a pre-treatment level [33.0 (29.9-36.5) kg/m^2] to month 6 [31.0 (27.6-34.8) kg/m^2] and month 12 months [31.6 (28.7-34.5) kg/m^2] after NSM (Table 6.1 and Figure 6.1). Table 6.1 also presents the number of subjects within each BMI category and shows that at month 6 and month 12 post-treatment, there was a reduction in the number of obese and morbidly obese individuals seen, without any concurrent increase in number of subjects classified as normal and overweight. This would suggest that the apparent reduction in BMI seen in patients with T2DM following NSM is not due to actual weight loss, but rather as a result of drop-out of obese and morbidly obese subjects (Table 6.1).

Table 6.2 and Figure 6.2 presents blood pressure data following NSM for patients with T2DM and periodontitis and non-diabetic patients with periodontitis. No significant differences between T2DM patients and non-diabetic patients were found at any time point and no significant changes were found following NSM (Table 6.2 and Figure 6.2).

Interestingly, from data collected at month 12, it would appear that there were minimal changes to the medical care for subjects over the study period. Out of the 29 T2DM subjects and 37 non-diabetic subjects reviewed at month 12, a change in medical history was reported in only 4 (13.8%) and 1 (2.7%) of subjects respectively.

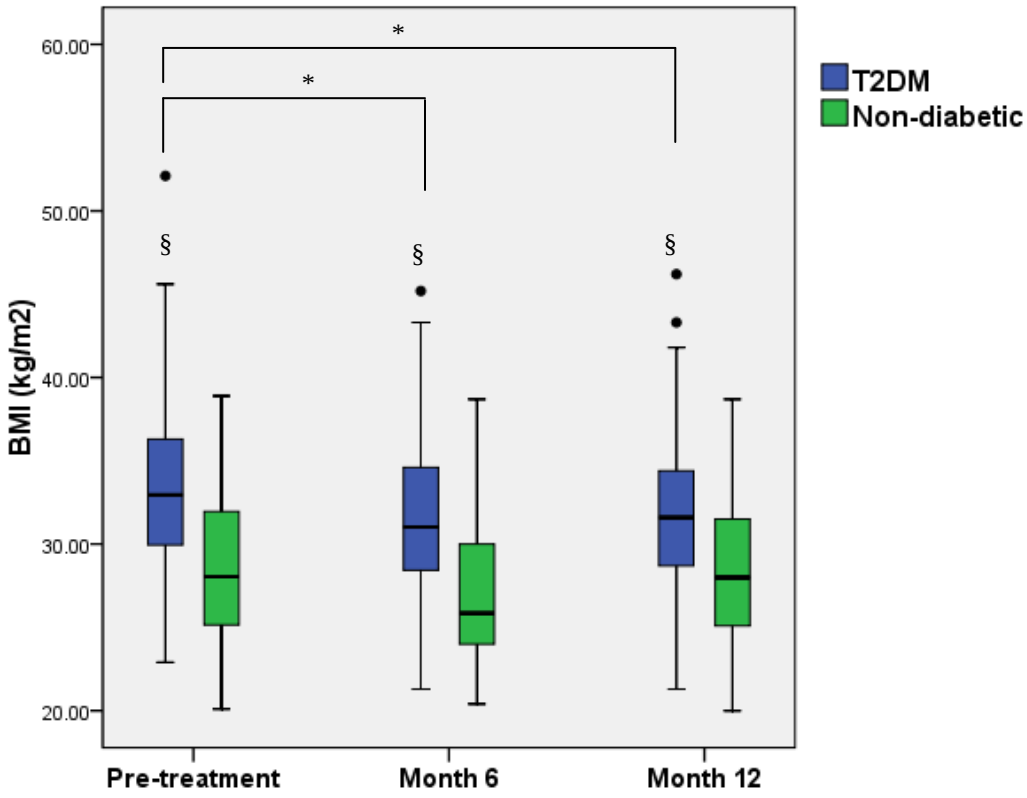
Table 6.1 BMI data in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects

	Pre-treatment		Month 6		Month 12		p-value
	T2DM subjects (n=48)	**Non-diabetic subjects (n= 46)**	**T2DM subjects (n=29)**	**Non-diabetic subjects (n= 22)**	**T2DM subjects (n=29)**	**Non-diabetic subjects (n= 37)**	
BMI (kg/m^2)	33.0 (29.9-36.5) [¶,†,$]	28.1 (25.1-32.0)	31.0 (27.6-34.8)[$]	25.9 (23.9-30.3)	31.6 (28.7-34.5)[$]	28.0 (25.1-32.9)	[¶,†,$] <0.05
BMI status (n(%))							
Normal weight (18.5-24.9)	3 (6.3)	10 (21.7)	2 (6.9)	8 (36.4)	2 (6.9)	8 (21.6)	
Overweight (25.0-29.9)	9 (18.8)	23 (50.0)	8 (27.6)	8 (36.4)	9 (31.0)	18 (70.3)	NS
Obese (30.0-34.9)	20 (41.7)	5 (10.9)	12 (41.4)	3 (13.6)	13 (44.8)	3 (78.4)	
Morbidly obese (>35.0)	16 (33.3)	8 (17.4)	7 (24.1)	3 (13.6)	5 (17.2)	8 (21.6)	

P-values were determined using chi-squared test for discrete variable, Friedman test with Wilcoxon post hoc test for continuous parametric variable compared over time and Mann-Whitney U test for continuous non-parametric data compared at each time point. Median (IQR) is presented as all the data are non-parametric.

[$] indicates a comparison within rows between of T2DM and non-diabetic groups at a specific time point
[¶] indicates a comparison within rows between pre-treatment and month 6 within either T2DM or non-diabetic groups
[†] indicates a comparison within rows between pre-treatment and month 12 within either T2DM or non-diabetic groups

Figure 6.1 BMI in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



Boxplots of BMI pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=48, month 6 n=29, month 12 n=29) and non-diabetic subjects (pre-treatment n=46, month 6 n=22, month 12 n=37)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

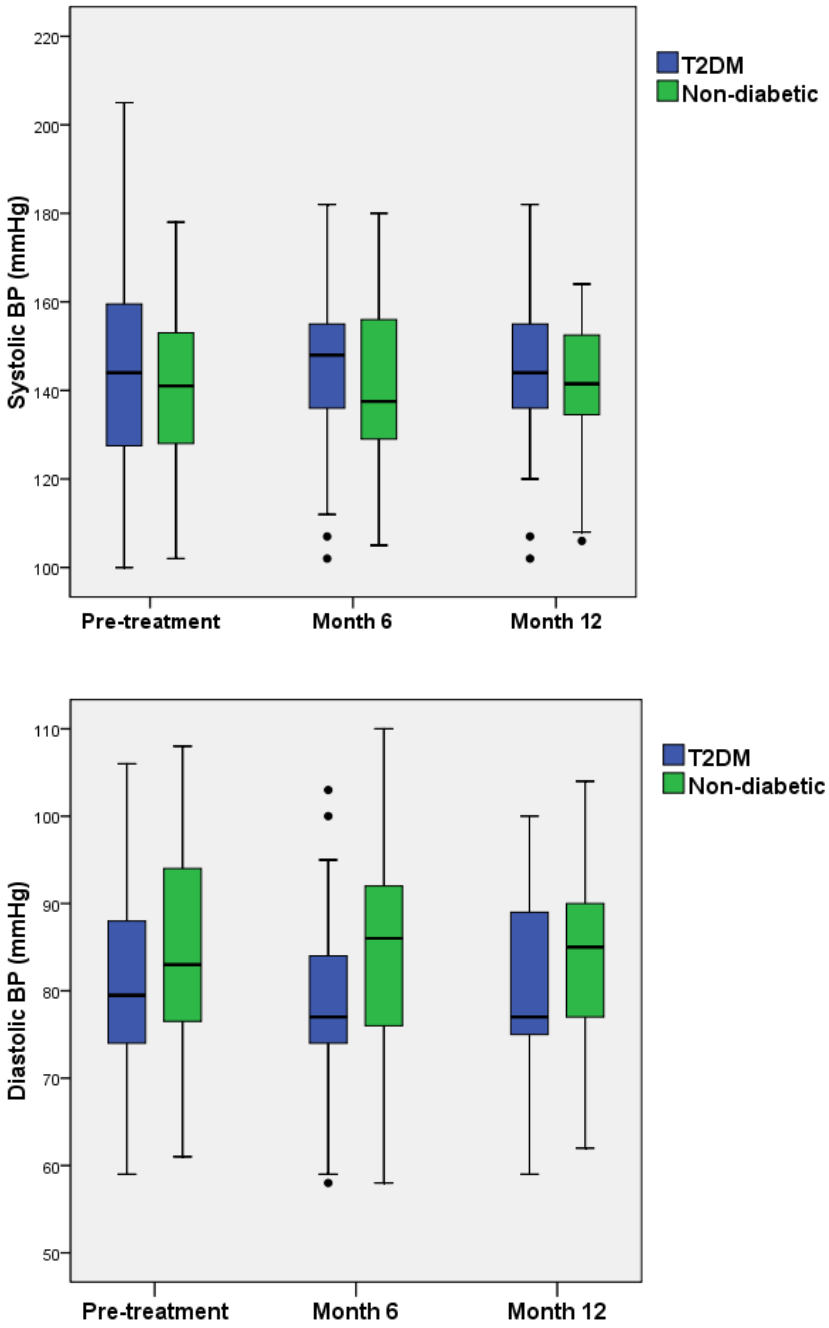
Table 6.2 Blood pressure data in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects

	Pre-treatment		Month 6		Month 12		p-value
	T2DM subjects (n=48)	**Non-diabetic subjects (n= 46)**	**T2DM subjects (n=29)**	**Non-diabetic subjects (n= 22)**	**T2DM subjects (n=29)**	**Non-diabetic subjects (n= 36)**	
Systolic BP	144.0 (127.3-159.8)	143.0 (127.5-153.0)	148.0 (135.5-155.5)	137.5 (128.5-156.3)	144.0 (135.5-155.5)	141.5 (134.3-152.8)	NS
Diastolic BP	79.5 (74.0-88.8)	83.0 (76.3-94.5)	77.0 (74.0-84.0)	86.0 (75.8-92.0)	77.0 (74.5-90.5)	85.0 (77.0-90.0)	NS

P-values were determined using Friedman test with Wilcoxon post hoc test for continuous parametric variables compared over time and Mann-Whitney U test for continuous non-parametric data compared at each time point. Median (IQR) is presented as all the data are non-parametric.

[$] indicates a comparison within rows between of T2DM and non-diabetic groups at a specific time point
[¶] indicates a comparison within rows between pre-treatment and month 6 within either T2DM or non-diabetic groups
[†] indicates a comparison within rows between pre-treatment and month 12 within either T2DM or non-diabetic groups

Figure 6.2 Blood pressure in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



Boxplots of systolic and diastolic BP pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=48, month 6 n=29, month 12 n=29) and non-diabetic subjects (pre-treatment n=46, month 6 n=22, month 12 n=36)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

6.2.2 Changes in HbA1c, hsCRP and lipids following non-surgical periodontal management

Table 6.3 and Figures 6.3 to 6.8 summarise the clinical biochemistry data following NSM for patients with T2DM and periodontitis and non-diabetic patients with periodontitis. As expected, at each time point, % HbA1c was significantly higher in patients with T2DM compared to non-diabetic subjects. In T2DM patients, pre-treatment % HbA1c levels [7.5 (6.7-9.2)%] showed a reduction at month 3 [7.05 (6.6-9.5)%] and month 12 [7.1 (6.6-7.4)%], which is a reduction of 0.455 and 0.40% respectively. The reductions in HbA1c did not however reach statistical significance.

When considering levels of triglycerides, subjects with T2DM had significantly higher levels compared to non-diabetic subjects at pre-treatment, month 3 and month 12. Also, compared to pre-treatment levels, triglyceride levels showed no significant changes following NSM in either subjects with T2DM or non-diabetic subjects at any time-point (Table 6.3 and Figure 6.4). For levels of HDL, non-diabetic subjects had significantly higher levels compared to subjects with T2DM, at each time point. Also, compared to pre-treatment levels, HDL levels showed no significant changes following NSM in either subjects with T2DM or non-diabetic subjects at any time-point (Table 6.3 and Figure 6.5). For levels of non-HDL, non-diabetic subjects had significantly higher levels compared to subjects with T2DM, at each time point. Also, in non-diabetic subjects, non-HDL levels demonstrated a significant reduction between pre-treatment [4.2 (3.6-4.6) mmol/L] and 6 month [4.0 (3.2-4.6) mmol/L]. For levels of total cholesterol, non-diabetic subjects had significantly higher levels compared to subjects with T2DM, at each time point. Also, in non-diabetic patients, a reduction was demonstrated in cholesterol levels between pre-treatment [5.5 (5.0-6.1) mmol//L] and 6 month [5.3 (4.9-6.0) mmol/L], although following the Bonferroni-Holm correction of p-values for multiple comparisons placed the critical p-value at 0.017, therefore, this

difference was detected as a trend (p=0.05) (Table 6.3 and Figure 6.7). For hsCRP levels, no significant differences between T2DM patients and non-diabetic patients were found at any time point. Also, in subjects with T2DM, hsCRP levels demonstrated a non-significant reduction between pre-treatment [2.1 (0.9-4.6) mg/L] and month 12 [1.5 (0.7-3.1) mg/L], which is a reduction of 0.6 mg/L. Similarly, in non-diabetic subjects hsCRP levels demonstrated a non-significant reduction between pre-treatment [2.3 (1.1-4.3) mg/L] and month 12 [1.6 (0.7-2.9) mg/L], which is a reduction of 0.7 mg/L, however both these reductions failed to reach statistical significance (Table 6.3 and Figure 6.8).

Table 6.4 presents the number of subjects within each category of glycaemic control. It shows that at month 12 post-treatment, there was a reduction in the number subjects categorised as having good glycaemic control, a reduction in the number subjects categorised as having poor glycaemic control and an increase in the number of subjects categorised as having moderate glycaemic control. This would suggest that the apparent reduction in HbA1c levels seen in subjects with T2DM following NSM is not a result of drop out of subjects but is more likely due to actual improvements in glycaemic control.

Following stratification of the data based on subjects' initial level of glycaemic control, Table 6.5 summarises the HbA1c levels following NSM for patients with T2DM and periodontitis. In T2DM patients with poorer initial glycaemic control, as indicated by HbA1c $\geq$ 7.5%, there was a 1.9 % reduction in HbA1c levels from pre-treatment [9.2 (8.3-10.2)%] to month 12 [7.3 (7.1-8.7)%], although, the reduction did not reach statistical significance. Figure 6.9 shows a line and scatter plot of HbA1c levels for individual subjects with T2DM and periodontitis at pre-treatment and month 12, with the lines highlighting that for the majority of subjects, a reduction in % HbA1c was demonstrated over this time period. Figures 6.10 and 6.11 presents the

same data as line and scatter plots following stratification based on subjects' initial level of glycaemic control. Interestingly, of those subjects with an initial HbA1c of 7.5% or greater, the majority show a reduction in HbA1c at month 12. In contrast, subjects with an initial HbA1c of less that 7.5% show a less clear response in HbA1c at month 12, with HbA1c levels for some subjects increasing and some decreasing (Figures 6.10 and 6.11).

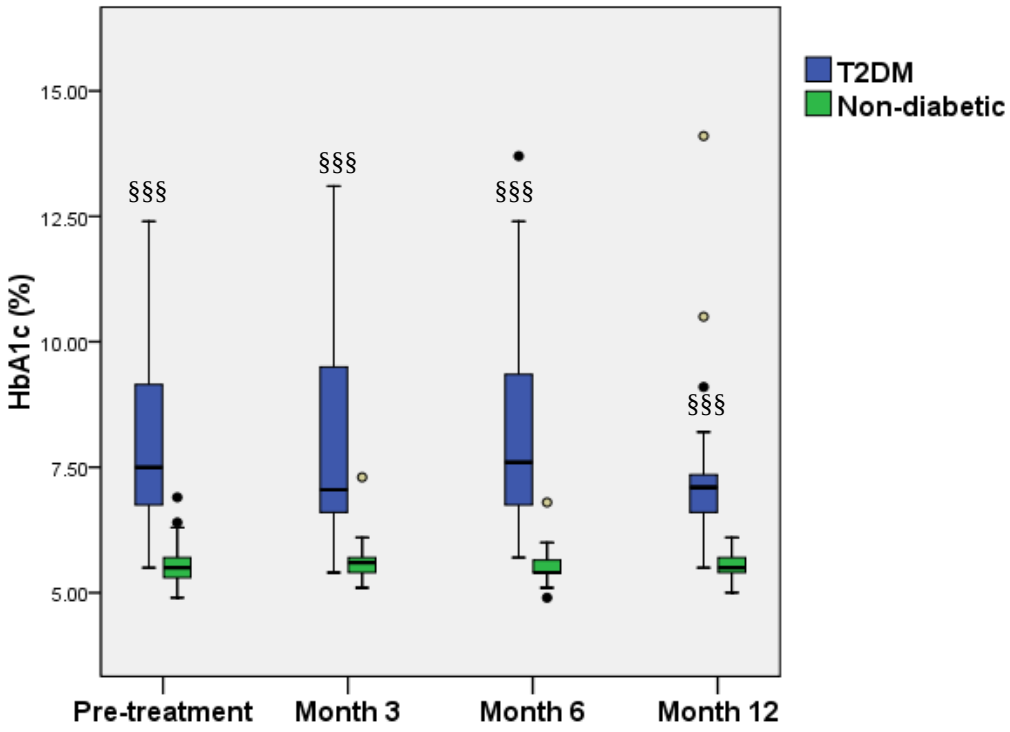
Table 6.3 Clinical biochemistry data in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects

	Pre-treatment		Month 3		Month 6		Month 12		
	T2DM subjects (n=47)	**Non-diabetic subjects (n= 48)**	**T2DM subjects (n=36)**	**Non-diabetic subjects (n= 42)**	**T2DM subjects (n=35)**	**Non-diabetic subjects (n= 39)**	**T2DM subjects (n=27)**	**Non-diabetic subjects (n= 33)**	**p-value**
HbA1c (%)	7.5 (6.7-9.2)$^{\$}$	5.5 (5.3-5.7)	7.05 (6.6-9.5)$^{\$}$	5.6 (5.4-5.7)	7.6 (6.7-9.4)$^{\$}$	5.4 (5.4-5.7)	7.1 (6.6-7.4)$^{\$}$	5.5 (5.4-5.7)	$^{\$}$ <0.001
Triglycerides (mmol/L)	2.5 (1.7-4.2)$^{\$}$	1.7 (1.2-2.7)	2.4 (1.4-3.4)$^{\$}$	1.6 (1.1-2.2)	2.3 (1.4-3.2)	1.6 (1.1-2.5)	2.0 (1.4-2.8)$^{\$}$	1.5 (1.1-1.9)	$^{\$}$ <0.05
HDL (mmol/L)	1.1 (1.0-1.3)$^{\$}$	1.4 (1.1-1.6)	1.1 (1.0-1.4)$^{\$}$	1.5 (1.2-1.8)	1.2 (1.0-1.4)$^{\$}$	1.4 (1.2-1.8)	1.1 (1.0-1.2)$^{\$}$	1.4 (1.1-1.7)	$^{\$}$<0.01
Non-HDL (mmol/L)	3.3 (2.8-3.9)$^{\$}$	4.2 (3.6-4.6)¶	2.9 (2.3-3.5)$^{\$}$	4.0 (3.6-4.7)	3.1 (2.5-3.6)$^{\$}$	4.0 (3.2-4.6)	2.7 (2.3-3.4)$^{\$}$	4.1 (3.5-4.8)	¶<0.01, $^{\$}$<0.01
Cholesterol (mmol/L)	4.4 (4.0-5.3)$^{\$}$	5.5 (5.0-6.1)¶	4.0 (3.6-5.1)$^{\$}$	5.5 (4.9-6.3)	4.4 (3.6-4.8)$^{\$}$	5.3 (4.9-6.0)	3.9 (3.4-4.8)$^{\$}$	5.5 (4.9-6.3)	¶<0.05, $^{\$}$<0.01
Hs-CRP (mg/L)	2.1 (0.9-4.6)	2.3 (1.1-4.3)	2.0 (1.1-5.1)	1.8 (0.8-3.1)	2.0 (0.8-4.6)	1.7 (0.9-3.2)	1.5 (0.7-3.1)	1.6 (0.7-2.9)	NS

P-values were determined using Friedman test with Wilcoxon post hoc test for continuous parametric variables compared over time and Mann-Whitney U test for continuous non-parametric data compared at each time point. Median (IQR) is presented as all the data is non-parametric.

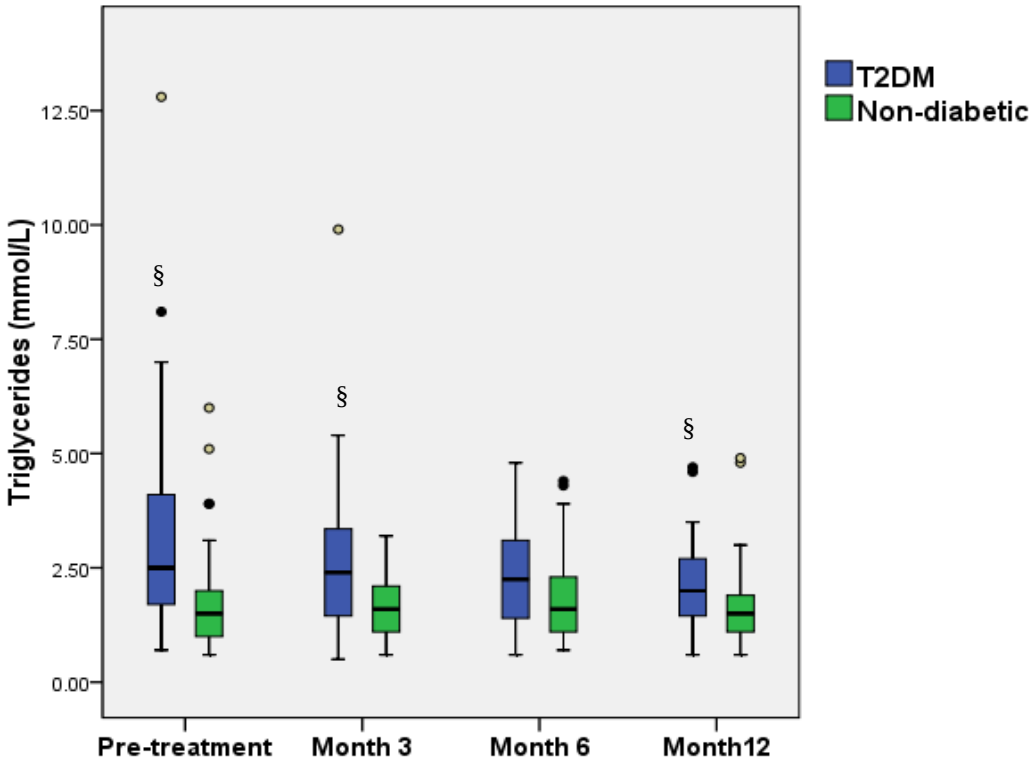
$^{\$}$ indicates a comparison within rows between of T2DM and non-diabetic groups at a specific time point
$^{\#}$ indicates a comparison within rows between pre-treatment and month 3 within either T2DM or non-diabetic groups
¶ indicates a comparison within rows between pre-treatment and month 6 within either T2DM or non-diabetic groups
† indicates a comparison within rows between pre-treatment and month 12 within either T2DM or non-diabetic groups

Figure 6.3 HbA1c levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



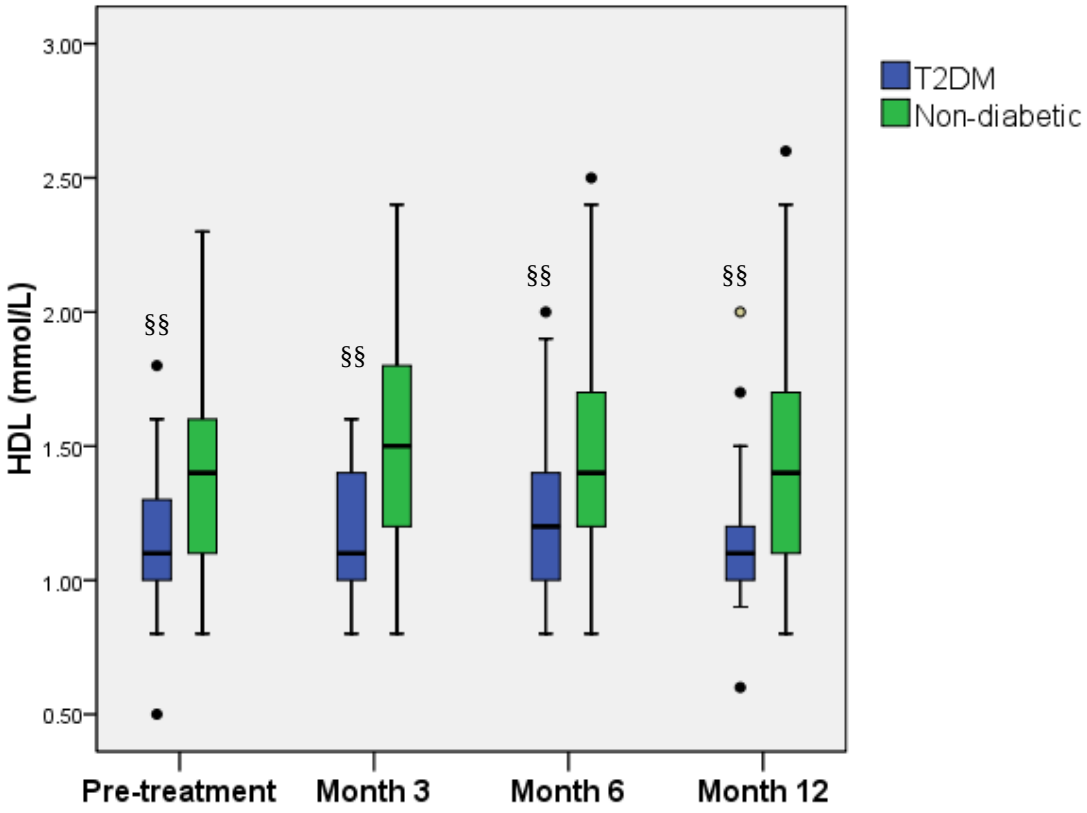
Boxplots of HbA1c levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=47, month 3 n=36, month 6 n=35, month 12 n=27) and non-diabetic subjects (pre-treatment n=48, month 3 n=42, month 6 n=39, month 12 n=33)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.4 Triglyceride levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



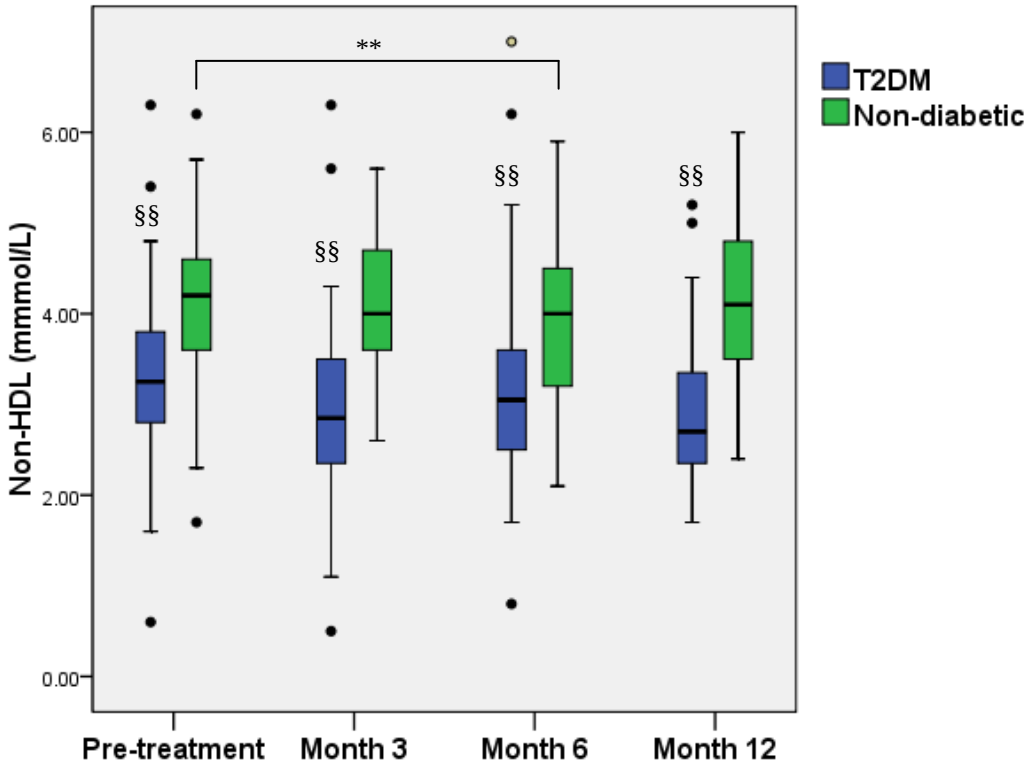
Boxplots of triglyceride levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=47, month 3 n=36, month 6 n=35, month 12 n=27) and non-diabetic subjects (pre-treatment n=48, month 3 n=42, month 6 n=39, month 12 n=33)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.5 HDL levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



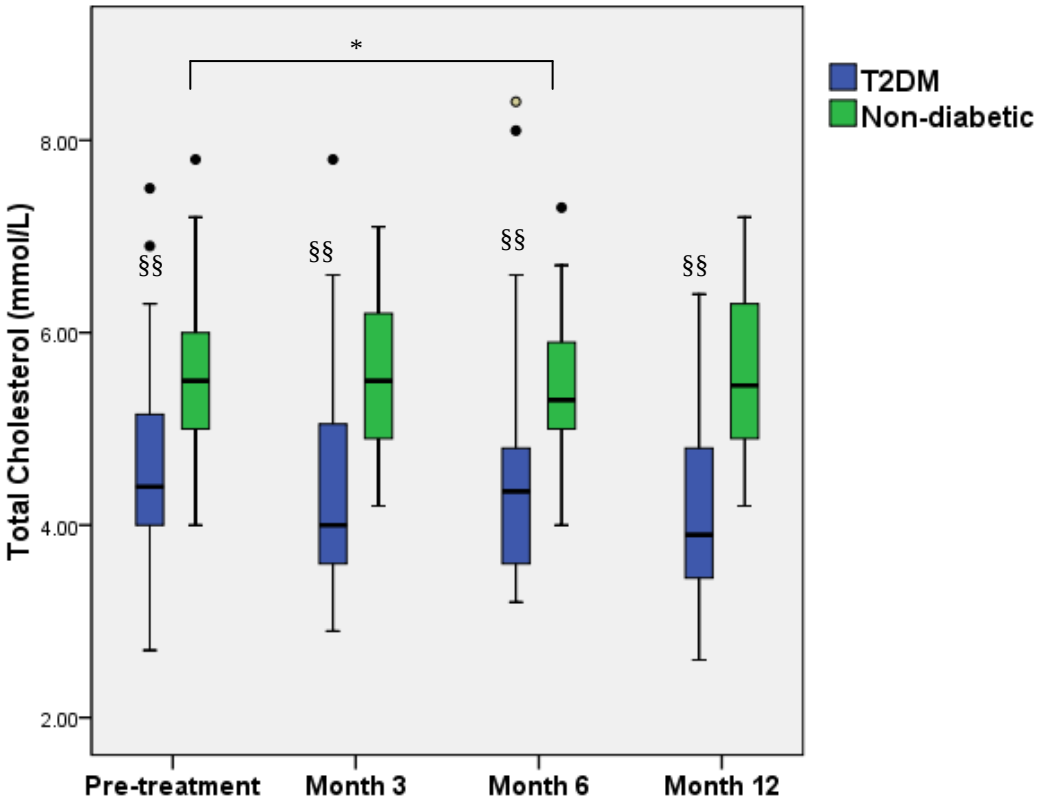
Boxplot of HDL levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=47, month 3 n=36, month 6 n=35, month 12 n=27) and non-diabetic subjects (pre-treatment n=48, month 3 n=42, month 6 n=39, month 12 n=33)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.6 Non-HDL levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



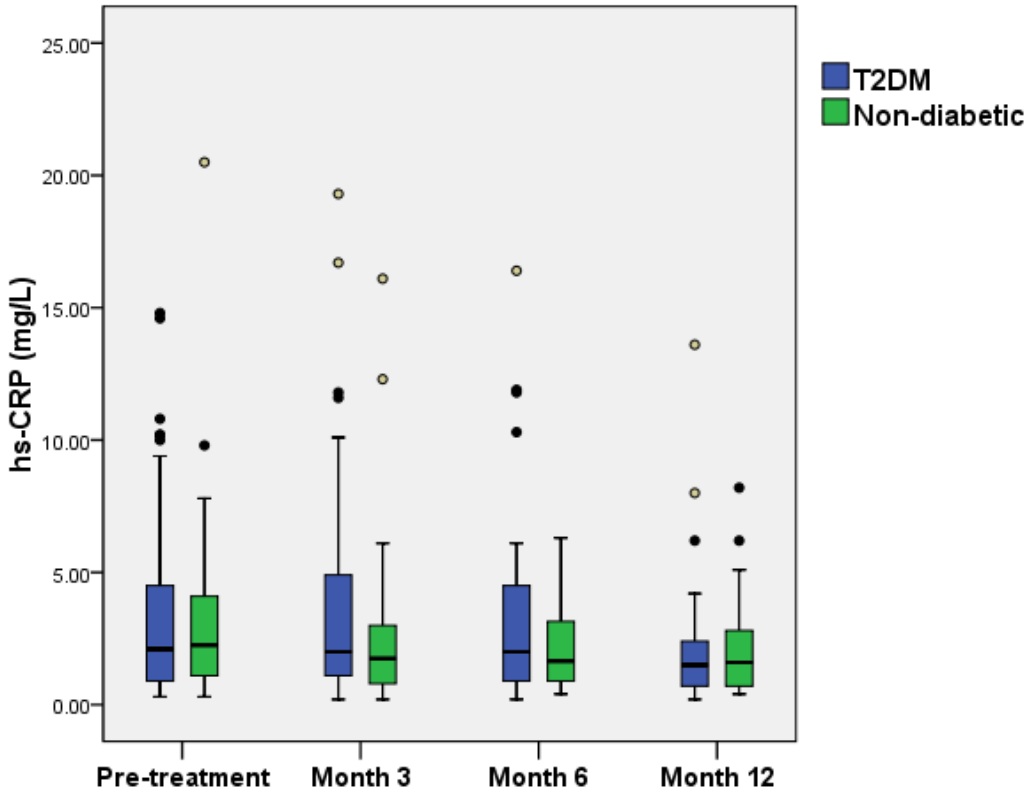
Boxplots of non-HDL levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=47, month 3 n=36, month 6 n=35, month 12 n=27) and non-diabetic subjects (pre-treatment n=48, month 3 n=42, month 6 n=39, month 12 n=33)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.7 Total cholesterol levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



Boxplots of total cholesterol levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=47, month 3 n=36, month 6 n=35, month 12 n=27) and non-diabetic subjects (pre-treatment n=48, month 3 n=42, month 6 n=39, month 12 n=33)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.8 HsCRP levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



Boxplots of hsCRP levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=47, month 3 n=36, month 6 n=35, month 12 n=27) and non-diabetic subjects (pre-treatment n=48, month 3 n=42, month 6 n=39, month 12 n=33)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Table 6.4 Glycaemic control in T2DM subjects with periodontitis pre- and post NSM

	Pre-treatment (n=47)	Month 3 (n=36)	Month 6 (n=35)	Month 12 (n=27)	p-value
Glycaemic control categories (n(%))					
Good (<7.0%)	18 (38.3)	15 (41.7)	17 (48.6)	7 (25.9)	
Moderate (7.0-8.5%)	12 (25.5)	7 (19.4)	3 (8.6)	16 (59.3)	NS
Poor (>8.5%)	17 (36.2)	14 (38.9)	15 (42.9)	4 (14.8)	
Glycaemic control target met (≤6.5%) (n(%))	7 (14.9)	9 (25.0)	7 (20.0)	6 (22.2)	NS

P-values were determined using chi-squared test for discrete variable.

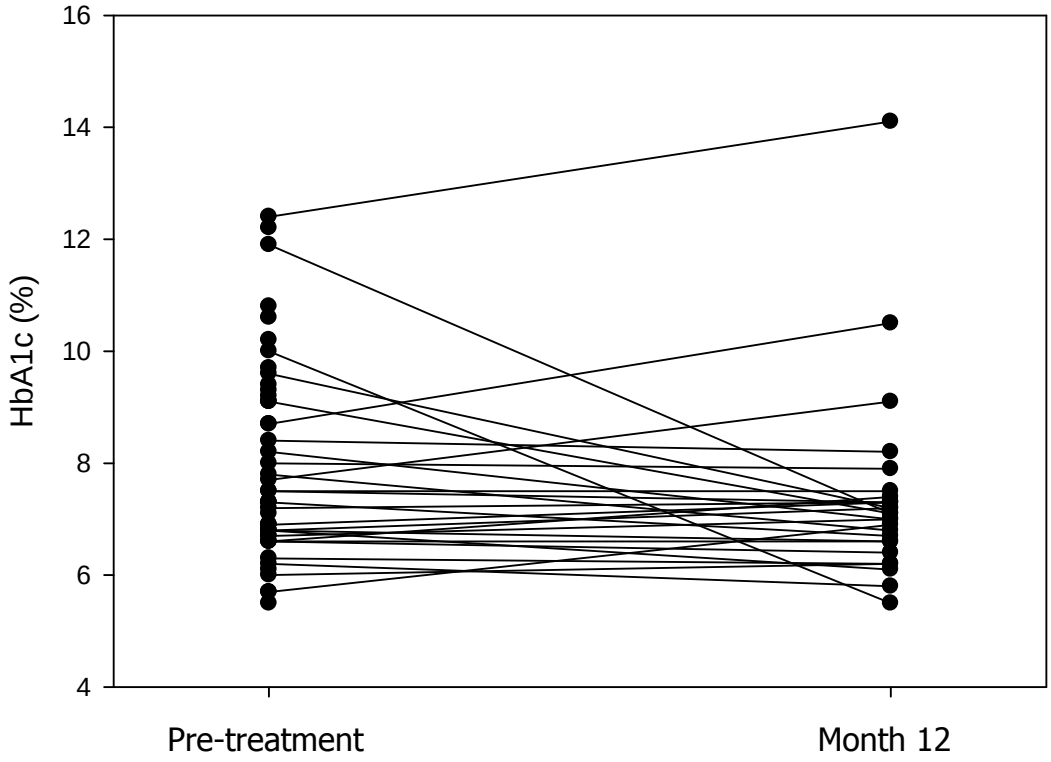
Table 6.5 HbA1c in T2DM subjects pre- and post NSM categorised on initial HbA1c level

Initial HbA1c	Pre-treatment	Month 3	Month 6	Month 12	p-value
>7.5%	9.2 (8.3-10.2)[$]	9.5 (8.4-9.8)[$]	9.2 (7.8-9.9)[$]	7.3 (7.1-8.7) [$]	[$] <0.01
<7.5%	6.7 (6.2-6.9)	6.8 (6.2-7.0)	6.8 (6.1-6.9)	6.7 (6.2-7.2)	

P-values were determined using Friedman test with Wilcoxon post hoc test for continuous parametric variables compared over time and Mann-Whitney U test for continuous non-parametric data compared at each time point. Median (IQR) is presented as all the data are non-parametric. Subjects with initial HbA1c≥ (pre-treatment n=24, month 3 n=17, month 6 n=18 and month 12 n=13) and initial HbA1c <7.5% (pre-treatment n=23, month 3 n=18, month 6 n=16, and month 12 n=14)

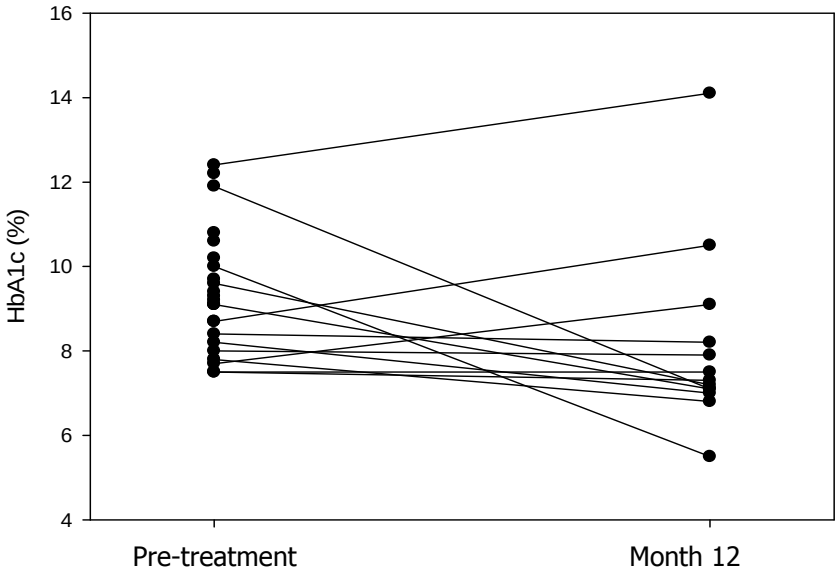
[$] indicates a comparison at a specific time point between rows
[#] indicates a comparison between pre-treatment and month 3 within rows
[¶] indicates a comparison between pre-treatment and month 6 within rows
[†] indicates a comparison between pre-treatment and month 12 within

Figure 6.9 HbA1c levels in individual subjects with periodontitis and T2DM pre-treatment and month 12



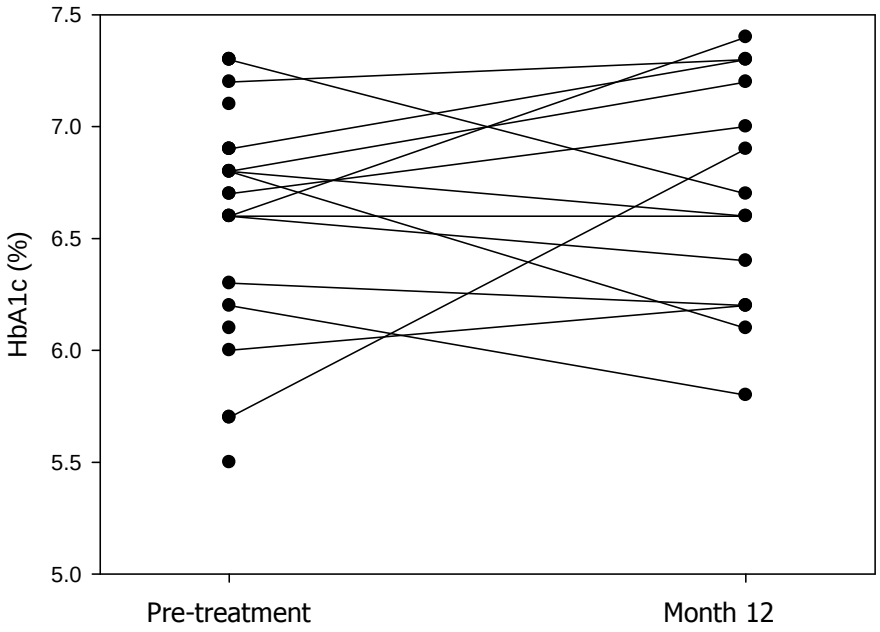
Line and scatter plot of HbA1c levels for individual subjects with T2DM and periodontitis at pre-treatment (n=47) and month 12 (n=27). Lines highlight the direction of change of HbA1c.

Figure 6.10 HbA1c levels in individual subjects with periodontitis and T2DM pre-treatment and month 12 with initial HbA1c ≥7.5%



Line and scatter plots of HbA1c levels for individual subjects with T2DM and periodontitis and with initial HbA1c levels ≥7.5%: pre-treatment (n=24) and month 12 (n=13). Lines highlight the direction of change of HbA1c.

Figure 6.11 HbA1c levels in individual subjects with periodontitis and T2DM pre-treatment and month 12 with initial HbA1c <7.5%



Line and scatter plots of HbA1c levels for individual subjects with T2DM and periodontitis and with initial HbA1c levels <7.5%: pre-treatment (n=23) and month 12 (n=14). Lines highlight the direction of change of HbA1c.

6.2.3 Changes in clinical periodontal data following non-surgical periodontal management

Table 6.6 and Figures 6.12 to 6.20 summarises the clinical periodontal data following non-surgical periodontal management for patients with T2DM and periodontitis and non-diabetic patients with periodontitis.

When considering the % of sites exhibiting BOP, no significant differences in % BOP were found between subjects with T2DM and non-diabetic subjects at any time point. Also, in subjects with T2DM, compared to pre-treatment levels [46.0 (30.0-60.7)%], % BOP showed significant reductions following NSM at 3 months [18.5 (8.8-38.0)%], 6 months [15.3 (8.3-39.6)%] and 12 months [17.6 (6.5-28.4)%]. Similarly, in non-diabetic subjects, compared to pre-treatment levels [43.0 (29.4-56.7)%], % BOP showed significant reductions following NSM at 3 months [14.5 (10.3-27.0)%], 6 months [14.7 (7.2-25.0)%] and 12 months [10.0 (5.1-24.2)%] (Table 6.6 and Figure 6.12).

For gingival inflammation, no significant differences in mGI were found between subjects with T2DM and non-diabetic subjects at any time point. Also, in subjects with T2DM, compared to pre-treatment levels [2.0 (1.5-2.7)], mGI showed significant reductions following NSM at 3 months [1.4 (0.7-2.0)], 6 months [1.5 (0.9-2.0)] and 12 months [1.3 (0.8-1.7)]. Similarly, in non-diabetic subjects, compared to pre-treatment levels [2.4 (2.0-2.7)], mGI showed significant reductions following NSM at 3 months [1.6 (0.9-2.2)], 6 months [1.5 (0.7-2.0)] and 12 months [1.4 (0.9-2.3)] (Table 6.6 and Figure 6.13).

When considering plaque levels, subjects with T2DM had significantly higher PI at pre-treatment [0.8 (0.5-1.1)], month 3 [0.5 (0.3-0.7)], month 6 [0.6 (0.3-0.8)] and month 12 [0.5 (0.3-0.7)] compared to non-diabetic subjects at pre-treatment [0.6 (0.4-

0.8)], month 3 [0.3 (0.1-0.6)], month 6 [0.3 (0.1-0.6)] and month 12 [0.4 (0.2-0.8)]. When the impact of the pre-treatment difference between subjects with T2DM and non-diabetic subjects was taken into account, the differences found between subjects with T2DM and non-diabetic subjects at month 3, month 6 and month 12 failed to reach significance. In subjects withT2DM, compared to pre-treatment levels [0.8 (0.5-1.1)], PI showed significant reductions following NSM at 3 months [0.5 (0.3-0.7)], 6 months [0.6 (0.3-0.8)] and 12 months [0.5 (0.3-0.7)]. Similarly, in non-diabetic subjects, compared to pre-treatment levels [0.6 (0.4-0.8)], PI showed significant reductions following NSM at 3 months [0.3 (0.1-0.6)], 6 months [0.3 (0.1-0.6)] and 12 months [0.4 (0.2-0.8)] (Table 6.6 and Figure 6.14).

For mean PD, no significant differences were found between subjects with T2DM and non-diabetic subjects at any time point. In subjects withT2DM, compared to pre-treatment levels [2.8 (2.4-3.2) mm], mean PD showed significant reductions following NSM at 3 months [2.4 (2.2-2.9) mm], 6 months [2.2 (2.0-2.7) mm] and 12 months [2.2 (1.9-2.8) mm]. Similarly, in non-diabetic subjects, compared to pre-treatment levels [2.9 (2.5-3.5) mm], mean PD showed significant reductions following NSM at 3 months [2.6 (2.2-2.9) mm], 6 months [2.5 (2.1-2.9) mm] and 12 months [2.3 (1.9-2.7) mm] (Table 6.6 and Figure 6.15).

For mean LOA, no significant differences were found between subjects with T2DM and non-diabetic subjects at any time point. In subjects withT2DM, compared to pre-treatment levels [3.1 (2.8-3.9) mm], mean LOA showed significant reductions following NSM at 3 months [3.0 (2.6-3.6) mm], 6 months [2.8 (2.4-3.7) mm] and 12 months [2.8 (2.4-3.6) mm]. Similarly, in non-diabetic subjects, compared to pre-treatment levels [3.4 (2.9-4.2) mm], mean LOA showed significant reductions following NSM at 3 months [3.3 (2.8-3.9) mm], 6 months [3.1 (2.6-4.1) mm] and 12 months [3.1 (2.5-3.7) mm] (Table 6.6).

For the % of PD sites ≥ 5 mm, at pre-treatment, despite the apparent higher % of PD sites ≥ 5 mm recorded in non-diabetic subjects [16.0 (8.0-30.8)%] compared to subjects with T2DM [10.9 (8.1-18.2)%], the difference failed to reach statistical significance. After treatment, the % of PD sites ≥ 5 mm were significantly higher in non-diabetic subjects at month 3 [10.1 (5.8)%] and month 6 [9.0 (3.2-19.5)%] compared to subjects with T2DM at month 3[5.3 (1.8-9.9)%] and month 6 [5.3 (1.9-9.7)%]. When the impact of the pre-treatment difference between subjects with T2DM and non-diabetic subjects was taken into account, the differences found between subjects with T2DM and non-diabetic subjects at month 3 and month 6 failed to reach significance. In subjects with T2DM, compared to pre-treatment levels [10.9 (8.1-18.2)%], % of PD sites ≥5mm showed significant reductions following NSM at 3 months [5.3 (1.8-9.9)%], 6 months [5.3 (1.9-9.7)%] and 12 months [3.0 (0.9-8.9)%]. Similarly, in non-diabetic subjects, compared to pre-treatment levels [16.0 (8.0-30.8)%], % of PD sites ≥5mm showed significant reductions following NSM at 3 months [10.1 (5.8-19.0)%], 6 months [9.0 (3.2-19.5)%] and 12 months [3.2 (1.3-13.4)%] (Table 6.6 and Figure 6.16).

When considering periodontal surface area, pre-treatment PESA was significantly higher in non-diabetic subjects [1711.0 (1439.4-2096.5) mm^2] compared to subjects with T2DM [1401.5 (1144.3-1733.7) mm^2]. At month 3, 6 and 12, non-diabetic subjects had apparently higher PESA compared to non-diabetic subjects, however the differences failed to reach statistical significance. In subjects withT2DM, compared to pre-treatment levels [1401.5 (1144.3-1733.7) mm^2], PESA showed significant reductions following NSM at 3 months [1238.2 (1092.4-1543.9) mm^2], 6 months [1159.7 (1010.1-509.0) mm^2] and 12 months [1181.3 (930.3-1374.7) mm^2]. Similarly, in non-diabetic subjects, compared to pre-treatment levels [1711.0 (1439.4-2096.5) mm^2], PESA showed significant reductions following NSM at 3 months

[1464.2 (1192.0-1686.2) mm^2], 6 months [1402.9 (1139.8-1698.3) mm^2] and 12 months [1260.2 (983.7-1566.8) mm^2] (Table 6.6 and Figure 6.17).

For PISA, no significant differences were found between subjects with T2DM and non-diabetic subjects at any time point. In subjects withT2DM, compared to pre-treatment levels [683.0 (439.1-1085.5) mm^2], PISA showed significant reductions following NSM at 3 months [339.0 (145.4-688.3) mm^2], 6 months [340.1 (101.0-628.1) mm^2] and 12 months [216.5 (87.1-539.4) mm^2]. Similarly, in non-diabetic subjects, compared to pre-treatment levels [897.3 (683.6-1232.9) mm^2], PISA showed significant reductions following NSM at 3 months [299.4 (159.5-536.5) mm^2], 6 months [262.6 (89.0-514.8) mm^2] and 12 months [215.1 (62.5-520.3) mm^2] (Table 6.6 and Figure 6.18).

Table 6.7 and Figures 6.19 and 6.20 summarises the % of sites displaying a reduction in PD or LOA for patients with T2DM and periodontitis and non-diabetic patients with periodontitis. When considering the % of sites demonstrating a reduction in PD of ≥ 2 mm during the 0 to 3 month period, no significant differences were found when comparing subjects with T2DM [11.0 (5.3-17.2)%] and non-diabetic subjects [12.3 (8.3-20.8)%]. Similarly, during the 0 to 6 month period, no significant differences were found in the number of sites demonstrating a reduction in PD of ≥2mm when comparing subjects with T2DM [12.2 (9.3-19.1)%] and non-diabetic subjects [14.0 (9.3-19.4)%]. Likewise, during the 0 to 12 month period, no significant differences were found in the % of sites demonstrating a reduction in PD of ≥ 2 mm when comparing subjects with T2DM [13.6 (10.5-24.0)%] and non-diabetic subjects [18.0 (10.2-23.2)%] (Table 6.7 and Figure 6.19).

When considering the % of sites demonstrating a reduction in PD of ≥ 3 mm during the 0 to 3 month period, no significant differences were found when comparing

subjects with T2DM [3.3 (1.0-6.6)%] and non-diabetic subjects [4.3 (2.4-8.3)%]. Similarly, during the 0 to 6 month period, no significant differences were found in the % of sites demonstrating a reduction in PD of ≥ 3 mm when comparing subjects with T2DM [4.7 (2.8-8.3)%] and non-diabetic subjects [5.1 (2.9-10.4)%]. Likewise, during the 0 to 12 month period, no significant differences were found in the % of sites demonstrating a reduction in PD of ≥ 3 mm when comparing subjects with T2DM [6.6 (2.7-11.4)%] and non-diabetic subjects [6.0 (3.8-11.7)%] (Table 6.7 and Figure 6.20).

Table 6.6 Clinical periodontal data in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects

	Pre-treatment		Month 3		Month 6		Month 12		
	T2DM subjects (n=47)	**Non-diabetic subjects (n= 48)**	**T2DM subjects (n=37)**	**Non-diabetic subjects (n= 42)**	**T2DM subjects (n=35)**	**Non-diabetic subjects (n= 40)**	**T2DM subjects (n=29)**	**Non-diabetic subjects (n= 37)**	**p-value**
BOP (%)	46.0 (30.0-60.7)[#,¶,†]	43.0 (29.4-56.7)[#,¶,†]	18.5 (8.8-38.0)	14.5 (10.3-27.0)	15.3 (8.3-39.6)	14.7 (7.2-25.0)	17.6 (6.5-28.4)	10.0 (5.1-24.2)	[#,¶,†]<0.001
mGI	2.0 (1.5-2.7)[#,¶,†]	2.4 (2.0-2.7)[#,¶,†]	1.4 (0.7-2.0)	1.6 (0.9-2.2)	1.5 (0.9-2.0)	1.5 (0.7-2.0)	1.3 (0.8-1.7)	1.4 (0.9-2.3)	[#,¶,†]<0.001
PI	0.8 (0.6-1.1)[#,¶,†,$]	0.6 (0.4-0.8)[#,¶,†]	0.5 (0.3-0.7)[$]	0.3 (0.1-0.6)	0.6 (0.3-0.8)[$]	0.3 (0.1-0.6)	0.5 (0.3-0.7)[$]	0.4 (0.2-0.8)	[#,¶]<0.001 [†,$]<0.05
Mean PD (mm)	2.8 (2.4-3.2)[#,¶,†]	2.9 (2.5-3.5)[#,¶,†]	2.4 (2.2-2.9)	2.6 (2.2-2.9)	2.2 (2.0-2.7)	2.5 (2.1-2.9)	2.2 (1.9-2.8)	2.3 (1.9-2.7)	[#,¶,†]<0.001
Mean recession (mm)	0.4 (0.3-0.7)[#,¶,†]	0.4 (0.2-0.9)[#,¶,†]	0.7 (0.4-0.8)	0.6 (0.3-1.0)	0.5 (0.4-0.8)	0.6 (0.3-1.0)	0.6 (0.4-0.9)	0.7 (0.4-1.2)	[#,¶,†]<0.001
Mean LOA (mm)	3.1 (2.8-3.9)[#,¶,†]	3.4 (2.9-4.2)[#,¶,†]	3.0 (2.6-3.6)	3.3 (2.8-3.9)	2.8 (2.4-3.7)	3.1 (2.6-4.1)	2.8 (2.4-3.6)	3.1 (2.5-3.7)	[#,¶,†]<0.001
% PD sites ≥5mm	10.9 (8.1-18.2)[#,¶,†]	16.0 (8.0-30.8)[#,¶,†]	5.3 (1.8-9.9)[$]	10.1 (5.8-19.0)	5.3 (1.9-9.7)[$]	9.0 (3.2-19.5)	3.0 (0.9-8.9)	3.2 (1.3-13.4)	[#,¶,†]<0.001

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	Pre-treatment		Month 3		Month 6		Month 12		
	T2DM subjects (n=47)	**Non-diabetic subjects (n= 48)**	**T2DM subjects (n=37)**	**Non-diabetic subjects (n= 42)**	**T2DM subjects (n=35)**	**T2DM subjects (n=47)**	**T2DM subjects (n=29)**	**Non-diabetic subjects (n= 37)**	**p value**
PESA (mm^2)	1401.5 (1144.3-1733.7)[#,¶,†]	1711.0 (1439.4-2096.5)[#,¶,†,$]	1238.2 (1092.4-1543.9)	1464.2 (1192.0-1686.2)	1159.7 (1010.1-509.0)	1402.9 (1139.8-1698.3)	1181.3 (930.3-1374.7)	1260.2 (983.7-1566.8)	[#,¶,†]<0.001 [$]<0.01
PISA (mm^2)	683.0 (439.1-1085.5)[#,¶,†]	897.3 (683.6-1232.9)[#,¶,†]	339.0 (145.4-688.3)	299.4 (159.5-536.5)	340.1 (101.0-628.1)	262.6 (89.0-514.8)	216.5 (87.1-539.4)	215.1 (62.5-520.3)	[#,¶,†]<0.001

P-values were determined using Friedman test with Wilcoxon post hoc test for continuous parametric variables compared over time and Mann-Whitney U test for continuous non-parametric data compared at each time point. Median (IQR) is presented as all the data were non-parametric.

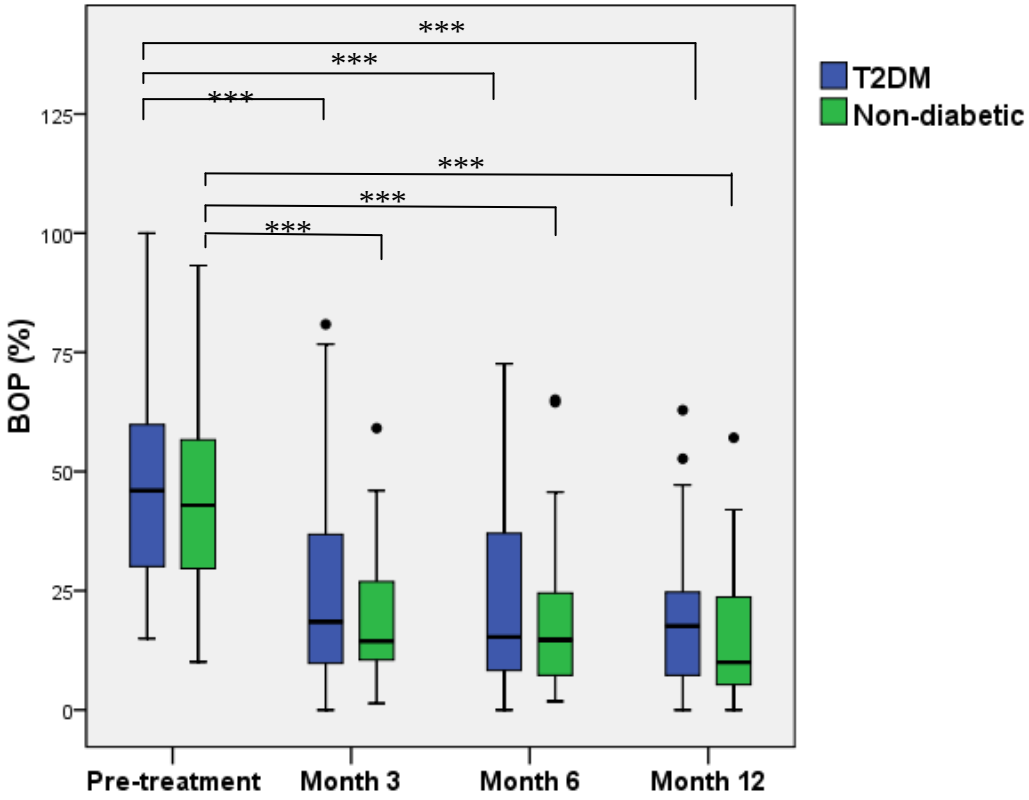
[$] indicates a comparison within rows between of T2DM and non-diabetic groups at a specific time point
[#] indicates a comparison within rows between pre-treatment and month 3 within either T2DM or non-diabetic groups
[¶] indicates a comparison within rows between pre-treatment and month 6 within either T2DM or non-diabetic groups
[†] indicates a comparison within rows between pre-treatment and month 12 within either T2DM or non-diabetic groups

Table 6.7 The change in PD and LOA after NSM for both T2DM and non-diabetic subjects

	0-3 months		0-6 months		0-12 months		
	T2DM subjects (n=36)	**Non-diabetic subjects (n= 41)**	**T2DM subjects (n=35)**	**Non-diabetic subjects (n= 40)**	**T2DM subjects (n=28)**	**Non-diabetic subjects (n= 37)**	**p value**
% PD sites reduced ≥2mm	11.0 (5.3-17.2)	12.3 (8.3-20.8)	12.2 (9.3-19.1)	14.0 (9.3-19.4)	13.6 (10.5-24.0)	18.0 (10.2-23.2)	NS
% PD sites reduced ≥3mm	3.3 (1.0-6.6)	4.3 (2.4-8.3)	4.7 (2.8-8.3)	5.1 (2.9-10.4)	6.6 (2.7-11.4)	6.0 (3.8-11.7)	NS
% LOA sites reduced ≥2mm	11.1 (8.3-14.6)	11.7 (8.3-17.8)	13.8 (9.7-19.5)	12.6 (8.7-17.9)	15.5 (9.3-22.2)	14.2 (10.6-19.7)	NS
% LOA sites reduced ≥3mm	3.3 (0.8-5.6)	4.2 (2.4-7.8)	4.9 (1.9-6.8)	5.1 (2.6-9.4)	5.3 (1.9-8.3)	5.6 (2.9-8.3)	NS

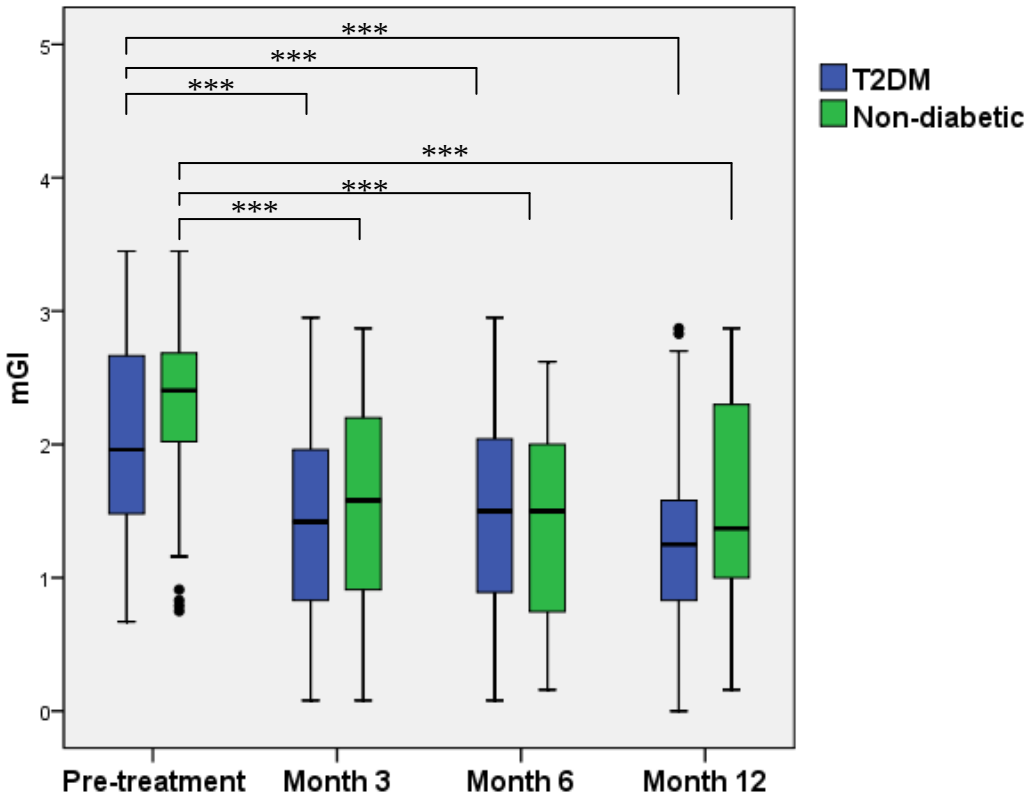
P-values were determined using Mann-Whitney U test for continuous non-parametric data compared at each time point. Median (IQR) is presented as all the data were non-parametric. $^{\$}$ indicates a comparison within rows between of T2DM and non-diabetic groups at a specific time point

Figure 6.12 BOP in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



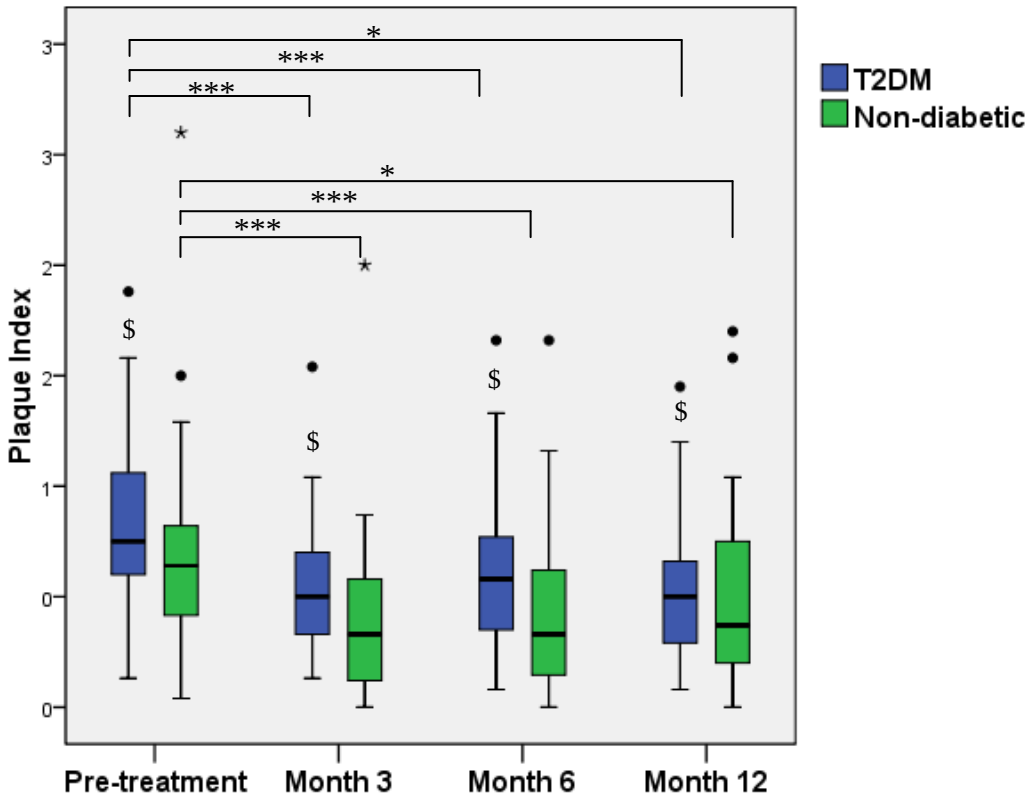
Boxplots of % of sites showing BOP pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=47, month 3 n=37, month 6 n=35, month 12 n=29) and non-diabetic subjects (pre-treatment n=48, month 3 n=42, month 6 n=40, month 12 n=37)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.13 mGI in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



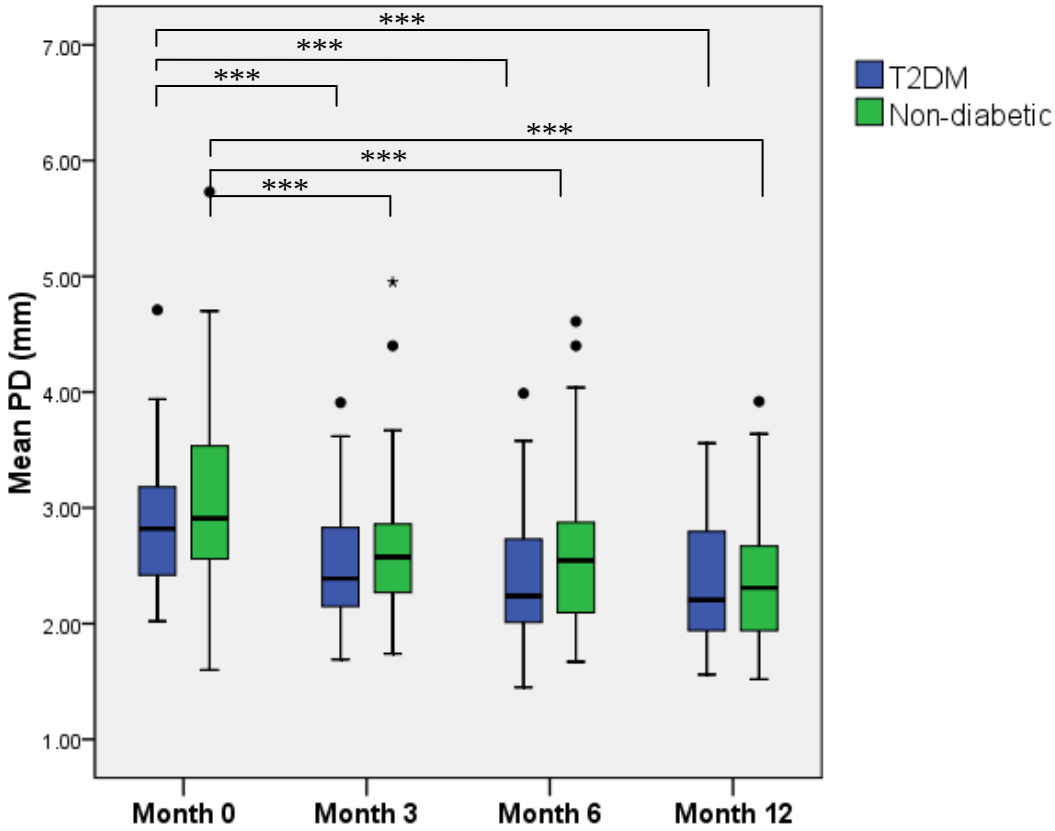
Boxplots of mGI pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=47, month 3 n=37, month 6 n=35, month 12 n=29) and non-diabetic subjects (pre-treatment n=48, month 3 n=42, month 6 n=40, month 12 n=37)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.14 PI in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



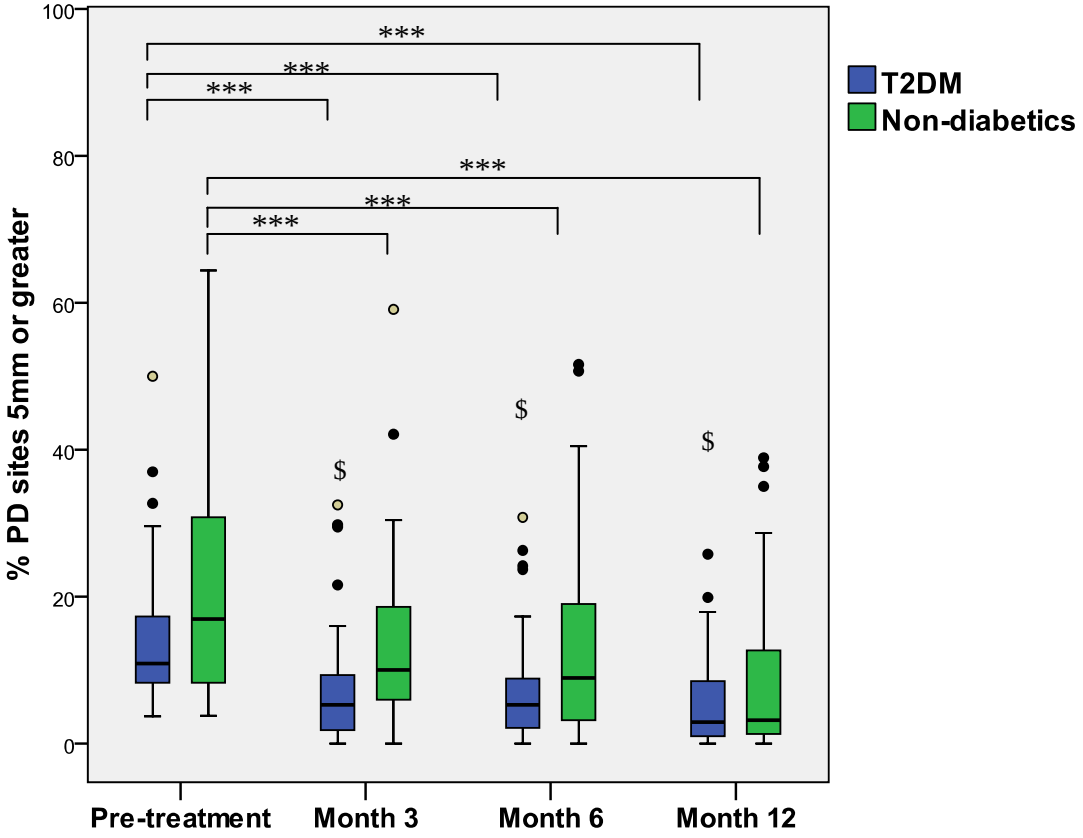
Boxplots of PI pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=47, month 3 n=37, month 6 n=35, month 12 n=29) and non-diabetic subjects (pre-treatment n=48, month 3 n=42, month 6 n=40, month 12 n=37)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.15 Mean PD in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



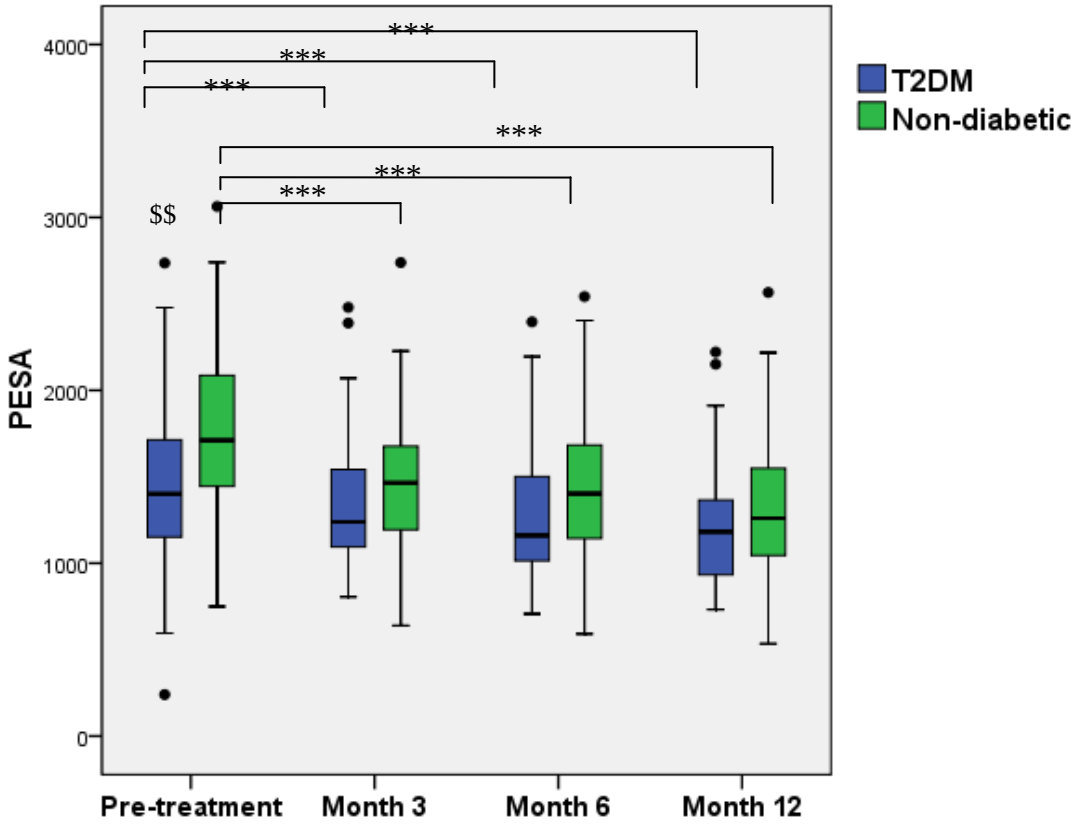
Boxplots of mean PD pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=47, month 3 n=37, month 6 n=35, month 12 n=29) and non-diabetic subjects (pre-treatment n=48, month 3 n=42, month 6 n=40, month 12 n=37)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.16 % of PD sites ≥5mm in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



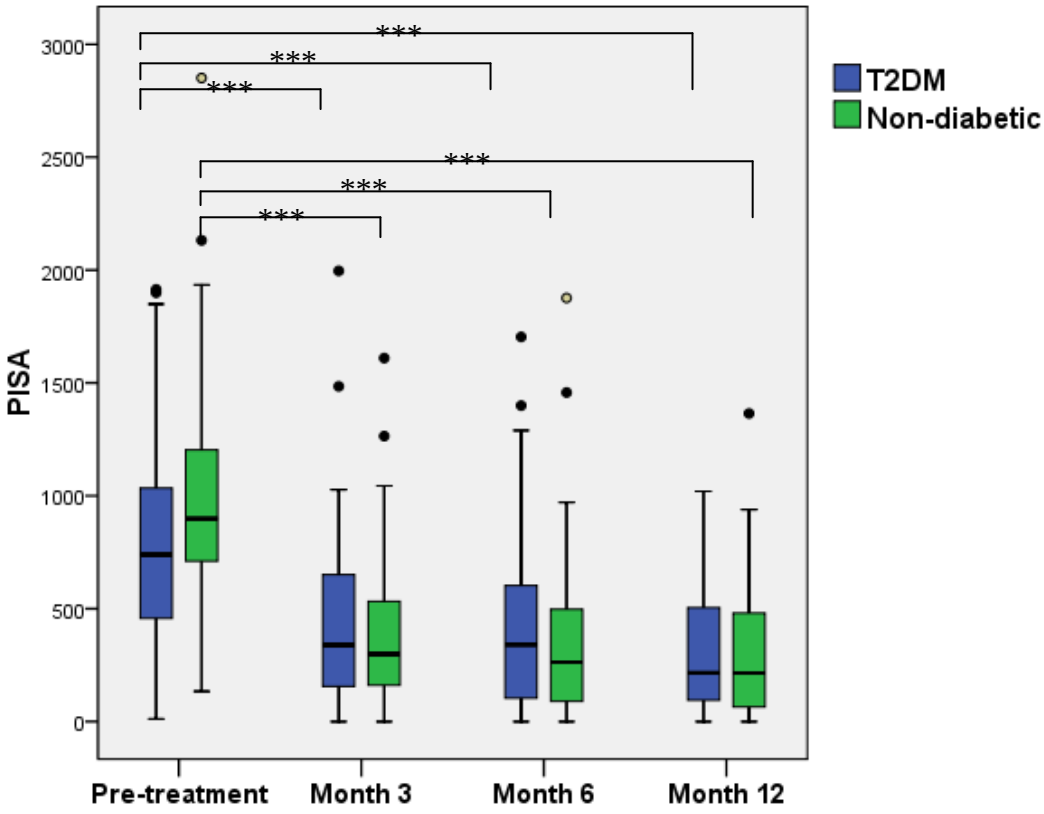
Boxplots of % of PD sites ≥5mm pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=47, month 3 n=37, month 6 n=35, month 12 n=29) and non-diabetic subjects (pre-treatment n=48, month 3 n=42, month 6 n=40, month 12 n=37)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.17 Mean PESA in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



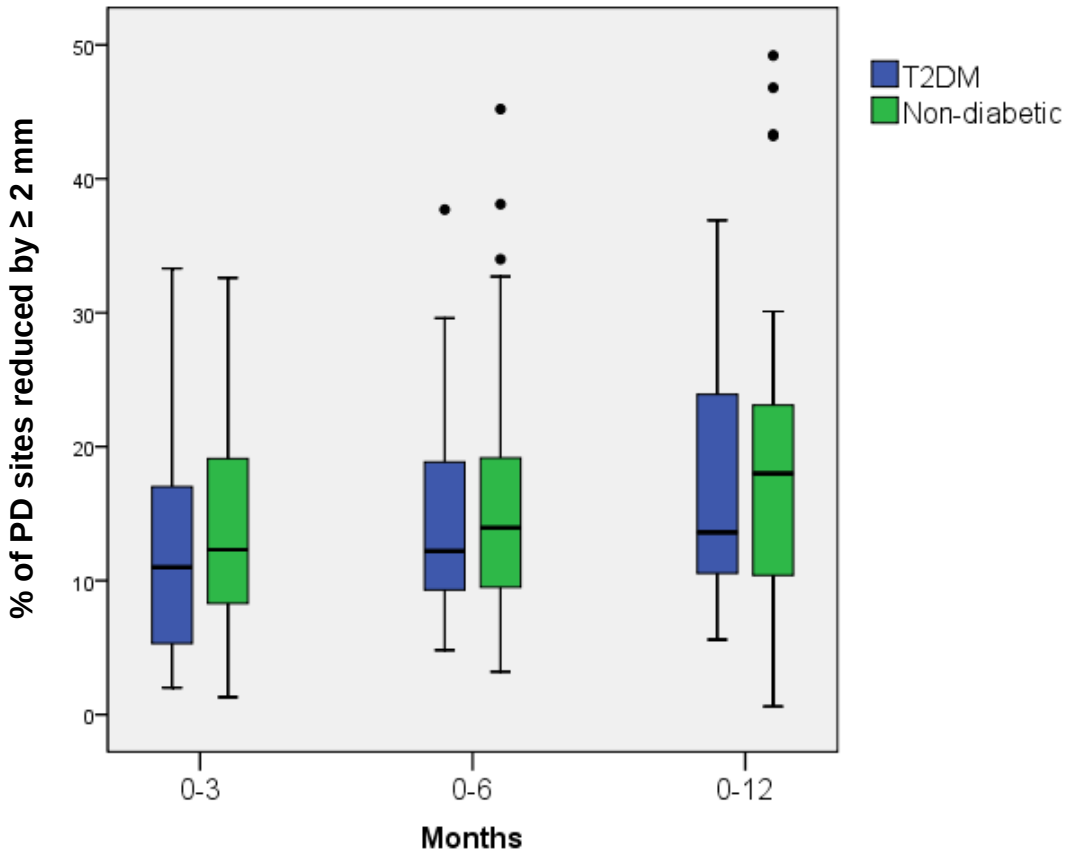
Boxplots of PESA pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=47, month 3 n=37, month 6 n=35, month 12 n=29) and non-diabetic subjects (pre-treatment n=48, month 3 n=42, month 6 n=40, month 12 n=37)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.18 Mean PISA in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



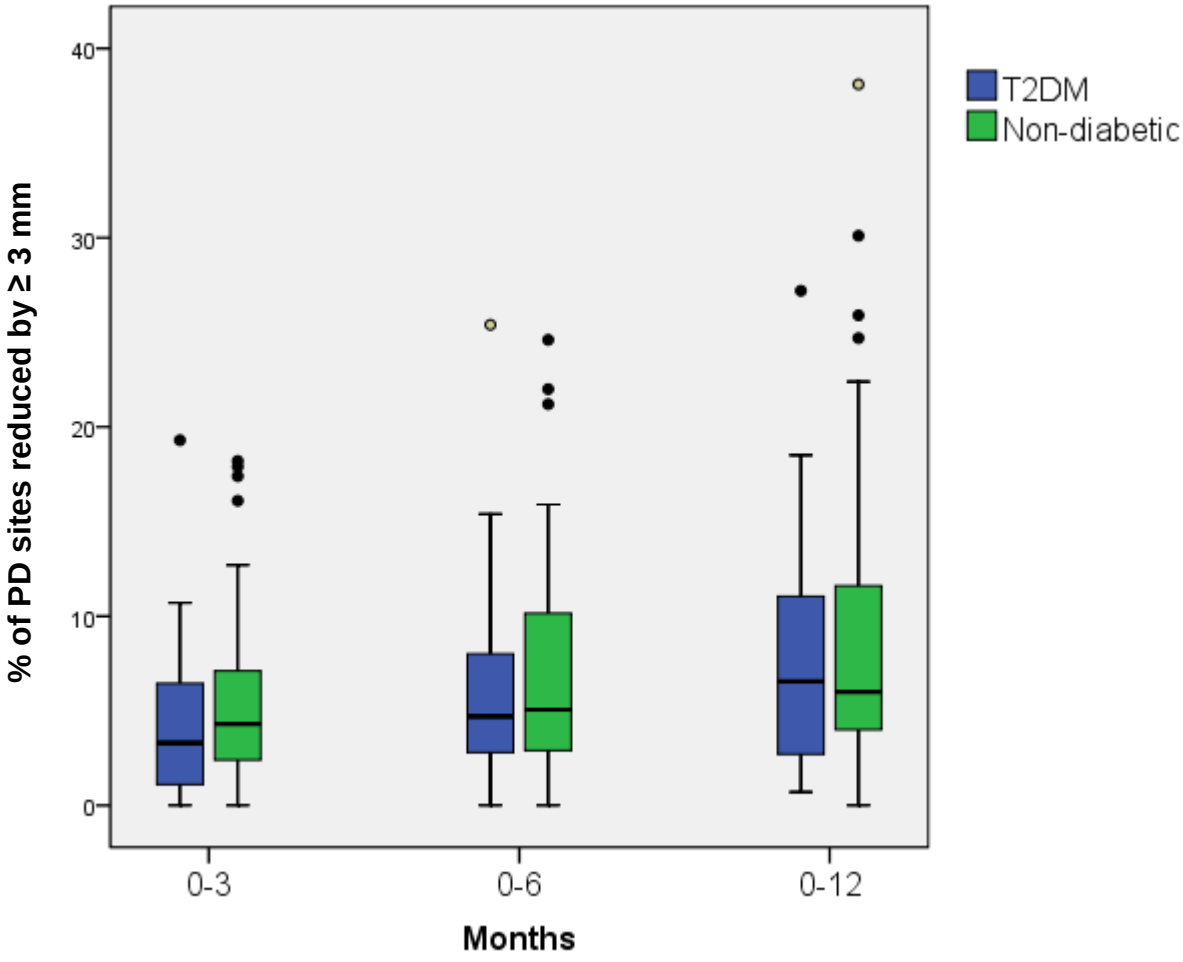
Boxplots of PISA pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=47, month 3 n=37, month 6 n=35, month 12 n=29) and non-diabetic subjects (pre-treatment n=48, month 3 n=42, month 6 n=40, month 12 n=37)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.19 % of PD sites showing ≥ 2 mm reduction in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



Boxplots of % of PD sites showing ≥ 2 mm reduction pre- and post NSM in subjects with periodontitis in both T2DM subjects (0-3 months n=36, 0-6 months n=35, 0-12 months n=37) and non-diabetic subjects (0-3months n=41, 0-6 months n=40, 0-12 months n=40)**.** Statistics: Mann-Whitney U test § $p<0.05$, §§ $p<0.01$, $^{\S\S\S}p<0.001$ (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.20 % of PD sites showing ≥ 3 mm reduction in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



Boxplots of % of PD sites showing ≥ 3 mm reduction pre- and post NSM in subjects with periodontitis in both T2DM subjects (0-3 months n=36, 0-6 months n=35, 0-12 months n=37) and non-diabetic subjects (0-3months n=41, 0-6 months n=40, 0-12 months n=40). Statistics: Mann-Whitney U test § $p<0.05$, §§ $p<0.01$, $^{§§§}p<0.001$ (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

6.2.4 Changes in local and systemic cytokine data following non-surgical periodontal management

6.2.4.1 Serum cytokine data

Table 6.8 and Figures 6.21 to 6.24 summarise the serum cytokine data following non-surgical periodontal management for patients with T2DM and periodontitis and non-diabetic patients with periodontitis.

When considering levels of IL-6 in serum, no significant differences were found between subjects with T2DM and non-diabetic subjects at pre-treatment, month 3 or month 6. At month 12, subjects with T2DM had significantly higher levels of serum IL-6 [0.91 (0.53-1.56) pg/ml] compared to non-diabetic subjects [0.61 (0.41-1.96) pg/ml]. Also, in subjects with T2DM and in non-diabetic subjects, no significant changes in serum IL-6 levels were demonstrated following NSM at any time-point. Furthermore, serum levels of IL-6 remained consistently around the lower limits of detection for the assay used (Table 6.8 and Figure 6.21).

With regards to serum TNF-α, pre-treatment levels were significantly higher in subjects with T2DM [7.10 (3.25-9.30) pg/ml] compared to non-diabetic subjects [3.44 (2.34-7.24) pg/ml]. However, at month 3 and 6, this situation reversed, with significantly higher levels of serum TNF-α levels in the non-diabetic subjects [6.43 (3.16-9.13) pg/ml and 7.06 (5.59-8.79) pg/ml] compared to the subjects withT2DM [3.70 (2.21-6.72) pg/ml and 3.86 (2.15-7.33) pg/ml]. At month 12, No significant differences in serum TNF-α levels were found between subjects with T2DM and non-diabetic subjects. In subjects withT2DM, compared to pre-treatment levels [7.10 (3.25-9.30) pg/ml], serum TNF-α levels, showed significant reductions following NSM at 3 months [3.70 (2.21-6.72) pg/ml] and 6 months [3.86 (2.15-7.33) pg/ml], with a non-significant increase being noted at month 12 [8.03 (5.28-9.62) pg/ml]. In non-diabetic

subjects, compared to pre-treatment levels [3.44 (2.34-7.24) pg/ml], serum TNF-α levels, showed significant increases following NSM at 6 months [7.06 (5.59-8.79) pg/ml] and 12 months [7.70 (6.71-10.20) pg/ml] (Table 6.8 and Figure 6.22).

For levels of IL-1β in serum, no significant differences were found between subjects with T2DM and non-diabetic subjects at month 3 or month 6. However, pre-treatment and at month 12, subjects with T2DM had significantly higher levels of serum IL-1β compared to non-diabetic subjects. In subjects withT2DM, compared to pre-treatment levels [0.08 (0.00-0.34) pg/ml], serum IL-1β levels showed significant reductions following NSM at 3 months [0.00 (0.00-1.15) pg/ml], 6 months [0.00 (0.00-0.13) pg/ml] and month 12 [0.04 (0.00-0.15) pg/ml]. In non-diabetic subjects, compared to pre-treatment levels, no changes in serum IL-1β was demonstrated at 6 and 12 months. At month 3 [0.03 (0.00-0.09) pg/ml] an increase was found in IL-1β levels compared to pre-treatment levels [0.00 (0.00-0.05) pg/ml], however, the Bonferroni-Holm correction of p-values for multiple comparisons placed the critical p-value at 0.017, therefore, this difference was reported as a trend (p=0.027). Furthermore, serum levels of IL-1β remained consistently around the lower limits of detection for the assay used (Table 6.8 and Figure 6.23).

For levels of IFN-γ in serum, no significant differences were found between subjects with T2DM and non-diabetic subjects at pre-treatment, month 3, month 6 and month 12. Also, in subjects with T2DM and in non-diabetic subjects, no significant changes in serum IFN-γ levels were seen following NSM at any time-point. Furthermore, serum levels of IFN-γ remained consistently around the lower limits of detection for the assay used (Table 6.8 and Figure 6.24).

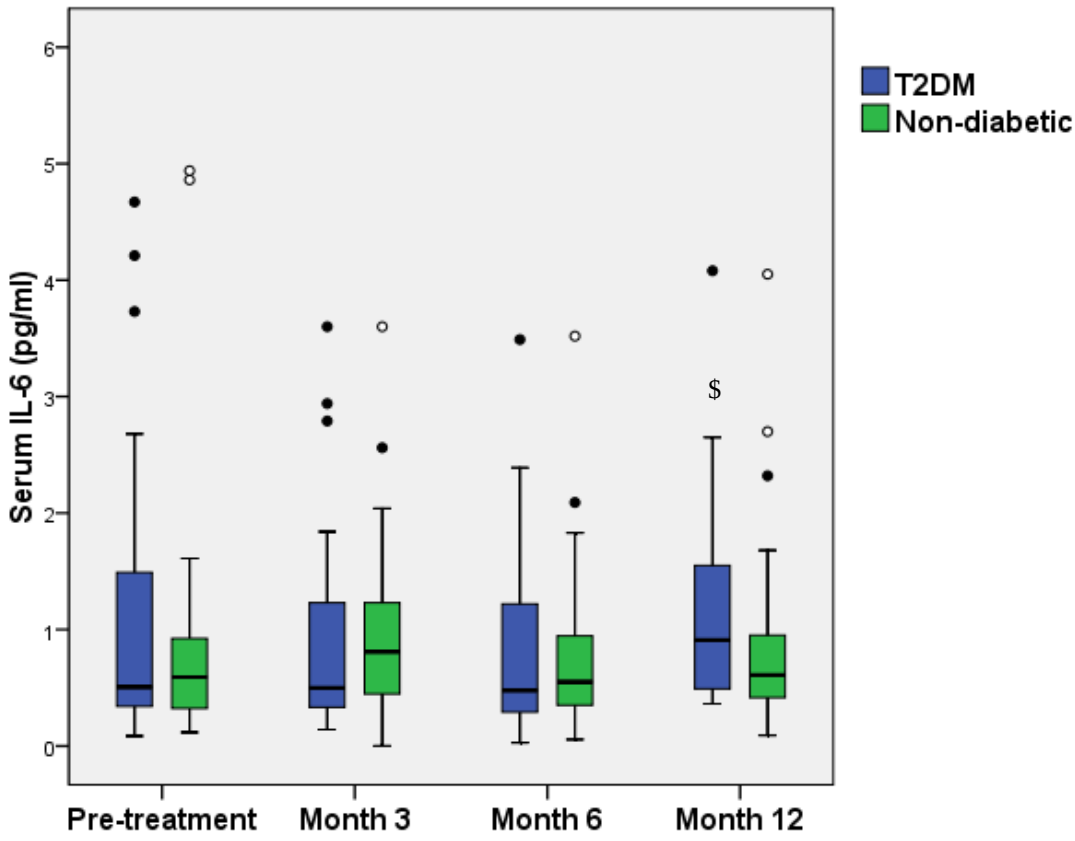
Table 6.8 Serum cytokine data in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects

	Pre-treatment		Month 3		Month 6		Month 12		
	T2DM subjects (n=46)	**Non-diabetic subjects (n= 45)**	**T2DM subjects (n=32)**	**Non-diabetic subjects (n= 38)**	**T2DM subjects (n=30)**	**Non-diabetic subjects (n= 38)**	**T2DM subjects (n=26)**	**Non-diabetic subjects (n= 34)**	**p-value**
Serum IL-6 (pg/ml)	0.51 (0.34-1.50)	0.59 (0.32-0.93)	0.50 (0.33-1.24)	0.81 (0.41-1.24)	0.47 (0.29-1.31)	0.55 (0.35-0.95)	0.91 (0.53-1.56)$	0.61 (0.41-1.96)	$<0.05
Serum TNF-α (pg/ml)	7.10 (3.25-9.30)#,¶,$	3.44 (2.34-7.24)¶,†	3.70 (2.21-6.72)$	6.43 (3.16-9.13)	3.86 (2.15-7.33)$	7.06 (5.59-8.79)	8.03 (5.28-9.62)	7.70 (6.71-10.20)	#,¶,$<0.05 †<0.001
Serum IL-1β (pg/ml)	0.08 (0.00-0.34)#,¶,†,$	0.00 (0.00-0.05)	0.00 (0.00-1.15)	0.03 (0.00-0.09)	0.00 (0.00-0.13)	0.00 (0.00-0.07)	0.04 (0.00-0.15)$	0.00 (0.00-0.00)	#,¶,†<0.05 $<0.001
Serum IFN-γ (pg/ml)	1.09 (0.56-2.50)$	0.57 (0.26-1.33)	0.74 (0.39-1.44)	1.07 (0.27-1.81)	0.90 (0.19-1.50)	1.11 (0.33-1.57)	1.08 (0.83-1.44)	1.11 (0.65-1.63)	$<0.01

P-values were determined using Friedman test with Wilcoxon post hoc test for continuous parametric variables compared over time and Mann-Whitney U test for continuous non-parametric data compared at each time point. Median (IQR) is presented as all the data were non-parametric.

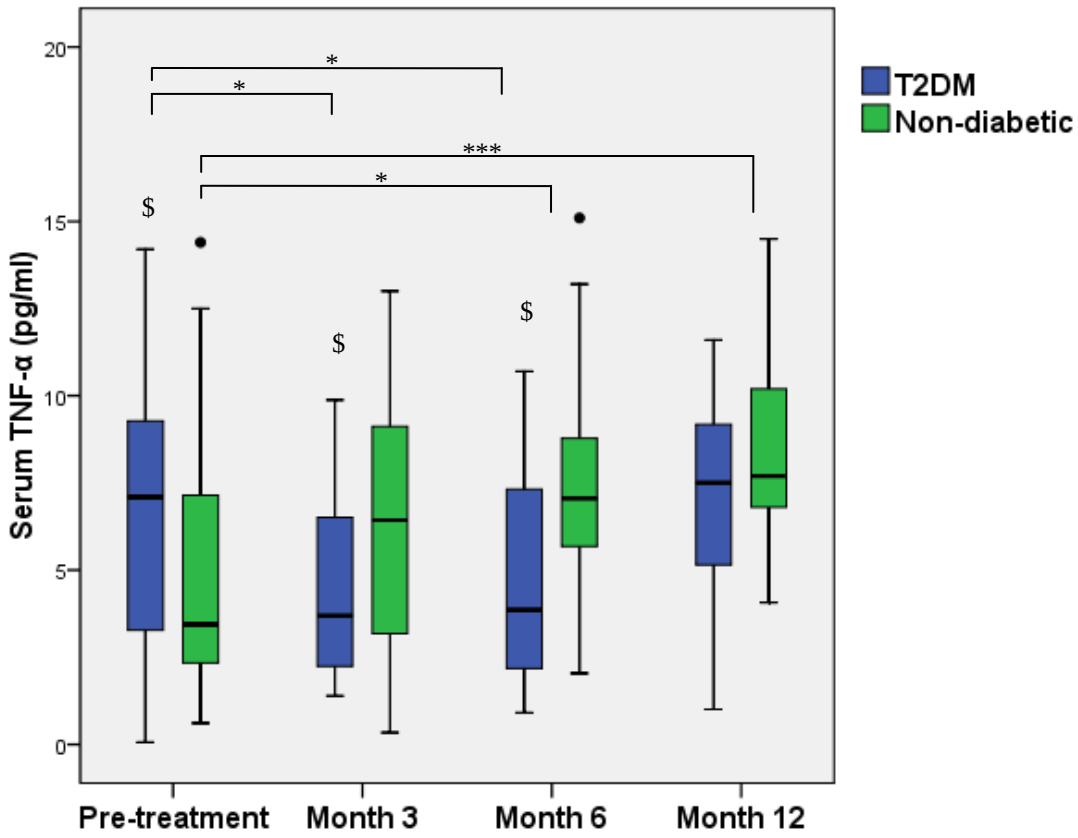
$ indicates a comparison within rows between of T2DM and non-diabetic groups at a specific time point
# indicates a comparison within rows between pre-treatment and month 3 within either T2DM or non-diabetic groups
¶ indicates a comparison within rows between pre-treatment and month 6 within either T2DM or non-diabetic groups
† indicates a comparison within rows between pre-treatment and month 12 within either T2DM or non-diabetic groups

Figure 6.21 Serum IL-6 levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



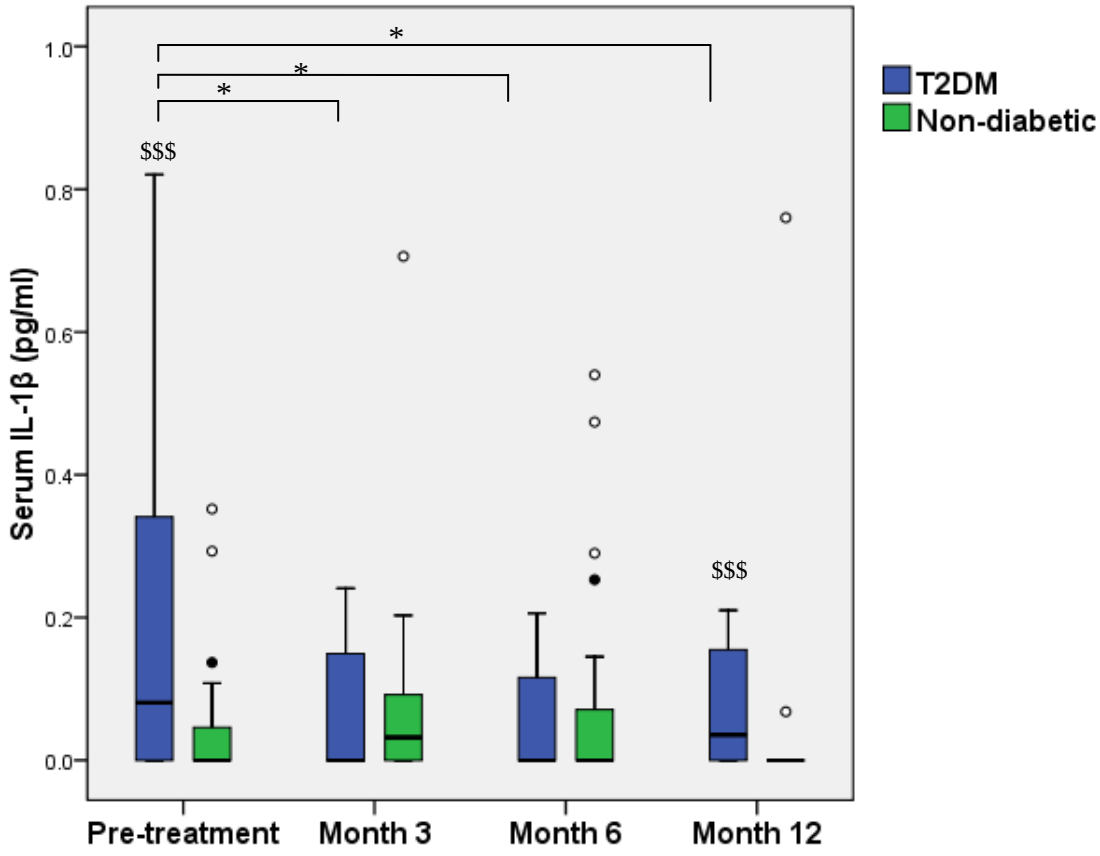
Boxplots of serum IL-6 levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=46, month 3 n=32, month 6 n=30, month 12 n=26) and non-diabetic subjects (pre-treatment n=45, month 3 n=38, month 6 n=38, month 12 n=34)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.22 Serum TNF-α levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



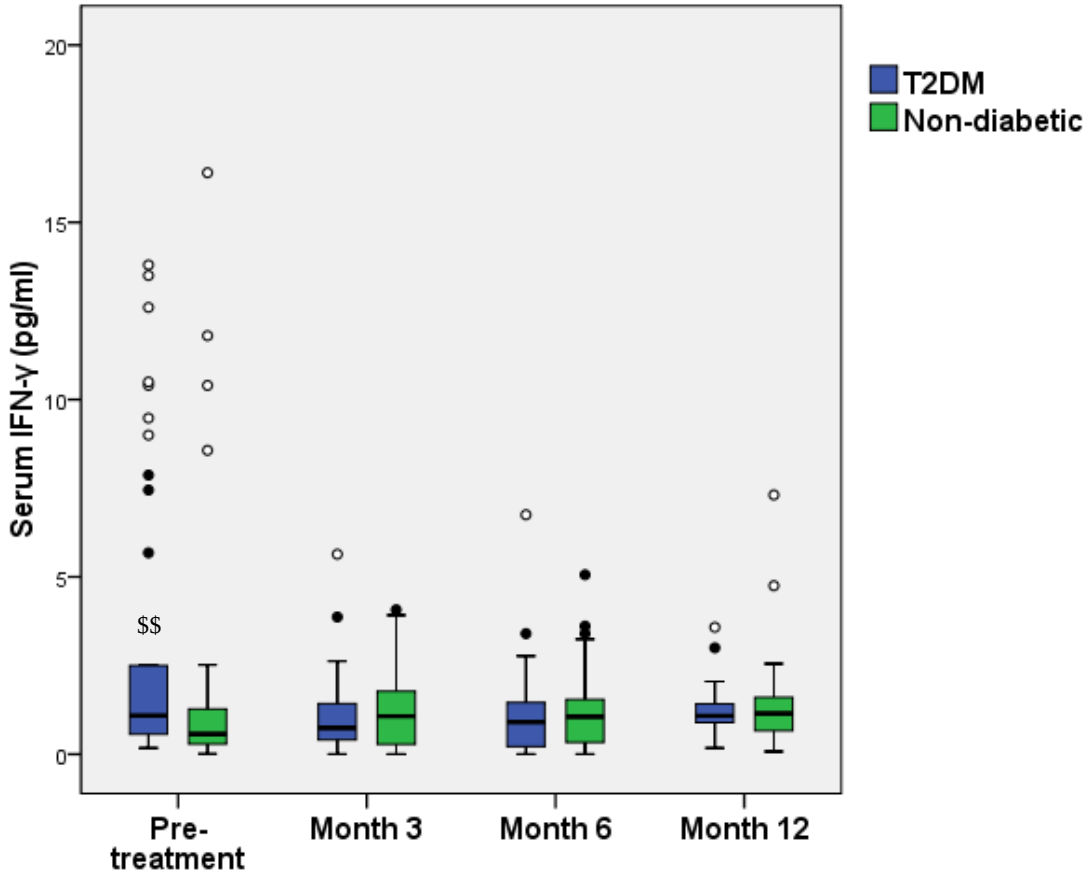
Boxplots of serum TNF-α levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=46, month 3 n=32, month 6 n=30, month 12 n=26) and non-diabetic subjects (pre-treatment n=45, month 3 n=38, month 6 n=38, month 12 n=34)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.23 Serum IL-1β levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



Boxplots of serum IL-1β levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=46, month 3 n=32, month 6 n=30, month 12 n=26) and non-diabetic subjects (pre-treatment n=45, month 3 n=38, month 6 n=38, month 12 n=34)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Serum 6.24 Serum IFN-γ levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



Boxplots of serum INF-γ levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=46, month 3 n=32, month 6 n=30, month 12 n=26) and non-diabetic subjects (pre-treatment n=45, month 3 n=38, month 6 n=38, month 12 n=34)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

6.2.4.2 Saliva cytokine data

Table 6.9 and Figures 6.28 to 6.28 summarise the saliva cytokine data following non-surgical periodontal management for patients with T2DM and periodontitis and non-diabetic patients with periodontitis.

When considering Il-6 levels in saliva, no significant differences were found between T2DM and non-diabetic subjects, except at month 3, when non-diabetic subjects [1.96 (1.17-8.07) pg/ml] had significantly higher levels of IL-6 in saliva compared to subjects with T2DM [0.90 (0.43-3.79) pg/ml]. In subjects with T2DM, compared to pre-treatment levels, no changes in saliva IL-6 were demonstrated at 6 and 12 months. In subjects with T2DM, at month 12 [4.06 (1.62-6.96) pg/ml] an apparent increase was found in IL-6 levels compared to pre-treatment levels [2.29 (1.27-4.83) pg/ml], however, the Bonferroni-Holm correction of p-values for multiple comparisons placed the critical p-value at 0.017, therefore, this difference was reported as a trend (p=0.04). In non-diabetic subjects, no significant changes in serum IL-6 levels were demonstrated following NSM at any time-point (Table 6.9 and Figure 6.25).

With regards to saliva TNF-α, pre-treatment and month 3 levels were significantly higher in non-diabetic subjects [2.59 (1.18-3.97) pg/ml and 2.58 (1.62-4.52) pg/ml] compared to subjects with T2DM [1.58 (0.61-3.08) pg/ml and 0.66 (0.26-1.53) pg/ml]. At month 6 and month 12, no differences in saliva TNF-α levels were found between T2DM and non-diabetic subjects. In subjects withT2DM, compared to pre-treatment levels [1.58 (0.61-3.08) pg/ml], saliva TNF-α levels showed significant reductions following NSM at 3 months [0.66 (0.26-1.53) pg/ml], however conversely a significant increase was noted at month 12 [2.88 (1.25-4.32) pg/ml]. Similarly, in non-diabetic subjects, compared to pre-treatment levels [2.59 (1.18-3.97) pg/ml], saliva TNF-α levels, showed a reduction at month 6 [1.59 (1.00-3.83) pg/ml], however, this change failed to reach statistical significance (Table 6.9 and Figure 6.26).

With regards to saliva IL-1β, pre-treatment and month 3 levels were significantly higher in non-diabetic subjects [62.60 (39.20-97.10) pg/ml and 55.75 (21.03-96.15) pg/ml] compared to T2DM subjects [38.65 (20.45-68.28) pg/ml and 19.00 (6.96-50.55) pg/ml]. For T2DM subjects, compared to pre-treatment levels [38.65(20.45-68.28)], saliva IL-1β showed a reduction at month 3 [19.00 (6.96-50.55) pg/ml], however, the Bonferroni-Holm correction of p-values for multiple comparisons placed the critical p-value at 0.017, therefore, this difference was reported as a trend (p=0.043). In non-diabetic subjects, compared to pre-treatment levels [62.60 (39.20-97.10) pg/ml], saliva IL-1β showed apparent reductions at month 3[55.75 (21.03-96.15) pg/ml], month 6 [38.55 (21.43-67.80) pg/ml] and month 12 [48.20 (27.10-86.20) pg/ml], although these differences failed to reach statistical significance. (Table 6.9 and figure 6.27).

For saliva IFN-γ, pre-treatment and month 3 levels, were significantly higher in non-diabetic subjects [1.55 (0.86-2.24) pg/ml and 1.26 (0.86-1.85) pg/ml] compared to subjects with T2DM [0.86 (0.47-1.60) pg/ml and 0.43 (0.25-0.78) pg/ml]. In subjects with T2DM, compared to pre-treatment [0.86 (0.47-1.60) pg/ml], saliva IFN-γ levels were significantly reduced at 3 months [0.43 (0.25-0.78) pg/ml], however conversely a significant increase was noted at month 12 [1.61 (1.03-2.69) pg/ml]. For non-diabetic subjects, compared to pre-treatment [1.55 (0.86-2.24) pg/ml], saliva IFN-γ levels were reduced at 6 months [0.83 (0.42-1.88) pg/ml], however, the Bonferroni-Holm correction of p-values for multiple comparisons placed the critical p-value at 0.017, therefore, this difference was reported as a trend (p=0.04) (Table 6.9 and Figure 6.28).

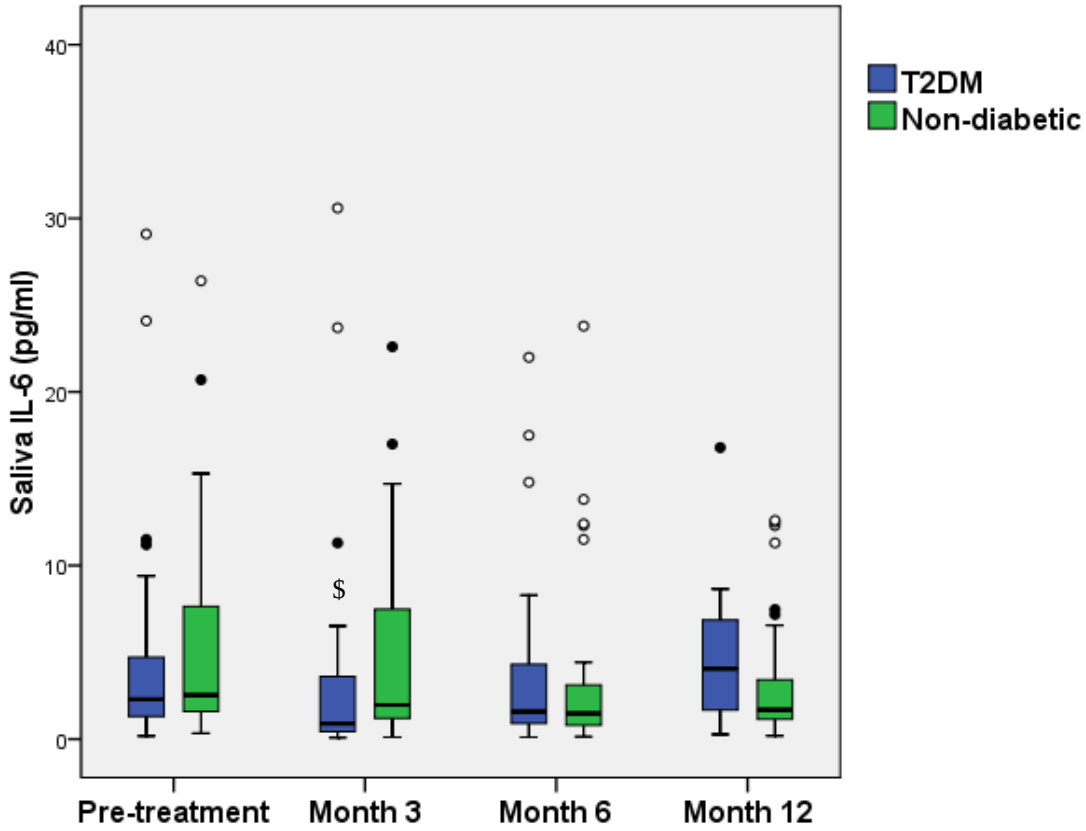
Table 6.9 Saliva cytokine data in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects

	Pre-treatment		Month 3		Month 6		Month 12		
	T2DM subjects (n=48)	**Non-diabetic subjects (n= 43)**	**T2DM subjects (n=33)**	**Non-diabetic subjects (n= 39)**	**T2DM subjects (n=30)**	**Non-diabetic subjects (n= 38)**	**T2DM subjects (n=26)**	**Non-diabetic subjects (n= 35)**	**p-value**
Saliva IL-6 (pg/ml)	2.29 (1.27-4.83)	2.41 (0.95-7.29)	0.90 (0.43-3.79)$^{\$}$	1.96 (1.17-8.07)	1.59 (0.87-4.35)	1.48 (0.79-3.21)	4.06 (1.62-6.96)	1.68 (1.13-3.42)	$^{\$}$<0.05
Saliva TNF-α (pg/ml)	1.58 (0.61-3.08)$^{\#,\dagger,\$}$	2.59 (1.18-3.97)	0.66 (0.26-1.53)$^{\$}$	2.58 (1.62-4.52)	1.45 (0.93-2.36)	1.59 (1.00-3.83)	2.88 (1.25-4.32)	2.73 (1.41-3.54)	$^{\#,\dagger,\$}$<0.05
Saliva IL-1β (pg/ml)	38.65 (20.45-68.28)$^{\$}$	62.60 (39.20-97.10)	19.00 (6.96-50.55)$^{\$}$	55.75 (21.03-96.15)	32.45 (14.28-88.40)	38.55 (21.43-67.80)	64.60 (26.88-121.50)	48.20 (27.10-86.20)	$^{\$}$<0.05
Saliva IFN-γ (pg/ml)	0.86 (0.47-1.60)$^{\#,\dagger,\$}$	1.55 (0.86-2.24)	0.43 (0.25-0.78)$^{\$}$	1.26 (0.86-1.85)	0.88 (0.56-1.64)	0.83 (0.42-1.88)	1.61 (1.03-2.69)	1.30 (0.80-1.96)	†<0.001 $^{\$,\#}$<0.01

P-values were determined using Friedman test with Wilcoxon post hoc test for continuous parametric variables compared over time and Mann-Whitney U test for continuous non-parametric data compared at each time point. Median (IQR) is presented as all the data were non-parametric.

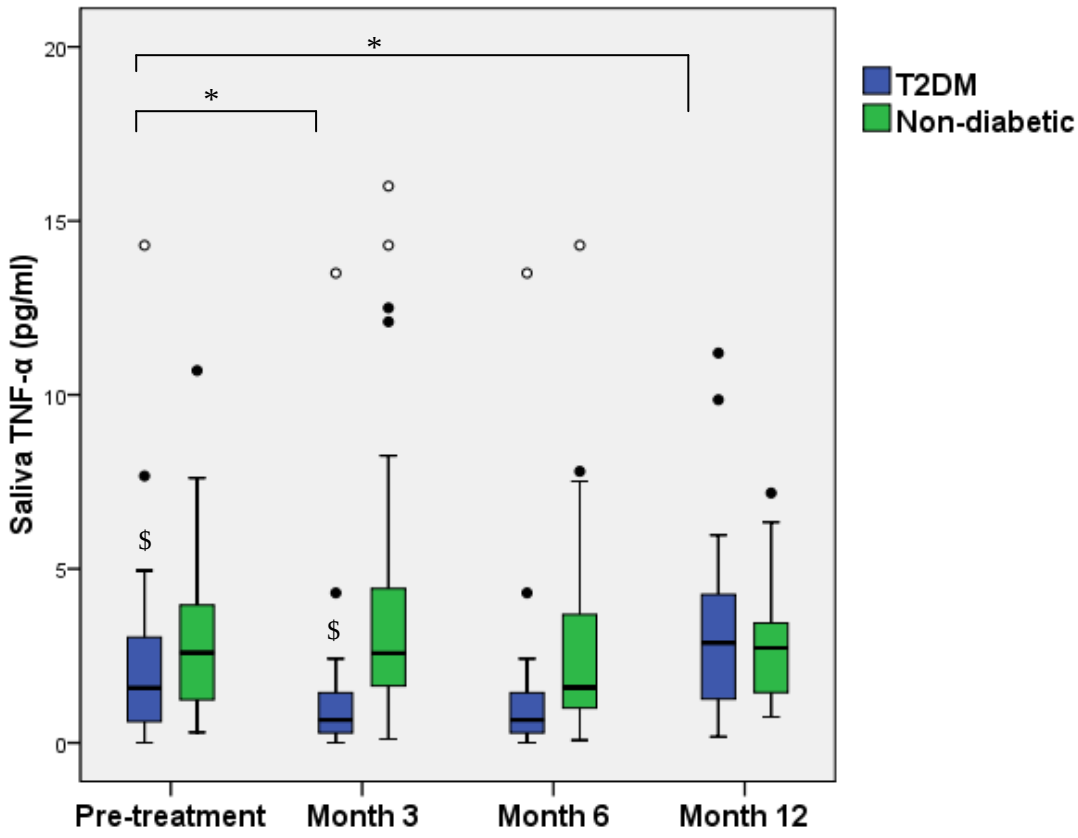
$^{\$}$ indicates a comparison within rows between of T2DM and non-diabetic groups at a specific time point
$^{\#}$ indicates a comparison within rows between pre-treatment and month 3 within either T2DM or non-diabetic groups
¶ indicates a comparison within rows between pre-treatment and month 6 within either T2DM or non-diabetic groups
† indicates a comparison within rows between pre-treatment and month 12 within either T2DM or non-diabetic groups

Figure 6.25 Saliva IL-6 levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



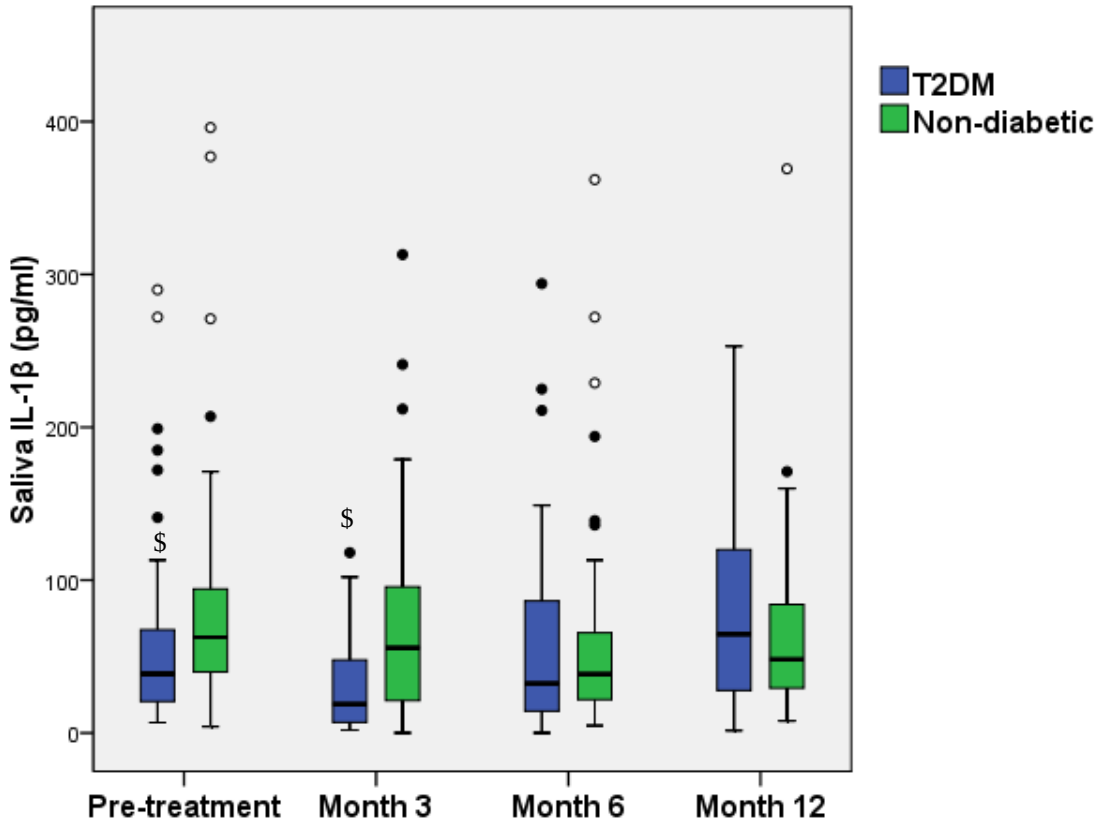
Boxplots of saliva IL-6 levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=48, month 3 n=33, month 6 n=30, month 12 n=26) and non-diabetic subjects (pre-treatment n=43, month 3 n=39, month 6 n=38, month 12 n=35)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.26 Saliva TNF-α levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



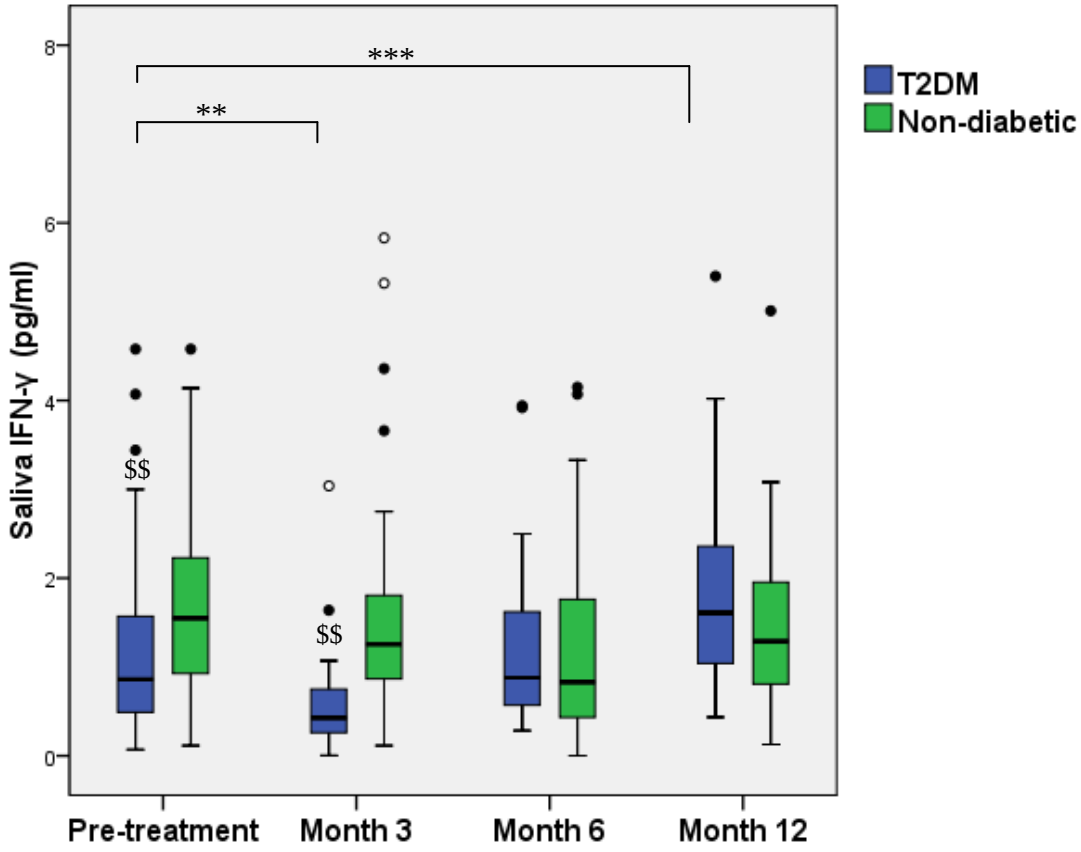
Boxplots of saliva TNF-α levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=48, month 3 n=33, month 6 n=30, month 12 n=26) and non-diabetic subjects (pre-treatment n=43, month 3 n=39, month 6 n=38, month 12 n=35)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figures 6.27 Saliva IL-1β levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



Boxplots of saliva IL-1β levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=48, month 3 n=33, month 6 n=30, month 12 n=26) and non-diabetic subjects (pre-treatment n=43, month 3 n=39, month 6 n=38, month 12 n=35)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.28 Saliva IFN-γ levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



Boxplots of saliva IFN-γ levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=48, month 3 n=33, month 6 n=30, month 12 n=26) and non-diabetic subjects (pre-treatment n=43, month 3 n=39, month 6 n=38, month 12 n=35)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

6.2.4.3 GCF cytokine data

Table 6.10 and Figures 6.29 to 6.32 summarise the GCF cytokine data following non-surgical periodontal management for patients with T2DM and periodontitis and non-diabetic patients with periodontitis.

When considering IL-6 levels in GCF, no significant differences were found between T2DM and non-diabetic subjects, except at month 6, when non-diabetic subjects [1.51 (0.94-2.34) pg/ml] had significantly higher levels of IL-6 in GCF compared to subjects with T2DM [0.89 (0.14-1.80) pg/ml]. In subjects with T2DM, compared to pre-treatment levels [1.97 (0.98-5.17) pg/ml], IL-6 in GCF showed significant reductions at month 3 [1.03 (0.51-2.70) pg/ml], month 6 [0.89(0.14-1.80)] and month 12 [1.03 (0.34-2.17) pg/ml]. In non-diabetic subjects, compared to pre-treatment levels [2.25(1.12-3.37)] IL-6 in GCF showed significant reductions at month 6 [1.51 (0.94-2.34) pg/ml] and non-significant reductions was also seen at month 3 [1.96 (0.93-3.32) pg/ml] and month 12 [1.28 (0.56-3.77) pg/ml] (Table 6.10 and Figure 6.29).

For TNF-α levels in GCF, no significant differences were found between T2DM and non-diabetic subjects, except at month 6, when non-diabetic subjects [4.36 (2.21-7.55) pg/ml] had significantly higher levels of IL-6 in GCF compared to subjects with T2DM [2.03 (0.65-3.80) pg/ml]. In subjects with T2DM, compared to pre-treatment levels [4.16 (2.72-6.69) pg/ml], TNF-α in GCF showed significant reductions at month 3 [3.59 (1.28-10.37) pg/ml], month 6 [2.03 (0.65-3.80) pg/ml] and month 12 [1.74 (0.96-3.45) pg/ml]. In non-diabetic subjects, compared to pre-treatment levels [4.49 (2.55-7.04) pg/ml], saliva IL-1β showed apparent reduction at month 12 [2.35 (1.09-3.55) pg/ml], although this difference failed to reach statistical significance (Table 6.10 and Figure 6.30).

When considering IL-1β levels in GCF, no significant differences were found between T2DM and non-diabetic subjects at any time-points. In subjects with T2DM compared to pre-treatment levels [344.33 (156.16-572.50) pg/ml], IL-1β in GCF showed significant reductions at month 3 [180.05 (68.98-382.93) pg/ml], month 6 [116.35 (48.71-206.56) pg/ml] and month 12 [175.65 (58.54-366.95) pg/ml]. Similarly, in non-diabetic subjects compared to pre-treatment levels [413.38 (213.83-770.56) pg/ml] IL-1β in GCF showed significant reductions at month 3 [166.25 (90.85-350.75) pg/ml], month 6 [150.60 (77.56-268.32) pg/ml] and month 12 [184.25 (77.51-291.71) pg/ml] (Table 6.10 and Figure 6.31).

For IFN-γ levels in GCF, no significant differences were found between T2DM and non-diabetic subjects, except before treatment, when non-diabetic subjects [4.40 (2.18-7.09) pg/ml] had significantly higher levels of INF-γ in GCF compared to subjects with T2DM [2.15 (1.11-5.24) pg/ml]. In subjects with T2DM, compared to pre-treatment levels [2.15 (1.11-5.24) pg/ml], saliva IFN-γ showed a significant reduction at month 6 [0.85 (0.23-1.89) pg/ml] and a non-significant reduction at month 3 [1.29 (0.35-3.46) pg/ml] and month 12 [1.62 (0.64-3.37) pg/ml]. In non-diabetic subjects, compared to pre-treatment levels [4.40 (2.18-7.09) pg/ml], saliva IFN-γ showed a significant reduction at month 3 [2.54 (0.92-4.87) pg/ml], month 6 [2.21 (1.41-4.23) pg/ml] and month 12 [1.43 (0.50-2.46) pg/ml] (Table 6.10 and Figure 6.32).

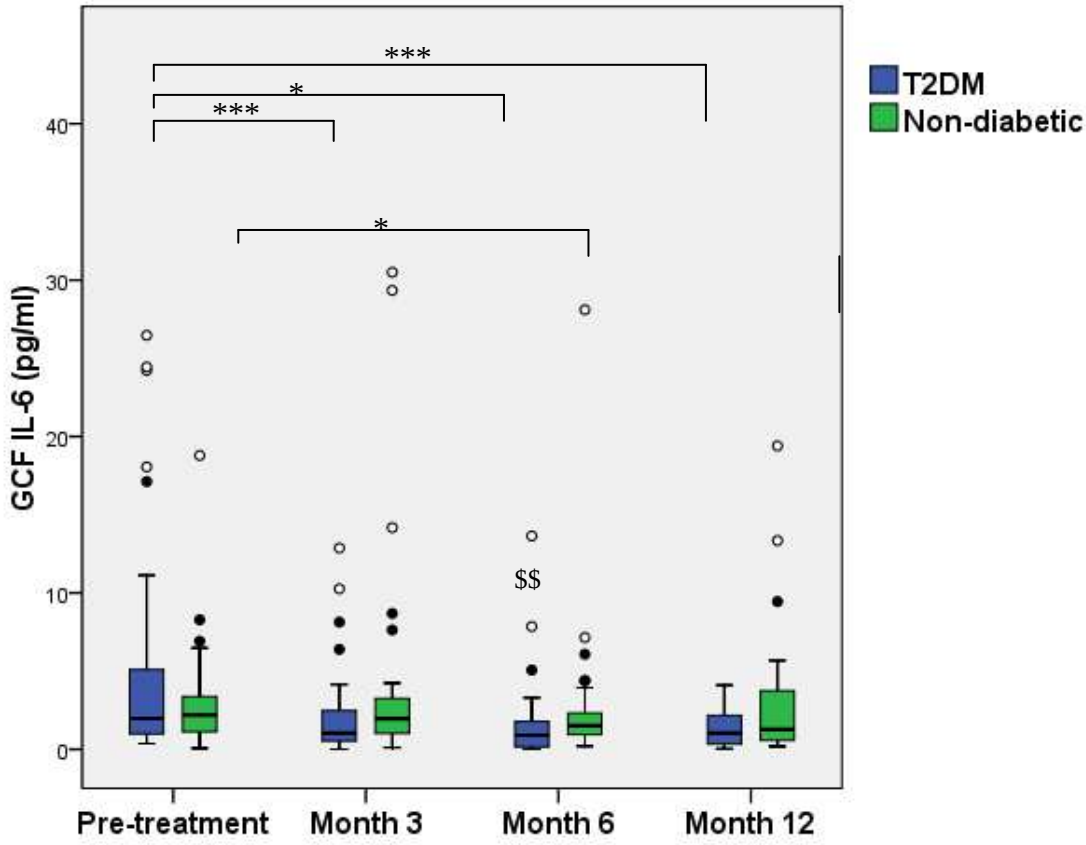
Table 6.10 GCF cytokine data in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects

	Pre-treatment		Month 3		Month 6		Month 12		
	T2DM subjects (n=48)	**Non-diabetic subjects (n= 47)**	**T2DM subjects (n=35)**	**Non-diabetic subjects (n= 38)**	**T2DM subjects (n=36)**	**Non-diabetic subjects (n= 38)**	**T2DM subjects (n=28)**	**Non-diabetic subjects (n= 36)**	**p-value**
GCF vol (µl)	0.50 (0.38-0.67)[¶,#]	0.51(0.34-0.68)	0.29 (0.20-0.46)	0.46 (0.28-0.59)	0.41 (0.34-0.53)	0.34 (0.23-0.66)	0.40 (0.29-0.58)	0.43 (0.30-0.64)	[¶,]<0.01 [#,]<0.001
GCF IL-6 (pg/ml)	1.97 (0.98-5.17)[#,¶,†]	2.25 (1.12-3.37)[¶]	1.03 (0.51-2.70)	1.96 (0.93-3.32)	0.89 (0.14-1.80)[$]	1.51 (0.94-2.34)	1.03 (0.34-2.17)	1.28 (0.56-3.77)	[¶,$]<0.05 [#,†]<0.001
GCF TNF-α (pg/ml)	4.16 (2.72-6.69)[¶,†]	4.49 (2.55-7.04)[†]	3.59 (1.28-10.37)	4.82 (2.45-7.56)	2.03 (0.65-3.80)[$]	4.36 (2.21-7.55)	1.74 (0.96-3.45)	2.35 (1.09-3.55)	[¶,†]<0.001
GCF IL-1β (pg/ml)	344.33 (156.16-572.50)[#,¶,†]	413.38 (213.83-770.56)[#,¶,†]	180.05 (68.98-382.93)	166.25 (90.85-350.75)	116.35 (48.71-206.56)	150.60 (77.56-268.32)	175.65 (58.54-366.95)	184.25 (77.51-291.71)	[¶]<0.001 [#,†]<0.01
GCF IFN-γ (pg/ml)	2.51 (1.11-5.24)[¶,$]	4.40 (2.18-7.09)[#,¶,†]	1.29 (0.35-3.46)	2.54 (0.92-4.87)	0.85 (0.23-1.89)[$]	2.21 (1.41-4.23)	1.62 (0.64-3.37)	1.43 (0.50-2.46)	[¶,$]<0.05 [†,#]<0.01

P-values were determined using Friedman test with Wilcoxon post hoc test for continuous parametric variables compared over time and Mann-Whitney U test for continuous non-parametric data compared at each time point. Median (IQR) is presented as all the data were non-parametric.

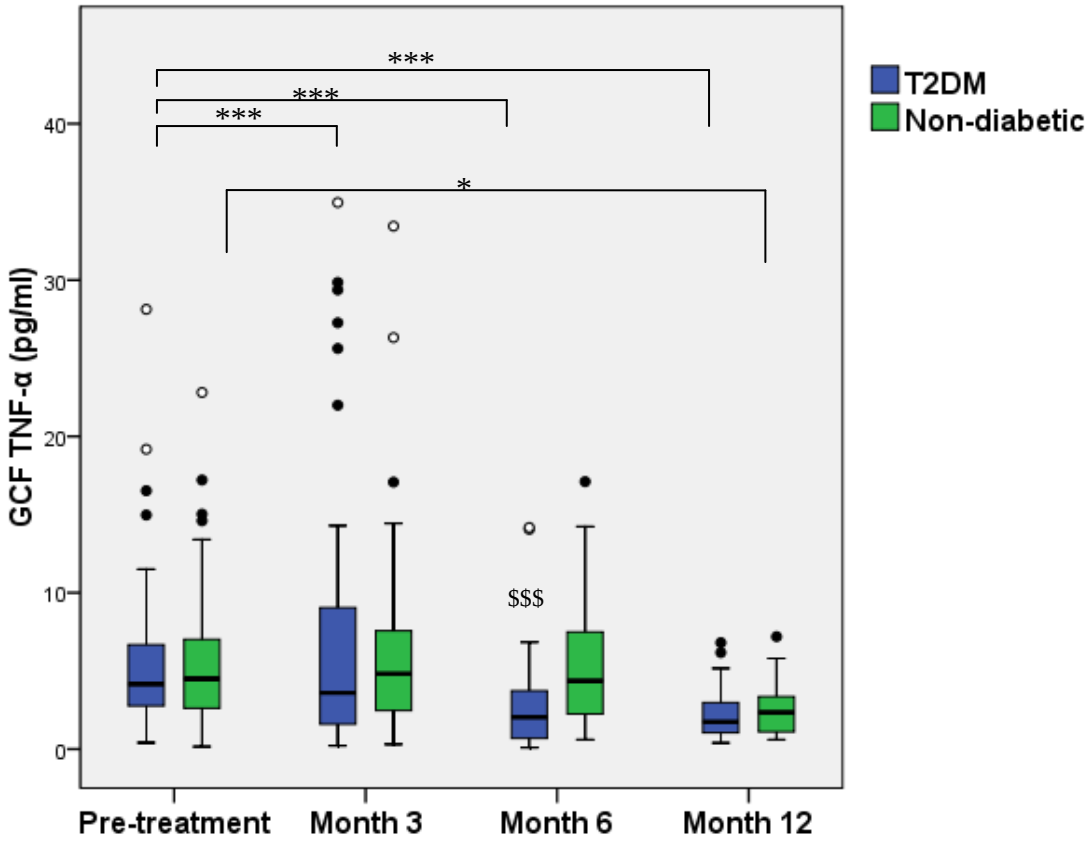
[$] indicates a comparison within rows between of T2DM and non-diabetic groups at a specific time point
[#] indicates a comparison within rows between pre-treatment and month 3 within either T2DM or non-diabetic groups
[¶] indicates a comparison within rows between pre-treatment and month 6 within either T2DM or non-diabetic groups
[†] indicates a comparison within rows between pre-treatment and month 12 within either T2DM or non-diabetic groups

Figure 6.29 GCF IL-6 levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



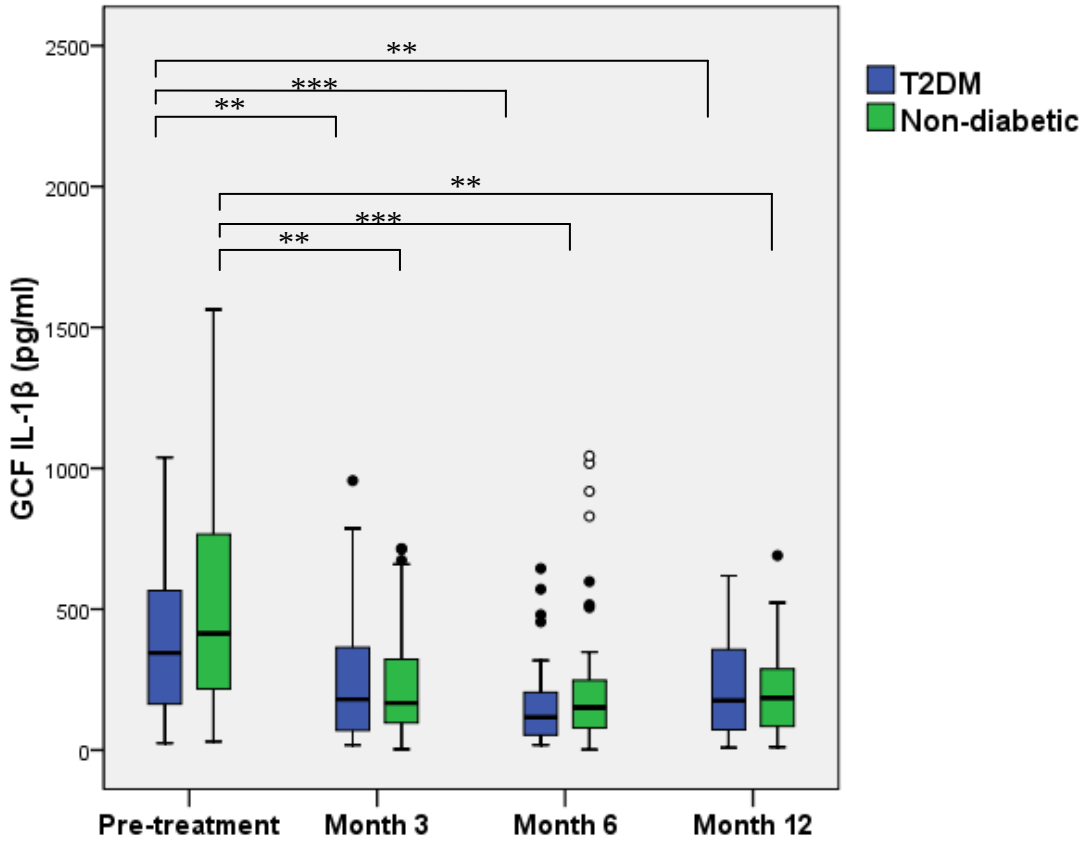
Boxplots of GCF IL-6 levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=48, month 3 n=35, month 6 n=36, month 12 n=28) and non-diabetic subjects (pre-treatment n=47, month 3 n=38, month 6 n=38, month 12 n=36)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.30 GCF TNF-α levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



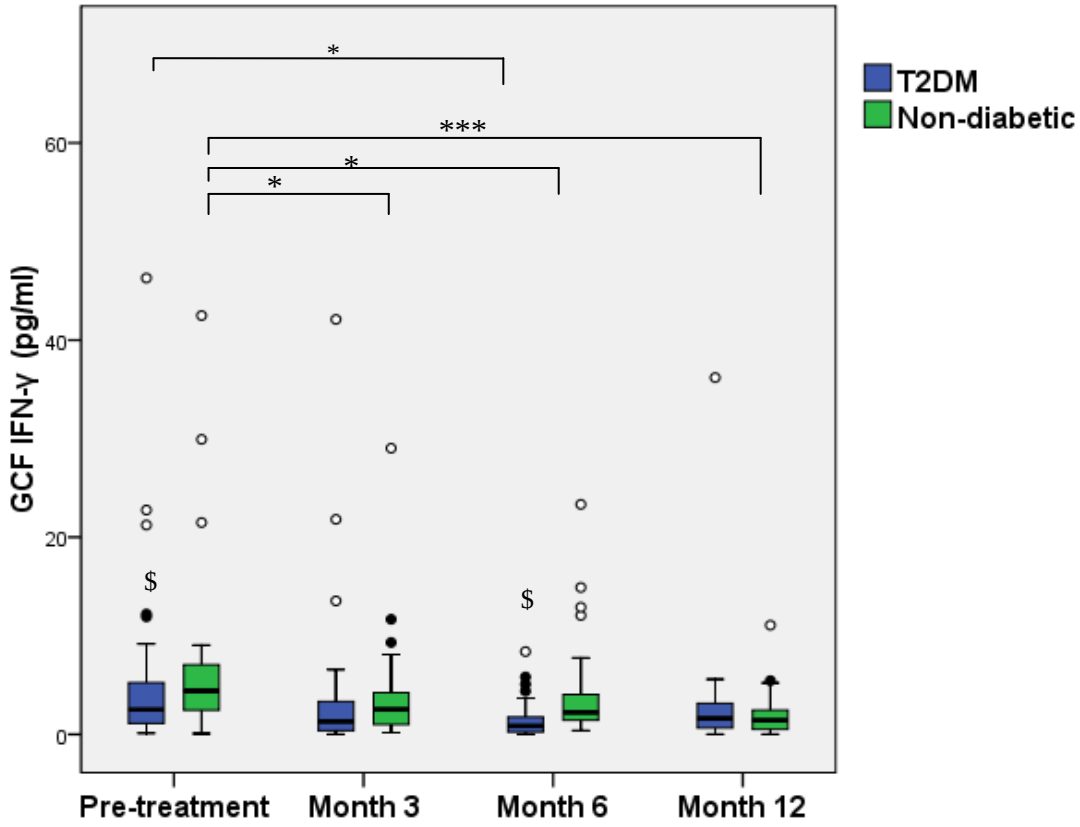
Boxplots of GCF TNF-α levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=48, month 3 n=35, month 6 n=36, month 12 n=28) and non-diabetic subjects (pre-treatment n=47, month 3 n=38, month 6 n=38, month 12 n=36)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.31 GCF IL-1β levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



Boxplots of GCF IL-1β levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=48, month 3 n=35, month 6 n=36, month 12 n=28) and non-diabetic subjects (pre-treatment n=47, month 3 n=38, month 6 n=38, month 12 n=36)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

Figure 6.32 GCF IFN-γ levels in subjects with periodontitis pre- and post NSM for both T2DM and non-diabetic subjects



Boxplots of GCF IFN-γ levels pre- and post NSM in subjects with periodontitis in both T2DM subjects (pre-treatment n=48, month 3 n=35, month 6 n=36, month 12 n=28) and non-diabetic subjects (pre-treatment n=47, month 3 n=38, month 6 n=38, month 12 n=36)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001(according to time within T2DM or non-diabetic group); Mann-Whitney U test § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups at each time point). ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

6.3 Discussion

Changes in BMI

In this study, it is clear that obesity, as defined by BMI, is closely linked to the presence of T2DM. At each time point, BMI was significantly higher in subjects with T2DM compared to non-diabetic subjects (Table 6.1 and Figure 6.1). Similarly, a greater proportion of subjects classified as obese and morbidly obese were found in the T2DM group compared to the non-diabetic group at each time point (Table 6.1). This reflects previous research that clearly demonstrates obesity as a risk factor for T2DM (Lundgren *et al.*, 1989; Chan *et al.*, 1994; Carey *et al.*, 1997; Wang *et al.*, 2005). Furthermore, in the present study, subjects with T2DM show a significant reduction in BMI from pre-treatment [33.0 (29.9-36.5) kg/m^2] to month 6 [31.0 (27.6-34.8) kg/m^2] and month 12 [31.6 (28.7-34.5) kg/m^2]. However, further analysis of the number of subjects within each BMI category at each time point suggests there is potentially a higher drop-out of obese and morbidly obese subjects compared to the non-obese subjects, perhaps contributing to the apparent reduction in BMI seen in subjects with T2DM following periodontal management. It is therefore difficult to draw clear conclusions about actual weight loss following periodontal treatment, however, the impact of periodontal treatment on weight reduction is unlikely to be significant.

Changes in biochemistry data

Within T2DM subjects, the risk of diabetic complications is strongly associated with previous hyperglycaemia and a 1% reduction in HbA1c has been associated with a 21% risk reduction in diabetic complications (Stratton 2000). In the present study pre-treatment HbA1c levels [7.5 (6.7-9.2)%] showed a reduction at month 3 [7.05 (6.6-9.5)%] and month 12 [7.1 (6.6-7.4)%], which is a reduction of 0.45% and 0.40%

respectively, although these reductions did not reach statistical significance (Table 6.3 and Figure 6.3). This is in line with past studies (Promsudthi *et al.*, 2005; Jones *et al.*, 2007; Correa *et al.*, 2010). In one study, pre-treatment HbA1c levels [8.98±0.88%] showed a non-significant decrease in HbA1c at 3 months [8.79±1.24%] in subjects receiving periodontal treatment (n=27) and a non-significant rise from pre-treatment [9.17±1.02%] to month 3 [9.28±1.50%] in subjects receiving no treatment (n=25) (Promsudthi *et al.*, 2005). In a larger RCT, at 4 months no significant differences in the change in HbA1c was showed when subjects receiving periodontal treatment (n=83) were compared against untreated subjects (n=82), such that, the mean HbA1c change in the treatment group was -0.65% compared to -0.51% in the untreated group (Jones *et al.*, 2007). However, other studies suggest that control of periodontal infection significantly does improve metabolic control (Stewart *et al.*, 2001; Rodrigues *et al.*, 2003; Kiran *et al.*, 2005; O'Connell *et al.*, 2008; Kardesler *et al.*, 2011). In one such study, pre-treatment HbA1c levels [7.31±0.74%] showed a significant decrease at 3 months [6.51±0.80%] in subjects receiving periodontal treatment (n=22) and a non-significant rise from pre-treatment [7.00±0.72%] to month 3 [7.31±2.08%] in subjects receiving no treatment (n=22) (Kiran *et al.*, 2005). Interestingly, the 0.45 % and 0.40% reductions in HbA1c seen in this study following NSM are comparable to data from the most recent meta-analysis which showed that when compared against no treatment or usual treatment, NSM gave a statistically significant 0.40% HbA1c reduction (p=0.04) (Simpson *et al.*, 2010). Within the current study, at month 12 there was a reduction in the number of T2DM subjects categorised as having poor glycaemic along with a concurrent increase in the number of subjects categorised as having moderate glycaemic control, making it unlikely that the reduction in HbA1c seen at month 12 was due to drop of subjects categorised as having poor glycaemic control.

In some studies, changes in HbA1c data following periodontal management were assessed separately in well controlled and poorly controlled T2DM patients (Dag *et al.*, 2009; Kardesler *et al.*, 2010; Santos *et al.*, 2010), with one study demonstrating significant reductions only in well controlled T2DM subjects (Dag et al., 2009), another study demonstrating significant reductions only in poorly controlled T2DM subjects (Kardesler *et al.*, 2010) and a further study showing no change in HbA1c in either group (Santos et al., 2010). In our study, in T2DM patients with poorer initial glycaemic control, as indicated by HbA1c ≥ 7.5%, there was a 1.9 % reduction in HbA1c levels from pre-treatment [9.2 (8.3-10.2)%] to month 12 [7.3 (7.1-8.7)%], although, the reduction did not reach statistical significance. Stratification of subjects based on HbA1c was not built into the recruitment strategy for the current study and to prevent low n values in groups, data were not subsequently stratified when analyses were performed.

Taken collectively, data from this present study and previous studies would appear to support that non-surgical periodontal management can produce an overall positive effect on glycaemic control. However, this would need to be confirmed in an interventional study with sufficient number of subjects to account for variations inherent in HbA1c between patients. Additionally, the method of periodontal therapy used in the future studies should also be carefully considered. In some of previous studies evaluating the impact of periodontal therapy on HbA1c, the NSM was supplemented with local (Iwamoto *et al.*, 2001) or systemic (O'Connell *et al.*, 2008) antibiotic administration. In this regard it is important to highlight the fact that tetracycline and its derivatives may act directly on insulin production (Qin *et al.*, 2002) and thus some of the reductions of HbA1c following therapy in some studies could be due to the antibiotic per se not only due to improvements in periodontal health. Also, the current

mainstay of periodontal management, particularly in the UK, is non-surgical management and given there is insufficient evidence to recommend the routine use of systemic antibiotics in the treatment of periodontitis (Sanz and Teughels, 2008), the method of periodontal therapy used in research should reflect this and thus allow generalisation of the results to the general population.

Data from the present study showed that, at each time point, HDL, non-HDL and total cholesterol were significantly higher in non-diabetic subjects compared to subjects with T2DM (Table 6.3). This is not surprising, given that the management of raised levels of serum lipids is a key priority within the national management guidelines for T2DM (NICE, 2008). Conversely, levels of triglycerides were significantly higher in subjects with T2DM compared to non-diabetic subjects at pre-treatment, month 3 and month 12. Caution must, however, be used when interpreting this result given that the serum samples taken as part of this study were not fasting samples, and food intake can lead to sharp rises in triglycerides levels. Potentially, T2DM subjects may have demonstrated raised levels of triglycerides due to more regular eating patterns. However, this cannot be confirmed within the present study as data regarding dietary intake were not collected. Data from the present study did not however, confirm previous research. One study found no significant differences between T2DM (well and poorly controlled) and non-diabetic subjects at any time points, except for LDL which was lower in well controlled T2DM compared to poorly controlled T2DM patients (Kardesler *et al.*, 2010) and another study found no differences in triglyceride and total cholesterol when diabetic subjects were compared to non-diabetic subjects (Christgau et al., 1998).

The present study also assessed the effect of improved periodontal health on serum lipid profiles in subjects with T2DM and periodontitis, however, no changes

were demonstrated in levels of triglycerides, HDL, non-HDL and total cholesterol following NSM (Table 6.3). This corroborates data from previous studies that demonstrated minimal changes in lipid profiles in subjects withT2DM following NSM (Christgau *et al.*, 1998; Kiran *et al.*, 2005; Kardesler *et al.*, 2010). Given that most T2DM subjects are likely to already receive intensive management of raised serum lipid levels as part of their T2DM management, the scope for further improvements in serum lipid profiles as a result of improved periodontal health is likely to be minimal. Interestingly, in the current study, a significant reduction in non-HDL and total cholesterol was demonstrated in non-diabetic subjects 6 months after treatment (Table 6.3). This supported a recent small study of subjects with hyperlipidemia and periodontitis that found that significant reductions in LDL and total cholesterol were demonstrated after NSM (Fentoglu et al., 2010). Given the potential role of serum lipids in the relationship between systemic disease and periodontitis (Iacopino and Cutler, 2000), further studies are required to clarify the effect of periodontal healing on lipid profiles in subjects with and without diabetes.

Data from the present study demonstrated a reduction in hsCRP levels at month 12 compared to pre-treatment levels in subjects with T2DM (2.1 (0.9-4.6) mg/L to 1.5(0.7-3.1) mg/L and non-diabetic subjects (2.3 (1.1-4.3) mg/L to 1.6 (0.7-2.9) mg/L, however, the reductions failed to reach statistical significance (Table 6.3 and Figure 6.8). This is in line with previous studies that demonstrated similar non-significant reductions in hsCRP following periodontal therapy in T2DM subjects (Christgau *et al.*, 1998; Correa *et al.*, 2010; Kardesler *et al.*, 2010). The wide variations in CRP levels seen in the current study are likely to be due to the non-specific nature of CRP as marker of systemic inflammation. In addition to T2DM and periodontitis, many other systemic conditions may impact upon CRP levels and transient increases in CRP can

occur with acute illness and thus a single measurement may not represent the true basal levels of CRP. Although at recruitment stage, patients with serious systemic illnesses were excluded from the study, extending exclusion criteria has to be weighed up against the effect it will have on recruitment of subjects and how effectively the research results can then be generalised to larger populations. Therefore, to clarify the effect of periodontal healing on systemic CRP levels in subjects with T2DM, it would be more appropriate to account for the variations seen in CRP by using the data generated from this and other studies to estimate, more accurately, the number of subjects required in a future studies.

Changes in Periodontal parameters

The significant reductions in all clinical periodontal parameters (BOP, mGI, PI, mean PD, PESA, PISA, % PD sites ≥5mm) at each post- treatment follow-up time point, in both diabetic and non-diabetic subjects, indicate that non-surgical periodontal treatment was effective and this post-treatment response was maintained for 12 months following initial treatment. Furthermore, in addition to the significant reductions in all clinical periodontal parameters seen in both T2DM and non-diabetic patients, few differences were found when comparing between groups each time point, confirming that NSM was effective regardless of diabetes status. This is in line with data from previous research that demonstrates significant improvements in clinical periodontal parameters following periodontal management in subjects with T2DM (Rodrigues *et al.*, 2003; Kiran *et al.*, 2005; Correa *et al.*, 2008; O'Connell *et al.*, 2008; Dag *et al.*, 2009; Correa *et al.*, 2010; Kardesler *et al.*, 2010; Santos *et al.*, 2010; Kardesler *et al.*, 2011).

Previously, in a number of small studies, the clinical periodontal response to different treatment therapies has been investigated in subjects with T2DM (Rodrigues *et al.*, 2003; O'Connell *et al.*, 2008; Correa *et al.*, 2010). One study of subjects with T2DM

and periodontitis (n=23) showed after NSM, compared to pre-treatment levels of BOP [90.4±10.5%], mean PD [4.2±1.0 mm], mean CAL [5.4±1.3 mm], % PD sites ≥ 5 mm [38.0±22.8 %] significant reductions were seen at 3 months post treatment in BOP [28.9±16.5%], mean PD [2.8±0.4 mm], mean CAL [4.6±1.1 mm], % PD sites ≥ 5 mm [7.5±6.8%] (Correa *et al.*, 2010). Another small study of 30 T2DM subjects comparing the effect of 2 periodontal treatment therapies showed significant improvements in periodontal parameters at the 3 month follow up (Rodrigues et al., 2003). In the NSM group, pre-treatment mean PD [2.7±0.7 mm] and BOP [38±13%] were significantly reduced at month 3 with mean PD [1.9±0.4 mm] and BOP [15±9%] being recorded. In the group receiving NSM plus antibiotics, pre-treatment mean PD [3.2±0.8 mm] and BOP [32±15%] were significantly reduced at month 3 with mean PD [2.3±0.5 mm] and BOP [11±7%] being recorded. Furthermore, a comparison between the groups also failed to demonstrate significant differences in any of the clinical outcomes (Rodrigues et al., 2003). A further small study of 30 T2DM subjects comparing 2 periodontal treatment therapies showed significant improvements in periodontal parameters at the 3 month follow-up (O'Connell *et al.*, 2008). Once again, in the NSM group, pre-treatment mean PD [2.9±0.8 mm], number of PD sites ≥ 6 mm [12.6±14.1] and BOP [49.1±18.5%] were significantly reduced at month 3 with mean PD [2.1.9±0.3 mm], number of PD sites ≥6mm [3.7±2.6] and BOP [14.2±12.5%] being recorded. In the group receiving NSM plus antibiotics, pre-treatment mean PD [3.0±0.5mm], number of PD sites ≥6mm [7.5±5.2] and BOP [51.3±14.9%] were significantly reduced at month 3 with mean PD [1.9±0.3mm], number of PD sites ≥6mm [2.5±0.7] and BOP [8.9±4.8%] being recorded (O'Connell *et al.*, 2008). In a further study, 44 T2DM subjects were randomised to the treatment group, who received non-surgical periodontal management (n=22) or the control group that had no periodontal treatment (n=22) (Kiran et al.,

2005). At the 3 month time-point mean PD [2.3±0.5], mean LOA [3.2±1.1] and BOP [54.4±18.8], showed a significant improvement compared to pre-treatment mean PD [1.8±0.3], mean LOA [2.8±1.0] and BOP [23.9±12.7] in the treatment group but not in the control group (Kiran et al., 2005).

Overall, data from these studies demonstrate a good clinical response to periodontal therapy in subjects with T2DM and this is in line with data from the current study which shows that in subjects with T2DM compared to pre-treatment levels BOP [46.0(30.0-60.7)], mean PD [2.8(2.4-3.2)], mean LOA [3.1(2.8-3.9)] and % of PD sites ≥5mm [10.9(8.1-18.2)] were significantly reduced at month 3 with BOP [18.5(8.8-38.0)] mean PD [2.4(2.2-2.9)], mean LOA [3.0(2.6-3.6)] and % of PD sites ≥5mm [5.3(1.8-9.9)]. However, the lack of a non-diabetic group for comparison is a limitation of these previous studies and prevents conclusions from being made regarding the clinical periodontal response to NSM in subjects with T2DM compared to non-diabetic subjects. Furthermore, many of these studies relied on mean PD and mean CAL data to describe the clinical response within the periodontal tissues. Means fail to present data about the either the % or number of sites with deeper PD sites or the % of sites displaying a reduction in PD and thus conclusions about the severity and extent of periodontal disease in each group and how this changes with periodontal treatment cannot be made.

A number of small studies have also investigated the clinical periodontal response to non-surgical periodontal therapy in subjects with T2DM compared to non-diabetic subjects (Correa *et al.*, 2008; Dag *et al.*, 2009; Kardesler *et al.*, 2010; Kardesler *et al.*, 2011). In a study of 23 subjects with T2DM and periodontitis and 26 non-diabetic subjects with periodontitis, both groups showed significant improvements in the clinical periodontal parameters after NSM. Therefore, in subjects with T2DM, pre-treatment

BOP [94.4 (80.0-100.0)%], mean PD [3.8 (3.4-5.3)mm] and mean LOA [5.4 (4.4-6.4)mm] showed a significant reduction at 90 day after NSM to BOP [25.6 (17.9-32.7)%], mean PD [2.7 (2.4-3.0)mm] and mean LOA [4.9 (3.7-5.3)mm]. Likewise, in non-diabetic subjects pre-treatment BOP [74.8 (65.4-89.1)%], mean PD [3.6 (3.2-4.0) mm] and mean LOA [4.5 (3.8-5.1) mm] showed a significant reduction at 90 day after treatment BOP [14.0 (9.1-19.2)%], mean PD [2.4 (2.3-2.6) mm] and mean LOA [3.7 (3.5-4.5) mm] (Correa *et al.*, 2008). Another study of 20 subjects with T2DM and 22 non-diabetic patients all with periodontitis showed similar improvements in clinical periodontal variables at month 1 and month 3 after NSM. In subjects with T2DM, pre-treatment data [mean PD 3.9±0.5 mm, mean LOA 4.3±0.9 mm, PI 99.7±1.2 and BOP 84.5±10.3%] showed significant improvements at month 1 [mean PD 3.0±0.8 mm, mean LOA 4.1±1.0 mm, PI 38.3±9.5 and BOP 32.5±10.7%] and month 3 [mean PD 2.9±0.6 mm, mean LOA 4.1±0.9 mm, PI 39.8±16.3 and BOP 31.5±10.5%]. Also in non-diabetic subjects pre-treatment data [mean PD 3.9±0.5 mm, mean LOA 4.2±0.6 mm, PI 95.5±9.4 and BOP 67.2±13.5%] showed significant improvements at month 1 [mean PD 2.8±0.4 mm, mean LOA 3.5±0.6 mm, PI 48.7±5.6 and BOP 31.5±8.5 %] and month 3 [mean PD 2.7±0.3 mm, mean LOA 3.5±0.6 mm, PI 38.9±9.2 and BOP 30.1±8.5%] (Kardesler *et al.*, 2011).

In 2 further studies, the subjects with T2DM were also stratified based on initial glycaemic control (Dag *et al.*, 2009; Kardesler *et al.*, 2011). In one of these studies of 13 well controlled and 12 poorly controlled patients with T2DM and periodontitis and 15 non-diabetic patients with periodontitis, there were similar improvements in clinical periodontal variables at month 1 and month 3 after NSM. Therefore, in well controlled T2DM subjects pre-treatment data [PD 3.7±0.4 mm, PI 97.4±5.4% and BOP 81.5±7.9%] showed significant improvements at month 1 [PD 2.7±0.4 mm, PI

37.9±11.1% and BOP 26.3±10.4%] and month 3 [PD 2.8±0.4 mm, PI 35.6±8.7% and BOP 25.4±6.9%]. Likewise in poorly controlled T2DM subjects, pre-treatment data [PD 4.1±1.0 mm, PI 99.7±1.2% and BOP 86.0±12.3%] showed significant improvements at month 1 [PD 3.0±0.8 mm, PI 43.3±9.5 % and BOP 36.8±9.8 %] and month 3 [PD 3.1 ±0.6 mm, PI 44.2±6.3 % and BOP 40.6±8.2 %]. Also in non-diabetic subjects, pre-treatment data [PD 3.9±0.6 mm, PI 95.5±9.4% and BOP 78.8±16.5%] showed significant improvements at month 1 [PD 2.8±0.4 mm, PI 46.7±8.9 % and BOP 31.8±11.5%] and month 3 [PD 2.7±0.3 mm, PI 39.5±13.5% and BOP 28.1±9.2%]. When the groups were compared, no significant differences were demonstrated at any of the time points, except at month 1 and 3 subjects with poorly controlled T2DM patients demonstrating significantly higher BOP than the other 2 groups (Kardesler *et al.*, 2010). In the other study, 15 well controlled and 15 poorly controlled patients with T2DM and periodontitis and 15 non-diabetic patients with periodontitis showed similar improvements in clinical periodontal variables at month 3 after NSM. Therefore, in well controlled T2DM subjects pre-treatment data [mean PD 2.7±0.5 mm, mean LOA 4.3±0.8 mm, PI 1.8±0.7 and GI 1.0±0.3] showed significant improvements at month 3 [mean PD 2.3±0.4 mm, mean LOA 3.0±0.8 mm, PI 0.2±0.1 and GI 0.1±0.1]. Likewise in poorly controlled T2DM subjects, pre-treatment data [mean PD 2.8±0.7 mm, mean LOA 4.3±1.0 mm, PI 2.1±0.7 and GI 1.3±0.4] showed significant improvements at month 3 [mean PD 2.4±0.6 mm, mean LOA 3.0±0.8 mm, PI 0.3±0.2 and GI 0.1±0.1]. Also in non-diabetic subjects pre-treatment data [mean PD 2.6±0.4 mm, mean LOA 4.3±0.6 mm, PI 2.3±0.5 and GI 1.2±0.4] showed significant improvements at month 3 [mean PD 2.4±0.7 mm, mean LOA 2.9±0.6 mm, PI 0.2±0.3 and GI 0.1±0.03] (Dag *et al.*, 2009).

Collectively, the data from previous research indicates that NSM was equally as effective in subjects with T2DM subjects as in non-diabetic subjects. This is in line with data from the current study which showed similar improvements in clinical periodontal variables at month 3, month 6 and month 12 after NSM. For example, in subjects with T2DM, pre-treatment data [mean PD 2.8 (2.4-3.2) mm, mean LOA 3.1 (2.8-3.9) mm, PI 0.8 (0.5-1.1) and BOP 46.0 (30.0-60.7)%] showed significant improvements at month 3 [mean PD 2.4 (2.2-2.9) mm, mean LOA 3.0 (2.6-3.6) mm, PI 0.5 (0.3-0.7) and BOP 18.5(8.8-38.0) %], month 6 [mean PD 2.2 (2.0-2.7) mm, mean LOA 2.8 (2.4-3.7) mm, PI 0.6 (0.3-0.8) and BOP 15.3 (8.3-39.6) %] and month 12 [mean PD 2.2 (1.9-2.8) mm, mean LOA 2.8 (2.4-3.6) mm, PI 0.5 (0.3-0.7) and BOP 17.6 (6.5-28.4) %]. Likewise, in non-diabetic subjects pre-treatment data [mean PD 2.9 (2.5-3.5) mm, mean LOA 3.5 (2.9-4.2) mm, PI 0.6 (0.4-0.8) and BOP 43.0 (29.4-56.7) %] showed significant improvements at month 3 [mean PD 2.6 (2.2-2.9) mm, mean LOA 3.3 (2.8-3.9) mm, PI0.3 (0.1-0.6) and BOP 14.5 (10.3-27.0) %], month 6 [mean PD 2.5 (2.1-2.9) mm, mean LOA 3.1 (2.6-4.1) mm, PI 0.3 (0.1-0.6) and BOP 14.7 (7.2-25.0) %] and month 12 [mean PD 2.3 (1.9-2.7) mm, mean LOA 3.1 (2.5-3.7) mm, PI 0.4 (0.2-0.8) and BOP 10.0 (5.1-24.2) %] (Table 6.6)

Within the current study, when subjects with T2DM were compared to non-diabetic subjects mean PD, mean LOA and BOP showed no significant differences between groups at any time point. This corroborates data from 1 previous study that showed no significant differences between subjects with and without T2DM at either time points (Dag *et al.*, 2009). However, the current study does not support data from 2 previous studies that showed significantly higher BOP, mean PD and mean LOA in subjects with T2DM compared to non-diabetic subjects both before and after NSM (Correa *et al.*, 2008) and significantly higher BOP, mean PD measurements and mean

LOA in subjects with T2DM compared to non-diabetic subjects at various time points within the study (Kardesler *et al.*, 2011). Furthermore, within the present study, despite the reductions in PI seen in both T2DM and non-diabetic subjects, PI was significantly higher in T2DM subjects compared to non-diabetic subjects at each time point, indicating poorer oral care or increased plaque accumulation in T2DM subjects. This is in line with a previous study which demonstrated better improvements in plaque levels, gingival inflammation and BOP within the non-diabetic subjects compared to the T2DM subjects (Correa *et al.*, 2008). Caution must, however, be used when interpreting this result given the limitation that PI data, from the current study, do not represent full mouth assessments, but rather PI scores from 6 sites on 4 target teeth within each subject. The higher PI level in T2DM may, however represent a surrogate marker for poorer lifestyle and general health management, or how living with T2DM may negatively impact on a patient's motivation for optimal oral hygiene, given the patient's other competing healthcare needs.

Taken collectively, previous research and data from the current study show that NSM lead to significant improvements in the clinical periodontal status both in subjects with and without T2DM (Correa *et al.*, 2008; Dag *et al.*, 2009; Kardesler *et al.*, 2010; Kardesler *et al.*, 2011). However, in the majority of previous studies evaluating the clinical response to NSM in subjects with T2DM, the review period is 3 months (Kiran *et al.*, 2005; Correa *et al.*, 2008; O'Connell *et al.*, 2008; Dag *et al.*, 2009; Kardesler *et al.*, 2010; Kardesler *et al.*, 2011) with only one previously published study using a 6 month follow-up period (Santos *et al.*, 2010). Therefore conclusions from the currently published literature regarding the effect of NSM on clinical periodontal health are limited to the short term. From the current literature, no conclusions can be made about long term clinical response to periodontal therapy in subjects with T2DM. Given that

periodontitis is a chronic condition, the lack of data beyond 3 months is a limitation. Within the current study, the clinical periodontal response to NSM was monitored at months 3, 6 and 12 and, compared to pre-treatment data. Significant improvements were demonstrated at each time point for both T2DM subjects and non-diabetic subjects (Table 6.6).

Furthermore, previously published studies describe the clinical periodontal response to NSM in subjects with T2DM using mean PD, mean LOA and % BOP (Kiran *et al.*, 2005; O'Connell *et al.*, 2008; Dag *et al.*, 2009; Kardesler *et al.*, 2010; Santos *et al.*, 2010; Kardesler *et al.*, 2011). Whilst means provide a useful summary of the data, they provide no specific information about the extent and severity of the periodontitis. Only two previous studies supplemented the mean clinical data with data about the % of sites affected by specific probing depths (Correa *et al.*, 2008; Correa *et al.*, 2010). Therefore, one study showed that in subjects with T2DM, the % PD sites ≥7mm at pre-treatment [9.0 (1.0-22.0)%] was significantly reduced 90 days after NSM [0.0 (0.0-1.0)%]. Likewise, in non-diabetic subjects, the % PD sites ≥7mm at pre-treatment [5.5 (3.0-14.0)%] were significantly reduced 90 days after to [0.0 (0.0-0.0)%] (Correa *et al.*, 2008). Additionally, another study showed that in subjects with T2DM, the % PD sites ≥5mm at pre-treatment [38.0±22.8%] was significantly reduced at 3 months after NSM [7.5±6.8%]. Data from the current study shows that in subjects with T2DM, the % of PD sites ≥ 5 mm at pre-treatment [10.9 (8.1-18.2)%] was significantly reduced at month 3 [5.3 (1.8-9.9)%], month 6 [5.3 (1.9-9.7)%] and month 12 [3.0 (0.9-8.9)%]. Similarly, in non-diabetic subjects, the % of PD sites ≥ 5 mm at pre-treatment [16.0 (8.0-30.8)%] was significantly reduced at month 3 [10.1 (5.8-19.0)%], month 6 [9.0 (3.2-19.5)%] and month 12 [3.2 (1.3-13.4)%] (Table 6.6). At pre-treatment, there was an apparently higher % of PD sites ≥ 5 mm recorded in non-diabetic subjects [16.0

(8.0-30.8)%] compared to subjects with T2DM [10.9 (8.1-18.2)%], although the difference failed to reach significance. After treatment, the % of PD sites ≥ 5 mm were significantly higher in non-diabetic subjects at month 3 [10.1 (5.8)%] and month 6 [9.0 (3.2-19.5)%] compared to subjects with T2DM at month 3[5.3 (1.8-9.9)%] and month 6 [5.3 (1.9-9.7)%]. When the impact of the pre-treatment difference between subjects with T2DM and non-diabetic subjects was taken into account, the differences found between subjects with T2DM and non-diabetic subjects at month 3 and month 6 failed to reach significance (Table 6.6).

In an attempt to more fully describe the clinical impact of NSM in subjects with T2DM compared to non-diabetic subjects data relating to the % of sites displaying changes in PD and LOA were also presented in the present study (Table 6.7). For example, in subjects with T2DM, the % of PD sites that reduced by ≥ 2 mm from pre-treatment to month 3 was [11.0 (5.3-17.2)%], from pre-treatment to month 6 was [12.0 (9.3-19.1)%] and from pre-treatment to month 12 was [13.6 (10.5-24.0)%]. Likewise in non-diabetic subjects, the % of PD sites that reduced by ≥ 2 mm from pre-treatment to month 3 was [12.3 (8.3-20.8)%], from pre-treatment to month 6 was [14.0 (9.3-19.4)%] and from pre-treatment to month 12 was [18.0 (10.2-23.2)%]. When the changes in subjects with T2DM were compared to those in non-diabetic subjects, despite the apparently higher % of reducing sites shown in non-diabetic subjects, no significant differences were identified for any time period (Table 6.7). Given that previous research has demonstrated that greater reductions in PD are seen in deeper pockets (Cobb, 2002), it is not unreasonable to propose that the more severe periodontal disease present in the non-diabetic subjects compared to subjects with T2DM (Table 4.14) has led to the apparently higher % of PD sites reducing by ≥ 2 mm seen in non-diabetic subjects compared to subjects with T2DM.

As previously shown within this current study, PESA data were able to detect subtle differences in PD data that mean PD data failed to detect (Table 4.14). Furthermore, in the current study, PISA appears to reflect both PD and BOP data within a composite measure (Table 4.14). Given that inflammation within the periodontium has the potential to contribute to the systemic inflammatory burden and thus influence the pathogenic mechanisms present in T2DM, both PESA and PISA data have been used in this study to describe the clinical response to NSM. In subjects with T2DM, pre-treatment data [PESA 1401.5 (1144.3-1733.7) mm^2 and PISA 683.0 (439.1-1085.5) mm^2] showed significant reductions at month 3 [PESA 1238.2 (1092.4-1543.9) mm^2 and PISA 339.0 (145.4-688.3) mm^2], month 6 [PESA 1159.7 (1010.1-509.0) mm^2 and PISA 340.1 (101.0-628.1) mm^2] and month 12 [PESA 1181.3 (930.3-1374.7) mm^2] and PISA 216.5 (87.1-539.4) mm^2]. Similarly, in non-diabetic subjects, pre-treatment data [PESA 1711.0 (1439.4-2096.5) mm^2 and PISA 897.3 (683.6-1232.9) mm^2] showed significant reductions at month 3 [PESA 1464.2 (1192.0-1686.2) mm^2 and PISA 299.4 (159.5-536.5) mm^2], month 6 [PESA 1402.9 (1139.8-1698.3) mm^2 and PISA 262.6 (89.0-514.8) mm^2] and month 12 [PESA 1260 (983.7-1566.8) mm^2 and PISA 215.1 (62.5-520.3) mm^2]. Also, when subjects with T2DM were compared to non-diabetic subjects PESA and PISA showed no significant differences between groups at any time point (Table 6.6). It would appear therefore, that in this study, PESA and PISA data support the mean clinical data, demonstrating improvements in clinical periodontal variables at month 3, month 6 and month 12 after NSM that were similar in subjects with and without diabetes. Currently, published data relating to PESA and PISA is cross-sectional, with no published studies currently using PESA to describe the impact of NSM upon the health of the clinical periodontal tissues.

Changes in serum cytokines

Previous studies have begun to describe the effect of reducing periodontal inflammation on circulating inflammatory cytokine levels (Ide *et al.*, 2003; D'Aiuto *et al.*, 2004; Marcaccini *et al.*, 2009), however, until recently few studies had evaluated the impact of periodontal therapy on circulating levels of inflammatory cytokines in subjects with T2DM (Iwamoto *et al.*, 2001; O'Connell *et al.*, 2008; Dag *et al.*, 2009; Correa *et al.*, 2010; Kardesler *et al.*, 2010).

In the current study, in subjects with T2DM and non-diabetic subjects, pre-treatment levels of IL-6 [0.51 (0.35-1.50) pg/ml and 0.59 (0.32-0.93) pg/ml] showed no significant changes after NSM (Table 6.8). This does not support results from some previous studies in subjects with T2DM, which show significant reductions in serum IL-6 from pre-treatment level [2.1±0.3pg/ml] to 3 months post-treatment [1.1±0.2pg/ml] (O'Connell *et al.*, 2008) or apparent reductions in pre-treatment IL-6 levels [3.1 (2.1-4.2) pg/ml] to month 3 [2.3 (1.2-4.5) pg/ml], although the changes failed to reach statistical significance (Correa *et al.*, 2010). An additional study showed that in subjects with well controlled T2DM, pre-treatment levels of serum IL-6 were significantly reduced at month 1 and 3 post-treatment and non-diabetic subjects also showed a significant reduction in serum IL-6 levels at month 3, with poorly controlled T2DM subjects also demonstrating non-significant reductions at month 1 and 3 (Kardesler *et al.*, 2010). Interestingly, within the current study, serum levels of IL-6 remained consistently around the lower limits of detection for the assay used, at a lower level than reported in previous studies (O'Connell *et al.*, 2008; Correa *et al.*, 2010; Kardesler *et al.*, 2010).

Within the present study, serum TNF-α pre-treatment levels [7.10 (3.25-9.30) pg/ml] in subjects withT2DM showed significant reductions at 3 months [3.70 (2.21-6.72) pg/ml] and 6 months [3.86 (2.15-7.33) pg/ml] (Table 6.8). This is in line with a

previous study which shows pre-treatment serum TNF-α levels (9.2±3.33 pg/ml) and (12.44±5.27 pg/ml) showed significant reductions at 3 months after NSM (7.75±2.52 pg/ml) and (10.25±3.68 pg/ml), in subjects with well controlled and poorly controlled T2DM respectively (Dag et al., 2009). In another study, in subjects with T2DM, serum TNF-α levels showed a significant reduction from pre-treatment [3.77±1.03pg/ml] to 1 month post-treatment [3.28±0.86 pg/ml] (Iwamoto et al., 2001). In a separate study in subjects with T2DM, pre-treatment plasma levels of TNF-α [5.6 (4.3-7.4) pg/ml] showed a significant reduction at 3 months post-treatment [4.8 (2.7-6.7) pg/ml] (Correa *et al.*, 2010). However, a further study failed to showed a significant change in serum TNF-α level from pre-treatment [0.0±0.3 pg/ml] to 3 months after NSM [0.0±0.2 pg/ml] (O'Connell *et al.*, 2008). In the current study however, non-diabetic subjects shows significant increases at 6 months [7.06 (5.59-8.79) pg/ml] and 12 months [7.70 (6.71-10.20) pg/ml] compared to pre-treatment serum TNF-α levels [3.44 (2.34-7.24) pg/ml] (Table 6.8). This is not supported by data from previous studies which show, in non-diabetic subjects, either reductions in serum TNF-α levels from pre-treatment (10.42±4.80pg/ml) to 3 months after NSM (7.72±4.53pg/ml)] (Dag et al., 2009) or no significant changes after NSM (Ide *et al.*, 2003; Kardesler *et al.*, 2010). Possible reasons for the relatively low pre-treatment TNF-α levels in serum being measured in non-diabetic subjects could include seasonal variations in room temperature that occurred during the quantification assays. Interestingly, the majority of non-diabetic patients were recruited within a short period of time, whereas recruitment of subjects with T2DM was slower and therefore spread over a longer period. However, as part of this study data about the timing of blood sampling and serum cytokine analysis has not been recorded and thus clear conclusions about the impact of seasonal temperature variations cannot be made.

Overall, data from previous studies and the current study would appear to suggest that in T2DM, a reduction in periodontal inflammation may be associated with a reduction in serum TNF-α, which is potentially an important finding given the role of inflammatory cytokines in the pathogenesis of T2DM and modulation of glycaemic control. However, when comparing the pre-treatment levels from published studies as well as from the current study, there are clear variations in the levels of TNF-α presented. The use of different assays could potentially account for some of these variations. Additionally, some studies used serum samples whilst others used plasma samples for quantifying circulating cytokine levels, with the possibility of the release of blood cell contents potentially contributing to the level of cytokines in plasma samples, providing another source for inter-study variations in cytokine levels.

Changes in cytokines in saliva

Despite the recognition that saliva has the potential to provide important complimentary information with regards to diagnosis and monitoring of periodontitis (Miller *et al.*, 2010), there is currently limited published longitudinal data evaluating the impact of NSM on inflammatory cytokines in saliva (Sexton *et al.*, 2011).

In the present study, both non-diabetic subjects and subjects with T2DM showed that pre-treatment IL-6 levels in saliva [2.29 (1.27-4.38) pg/ml and 2.41 (0.95-7.29) pg/ml] did not change significantly after NSM (Table 6.9). Although pre-treatment levels of IL-6 in saliva appear similar to previously published data in systemically healthy subjects with periodontitis [3.6±5.9pg/ml] (Gursoy *et al.*, 2009), there are currently no published longitudinal data, investigating the impact of NSM on levels of IL-6 in saliva.

In the current study, in subjects with T2DM, pre-treatment levels of TNF-α in saliva [1.58 (0.61-3.08) pg/ml] were significantly reduced at month 3 [0.66 (0.26-1.53) pg/ml], although at month 12 the level was significantly increased [2.88 (1.25-4.32) pg/ml]. In non-diabetic subjects, compared to pre-treatment levels of TNF-α in saliva [2.59 (1.18-3.97) pg/ml] no significant changes were detected after NSM (Table 6.9). In comparison, one study evaluating changes in levels of TNF-α in saliva in systemically healthy subjects with periodontitis, reported significant reductions in TNF-α in saliva at month 4 in subjects receiving OHI alone and subjects receiving NSM, although numerical values were not published and the data were presented graphically, preventing a direct comparison with data from the current study (Sexton *et al.*, 2011).

Additionally, in the present study, in subjects with T2DM, pre-treatment levels of IL-1β in saliva [38.65 (20.45-68.28) pg/ml] were reduced at month 3 [19.00 (6.96-50.55) pg/ml] (detected as a trend following correction of p-values for multiple comparisons), although there was a non-significant increase at month 12 [64.60 (26.88-121.50) pg/ml]. In non-diabetic subjects, compared to pre-treatment levels [62.60 (39.20-97.10) pg/ml], saliva IL-1β showed apparent reduction at month 6 [38.55 (21.43-67.80) pg/ml] and month 12 [48.20 (27.10-86.20) pg/ml], although these differences failed to reach statistical significance (Table 6.9). In comparison, one study evaluating changes in levels of IL-1β in saliva in systemically healthy subjects with periodontitis reported significant reductions in IL-1β in saliva at month 4 in subjects receiving OHI alone and in subjects receiving NSM, although numerical values were not published and the data were presented graphically, preventing a direct comparison with data from the current study (Sexton *et al.*, 2011).

Finally, in the current study, in subjects with T2DM pre-treatment levels of IFN-γ in saliva [0.86(0.47-1.60)pg/ml] were significantly reduced at month 3 [0.43 (0.25-

0.78) pg/ml], although there was a significant increase at month 12 [1.61 (1.03-2.69) pg/ml]. In non-diabetic subjects, compared to pre-treatment levels [1.55 (0.86-2.24) pg/ml], saliva IFN-γ showed reductions at month 6 [0.83 (0.42-1.88) pg/ml] (detected as a trend following correction of p-values for multiple comparisons) (Table 6.9). However, levels of IFN-γ in saliva remained consistently around the lower limits of detection for the assay used and currently no published data is available for comparison.

Overall, data from previous studies and the current study would appear to suggests that in subjects with periodontitis, a reduction in periodontal inflammation may be associated with a reductions in levels of TNF-α and IL-1β in saliva (Sexton *et al.*, 2011). This would suggest that selected salivary biomarkers may reflect the clinical response to periodontal therapy. However, further longitudinal studies would be required to clarify this.

Changes in cytokines in GCF

In subjects with T2DM, the current study demonstrated that pre-treatment GCF levels of IL-6 [1.97 (0.98-5.17) pg/ml] reduced significantly at month 3 [1.03 (0.51-2.70) pg/ml], month 6 [0.89 (0.14-1.80)pg/ml] and month 12 [1.03 (0.34-2.17)pg/ml]. Also pre-treatment GCF levels of TNF-α [4.16 (2.72-6.69) pg/ml] also reduced significantly at month 3 [3.59 (1.28-10.37) pg/ml], month 6 [2.03 (0.65-3.80) pg/ml] and month 12 [1.74(0.96-3.45)pg/ml]. Similarly, pre-treatment GCF levels of IL-1β [344.33 (156.16-572.50) pg/ml] reduced significantly at month 3 [180.05 (68.98-382.93) pg/ml], month 6 [116.35 (48.71-206.56) pg/ml] and month 12 [175.65 (58.54-366.95) pg/ml]. Also, in T2DM subjects, the pre-treatment GCF level of IFN-γ [2.51 (1.11-5.24) pg/ml] also showed a significant reduction but only at month 6 [0.85 (0.23-1.89) pg/ml].

In non-diabetic subjects, the current study demonstrated that pre-treatment GCF levels of IL-1β [413.38 (213.83-770.56) pg/ml] reduced significantly at month 3 [166.25 (90.85-350.75) pg/ml], month 6 [150.60 (77.56-268.32) pg/ml] and month 12 [184.25 (77.51-291.71) pg/ml]. Also, pre-treatment GCF levels of IFN-γ [4.40 (2.18-7.09) pg/ml] reduced significantly at months 3 [2.54 (0.92-4.87) pg/ml], month 6 [2.21 (1.41-4.23) pg/ml] and month 12 [1.43 (0.50-2.46) pg/ml]. Also in non-diabetic subjects, pre-treatment GCF levels of IL-6 [2.25 (1.12-3.37) pg/ml] reduced significantly at month 6 [1.51 (0.94-2.34) pg/ml] and pre-treatment levels of TNF-α [4.49 (2.55-7.04) pg/ml] reduced significantly at month 12 [2.35 (1.09-3.55) pg/ml]. Overall, in both T2DM and non-diabetic subjects, reductions in GCF levels of these 4 selected pro-inflammatory cytokines would appear to mirror the improvements in periodontal health seen within this study following periodontal therapy.

Currently published data investigating the changes in pro-inflammatory cytokines levels in GCF following non-surgical periodontal therapy in T2DM subjects is limited to three studies (Correa *et al.*, 2008; Kardesler *et al.*, 2010; Santos *et al.*, 2010). In one study, the impact of NSM on levels of IL-1β was assessed in patients with (n=23) and without (n=26) T2DM (Correa et al., 2008). After treatment, a significant improvement in clinical periodontal parameters in both groups was accompanied by significant reductions in GCF volume and the total amount of IL-1β (Correa et al., 2008). In another study, the periodontal treatment effects on GCF IL-6 levels in T2DM (n=20) and non-diabetic subjects (n=22) were evaluated (Kardesler et al., 2011). At 3 months post-treatment, reductions in the total amount of GCF IL-6 were demonstrated graphically in both T2DM and non-diabetic subjects, however, these reductions failed to reach significance and this studies did not publish the actual values for the cytokine levels pre- and post-treatment, making interpretation of the results difficult (Kardesler et

al., 2011). In a further study, the response of TNF-α, IFN-γ, IL-4 and IL-17 in GCF was assessed in patients with well controlled T2DM (n=18) and in patients with poorly controlled T2DM (n=20) (Santos et al., 2010), showing no significant changes in the total amounts of TNF-α, IFN-γ, IL-4 and IL-17 in GCF at 3 and 6 months post-treatment. Again, failure to publish the actual values for the cytokine levels pre- and post-treatment makes the interpretation of the results difficult. Furthermore, a lack of non-diabetic subjects limits possible conclusions about the response in subjects with T2DM compared to non-diabetic subjects.

Given the paucity of the available data regarding changes in GCF cytokines following NSM in subjects with T2DM, it's worthwhile comparing data from the present study to studies evaluating the cytokine response to periodontal therapy, in systemically healthy subjects (Engebretson *et al.*, 2002; Thunell *et al.*, 2010). A follow-up study of systemically healthy subjects (n=29) showed that following non-surgical periodontal treatment, in patients with mild to moderate periodontitis and in patients with severe periodontitis the IL-1β levels in GCF were reduced 2 weeks post-treatment (Engebretson et al., 2002). However, at 6 months, the significant post-treatment reduction in GCF IL-1β levels was maintained only in patients with mild-moderate periodontal disease, with IL-1β returning to baseline levels in patients with severe disease (Engebretson et al., 2002). In a more recent study, a multiplex immunoassay was used to evaluate the effect of periodontal therapy on a range of 22 GCF mediators, including IL-1β, IL-6, TNF-α and IFN-γ in a small number of systemically healthy subjects (n=6) (Thunell et al., 2010). Following periodontal therapy, significant reductions were detected in 13 of the 16 detectable cytokines, including IL-1β, IL-6 and IFN-γ. However, interestingly, TNF-α was not found to be detectable in the GCF samples taken in this study.

Overall, data from previous studies and the current study would appear to suggest that in subjects with periodontitis, regardless of diabetes status, a reduction in periodontal inflammation may be associated with a reductions in levels of IL-1β, and possibly IL-6, TNF-α and IFN-γ in GCF (Engebretson *et al.*, 2002; Correa *et al.*, 2008; Thunell *et al.*, 2010). Notwithstanding the differences in storage and elution protocols used within this field of research, data would suggest that in subjects with and without T2DM, selected GCF biomarkers may reflect the clinical response to periodontal therapy. However, further longitudinal studies, using standardized protocols for GCF sampling, elution and storage, would be required to clarify this.

Summary of key findings from chapter 6

- At each time point BMI was significantly higher in subjects with T2DM compared to non-diabetic subjects. Also, in subjects with T2DM a significant reduction in BMI was seen following NSM.
- In subjects with T2DM, HbA1c showed 0.45% and 0.40% reductions at month 3 and month 12 respectively, although these reductions did not reach statistical significance.
- At each time point HDL, non-HDL and cholesterol was significantly higher in non-diabetic subjects compared to subjects with T2DM. Also, in non-diabetic subjects a significant reduction in non-HDL and cholesterol was seen 6 months after NSM.
- In both diabetic and non-diabetic subjects reductions in hsCRP were seen 12 months after NSM, although these reductions did not reach statistical significance.

- In both diabetic and non-diabetic subjects significant reductions in % BOP, mGI, PI, mean PD, mean LOA, PESA and PISA were seen at 3, 6 and 12 months after NSM. Furthermore, mGI, % BOP, mean PD, mean LOA, PESA and PISA showed no significant differences between subjects with T2DM and non-diabetic subjects at any time point.
- In both diabetic and non-diabetic subjects significant reductions in % PD sites ≥ 5 mm were seen at 3, 6 and 12 months after NSM. Taking into account the pre-treatment difference in % PD sites ≥ 5 mm, the differences between subjects with T2DM and non-diabetic subjects at month 3 and month 6 failed to reach significance.
- The % of PD sites that reduced by ≥ 2 mm and ≥ 3 mm appeared higher in non-diabetic subjects compared to subjects with T2DM, although the differences failed to reach statistical significance.
- In subjects with T2DM serum TNF-α was significantly reduced 3 and 6 months after NSM. Conversely, in non-diabetic subjects serum TNF-α was significantly increased at 6 and 12 months after NSM.
- In subjects with T2DM a reduction in IL-1β in saliva was seen at 3 months after NSM was detected as a trend. In non-diabetic subjects reductions in IL-1β in saliva were seen at 6 and 12 months, although these differences failed to reach statistical significance.
- In both diabetic and non-diabetic subjects significant reductions in IL-1β in GCF were seen at 3, 6 and 12 months after NSM. Similarly, reductions in TNF-α and IL-6 in GCF were seen after NSM, although not at every time point.

Chapter 7 General Discussion

Diabetes is widely accepted as a major public health problem, associated with reduced life expectancy, increased morbidity and increased mortality (IDF, 2006; Mulnier *et al.*, 2006; Dale *et al.*, 2008). Between 2007 and 2025, the global predicted growth is 55% (IDF, 2006) and a recent UK health survey projects a 98% increase in the rate of obesity related diabetes by 2050 (Brown *et al.*, 2010). As a risk factor for periodontal disease, T2DM is associated with an increased incidence and severity of periodontal disease, with the risk for developing periodontitis being greater if diabetes control is poor (Nelson *et al.*, 1990; Emrich *et al.*, 1991; Taylor *et al.*, 1998a; Tsai *et al.*, 2002; Moles, 2006). Furthermore, evidence suggests that those with moderate levels of periodontal disease have a 2-fold increased risk for developing T2DM (Demmer *et al.*, 2008) and diabetics with severe periodontal disease are six times more likely to have poor glycaemic control (Taylor *et al.*, 1996), highlighting a possible bi-directional relationship between these two disease. Given the predicated future increase in diabetes and the likely impact on the future prevalence of periodontal disease, understanding the mechanistic connections between the two diseases is of increasing importance to health care.

In this study, subjects with gingivitis or periodontitis presented with a significantly longer history of diabetes compared to those with healthy periodontal tissues. Furthermore, a higher proportion of subjects with healthy periodontal tissues (64.3%) was found to meet the UK target for glycaemic control (HbA1c $\leq$ 6.5%) and progressively fewer subjects reached this target in the gingivitis (26.3%) and periodontitis (14.9%) groups, respectively. There was also a higher proportion of subjects with healthy periodontal tissues being managed by dietary intervention alone

and progressively fewer in the gingivitis and periodontitis groups, respectively. Conversely, a higher proportion of subjects with periodontitis (29.8%) required insulin, with progressively fewer subjects requiring insulin in the gingivitis (17.9%) and healthy periodontal tissues (0.0%) groups, respectively. Taken collectively, these findings highlight that the deterioration in glycaemic control appears to be mirrored by worsening periodontal health and that, as with other diabetes complications, the duration of diabetes and the degree of glycaemic control (UKPDS, 1998b) would appear to be important factors in the development of periodontitis in subjects with T2DM

Within T2DM subjects, the risk of diabetic complications is strongly associated with previous hyperglycaemia and a 1% reduction in HbA1c has been associated with a 21% risk reduction in diabetic complications (Stratton 2000). Consequently, national guidelines have therefore set an ideal target HbA1c of 6.5%, and recommend the use of dietary and lifestyle interventions, with medications, as required, to achieve and maintain this target HbA1c levels (NICE, 2008). A number of previous studies have investigated the impact of periodontal treatment on glycaemic control in subjects with T2DM (Stewart *et al.*, 2001; Kiran *et al.*, 2005; Promsudthi *et al.*, 2005; Jones *et al.*, 2007; O'Connell *et al.*, 2008) and meta-analyses of data from smaller interventional studies have concluded that periodontal treatment could improve glycaemic control (Darre *et al.*, 2008; Simpson *et al.*, 2010), with NSM producing a statistically significant 0.40% HbA1c reduction (p=0.04) when compared to no treatment or usual treatment (Simpson *et al.*, 2010). Interestingly, a similar magnitude of change in HbA1c was demonstrated in the present study which showed that after NSM, in subjects with T2DM, HbA1c was reduced by 0.45% and 0.40% at month 3 and month 12 respectively, although in the current study these reductions did not reach statistical significance. Given the potential for successful periodontal treatment to contribute to a

positive effect on glycaemic control, is it surprising that as yet, the maintenance of oral health does not currently feature within national management guidelines for T2DM (NICE, 2008). It is hoped that the data from this study will contribute to future meta-analyses to investigate this issue further.

It is increasingly recognised that inflammation plays a role in the development of diabetes and the association between elevated systemic inflammation and the development of T2DM has been previously demonstrated (Bertoni *et al.*, 2010). In subjects with T2DM, CRP has been proposed as a cardiovascular risk marker (Pfutzner and Forst, 2006) as well as a useful marker in predicting the risk of developing T2DM (Freeman *et al.*, 2002; Duncan *et al.*, 2003). In the current study, levels of hsCRP appeared higher in subjects with T2DM [2.3 (0.9-4.5) mg/l) compared to non-diabetic subjects [1.9 (0.8-3.9) mg/l), although the difference failed to achieve statistical significance. Interestingly, when subjects were further categorised based on periodontal status, subjects with T2DM and healthy periodontal tissues had significantly higher hsCRP [2.4 (0.8-5.5) mg/l) than non-diabetic subjects with healthy periodontal tissues [0.6 (0.2-1.5) mg/l). Evidence from a previously published meta-analysis also demonstrated that CRP is elevated in patients with periodontitis (Paraskevas *et al.*, 2008) and this was confirmed in the current study, which showed that in non-diabetic subjects, hsCRP was significantly lower in those with healthy periodontal tissues [0.6 (0.2-1.5) mg/l) compared to subjects with gingivitis [2.3 (0.8-4.2) mg/l] or periodontitis [2.3 (1.1-4.3) mg/l], although in subjects with T2DM, similar differences in hsCRP were not found. This might be because the impact of T2DM on CRP levels outweighs any impact of periodontal inflammation. Furthermore, previous studies have demonstrated that reduction in CRP levels follows periodontal management (D'Aiuto *et al.*, 2004; Marcaccini *et al.*, 2009), although this is not a universal finding in the

literature (Ide *et al.*, 2003), and the most recent meta-analysis concluded that moderate evidence exists for an effect of periodontal therapy in lowering the levels of CRP (Paraskevas *et al.*, 2008). Within the current study, the effect of NSM on CRP levels was investigated in subjects with T2DM. In both diabetic and non-diabetic subjects non-significant reductions in hsCRP were seen 12 months after NSM, confirming data from previous studies that also demonstrate non-significant reductions in hsCRP in subjects with T2DM (Christgau *et al.*, 1998; Correa *et al.*, 2010; Kardesler *et al.*, 2010). The wide variations in CRP levels are likely to be due to the non-specific nature of CRP as a marker of inflammation and future studies investigating the impact of NSM on CRP levels in T2DM need to be adequately powered to account for such variation.

Obesity is a powerful risk factor for T2DM (Chan *et al.*, 1994), with BMI predicting the risk of T2DM (Wang *et al.*, 2005) and obesity being proposed as the leading cause of insulin resistance (Kahn *et al.*, 2006). The link between diabetes and obesity was demonstrated in the current study, which showed that, at each time point, subjects with T2DM had significantly higher BMI compared to the non-diabetic patients. Furthermore, the T2DM group contained a higher proportion of obese (42.6 %) and morbidly obese (30.7%) subjects compared to the non-diabetic group (10.8% and 13.3%, respectively). Interestingly, in subjects with T2DM a significant reduction in BMI was seen following NSM, although the contribution made by the higher drop out of obese and morbidly obese subjects seen over time makes it difficult to draw clear conclusions about actual weight loss following periodontal treatment.

T2DM and hypertension are commonly associated conditions, both of which carry an increased risk of cardiovascular and renal disease (Garcia *et al.*, 1974; Turner *et al.*, 1998b). Despite evidence that tight control of BP in subjects with T2DM reduces diabetic complications (Turner *et al.*, 1998a) and the clear guidance for the intensive

management of hypertension in subjects with T2DM (NICE, 2008), the present study demonstrates that systolic BP was significantly higher in subjects with T2DM (146.9 mmHg) compared to non-diabetic subjects (136.6 mm Hg). This supports previous evidence showing higher prevalence of hypertension in T2DM patients than that observed in the general population (HDS, 1993).

In subjects with T2DM, hyperlipidemia is a risk factor for CVD (NICE, 2008). Furthermore, an association between hyperlipidemia and periodontitis has previously been demonstrated (Cutler *et al.*, 1999b; Losche *et al.*, 2000; Noack *et al.*, 2000; Fentoglu *et al.*, 2009; Fentoglu *et al.*, 2010) with hyperlipidemia described as one of the factors associated with diabetes-induced immune cell alterations (Iacopino and Cutler, 2000). Data from the present study showed that levels of non-HDL and cholesterol were significantly higher in non-diabetic subjects compared to subjects with T2DM, although this is not surprising, given that the management of raised lipid levels is a key priority for diabetes management (NICE, 2008). In subjects with T2DM and periodontitis, no changes in lipid levels were demonstrated following NSM, corroborating data from previous studies (Christgau *et al.*, 1998; Kiran *et al.*, 2005; Kardesler *et al.*, 2010) and perhaps reflecting that when raised serum lipid levels are already actively managed as part of T2DM treatment, further improvements are not gained through improvements in periodontal health. Interestingly, a significant reduction in non-HDL and cholesterol was seen 6 months after NSM in non-diabetic subjects, which supports data from a recent small study (Fentoglu *et al.*, 2010), although an appropriately designed RCT would be required to confirm this.

Previous epidemiological evidence has reported an increased prevalence and severity of periodontal disease in subjects with T2DM compared to non-diabetic subjects (Shlossman *et al.*, 1990; Sandberg *et al.*, 2000; Mattout *et al.*, 2006; Moles,

2006). In more recent interventional studies evaluating the response to periodontal therapy, the pre-treatment periodontal condition of subjects with and without diabetes was well matched (Dag *et al.*, 2009; Kardesler *et al.*, 2010) or the T2DM subjects had higher levels of BOP and mean PD (Correa et al., 2008). In the current study, when exploring the pre-treatment periodontal status of subjects with T2DM in comparison to those without diabetes, no significant differences were found in the mean PD, mean recession and mean LOA in subjects with T2DM and periodontitis compared to non-diabetic subjects with periodontitis. However, periodontal disease rarely affects all parts of the periodontium equally and whilst means provides a useful summary statistic of the data, they provide no specific information about the extent and severity of the periodontitis. In the current study, the percent of periodontal sites with PD $\geq$ 6 mm was significantly lower in subjects with T2DM and periodontitis compared to non-diabetic subjects with periodontitis, indicating more severe periodontal disease was present, at pre-treatment, in non-diabetic subjects compared to subjects with T2DM. This was contrary to previous epidemiological evidence (Shlossman *et al.*, 1990; Sandberg *et al.*, 2000; Mattout *et al.*, 2006; Moles, 2006) and was not replicated in the one previous study that supplemented the mean PD data with data on the percent of sites with advanced periodontal disease, and which showed no significant differences in the percent of periodontal sites with PD $\geq$ 7 mm between subjects with T2DM and non-diabetic subjects (Correa *et al.*, 2008). This most probably reflects the differences in the recruitment pools used for diabetic and non-diabetic subjects in the current study (and must be regarded, unfortunately, as a weakness in this study) and highlights the need to stratify periodontal case selection based on extent and severity of disease to ensure more robust matching of groups with regards to periodontal status in future studies.

Given that inflammation within the periodontium has the potential to contribute to the systemic inflammatory burden (Loos, 2005) and thus influence the pathogenic mechanisms present in T2DM (Hotamisligil *et al.*, 1996; Stumvoll *et al.*, 2005), it is interesting to note that in the present study, higher levels of gingival inflammation were seen in subjects with T2DM and gingivitis compared to non-diabetic subjects with gingivitis, although this difference is not replicated in subjects with periodontitis. Given that periodontitis was more advanced in the non-diabetic group compared to the T2DM group, the gingivitis groups offer the potential to study in more detail the effect of diabetes on gingival/periodontal inflammation. Given the tight criteria for defining gingivitis, the gingivitis patients in the T2DM and non-diabetic groups were very well matched from a clinical perspective. These groups therefore offer utility to study the impact of diabetes on gingival inflammation. It is noteworthy that gingival inflammation was significantly higher in the T2DM group with gingivitis, compared to the non-diabetic patients with gingivitis, and this could possibly reflect up-regulated inflammation as a result of diabetes. Interestingly, this concept is supported by the T2DM subjects with gingivitis having elevated levels of GCF IL-6 (1.44 (0.53-3.61) pg/ml), IL-1β (173.41 (77.60-268.19) pg/ml) and IFN-γ (2.17 (0.65-2.50) pg/ml) compared to the GCF levels of IL-6 (0.90 (0.39-2.12) pg/ml), IL-1β (116.25 (56.50-176.53) pg/ml) and IFN-γ (1.85 (1.01-2.44) pg/ml) in the non-diabetic subjects with gingivitis, although these differences didn't achieve statistical significance.

The current study also showed that PESA was able to represent key aspects of the PD data, highlighting differences in PD between subjects with different periodontal status and detecting differences in the extent and severity of periodontal disease between T2DM and non-diabetic subjects. PISA also reflected the differences in periodontal case definitions within the current study and given that PISA incorporates

both BOP and PD data into a single variable, data from the present study confirms PESA and PISA as valuable measures of the extent of periodontal disease and the periodontal inflammatory burden (Nesse *et al.*, 2008; Nesse *et al.*, 2009).

In the current study, both diabetic and non-diabetic subjects demonstrated significant reductions in % BOP, mGI, PI, mean PD, mean LOA, PESA and PISA at 3, 6 and 12 months after NSM. Furthermore, when diabetic and non-diabetic subjects were compared at each time point, no significant differences were identified in mGI, % BOP, mean PD, mean LOA, PESA and PISA. This supports previous research that has demonstrated significant improvements in clinical periodontal parameters following periodontal management in subjects with T2DM (Rodrigues *et al.*, 2003; Kiran *et al.*, 2005; Correa *et al.*, 2008; O'Connell *et al.*, 2008; Dag *et al.*, 2009; Correa *et al.*, 2010; Kardesler *et al.*, 2010; Santos *et al.*, 2010; Kardesler *et al.*, 2011). In both diabetic and non-diabetic subjects, the current study also showed significant reductions in % PD sites ≥ 5 mm at 3, 6 and 12 months after NSM, and taking into account the pre-treatment differences in % PD sites ≥ 5 mm, the differences between subjects with T2DM and non-diabetic subjects at month 3 and month 6 failed to reach statistical significance. This confirms previous research that demonstrates significant reductions in the % of PD sites exhibiting advanced disease following NSM in subjects with T2DM (Correa *et al.*, 2008; Correa *et al.*, 2010). The present study also showed that the % of PD sites that reduced by ≥ 2 mm and ≥ 3 mm appeared higher in non-diabetic subjects compared to subjects with T2DM, although the differences failed to reach statistical significance. Given that greater reductions in PD are seen in deeper pockets after NSM (Cobb, 2002), the apparently higher % of PD sites reducing by ≥ 2 mm and ≥ 3 mm seen in the non-diabetic subjects most probably reflects the increased severity of periodontal disease seen in non-diabetic subjects at baseline. Overall, the current study confirms that NSM

is effective in managing periodontitis in subjects with T2DM, and highlights the need for robust patient pathways to ensure delivery and uptake of periodontal screening, supplemented with therapy as required, within this disease susceptible population

In the current study, the group with T2DM contained a significantly higher percent of subjects with dry mouth compared to those without diabetes and the percentage of subjects with at least one carious tooth was over 4 times greater in subjects with T2DM compared to non-diabetic subjects, confirming data from previous studies that have highlighted poorer oral health in subjects with T2DM (Sandberg *et al.*, 2000; Hintao *et al.*, 2007; Borges *et al.*, 2010). Furthermore, in the present study, the T2DM group reported poorer oral health behaviours (including attendance at a dentist and oral hygiene practices) compared to the non-diabetic group. This corroborates previous research that demonstrates that the attitudes towards oral health of patients with diabetes are poor in comparison with findings from surveys of general populations (Allen *et al.*, 2008). In the current study, 95% of subjects with T2DM had received examinations of their feet and eyes within the past 12 months, clearly demonstrating the robust patient pathways that exist for screening for diabetic complications such as retinopathy and peripheral vascular disease. However, the same is not true regarding screening for oral complications in subjects with T2DM, with as many as one third of subjects with T2DM in this study reporting not seeing a dentist in the past 12 months. This clearly demonstrates that the opportunity for screening for oral complications is not currently taken up by all subjects within this disease-susceptible population. Taken collectively, this emphasises the importance of considering oral health within a wider perspective of maintaining systemic health, and the need for more robust care pathways to ensure good oral health in disease susceptible patients.

The role of inflammatory cytokines in the pathogenesis of periodontal disease is well recognised (Preshaw and Taylor, 2011). Likewise, inflammatory cytokines are key players in the relationship between inflammation and T2DM with cytokines, such as TNF-α, IL-1β and IL-6, contributing to the development of T2DM through obesity, insulin resistance and β-cell dysfunction (Donath *et al.*, 2003; Pickup, 2004; Wellen and Hotamisligil, 2005). It is proposed that alterations in immunologically active molecules as a result of T2DM have the potential to influence the cytokine network within the periodontium and thus contribute to local periodontal tissue destruction (Preshaw *et al.*, 2007). Furthermore, inflammation within the periodontium has the potential to contribute to the systemic inflammatory burden present in T2DM (Loos, 2005; Nesse *et al.*, 2009). It is therefore surprising that, until very recently, relatively few studies have investigated the role of inflammatory cytokines in patients with T2DM and periodontal disease (Cutler *et al.*, 1999a; Engebretson *et al.*, 2007; Correa *et al.*, 2008; Dag *et al.*, 2009; Correa *et al.*, 2010; Kardesler *et al.*, 2010; Santos *et al.*, 2010).

Previous research demonstrates significantly higher serum levels of IL-6 and TNF-α in subjects with T2DM compared to non-diabetic subjects (Pradhan *et al.*, 2001; Spranger *et al.*, 2003b; Bertoni *et al.*, 2010) and this was supported in the current study, which showed significantly higher serum levels of TNF-α, IL-1β and IFN-γ in subjects with T2DM compared to non-diabetic subjects and significantly higher serum levels of TNF-α, IL-1β and IFN-γ in T2DM subjects with periodontitis compared to non-diabetic subjects with periodontitis. Interestingly, in the present study, in subjects with T2DM and periodontitis, serum TNF-α was significantly reduced 3 and 6 months after NSM, supporting previously published data demonstrating short term reductions in circulating TNF-α after periodontal therapy in subjects with T2DM (Iwamoto *et al.*, 2001; Correa *et al.*, 2008; Dag *et al.*, 2009). Given the role of TNF-α in the pathogenesis of T2DM,

reductions in circulating TNF-α following NSM may, in part, explain the improvements in glycaemic control seen in subjects with T2DM after periodontal therapy. Conversely, in the current study, serum TNF-α was significantly increased at 6 and 12 months after NSM in non-diabetic subjects, with relatively low pre-treatment serum TNF-α levels being demonstrated in these subjects, potentially contributing to this apparent increase after NSM. Additionally, it would appear that data from the current study don't support the published evidence that subjects with periodontitis have higher levels of circulating inflammatory cytokines (Marcaccini *et al.,* 2009; Sun *et al.,* 2009) with significantly lower serum TNF-α, IL-1β and IFN-γ demonstrated in non-diabetic subjects with periodontitis compared to subjects with healthy periodontal tissues. However, low levels of cytokines present in serum (at the limits of detection of even the high sensitivity assay system used in this research), along with variations in levels, make it difficult to interpret results and prevent clear conclusions from being made. To clarify the role of circulating inflammatory cytokines in the relationship between T2DM and periodontitis, further studies with larger sample size would be required.

Our understanding of the specific roles of cytokines in periodontal disease is deepest for pro-inflammatory cytokines, for example IL-1β, IL-6 and TNF-α (Preshaw and Taylor, 2011) and previous data demonstrated increased levels of IL-1β and IL-6 in GCF from subjects with periodontitis (Preiss and Meyle, 1994; Figueredo *et al.*, 1999; Engebretson *et al.*, 2002; Zhong *et al.*, 2007) and in GCF sampled from diseased sites compared with healthy sites (Mogi *et al.*, 1999; Lin *et al.*, 2005; Thunell *et al.*, 2010). This is supported by data from the present study which demonstrated significantly higher GCF IL-1β in diabetic subjects with periodontitis [344.33 (156.16-572-50) pg/ml] compared to those with those with gingivitis [173.41 (77.60-268.19) pg/ml) and also those with healthy periodontal tissues [84.95 (40.66-130.61) pg/ml]. Similarly,

significantly higher GCF IL-1β was demonstrated in non-diabetic subjects with periodontitis [413.38 (213.84-770.56) pg/ml] compared to those with those with gingivitis [116.25 (56.50-176.53) pg/ml) and also those with healthy periodontal tissues [54.26 (25.79-100.65) pg/ml]. Also in non-diabetic subjects, significantly higher levels of GCF IL-6, TNF-α and IFN-γ were demonstrated in subjects with periodontitis compared to those with healthy periodontal tissues and in subjects with T2DM the same differences were detected as a trend. This confirms the utility of GCF IL-6, TNF-α, INF-γ and particularly IL-1β as indicators of inflammatory status in the periodontal tissues. Additionally, the findings of elevated circulating levels of these cytokines in T2DM patients and the less clear-cut finding of elevated GCF cytokines in T2DM patients with gingivitis may be a reflection of the underlying alterations to inflammatory networks that result from the diabetic state.

In the current study, no significant differences in GCF IL-6, TNF-α, IL-1β and IFN-γ levels were found between subjects with T2DM and non-diabetic subjects, and even following categorisation based on periodontal status, in non-diabetic subjects with periodontitis the apparently higher levels of GCF IL-6, TNF-α and IL-1β compared to T2DM subjects with periodontitis failed to reach statistical significance. This supports previous data that showed no differences in pre-treatment IL-1β levels between subjects with and without T2DM (Correa *et al.*, 2008) but doesn't support the findings of a further study that demonstrated higher levels of GCF IL-6 in T2DM subjects with periodontitis compared to non-diabetic subjects with periodontitis (Kardesler *et al.*, 2011). In the current study, the higher levels, albeit non-significant, of GCF cytokines in non-diabetic subjects with periodontitis compared to subjects with T2DM and periodontitis, may well be a reflection of the more severe periodontal disease that was present in non-diabetic subjects compared to the subjects with T2DM.

The relationship between increasing levels of pro-inflammatory cytokines in GCF and the presence of periodontal disease is further supported by significant positive correlations that were consistently demonstrated in the current study between mGI, mean PD, PESA and PISA and GCF levels of IL-6, TNF-α and IL-1β. Furthermore, the current study also demonstrated that GCF levels of IL-1β and IFN-γ were significant predictors of PISA, and a trend for GCF TNF-α levels as a predictor of PISA was also noted. Data from the current study therefore corroborate published research that demonstrated significant positive correlations between GCF levels of IL-1β and clinical periodontal parameters in subjects with T2DM (Engebretson *et al.*, 2004; Engebretson *et al.*, 2007).

Previous studies investigating the impact of periodontal therapy on GCF cytokine levels demonstrated significant reductions in GCF IL-1β, IL-6 and IFN-γ post-treatment (Engebretson *et al.*, 2002; Thunell *et al.*, 2010). Additionally, in patients withT2DM, although one study demonstrated significant reductions in IL-1β (Correa *et al.*, 2008), others showed a non-significant reduction in GCF IL-6 (Kardesler *et al.*, 2011) or no significant changes in GCF TNF-α after NSM (Santos *et al.*, 2010). In the current study, GCF samples from both diabetic and non-diabetic subjects showed significant reductions in IL-1β at 3, 6 and 12 months after NSM. Similarly, reductions in TNF-α and IL-6 in GCF were seen after NSM, although not at every time point. Overall, in both T2DM and non-diabetic subjects, reductions in GCF levels of selected pro-inflammatory cytokines would appear to mirror improvements in periodontal health seen following NSM.

It is now recognised that biomarkers in saliva have the potential to provide information to aid in the diagnosis and monitoring of periodontal disease (Miller *et al.*, 2010). Published data demonstrated higher levels of Il-1β in saliva from subjects with

periodontitis compared to those without periodontitis (Miller *et al.*, 2006; Gursoy *et al.*, 2009), and this is mirrored in the current study which showed that IL-1β levels in saliva were significantly higher in subjects with periodontitis compared to those with healthy periodontal tissues, in both diabetic and non-diabetic subjects. This would suggest that levels of IL-1β in saliva reflect the periodontal status. Similarly, IL-6 in saliva was significantly higher in subjects with periodontitis compared to those with healthy periodontal tissues, but the picture is less clear with differences only demonstrated in subjects with T2DM. Interestingly, when pre-treatment levels of cytokines in saliva from subjects with T2DM were compared to levels from non-diabetic subjects, TNF-α, IL-1β and IFN-γ were significantly higher in non-diabetic subjects compared to subjects with T2DM, most likely reflecting the more severe periodontal disease present in the non-diabetic subjects compared to subjects with T2DM seen in the current study. Significant positive correlations were also found in the current study between mGI, % BOP, PESA and PISA and saliva levels of IL-6 and IL-1β. Furthermore, salivary levels of IL-6 and IL-1β were significant predictors of PISA. This corroborates published research that demonstrated significant positive correlations between levels of IL-1β in saliva and clinical periodontal parameters (Miller *et al.*, 2006; Tobon-Arroyave *et al.*, 2008; Gursoy *et al.*, 2009), and reflects the potential importance of saliva as a diagnostic fluid for periodontal disease.

One previous study has demonstrated significant reductions in IL-1β in saliva after NSM (Sexton *et al.*, 2011). In the present study, a reduction in IL-1β in saliva was detected as a trend at 3 months after NSM in subjects with T2DM, and non-significant reductions in IL-1β in saliva were seen at 6 and 12 months in non-diabetic subjects. Taken collectively, it would suggest that IL-1β may reflect the clinical response to

periodontal therapy, although clearly, further investigations are warrant to confirm whether IL-1β in saliva is a useful biomarker for periodontitis.

In conclusion, the findings of the present study contribute to the knowledge of the clinical and biological response to periodontal therapy, both in diabetic and non-diabetic patients. Moreover, the current study highlights the importance of studying pro-inflammatory cytokines in both periodontal disease and diabetes, and more specifically, pro-inflammatory cytokines such as IL-6, TNF-α and IL-1β show potential as contributors to the pathogenic links between diabetes and periodontal disease, with the consideration of IL-1β in saliva and GCF as a prognostic marker for periodontitis in individuals with and without diabetes. It is encouraging that a reduction in HbA1c was observed following periodontal therapy in the diabetic patient group, although this failed to achieve statistical significance. Additional research is warranted to investigate this further, ensuring that groups are well matched for periodontal status.

Chapter 8 References

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