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PERIODONTAL CONDITIONS AND TREATMENT OUTCOMES FOR SUBJECTS WITH DIABETES MELLITUS: SPECIAL EMPHASIS ON HBA1C LEVELS AND T-CELLS

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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This book is dedicated to my amazing parents and family.

Anything is achievable with you by my side.

ABSTRACT

A two way relationship between periodontal disease (PD) and diabetes mellitus has previously been reported. This thesis was aimed to further analyze the influence of PD on blood glucose levels of subjects with type 2 diabetes mellitus (T2D) and vice versa. Periodontal conditions (bleeding on probing [BOP], plaque index [PI], periodontal pocket depth [PPD] [4mm < 6mm and ≥ 6mm]) and marginal bone levels) were investigated in subjects with pre-diabetes as well as those with or without diabetes mellitus. HbA1c levels in individuals with periodontal disease and diabetes mellitus were analyzed after non-surgical and surgical treatments. This thesis also assessed the influence of diabetes on infiltration of inflammatory cells, T lymphocytes and B lymphocytes in gingiva and the effect of glucose control on T-cell motility.

AIMS

Study I aimed to evaluate the difference in periodontal conditions between subjects with pre-diabetes, well or poorly controlled diabetes mellitus and non-diabetes. Hemoglobin A1c levels, BMI and WC were also measured.
Study II aimed to study the effect of periodontal treatment (non-surgical and surgical) on HbA1c levels in subjects with or without PD and with or without diabetes mellitus. HbA1c, BOP, PI and PPD 4mm <6mm; ≥6mm were recorded.
Study III aimed to investigate the infiltration of T and B lymphocytes in gingival biopsies of diabetic and non-diabetic subjects with or without periodontal disease.
Study IV examined the influence of D-glucose on T cell motility.

RESULTS

In *study I*, thirty-seven percent of all individuals who reported to be non-diabetics were pre-diabetics. An increase in the number of PPD ≥ 6mm was seen in subjects with poorly controlled diabetes as compared to pre-diabetics and non-diabetics (P<0.05).
In *study II*, periodontal inflammatory conditions in all groups improved with non-surgical and surgical treatment. Subjects with diabetes mellitus showed decreased levels in HbA1c over a period of 3 and 6 months. No changes were detected in PPD 4mm <6mm in diabetics after treatment. Subjects with diabetes require periodontal treatment for at least 6 months to reduce blood sugar levels.
In *study III*, the number of gingival CD4 and CD19 positive cells was reduced in diabetic individuals suggesting that diabetes inhibits lymphocyte entry into gingival tissues.
In *study IV*, it was demonstrated that D-glucose, at a high concentration, intially stimulated T-cell motility while subsequently causing inhibition. These effects were associated with increased cell surface expression of TSP-1 and LRP-1.

In conclusion,

This thesis shows that diabetes mellitus influences the periodontal conditions and treatment of periodontal disease has an influence on blood sugar status. Furthermore, diabetes mellitus influences the immunological status where high blood glucose sugar levels influence T cells. Overall, this thesis strenghtens the bidirectional relationship between PD and diabetes mellitus from both a pre-clinical and a clinical aspect.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following articles, which are referred to in the text by their Roman Numerals.

I. **Altamash M,** Arledal S, Klinge B, Engström P.E. Pre-diabetes and diabetes: Medical risk factors and periodontal conditions. Acta Odontologica Scandinavica 2013; 71:1625-31.

II. **Altamash M,** Klinge B, Engström P.E. Periodontal treatment and HbA1c levels in subjects with diabetes mellitus. Journal of Oral Rehabilitation 2016; 43:31-8.

III. **Altamash M,** Hasan SM, Engström P.E, Sundqvist K. Type 2 diabetes mellitus reduces the number of gingival T and B cells in patients with periodontitis.
(Manuscript)

IV. **Altamash M,** Engström P.E, Sundqvist K. Glucose control of T cell motility through endogenous thrombospondin-1 and its receptor low density lipoprotein receptor-related protein-1.
(Manuscript)

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CONTENTS

LIST OF ABBREVIATIONS

AGE	Advanced Glycation End Products
BMI	Body Mass Index
BOP	Bleeding on Probing
HbA1c	Hemoglobin A1c
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
FPG	Fasting Plasma Glucose
LRP-1	Lipoprotein receptor -Related Protein-1
MBL	Marginal Bone Level
OGTT	Oral Glucose Tolerance Test
PD	Periodontal disease
PPD	Periodontal Pocket Depth
PI	Plaque Index
RAGE	Human Receptor for Advanced Glycation Endproducts
RBGL	Random Blood Glucose
SD	Standard Deviation
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TSP-1	Thrombospondin-1
WC	Waist Circumference
WHO	World Health Organization

1 INTRODUCTION

The link between periodontal disease and diabetes mellitus will be highlighted in this thesis. Supporting evidence will be provided to illustrate how persisting hyperglycemia influences the immuno-inflammatory response to periodontal tissues and how periodontal treatment will have an influence on blood glucose level. Periodontal disease has been reported as a strong predictor of insulin resistance and is considered to be one of the six complications of diabetes (Löe, 1993; Demmer et al, 2012). Poorly controlled diabetes may trigger the severity of periodontal disease (Oppermann et al, 2012; Borgnakke et al, 2013). It has been reported that insulin resistance is linked to inflammation which contributes to periodontal disease (Mealey et al, 2006). Studies concerning the effect of non-surgical periodontal treatment on blood glucose levels have been performed. In this thesis we study the effect of non-surgical and surgical treatment of periodontal disease on HbA1c levels. Furthermore we also asses the influence of diabetes mellitus on infilterating inflammatory cells, T lymphocytes and B lymphocytes in gingiva and the effect of glucose on T-cell motility.

2 PERIODONTAL DISEASE

Periodontitis is an inflammatory disease of bacterial origin that destroys the tissues supporting the teeth; periodontal ligament, gingiva, cementum and alveolar bone (Nanci et al, 2006). Clinicians use both clinical and radiographic findings to diagnose periodontitis in patients (International Workshop for the Classification of Periodontal Diseases and Conditions 1999; Armitage, 2004). Clinical signs of periodontitis are manifested as change in color and texture in gingiva. Classical signs are redness and swelling (Figure 1A). In advanced stages, there is loss of supporting tissue surrounding the tooth in contrast to a healthy periodontium where there the texture is firm and coral pink in color with no loss of function (Ho et al, 2015) (Figure 1B). Clinically, disease presence and severity is examined by the measurement of deep periodontal pockets that enable settlement of micro-organisms and resulting inflammation is manifested by redness, gingival swelling and pain (Armitage, 2004).

2.1 WORLDWIDE PREVALENCE RATE

About 90% of the world's population is affected by inflammatory periodontal diseases making it the most common chronic inflammatory condition (Pihlstrom et al, 2005). Globally around 5-15% of the adult population is affected by severe periodontitis which refers to the presence of ≥ 6mm pocketing (Dye et al, 2012). In 2010 the prevalence of periodontal disease was reported to be 10.8% who were affected worldwide (Kassebaum et al, 2014).

2.2 PREVALENCE IN USA AND GREAT BRITAIN

In United States reports from 2009 to 2012 showed that 64.7 million individuals had periodontal disease, almost 8.9% of whom had severe periodontal conditions (Eke et al, 2015). An Adult Dental Health Survey (2009) conducted in England, Wales and Northern Ireland identified 8% of adults with at least one pocket of 6mm or deeper (White et al, 2011).

2.3   PREVALENCE IN PAKISTAN

So far, there is no research study done in Pakistan to assess the incidence and prevalence of periodontal diseases at a national level. A national oral health survey was conducted in 2003 in collaboration with WHO which was limited to 21 out of 157 districts only. Periodontal health was a minor component of this survey. The survey was not designed to assess incidence and prevalence of periodontal disease. However, this survey did show that periodontal health in Pakistan was very poor. Only 28% of 12 year olds had healthy gums and more than 93% of the 65 years old had some gingival or periodontal disease. These results indicate that periodontal disease including gingivitis is endemic in Pakistan. It has been reported that the incidence of periodontal disease is higher in the rural population (Oral health in Pakistan. A situation analysis, 2003). Studies conducted at regional and local level also show high prevalence of periodontal disease (Chauhdry et al, 2008).

Figure 1A Clinical signs of periodontal disease

Figure 1B Clinical signs of healthy gums

3 VARIABLES OF PERIODONTAL CONDITIONS

3.1 DENTAL PLAQUE/ ORAL BIOFILM

Oral biofilm that develops on the dental surfaces is known as dental plaque. A soft tenacious material found on tooth surfaces, not easily removed by rinsing with water, is termed as dental plaque (Dawes et al, 1963). Plaque beside the gingival border of the tooth surface is called supragingival plaque. Plaque found below the gingival margin in the gingival sulcus or in the periodontal pocket is called subgingival plaque. Subgingival plaque creates interproximal space due to edema and gives rise to inflammatory reactions and loss of periodontal attachment filled by gingival papilla (Theilade et al, 1966; Listgarten et al, 1975; Theilade et al, 1975; Listgarten et al, 1976a). Dental plaque has been defined as several species of bacteria embedded in a matrix of polymers surrounding the tooth surface (Marsh et al, 2011).

3.2 BLEEDING ON PROBING

Bleeding on probing, a reliable indicator of gingival inflammation in response to bacterial pathogens, is used to diagnose and monitor periodontal disease alongside other assessments as periodontal pocket depth, clinical attachment loss, and marginal bone loss (Caton et al, 1988; Abbas et al, 1990; Lang et al, 1990; Lang et al, 1991; Karayiannis et al, 1992).

3.3 PERIODONTAL POCKET DEPTH

Periodontal pocket is considered as the cardinal sign of periodontitis. It is an extension of the junctional epithelium along the root surface causing abnormal apical extension of gingival sulcus. Due to disease development, periodontal ligament is separated and pocket epithelium is formed (Page et al, 1976). Accurate measurement and identification of changes in periodontal pockets is necessary for evaluating the severity, progression and treatment of the disease (Taba et al, 2005).

4 ASSESSMENT OF POCKET DEPTH AND CLINICAL ATTACHMENT LOSS

When measuring pocket depth and clinical attachment loss it is important to note that the values depend on the inflammatory condition of the tissues. When a normal gingival tissue is probed it is restricted from the coronal to the apical extent of the junctional epithelium, which is at the cementoenamel junction (Listgarten et al, 1976b; Highfield, 2009). When inflammation is present in the gingiva and inflammatory cells have infiltrated the gingival connective tissue, there is less resistance to probe penetration and the periodontal probe generally passes apical to the level of the connective tissue attachment (Robinson and Vitek, 1979; Fowler et al, 1982). Therefore, PPD and clinical attachment level measurements may be overestimated in inflamed areas and underestimated in healthy tissues (Leroy et al, 2010).

5 RADIOLOGY

Radiographs are used in the diagnosis of periodontal disease. They can be utilised to assess bone loss and identification of other periodontal relevant features, such as calculus deposits, furcation lesions, and overhanging restoration margin. Periapicals, panoramic radiographs and horizontal and vertical bitewings can be used in periodontal assessment (Jeffcoat et al, 1992; Tugnait and Carmichael, 2005; Mol and Balasundaram, 2008). Panoramic radiography is an extra oral procedure which creates the picture of the entire maxillomandibular region on a single film (Choi, 2011). Panoramic examination have showed an improved detection rate of 31.9% for periodontal diseases as compared to clinical examination alone (Shin et al, 2010). Calculi deposition of 62.6% has been reported in screening panoramic radiographs (An et al, 2007). It has been reported that panoramic radiographs detect more periodontal bone loss than periapical radiographs (Galal et al, 1985) and is useful for dental health screening in detection of periodontal disease (Muhammed et al, 1982; Meister et al, 1997). Several studies identified that when clinical examination was supplemented by radiographic examinations, the overall number of subjects with periodontal disease that were detected improved substantially (Galal et al, 1985; An et al, 2007; Shin et al, 2010).

6 RISK FACTORS FOR PERIODONTAL DISEASE

A risk factor can be defined as an incident that has been associated with increased intensity of disease. It is imperative to identify the dissimilarities between risk factors that are correlated with a disease although they do not necessarily trigger the disease. Risk factors can be modifiable or non-modifiable. Modifiable risk factors are commonly environmental in nature while non-modifiable risk factors are typically inherent to the individuals (Van Dyke et al, 2005).

6.1 MODIFIABLE RISK FACTORS

6.1.1 Diabetes mellitus

Diabetes mellitus is considered a modifiable factor which can be treated although it cannot be cured. Previous studies have reported an association between periodontal disease and poor glycemic control (Tervonen et al, 1994; Cutler et al, 1999; Tsai et al, 2002; Guzman et al, 2003). Both diseases have a potential influence on one another, a relationship termed as 'bi-directional' (Taylor, 2001; Nishimura et al, 2003). Previous longitudinal studies have shown that subjects with diabetes demonstrated increased risk of periodontal destruction, in comparison to subjects without diabetes. It was also reported that poorly controlled diabetics respond less effectively to periodontal treatment as compared to well-controlled diabetics and non-diabetics (Westfelt et al, 1996; Tervonen and Karjalainen, 1997).

6.1.2 Obesity

Obesity is one of the most important health concerns in urbanized countries (Doll et al, 2002). Obesity is a well-known risk factor in the occurrence of periodontal disease. Factors that may trigger periodontal disease may be related to increased body mass index (BMI) (Elter et al, 2000; Grossi and Ho, 2000) and waist circumference (WC) (Saito et al, 1998; Grossi and Ho, 2000; Shuldiner et al, 2001). Furthermore, obesity could develop immunological mechanisms aggravating poor periodontal conditions (Zhu and Nikolajczyk, 2014).

6.1.3 Smoking

Different studies have reported the relationship between the amount and duration of smoking and the severity of periodontal disease (Tomar and Asma, 2000; Johnson and Gill, 2004).

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6.1.4 Socioeconomic Status

Periodontal disease is associated with poor socioeconomic status (Javed et al, 2007). It has been reported that poor education and low income are associated with severe periodontitis (Borrell et al, 2006).

6.1.5 Stress

Stress as a risk factor, may instigate onset and progression of periodontal disease (Genco et al, 1998; Johannsen et al, 2005; Rosania et al, 2009).

6.2 NON-MODIFIABLE RISK FACTORS

6.2.1 Age

It is important to consider the age of the subjects when evaluating periodontal disease progression using marginal bone level assessment. This is due to the fact that prevalence of periodontal disease increases with age (Abdellatif and Burt, 1987; Albandar, 1990; Velden, 1991; Persson et al, 1998).

6.2.2 Genetic Factors

Identical twins propose 50% of vulnerability to periodontal disease due to host factors, even though bacterial infection is a key etiological agent in periodontal disease (Michalowicz et al, 2000).

7 ROLE OF T AND B LYMPHOCYTES IN PERIODONTAL DISEASE

The complex interactions between periodontopathic bacteria and host immune system plays an important role in periodontal disease (Kinane, 2001). Immunohistochemical studies have shown that an intense recruitment of polymorphonuclear leukocytes, B cells, antibody producing plasma cells and T cells within periodontal tissues are characterized as a local inflammatory response (Wassenaar et al, 1996). A study reported that in periodontal disease, a depressed T helper/T suppresser ratio was seen when compared to healthy patients (Celenligil et al, 1993). Evidence showed that T cells contribute to chronic periodontal disease and thereby control B cell activation (Wassenaar et al, 1995; Yamazaki et al, 1995).

8 TREATMENT OF PERIODONTAL DISEASE

Periodontal disease prevention and management requires patient's participation in the treatment process. Studies regarding plaque control have demonstrated that regular education in self-care techniques resulted in lesser periodontal attachment loss and healthier tooth surfaces (Axelsson et al, 2004). The crucial aim of periodontal treatment is to inhibit the inflammatory disease progression. Treatment requires mechanical elimination of the subgingival biofilm. Clinical parameters include bleeding on probing (BOP) and periodontal pocket depth (PPD) measurements which were used to evaluate periodontal conditions. To recover periodontal health an ideal treatment goal would be reducing the following parameters, bleeding on probing (BOP) and periodontal pocket depth (PPD). Chronic periodontal disease can be effectively treated provided adequate plaque control which is maintained through non-surgical or surgical treatment (Lindhe and Nyman, 1975; Nyman et al, 1977; Axelsson and Lindhe, 1981).

8.1 NON-SURGICAL TREATMENT OF PERIODONTAL DISEASE

Non-surgical therapy includes scaling and root planning with manual and/or ultrasonic instruments in addition to oral hygiene instructions. Several studies have reported a significant reduction in PPD, PI and BOP after non-surgical therapy (Axelsson & Lindhe, 1981, Badersten et al, 1987).

8.2 SURGICAL TREATMENT OF PERIODONTAL DISEASE

The purpose of flap procedure is to attain access and visibility of root surface for complete instrumentation with debridement that eliminates pockets, plaque and pathological tissues (Heitz-Mayfield et al 2002). Surgical treatment consists of open flap debridement to treat periodontal disease that is reported to be successful in stabilization of periodontal pocket depths (Ramfjord et al, 1974; Aljateeli et al, 2014; Mailoa et al, 2015).

9 PRE-DIABETES

Pre-diabetes is defined as an increased blood glucose level above the normal range that does not determine the standard to diagnose diabetes mellitus (Nichols et al, 2007). Impaired fasting glucose levels (IFG), fasting plasma glucose (FPG) levels from100mg/dl (5.6mmol) to 125mg/dl (6.9mmol/l) and oral glucose tolerance test (OGTT) levels from 140 mg/dl (7.8mmol/l) to 199mg/dl (11.0mmol/l) are found in pre-diabetic individuals. HbA1c levels between 5.7–6.4% (39mmol/mol - 46mmol/mol) are also used to diagnose pre-diabetes (Lorenzo et al, 2010) (Abraham and Fox, 2013). The risk of development of T2D is high in pre-diabetics (Nathan et al, 2007), 50% of pre-diabetics may be at risk of developing T2D within a period of 5 years (Cowie et al, 2010; American Diabetes Association 2011). In the year 2012, 86 million Americans aged 20 and older had pre-diabetes (American Diabetes Association. Statistics About Diabetes, 2014).

10 DIABETES

Diabetes is a group of metabolic disorders characterized by hyperglycaemia due to defective insulin secretion, defective insulin action or both (American Diabetes Association, 2009).

10.1 TYPES OF DIABETES

The types of diabetes are type 1 diabetes (T1D), type 2 diabetes (T2D) and gestational diabetes. Type 1 diabetes (referred to as insulin dependent diabetes or juvenile diabetes in the past) is a disorder in which insulin producing β-cells fail to produce insulin. This occurs due to an autoimmune destruction of insulin producing β-cells in the pancreas (American Diabetes Association 2009). A major risk factor for type 1 diabetes is genetic susceptibility. Furthermore, the onset of T1D in susceptible individuals appears to be triggered by environmental factors, viral infections, nutrition and lifestyle (Von Herrath, 2009). T2D (referred to as non-insulin-dependent diabetes or adult onset diabetes) arises due to insulin resistance. This occurs when the cells in the body do not respond to insulin properly, thus reducing the capacity to transfer glucose out of circulation and into the cells. This results in hyperglycaemia. During the early stages, the β-cells of the pancreas may secrete a normal amount of insulin, but this can decrease over time, leading to insulin deficiency and insulin resistance. Almost 90-95% of all diabetic cases comprise of T2D. This disorder is commonly associated with risk factors relating to lifestyle, such as obesity, lack of exercise, poor dietary habits and genetic susceptibility (American Diabetes Association, 2010). Individuals with high waist circumference, in males ≥ 40 inches (102cm) and in females ≥ 35 inches (88cm) and BMI ≥ 30 are considered overweight or obese and are at increased risk of developing T2D (Lundgren et al, 1989; Ganz et al, 2014) In gestational diabetes, pregnant women without a history of diabetes develop hyperglycemia during gestation. It is characterized by both insulin resistance and decreased insulin secretion. While the symptoms usually improve after giving birth, a small number of women continue to have diabetes post pregnancy (Casanova et al, 2014).

10.2 PREVALENCE OF DIABETES

10.2.1 Worldwide Prevalence Rate

The prevalence of diabetes has increased in low and middle income countries. World health organization estimated global prevalence of diabetes at 8.5% percent among adults aged 18 years and above in the year 2014, with a projection that diabetes will become the seventh leading cause of death by 2030 (WHO Global report on diabetes, 2016; Mathers et al, 2006). According to World Health Organization, the rising incidence of diabetes is now considered an epidemic (WHO Global Oral Health, 2003). As discussed above, diabetes is a major public health issue reaching epidemic proportions globally, almost 6% of the world's population is presently affected by this metabolic disease (Adeghate, 2006).

10.2.2 Prevalence in Asia

It is estimated that number of T2D subjects will rise to 201.8 million by the year of 2035 in South Asia (Nanditha et al, 2016). T2D affects approximately 1.2% to 14.6% people in Asia, 4.6% to 40% people in the Middle East, and 1.3% to 14.5 % people in Iran (Azizi, 2003).

10.2.3 Prevalence in USA

Among 7.8 million diabetics in the US, 90–95% has T2D with the remaining having T1D (Centers for Disease Control and Prevention: National diabetes fact sheet United States, 2008). More than 95% of diabetics over 45 years of age have T2D (Kenny et al, 1995).

10.2.4 Prevalence in Pakistan

The prevalence of diabetes is high in Pakistan. Pakistan has four provinces named Sindh, Balochistan, Punjab and Khyber Pakhtoon Kahwa. As reported earlier in three surveys, the three provinces Sindh, Balochistan and Khyber Pukhtoon Khawa have a diabetes prevalence 13.9%, 8.6% and 11.7% respectively. The early surveys in Punjab, the fourth province of Pakistan, showed high prevalence rate of IGT (Shera et al, 2010). Population of Pakistan is estimated to be around 158 million with 10% having T2D (Nishtar, 2004). This is notably high when compared to Sweden where only an estimated 3% to 4% individuals have diabetes (Berger, 1998). It is estimated that there will be a dramatic increase in diabetes in the US from year 2000 to 2030, from 17.7 million to estimated 30.3 million people being affected. Pakistan will also have an estimated 13.9 million people becoming diabetic during the same period (Wild et al, 2004). The country with the highest prevalence of diabetes mellitus is Tokelau, which is 37.5% (International Diabetes Federation, IDF Diabetes Atlas, 2015).

11 RISK FACTORS FOR DIABETES MELLITUS

11.1 GENDER

It has been reported that higher number of males suffered from diabetes mellitus than females worldwide in the year of 2015 (215.2 million men vs 199.5 million woman). It is expected that there will be a decrease in diabetes in males by 15.1 million compared to females with diabetes by 2040 (328.4 million men vs 313.3 million women) (International Diabetes Federation, IDF Diabetes Atlas, 2015).

11.2 FAMILY HISTORY

Diabetes is associated with family history which can help in detection and prevention of the disease (Valdez et al, 2007).

11.3 SOCIOECONOMIC STATUS

Poor education and a low socioeconomic status is linked with a high prevalence of T2D (Larranaga, 2005).

11.4 OBESITY

Different studies favour central obesity as a great risk factor for developing insulin resistance and diabetes (Kahn et al, 2000, Guilherme et al, 2008).

11.5 STRESS

It has been reported that stress is considered as one of the risk factors in the development of T2D (Surwit et al, 2002). Oxidative stress has also been associated with the development of T2D (Maritim et al, 2003).

12 DIAGNOSTIC TESTS FOR DIABETES

The criteria for diagnosis of T2D are HbA1c $\geq$ 6.5% (48 mmol/mol) according to National Glyco-hemoglobin Standardization Program NGSP and SI units (mmol/mol). Fasting is defined as no caloric intake for at least 8 hours. However other criteria's for diagnosing diabetes can be performed by Oral Glucose Tolerance Test (OGTT) 2-hrs plasma glucose $\geq$ 200mg/dL (11.1mmol/L), a random plasma glucose $\geq$200 mg/dL (11.1mmol/L) and fasting plasma glucose (FPG) $\geq$126 mg/dL (7.0mmol/L) (International Expert Committee, 2009).However OGTT is not commonly carried out in clinical practice because of time, cost and lesser reproducibility (Ko et al, 1998). A more preferred and accurate test for measurnenet of HbA1c levels for diagnosing diabetes is SI units system according to the international HbA1c consensus committee (Jones et al, 2011). HbA1c is a measure of glycosylated hemoglobin over a period of 2-3 months and is correlated with FPG (Simon et al, 1989; Rohlfing et al, 2002; Woerle et al, 2004). HbA1c levels are more reproducible than FPG. It is reported that coefficients of variation between HbA1c and FPG are 1.7% and 5.7% respectively (Barr, 2002). Moreover, the measurement of HbA1c does not require the patient to fast. The use of HbA1c could better integrate chronic hyperglycemia than FPG. HbA1c is defined as a hemoglobin of an adult (HbA molecule) bound with glucose at its N-terminal valine that are residues of beta chain hemoglobin. Glycation of haemoglobin is mainly regulated by glucose concentrations, which occurs within erythrocytes and is a nonenzymatic process (Carruthers, 1990). A two-step Maillard reaction develops Glycation. This two-step procedure includes the preliminary formation of a labile Schiff base and further goes through Amadori reorganization to form Amadori Product which are called HbA1c (Gallagher et al, 2009).

13 ADVANCED GLYCATION END PRODUCTS (AGES)

The pathogenesis concerning the risk for developing periodontal disease in diabetic subjects is unknown, although it has been linked with high deposition of glucose leading to the formation of advanced glucose end products (AGEs) (Zizzi et al, 2013). AGEs are chemically characterized as N-carboxymethyl-lysine and pentosidine compounds produced by a non-enzymatic reaction for reducing sugars, proteins and lipids (Goh et al, 2008). Hyperglycemia, hyperlipidemia and oxidative stress accelerate AGEs levels which can further cause interference in the activity of glycated macromolecules, enzymes and surface binding receptors on AGEs (RAGE), triggering inflammatory responses via leukocytes (Rabbani et al, 2011; Beisswenger et al, 2012). The pathogenesis behind diabetic complication could be attributed to AGE formation resulting from persistent hyperglycemia (Brownlee, 1994; Vlassara, 1994). Diabetic patients with periodontitis have AGEs leading to an increase in oxidative stress (Schmidt et al, 1996). The findings of these studies have been reported to indicate that the increase in oxidative stress within gingival tissues is considered to contribute to periodontal tissue destruction in individuals with diabetes (Patil et al, 2016).

14 SYSTEMIC AND LOCAL EFFECTS OF DIABETES

Diabetes can lead to both systemic and local inflammation, hence contributing to diabetes associated morbidity and mortality (Al-Maskari et al, 2011). The systemic effects of diabetes result due to a non-regulated immuno-inflammatory process, which affects the tissues locally and systematically. Due to the microbial nature, secondary mediators are formed, which further aggravate inflammatory reactions. The source of such systemic infection is the oral cavity itself, which regularly releases bacteria in the blood stream thereby attacking the body at regular intervals. These patients therefore are at risk of developing end stage renal disease, atherosclerosis, cardiac and renal related complications (Berlanga-Acosta et al, 2013; Sharma et al, 2016).

15 EFFECT OF DIABETES ON ORAL HEALTH

Influence of diabetes on oral health conditions has been well documented. Oral conditions in patients with diabetes exhibit dental caries, xerostomia (dry mouth), tooth loss, gingivitis, chelitis, and increased glucose levels in saliva and periodontitis (Rees et al, 2000).

16 EFFECT OF DIABETES ON PERIODONTAL HEALTH

Studies have investigated the association between glycemic control in T2D and periodontal disease (Unal et al, 1993; Novaes et al, 1996; Taylor et al, 1998). A longitudinal epidemiological study of the Pima Indians in Arizona, USA (Taylor et al, 1996) found that subjects with T2D in good to moderate glycemic control diagnosed with severe periodontitis at baseline were approximately six times more likely to have poor glycemic control at approximately 2-years follow-up than those without severe periodontitis at baseline. In another observational study of adults with T2D, aged 58–77 years reported an association between advanced periodontal disease and impaired metabolic control (Collin et al, 1998). Periodontal disease has been linked with diabetes (Sharma et al, 2016). Studies show that diabetics are more likely to suffer from periodontal disease in both frequency and severity than non-diabetics. The length of the disease further contributes to oral health outcomes. Individuals with diabetes may experience onset of periodontal problems at an earlier age compared to non-diabetic individuals (Löe, 1993). Some case reports showed that there is an increased prevalence and severity of periodontal conditions in individuals with poorly controlled diabetes (Ainamo et al, 1990). Marginal bone loss around teeth is four times greater in individuals with T2D when compared to non-diabetic individuals (Taylor et al, 1998). Studies have reported that individuals had higher marginal bone loss, fulminated by poorly controlled diabetes (Javed et al, 2007). Evaluation of HbA1c levels at dental offices of undiagnosed diabetes mellitus and pre-diabetic patients will not only help in early diagnosis but will further prevent risk and complications of periodontal disease (Holm et al, 2016).

17 PERIODONTAL TREATMENT AND BLOOD GLUCOSE LEVELS

There are several studies that have reported improvement in blood glucose levels due to periodontal treatment (Katagiri et al, 2013). A meta-analysis was done where five studies were selected among 639 intervention studies. It was found that there was significant decrease of 0.4% in HbA1c levels in T2D patients after undergoing periodontal treatment (Teeuw et al, 2010). A study with 514 patients in a six month trial reported that non-surgical periodontal therapy did not improve glycemic control in diabetic patients with periodontitis (Engebretson et al, 2013). This trial did not support the results of smaller clinical trials and meta-analyses that showed improvements in HbA1c levels as a result of non-surgical periodontal therapy (Borgnakke et al, 2014).

18 QUESTIONNAIRES

Self-reported investigations have been found as valid means of surveillance of periodontal disease and several systemic diseases including diabetes mellitus, cardiovascular disease, cerebral vascular disease and other associated risk factors (Taylor et al, 2007). Questionnaires are helpful in investigating oral health attitudes of subjects (Halawany et al, 2015). Validity and reliability of questionnaires on oral health and disease are important for selection of self-assessed data (Klinge, 2006). It is also cost effective to gather large amount of data from subjects using "questionnaires".

19 AIMS OF THESIS

19.1 GENERAL AIM

The aim of the study is to characterize periodontal conditions in different stages of diabetes, to explore the periodontal treatment outcomes for patients with T2D with special emphasis on HbA1c levels, to investigate the gingival T cells presence in periodontal disease and influence of glucose on T cell motility.

19.2 SPECIFIC AIMS

Study I

To compare the risk factors, HbA1c, BMI and WC with periodontal conditions and to further evaluate the difference between subjects with pre-diabetes, T2D and non-diabetes. Hypothesis is that increased blood sugar levels in pre-diabetic and diabetic subjects will give rise to severe periodontal conditions.

Study II

To investigate non-surgical and surgical treatment outcomes on periodontal conditions, as well as blood glucose levels (HbA1c) in T2D and non-diabetic subjects with and without PD.

Study III

To investigate the influence of T2D on the presence of T and B cells in the gingiva of individuals with and without periodontal disease.

Study IV

To investigate the influence of D-glucose on motility and cell surface expression of endogenous thrombospondin-1(TSP-1) and its receptor low density lipoprotein receptor-related protein-1(LRP-1) in T cells.

20 ETHICAL CONSIDERATIONS

The research project was approved by the local ethical board review in Stockholm, Sweden and the ethical committee of Altamash Institute of Dental Medicine, Karachi, Pakistan. Before including subjects for the study, a written informed consent in the native language, Urdu was obtained.

21 MATERIAL AND METHODS

This thesis is based on four studies, which are in the text referred to their Roman numerals (Study I to Study IV). The data collection was obtained through questionnaires, clinical examination, radiological investigations and laboratory based techniques.

21.1 STUDY I

In **Study I,** questionnaires, periodontal examinations, radiographical investigations, measurements of RBGL, HbA1c, BMI and WC were obtained in a local dental hospital in Karachi, Pakistan.

21.2 STUDY II

In **Study II,** a study was designed for T2D subjects and non-diabetic subjects which were undergoing non-surgical periodontal treatment at baseline. The inclusion criteria was adults aged 25 years and above. Subjects were analyzed and assessed through a questionnaire. All subjects with and without PD underwent scaling therapy, which included oral hygiene instructions and plaque control assessment at baseline. After three months, diabetic and non-diabetic subjects with PD were treated by performing periodontal surgery for PPD $\geq$ 6mm with Modified Widman flap (Ramfjord and Nissele, 1974). Criteria for subgingival scaling after three months was PPD < 6mm. Post-op instructions for individuals receiving periodontal surgery was rinsing mouth thrice daily with 0.2% chlorhexidine mouthwash for 3 weeks. Periodontal conditions and blood glucose levels were evaluated after 3 and 6 months in subjects with and without PD. Diabetic and non-diabetic subjects without PD were also re-examined after 3 months.

21.3 STUDY III

In **Study III**, subjects who came for dental treatment to Altamash Institute of Dental Medicine, Karachi, Pakistan were examined. A medical and dental history questionnaire form was filled in by the patients and periodontal examination of full mouth was carried out. Forty-nine gingival punch biopsies were collected from diabetic and non-diabetic subjects with or without periodontal disease. Fifteen gingival punch biopsies were collected from diabetic subjects with periodontal disease, four biopsies from diabetic subjects without periodontal disease. Fifteen gingival punch biopsies were collected from non-diabetic subjects with periodontal disease and 15 from non-diabetic subjects without periodontal disease.

21.4 STUDY IV

In **Study IV**, blood lymphocyte cells were collected from healthy subjects and purified using lymphoprep, depletion of monocytes was done by treatment with carbonyl iron and magnetic removal of phagocytic cells. Antigen expression was detected with monoclonal antibodies and for intracellular antigens cells detection.

22 BLOOD GLUCOSE EXAMINATION

Venous blood samples were collected to determine HbA1c levels and were analyzed at Karachi Laboratory Diagnostic Center, Karachi, Pakistan. Roche TinaquantR Hemoglobin HbA1c II method was used to analyze blood glucose levels (Hitachi Modular P Analyzer Indianapolis, IN). In study I, II and III blood glucose levels were measured for all subjects. HbA1c levels were measured at baseline, three and six months post treatment in study II.

23 PERIODONTAL EXAMINATION

23.1 STUDY I

In **Study I**, initially fifteen subjects underwent measurement of periodontal pocket depth (PPD) which were recorded and calibrated to compare the results. Periodontal measurements of PI (Ainamo and Bay, 1975) and BOP (Mühlemann and Son, 1971) were also recorded. Full mouth assessment was carried out including maxillary and mandibular teeth excluding third molars. PI, BOP and PPD were measured at four sites (mesial, distal, buccal and lingual/palatal). Pocket depth was measured to the nearest millimetre by a graded probe (Hu-Friedy Manufacturing, Chicago, IL). Periodontal pocket depths were recorded between 4mm <6mm and $\geq$ 6mm and embedded root remnants were considered as missing teeth. Extra-oral panoramic radiographs (Kodak T-Mat, Eastman Kodak, Rochester, NY and Konica Minolta MG-SR PLUS NIF, finished in Mexico for Konica) of all the selected subjects were taken using panoramic tomography machine (Villa Sistemi Medicali, MR05, Buccinasco (MI), Italy and Yoshida Panovra, 10-C, Tokyo, Japan). Radiographs were further scanned (Epson Perfection V700 PHOTO (Dual Lens System) Tokyo, Japan) and monitored on a computer monitor (Eizo S1910 Tokyo, Japan) for measurements. Marginal bone loss was measured as the vertical distance from the cemento-enamel junction to the most apical part of the marginal bone. Measurements of MBL for molars and premolars (excluding third molars) in both arches were done. Marginal bone loss was assessed in pixels (ImageJ Tool 1.43, National Institutes of Health, Bethesda, Maryland, USA) and calibration of one pixel, in scanned radiographs was equal to 0.025 mm.

23.2 STUDY II

For calibration, the measurements of BOP, PI and PPD in fifteen subjects were compared as earlier reported (Altamash M et al, 2013). PPD was assessed with a graded probe to the nearest millimeter. PD was defined as three pockets ≥ 6 mm on three different teeth. PPD between 4mm <6mm and ≥ 6 mm were recorded. Embedded roots remnants were considered as teeth missing.

23.3 STUDY III

In **study III,** 49 participants were selected. 15 subjects with PD and diabetes, 15 with PD without diabetes, 4 without PD with diabetes, 15 without PD and diabetes. PPD, BOP, and PI were registered at four sites, mesial, buccal, distal and lingual. PPD was measured to the nearest millimetre by a graded probe. PPD were assessed 4mm <6mm and $\geq$ 6mm. Inclusion criteria was 25 years of age and above.

23.3.1 IMMUNOHISTOCHEMISTRY

Collection of gingival specimens was done during the period of periodontal surgery or tooth extraction. Using a 3 mm diameter tissue punch biopsy, the gingiva on the buccal zone of the tooth surface, 2-3mm beneath the interproximal papilla, was biopsied (Neobiotech, Busan, South Korea). For immunohistochemistry, the paraffin blocks were prepared after obtaining the biopsies, then immediately stained with hematoxylin-eosin, fixed in 10% buffered formalin and left overnight for fixation. For each biopsy preparation, a 3μm microns micrometer section was cut and mounted on FLEX IHC microscope coated slide Code K8020. The slides were fixed for 50 to 60 minutes in the oven at 58 to 60C .The retrieved slides from the oven were de-waxed in xylene and hydrated through series of alcohol dipping and washed through running tap water. Immunohistochemical staining specifically was performed for anti-CD4 (T helper cells) monoclonal mouse anti-human (in dilution range 1:40 to 1:80) (Code M7310) (Dako, Glostrup, Denmark), anti-CD3 (T-cell receptor) monoclonal mouse anti-human (in dilution range1:25 to 1:50) (Code M7254) (Dako, Glostrup, Denmark), anti-CD8 (Cytotoxic/supressor T cell) monoclonal mouse anti-human (in dilution range1:50 to 1:100) (Code M7103) (Dako, Glostrup, Denmark), anti-CD19 (B Lymphocytes) monoclonal mouse anti-human (in dilution range1:50 to 1:100) (Code M7296) (Dako, Glostrup, Denmark), anti-CD105 (anti-endoglin) monoclonal mouse anti-human SN6h (in dilution range 1:5 to 1:10) (Code M3527) (Dako, Glostrup, Denmark) and anti-Foxp3 (T regulatory cells) monoclonal mouse anti-human (in dilution range 1:50 to 1:500) (Code 2A11G9) (Santa Cruz Biotechnology, Santa Cruz, CA,USA). All immunohistochemical stainings were performed with EnVision™ FLEX, High pH, (Dako Autostainer/Autostainer Plus kit) Code K8010 method and Dako Antibody Diluent for the preparation of primary antibody dilutions and negative control reagents (Tris-HCI buffer containing stabilizing protein and 0.015 mol/L sodium azide) Code S0809. The sections were placed for antigen retrieval in a container with a water bath at 98-990C for 40 minutes with high pH 9.0. The water bath was removed from the container after 40 minutes and allowed to cool down at room

temperature for 20 minutes. Once cooled at room temperature they were placed with decant target retrieval solution and further rinsed two to three times with Dako washing buffer, pH 7.6. One to two drops of hydrogen blocking solution was placed on the sections and for 10 minutes incubated at room temperature in humidity chamber to block peroxidase and was washed twice with buffer solution for 5 minutes. Application of Primary antibody was done on the sections and diluted with DAKO antibody diluents optimized according to their dilution (Tris-HCl buffer containing stabilizing protein and 0.015 mol/L sodium azide) Code S0809. The sections were incubated for 30 minutes in humidity chamber at room temperature, later washed with buffer solution twice within five minutes. The sections after washing, were placed with DAB substrate chromogen solution and incubated for 2 minutes and was washed again with distilled water. Sections are counter stained with haematoxylin, 1 to 2 dips then rinsed gently under running tap water. They are further dehydrated in alcohol, xylene and distyrene plasticizer. A cover slip 24 mm x 40 mm Product No. CR12430 (Dako, Glostrup, Denmark) was placed.

23.4 STUDY IV

23.4.1 ANALYSIS OF T-CELL MIGRATION

23.4.2 Chemicals and Antibodies

Preparation and purification of rat tendon collagen type 1 were performed as described elsewhere (Elsdale T et al, 1972; Engvall E et al, 1977). Poly-L-lysine (molecular mass 5300) was bought from Miles-Veda Ltd (Rehovoth, Israel). Receptor-associated protein (RAP) was acquired from Oxford Biomedical Research (Oxford, MI). Anti-CD3 (clone SK7, IgGl) and anti-CD4 (clone SK3, IgGI) were attained from Becton Dickinson (Mountain View, CA; USA). Mouse IgG, anti-CD8 (CD8/144B), anti-CD19 (clone LE-CD19), and goat anti-mouse IgG were from Dako, (Glostrup, Denmark). Anti-TSP-1 clone A6.1 (also called TSP-Ab-4, IgG 1; was used for Western blotting and imnmunocytochemistry), and clone C 6.7 (also called TSP-Ab-3, IgG 1; was used for immunoprecipitation biotinylated cells) were from NEO-MARKERS (Fremont, CA. USA). Anti-CD91 (clone A2MR 2, IgG1) was bought from Santa Cruz Biotechnology (CA, USA). Biotinylated peroxidase and avidin were from Vector Laboratories (Burlingame, CA, USA).

23.4.3 Cells

Lymphoprep was used to purify blood lymphocytes and monocytes that were depleted by treatment with carbonyl iron and magnetic removal of phagocytic cells. Further enrichment was attained by depleting CD56-, CD19-, and CD14-positive cells. All cells were cultured in RPMI 1640 (Gibco Ltd., Paisley. Scotland) supplemented with 2 mM L-glutamine, 0, 16% sodium bicarbonate, 10 000 U/ml benzylpenicillin, 10 was used for 000 µg/ml streptomycin and 10% FCS or in serum-free AIM-V medium (Gibco). Glucose-free Dulbecco´s modified Eagles medium was used to perform the experiments.

23.4.4 Cell motility

Collagen type 1 was applied in plastic Petri-dishes 1ml/dish (30 mm; BD Biosciences) and diluted with serum-free RPMI 1640 and H_2O (8/1/1), which was allowed to polymerize at room temperature. A total of 1.0x106 cells in AIM-V medium was allowed to migrate for different times which were added to each well. Cytochalasin B, 10µg/ml prevented migration into the collagen showing that it is an active cellular process. The cells were fixed in 2.5 % glutaraldehyde or for immunocytochemistry in 2% paraformaldehyde and washed twice with PBS. Cell morphology and cell migration were evaluated in nine fixed positions in each well and at 50 µm intervals throughout the gel by the use of an inverted microscope (Nikon Eclipse TE300) and a digital depth meter (Heidenheim ND221). The results are given as mean number of infiltrating cells/field (x 20 objective) per infiltration depth (50 µm for the first two layers immediately beneath the gel surface and 100 µm for other layers further down), as total number of infiltrating cells throughout the gel (x 20 objective) or as maximal infiltration depth. The infiltrating cells were identified in situ in the collagen gels using immunocytochemistry after fixation in paraformaldehyde.

23.4.5 Quantitative immunocytochemistry

The expression of various antigens were analyzed in cells fixed in 2% paraformaldehyde at 4°C for 20 minutes attached to glass slides coated with Poly-L-lysine (10 µg/ml) at 4°C over night. Monoclonal antibodies and a complex of biotinylated peroxidase and avidin (Vector laboratories, Burlingame, CA) were used to detected antigen expression. Cells were fixed in 2% paraformaldehyde and permeabilizcd by 0.1% saponin for detection of intracellular antigens. The cells were examined in a Nikon Eclipse EI00OM microscope. Image processing and analysis program ImageJ was used to quantify the intensity of the immunocytochemical staining.

24 STATISTICAL METHODS

24.1 STUDY I

In study I, statistical analyses for MBL of molars and premolars concerning mesial and distal aspects were performed using software program SPSS17.0 software (SPSS, Chicago, IL). Mean of mandibular and maxillary arches along with 95% confidence interval were considered independent variables whereas dependent variables were considered for the mean of MBL in comparison to variables of pre-diabetes, well controlled T2D, poorly controlled T2D and non-diabetes. Age, gender, BMI, WC and PI, BOP, PDD were analysed by ANOVA. Bonferroni and post-hoc tests were performed for multiple comparisons.

24.2 STUDY II

In study II, statistical analyses were performed for all diabetic and non-diabetic subjects with or without PD. Quantitative variables PI, BOP, PPD and HbA1c levels were performed using SPSS17.0 software (SPSS, Chicago, IL). Mean (SD) were computed for all quantitative variables for PI, BOP, PPD, HbA1c and analysation was done by Repeated Measures Analysis of Variance (ANOVA) with a confidence interval of 95% between groups. Bonferroni was also used for variables PI, BOP and PPD.

24.3 STUDY III

In study III, Statistical version 19 (SPSS, Chicago, IL) were used to perform all statistical analysis. For each immunohistological variable mean and standard deviations were estimated. The comparisons among independent four groups and all pair wise comparisons were assessed by Kruskal-Wallis test with one way ANOVA test. For multiple comparisons between groups Mann Whitney U test was applied. $P<0.05$ was considered to be significant.

24.4 STUDY IV

In study IV, Cells were examined in a Nikon Eclipse EI00OM microscope. Image processing and analysis program ImageJ was used to quantify immunocytochemical staining intensity. Mean ± SD units were recorded for number of migrating cells. To evaluate the differences between groups Mann–Whitney U-test was performed and $P<0.01$ were considered statistically significant.

25 RESULTS

25.1 STUDY I

25.1.1 Interview questionnaire

A total of 213 subjects were selected, 96 were females and 117 were males, 92 individuals (43.2%) were diagnosed with T2D (47 females and 45 male). Out of the 92 individuals 68 reported diabetes in the questionnaire for the time period of 8 years as a mean value (range = 1–35 years). Mean age for debut of T2D was 50 years of age (range = 27–72 years).

25.1.2 HbA1c and RBGL

In poorly controlled T2D subjects, mean value of HbA1c was higher compared to well controlled T2D subjects, mean value of HbA1c in poorly controlled T2D subjects in males was at 9.8% and for females at 9.7% (range = 7–13.8% for males and range 7–19.4% for females) whereas mean value of HbA1c for subjects with well controlled diabetes was at 6.5% (range = 6.5– 6.9% for males and 5.3–6.9% for females). Mean value of HbA1c for pre-diabetic subjects was at 5.9% (range = 5.7–6.4% for both males and females) and non-diabetic subjects at 5.1% (range = 4.3–5.6% for males and 4.4–5.6% for females). According to HbA1c, subjects with poorly controlled T2D had higher levels of RBGL compared to subjects with well controlled T2D. Poorly controlled T2D subjects had a mean of RBGL at 252 mg/dl (14.2mmol/l) (range = 86–460 mg/dl [4.8–25.6mmol/l]) and subjects with well controlled T2D had a mean value of RBGL at 148 mg/dl (8.2mmol/l) (range = 84–268 mg/dl [4.7–14.9mmol/l]). Mean value of RBGL for non-diabetic subjects was at 109 mg/dl (6.07mmol/l) (range = 77–162 mg/dl [4.3–9 mmol/l]) and pre-diabetic subjects at 112 mg/dl (6.2mmol/l) (range = 81–202 mg/dl [4.5–11.2 mmol/l]).

25.1.3 Age, BMI and WC

The mean value of BMI for all the 213 subjects was 26 kg/m2. Mean value of BMI was higher at 28 kg/m2 (range = 14–47 kg/m2) for poorly controlled T2D subjects compared to well controlled T2D subjects which was 27 kg/m2 (range = 21–38 kg/m2). BMI for non-diabetic individuals had a mean value 25 kg/m2 (range = 15–60 kg/m2) and pre-diabetic subjects at 26 kg/m2 (range = 12–42 kg/m2). WC of poorly controlled T2D subjects was higher and found to be between 71–147 cm (mean value = 98 cm) as compared to well controlled T2D subjects which were between 71–127 cm (mean value = 88 cm). Non-diabetic subjects had a waist circumference between 64–134 cm (mean value = 97 cm) and pre-diabetics between 71–132 cm (mean value = 97 cm). The mean value of age for subjects with poorly controlled T2D were 48 years (range = 25–72 years) and for subjects with well controlled diabetes had a mean value of 48 years (range = 25–70 years). Mean value of age for non-diabetic subjects was 36 years (range = 25–73 years) and for pre-diabetics 41 years (range = 25–65 years).

25.1.4 Education

Sixty-four percentage of both poorly and well controlled T2D subjects were uneducated as well as 58% of subjects with pre-diabetes had no education.

25.1.5 Marginal Bone Level (MBL)

In subjects with poorly controlled T2D (n=51) Marginal bone level was recorded for molars with a mean value at 3.9 mm (range = 1.3– 11.2 mm) and for premolars with a mean value at 3.8 mm (range = 1.5–10.7 mm). For well controlled T2D subjects (n = 28), molars with a mean value at 3.5 mm (range = 1.8–5.3 mm) and for premolars with a mean value at 3.5 mm (range = 2.4–6.5 mm). For non-diabetic subjects (n = 38) MBL showed for molars a mean value at 3.1 mm (range = 1.5–5.9 mm) and for premolars with a mean value at 2.9 mm (range = 1.6–7 mm). Pre-diabetic subjects (n = 27) showed MBL in molars with a mean value at 2.9 mm (range = 1.3– 10.1 mm) and in premolars with a mean value at 3.0 mm (range = 1.3–5.5 mm).

25.1.6 BOP, PPD, PI and Number of Teeth

Highest level of BOP and the highest number of periodontal pocket depths were found in poorly controlled T2D subjects. Lowest level of BOP was in subjects with well controlled T2D. PI levels were highest in subjects with poorly controlled T2D and the lowest in subjects with well controlled T2D. Poorly controlled and well controlled T2D subjects both had a mean values of 24 teeth present (range 8–28 for poorly controlled T2D subjects, range 12–28 for well controlled T2D subjects) and non-diabetic subjects had a mean of 26 teeth present (range = 6–28). Pre-diabetic subjects had a mean value of 25 teeth (range = 8–28).

25.1.7 Smoking

Out of 213 subjects, 27 were smokers which was 26% of the poorly controlled T2D subjects, with a mean of 20 years since they have been smoking ranging from 6–30 years and a mean of 60 cigarettes per day (range = 20–120 cigarettes). Well controlled T2D subjects were 19% with a mean of 18 years since they have been smoking, range = 6–30 years, mean of 60 cigarettes per day and range = 20–140 cigarettes. Non-diabetic subjects were 37% with a mean of 10 years, range = 3–20 years and had a mean of 52 cigarettes per day, range = 20–100 cigarettes. Pre-diabetics were 19%, with a mean of 10 years since they have been smoking, range = 4–20 years and a mean of 60 cigarettes, range = 20–100 cigarettes.

25.1.8 Family History

Out of the selected 129 subjects of the study, thirty-one out of sixty-four were poorly controlled T2D subjects, twenty-six out of seventy-six were non-diabetic subjects, fifteen out of twenty-six well controlled T2D subjects and twenty out of forty-five pre-diabetic subjects reported family history of diabetes.

25.2 STUDY II

In this study, 330 individuals were examined at baseline, 129 subjects remained after six months. A low socioeconomic status lead to dropouts and were clinically examined and included in the study. Twenty-one subjects (12 males and 9 females) were diabetics with PD, eight subjects (5 males and 3 females) were non-diabetics with PD, 68 subjects were non-diabetics without PD (51 males and 17 females) and 32 subjects (15 males and 17 females) were diabetics without PD.

25.2.1 Age and Number of Teeth Present at Baseline

Mean age for diabetic subjects with PD, non-diabetics with PD, non-diabetics without PD and diabetics without PD were 53 (range 28-75), 46 (range 30-62), 32 (range 25- 71) and 44 (range 25-69). Mean number of teeth for diabetic subjects with PD, non-diabetics with PD, non-diabetics without PD and diabetics without PD were 23 (range 11-28), 24 (range 17-28), 26 (range 5-28) and 26 (range 15-28).

25.2.2 HbA1c Levels and Periodontal Conditions

Reductions of HbA1c levels were recorded after 3 months of non-surgical treatment in diabetics with and without PD with a mean value of 0.3%. After six months of surgical treatment, the mean for reduction recorded in HbA1c levels for diabetics with PD was 1% and diabetics without PD was 0.8%. At baseline, diabetics with PD had higher number of PPD $\geq$ 6mm compared to diabetics without PD ($P<0.01$) and non-diabetics without PD ($P<0.01$) whereas increased number of PPD $\geq$ 6mm was also recorded in non-diabetic subjects with PD compared to diabetics and non-diabetics without PD ($P<0.01$). After three months, higher levels of PPD $\geq$ 6mm were recorded in diabetics and non-diabetics with PD compared to diabetics ($P<0.01$) and non-diabetics without PD ($P<0.01$). At the end of sixth month higher occurrence of PPD $\geq$ 6mm were seen in diabetics with PD compared to diabetics without PD ($P<0.01$) and non-diabetics without PD. Increased levels of PPD $\geq$ 6mm were recorded in non-diabetics with PD compared to diabetics without PD ($P<0.05$). At base line diabetics with PD showed higher levels of PI compared to diabetics without PD ($P<0.05$) and non-diabetics without PD ($P<0.01$). After 3 months diabetics with PD showed increase in PI levels compared to diabetics without PD ($P<0.01$) and non-diabetics without PD ($P<0.05$). At baseline, BOP levels were higher in diabetics with PD compared to non-diabetics without PD ($P<0.01$) and diabetics without PD ($P<0.05$). After 3 months higher levels of BOP were recorded in diabetics with PD compared to diabetics without PD ($P<0.05$) and non-diabetics without PD ($P<0.01$). PPD 4mm <6mm had higher levels at base line in diabetics with PD compared to non-diabetics with PD ($P<0.01$). After 3 months PPD 4mm <6mm were high in diabetics with PD compared to diabetics without PD ($P<0.05$) and non-diabetics without PD ($P<0.05$). After 6 months, higher number of PPD 4mm <6mm were recorded in diabetics with PD, compared to diabetics without PD ($P<0.01$), higher levels of PPD 4mm <6mm were also found in diabetics without PD compared to non-diabetics without PD ($P<0.05$).

25.2.1 STATISTICAL ANALYSIS

25.2.1.1 Association of HbA1c with PI, BOP, PPD 4mm <6mm and PPD ≥ 6mm (n= 129) at baseline and 6 months.

A multiple linear regression was run to predict the association of HbA1c with PI, BOP, PPD 4mm <6mm and PPD ≥ 6mm (n= 129) in diabetics with PD and without PD at baseline and 6 months. The association of HbA1c with PI, BOP and PPD ≥ 6mm variables at baseline were statistically not significant when predicted by HbA1c ($P = 0.515$, $P = 0.346$, $P = 0.180$) respectively. The association of PPD 4mm <6mm measures with HbA1c (β= 0.036, P=<0.001) was markedly greater than the associations of PI (β=-0.004), BOP (β=0.007) and PPD ≥ 6mm (β=0.020) (Table 1). These variables PI, BOP, PPD 4mm <6mm and PPD ≥ 6mm (n= 129) were statistically not significant when predicted by HbA1c at 6 months, ($F = 1.543$, $P = 0.217$, $R^2 = 0.064$). Of note, the association of PPD 4mm <6mm measures with HbA1c (β= 0.021) was markedly greater than the associations of PI (β=0.008), BOP (β=0.018), and PPD ≥ 6mm (β=0.014) (Table 2).

25.2.1.2 Correlation between the HbA1c and PI, BOP, PPD 4mm <6mm and PPD ≥ 6mm at baseline and 6 months (n= 129).

Significant weak positive correlation was found between HbA1c and PI (r=0.216, P=0.014), BOP (r=0.389, P=<0.001), PPD 4mm <6mm (r=0.305, P=<0.001), PPD ≥ 6mm, (r=0.232, P=0.008), at baseline A very weak positive correlation was found between HbA1c and PI (r=0.108, P=0.222), BOP (r=0.127, P=0.153) at 6 months respectively. Interestingly, we observed a moderate positive correlation between HbA1C and PPD 4mm <6mm at 6 months (r=0.425, P=<0.001) and weak positive correlation with PPD ≥ 6mm (r=0.254, P=0.004) (Table 3).

Table 1: Association of HbA1c with PI, BOP, PPD 4mm <6mm and PPD ≥ 6mm (n= 129) at baseline.

	B Coefficients (Unstandardized)	P-value	95% CI
PI	-0.004	0.515	-0.017, 0.009
BOP	0.007	0.346	-0.007, 0.020
PPD 4mm <6mm	0.036	<0.001	0.019, 0.052
PPD ≥ 6mm	0.020	0.180	-0.009, 0.049
R = 0.443 **R² = 0.197** **Adjusted R² = 0.171** **F = 7.588 (P=<0.001)**			

Unstandardized regression coefficients, with 95 % confidence intervals. All independent variables were entered simultaneously into the models. Age and gender were controlled in all models.

Table 2: Association of HbA1c with PI, BOP, PPD 4mm <6mm and PPD ≥ 6mm (n= 129) at 6 months.

	B Coefficients (Unstandardized)	P-value	95% CI
PI	0.008	0.655	-0.030,0.046
BOP	0.018	0.423	-0.028,0.065
PPD 4mm <6mm	0.021	0.255	- 0.016, 0.059
PPD ≥ 6mm	0.014	0.488	-0.027, 0.056
R = 0.425 **R² = 0.181** **Adjusted R² = 0.064** **F = 1.543 (P=0.217)**			

Unstandardized regression coefficients, with 95 % confidence intervals. All independent variables were entered simultaneously into the models. Age and gender were controlled in all models.

Table 3: Correlation between the HbA1c and PI, BOP, PPD at baseline and 6 months (n= 129).

Correlation between the HbA1c and PI, BOP, PD at Baseline and 6 months (n=129)					
		PI	BOP	PPD 4mm< 6mm	PPD ≥ 6mm
HbA1C Baseline	Pearson Correlation	0.216	0.389	0.305	0.232
	p-value (2-tailed)	0.014	<0.001	<0.001	0.008
HbA1C 6 month	Pearson Correlation	0.108	0.127	0.425	0.254
	p-value (2-tailed)	0.222	0.153	<0.001	0.004

25.2.2 Smoking

Two subjects in each group of diabetics with PD and non-diabetic with PD were smokers. In diabetics without PD four subjects were smokers and in non-diabetics without PD 21 subjects were smokers.

25.2.3 Family History

Among 330 individuals, a total of fifty-four subjects out of the 163 diabetic subjects and eighteen out of 167 non-diabetic subjects, they have reported family history of diabetes.

25.2.4 Dropouts

A total of 330 individuals (Table 1) were included and examined in the study at baseline out of which, 186 individuals dropped out (Table 2). Subjects with diabetes without PD had the highest number of BOP, WC, BMI and HbA1c levels. Non-diabetic subjects without PD had the lowest number of PI, BOP, PPD 4mm <6mm, PPD ≥ 6mm, HbA1c levels and age, while number of teeth present where highest in this group. Diabetes with PD had the highest number of PI, PPD 4mm <6mm, PPD ≥ 6mm and age while WC, BMI and of teeth present were recorded the lowest in this group. In non-diabetics with PD PI and PPD ≥ 6mm were recorded the lowest.

Table 1.

Total of 330 individuals at baseline

PI, BOP, PPD, WC, BMI, HbA1c, Age and No of teeth (mean±SD) in Diabetics without PD, Non-diabetics without PD, Diabetics with PD and Non-diabetics with PD.

	Diabetics without PD	Non-diabetics without PD	Diabetics with PD	Non-diabetics with PD
	(n=71)	(n=108)	(n=92)	(n=59)
PI (%)	75.0±30.9	75.7±32.0	80.5±28.3	75.7±31.6
BOP (%)	62.2±36.3	59.1±37.2	71.8±33.6	63.2±36.6
PPD (4 <6 mm)	13.5±19.1	6.8±14.3	28.4±24.4	24.0±21.2
PPD (≥6 mm)	0.3±0.71	0.09±0.44	23.5±22.3	16.4±18.9
WC	96.6±14.1	86.0±14.0	93.0±12.5	93.5±11.6
BMI	27.4±5.4	24.7±5.9	26.5±5.6	25.5±3.87
HbA1c	8.4±2.1	5.1±0.39	10.3±19.8	5.7±0.56
Age	44±12.2	32±8.8	47±12.6	47±13.1
No of Teeth	25±4.1	27±3.1	23±5.0	23±5.2

Table 2.

Dropouts of 186 individuals at baseline to 3 months

PI, BOP, PPD, WC, BMI, HbA1c, Age and No of teeth (mean±SD) in Diabetics without PD, Non-diabetics without PD, Diabetics with PD and Non-diabetics with PD.

	Diabetics without PD	Non-diabetics without PD	Diabetics with PD	Non-diabetics with PD
	(n=39)	(n=40)	(n=65)	(n=42)
PI (%)	79.9±25.6	76.8±30.6	85.5±23.4	76.8±30.6
BOP (%)	62.2±34.8	60±34.8	77.1±29.8	60.1±34.8
PPD (4 < 6 mm)	14.7±20.0	9.6±16.9	29.3±21.2	9.6±16.9
PPD (≥ 6mm)	0.4±0.84	0.3±0.67	25.7±24.9	21.6±19.1
WC	99.6±14.4	93.8±12.3	92.4±11.9	93.8±12.3
BMI	27.5±5.7	27.2±7.5	26.9±5.7	27.2±7.5
HbA1c	9.5±1.7	5.1±0.3	8.9±2.4	5.5±0.4
Age	45±11.5	35±10.2	50 ±10.6	47±12.9
No of Teeth	25 ±4.2	26±3.5	23±5.2	23±5.5

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25.3 STUDY III

Gingival biopsies from subjects with PD without diabetes, had more CD4 (P=0.019) and CD19 (P=0.033) positive cells than biopsies from subjects without PD without diabetes. Subjects with PD with diabetes showed a lower number of CD4 (P=0.002) and CD19 (P=0.05) positive cells in gingival biopsies than in subjects with PD without diabetes. Gingival biopsies in subjects without PD with diabetes, had lower number of CD4 (P=0.049) positive cells than patients with PD without diabetes. No differences of CD3, CD8 and FoxP3 were shown between groups.

25.4 STUDY IV

High concentration (50mM) of D-glucose enhanced T cell motility were measured in collagen matrix by migration and development of polarized cell shape during first 10 minutes of exposure while subsequently causing inhibition. This resulted in increase of cell surface expression of TSP-1 and LRP-1. Exertion of variable stimulatory effect on the motility of the cells from separate individuals were triggered by insertion of glucose at a concentration of 1-20mM. T cell motility accompanied by inhibition is initially stimulated by insulin.

26 CONCLUSIONS AND DISCUSSIONS

Study I

The first study was able to ascertain the diagnosis of diabetes with the help of HbA1c and RBGL analysis of venous blood. In the study it were found that of the total individuals, around one in three who were diagnosed as non-diabetic were actually diagnosed as pre-diabetic according to HbA1c levels but not with the help of RBGL. HbA1c levels was found to be better predictors of pre-diabetes and diabetes. The presence of pre-diabetes and diabetes increases the risk of severe periodontal disease among patients, the prevalence of which has been near 10% in both US and Pakistan (Shera et al, 1995; Cowie et al, 2010). For Pakistan, this roughly translates to more than 13 million by the year 2030. Another prominent feature found among the patients with diabetes within the samples was their weight, with females showing an increased mean WC level compared to males in the group. The BMI of over 25kg/m2 should be considered another risk factor for periodontal disease and T2D (Pischon et al, 2007; Morita, 2011).The findings showed that periodontal conditions of pre-diabetic subjects were the same as non-diabetic subjects and severe periodontal conditions were found with poorly controlled diabetes.

Study II

In the second study we assessed the impact of periodontal treatment on HbA1c levels and periodontal health. Diabetics with PD showed more resistance towards healing of the periodontal structures. The HbA1c levels showed a reduction, which was similar to the finding reported by Simpson (Simpson et al, 2010). This particular finding indicates that periodontal health can influence blood glucose levels among diabetics, however, a phenomena such as the Hawthorne effect must be kept in mind (Janket et al, 2005; Watts, 2006). Stratton et al showed that even a reduction by a percent in HbA1c levels are enough to reduce microvascular complications and diabetes related death incidences significantly (Stratton et al, 2000). The importance of maintaining periodontal health among diabetics therefore may not be apparent at the local level (periodontal recovery) but may be a significant contributor to systemic health (Janket et al, 2005). In this study it was shown that the reduction in blood sugar levels in subjects with T2D after periodontal treatment requires at least six months.

Study III

In the third study we found increased presence of CD4 and CD19 positive cells in the gingival biopsies collected from non-diabetics with PD compared with diabetic subjects with PD. Therefore demonstrating how diabetes affects systematic response to infection and inhibits lymphocyte motility. This result supports the findings of the second study which showed that diabetic patients demonstrated a slower response to periodontal treatment compared to non-diabetic patients. In conclusion, the reduction in the number of CD4+ and CD19+ lymphocytes in gingival biopsies from diabetic subjects with PD probably reflects an effect of T2D on lymphocytes or endothelial cells.

Study IV

In the fourth study we assessed the influence of D-glucose on T cell motility and cell surface expression of endogenous thrombospondin-1 (TSP-1) and its receptor low density lipoprotein receptor /related protein-1(LRP-1) which have been shown to regulate T cell motility. D-glucose, at a high concentration induced rapid enhancement of T cell motility accompanied by a decrease and increased cell surface expression of TSP-1 and LRP-1. These findings suggest that D-glucose controls T cell motility through LRP-1 and TSP-1 and may provide better understanding of the function of the immune system in diabetic subjects. Hence, continuous T-cell exposure to high blood sugar levels may inhibit T cell motility.

27 SUMMARY

Subjects with diabetes mellitus and with high blood glucose levels showed severe periodontal conditions. Periodontal treatment, non-surgical and surgical improved blood glucose levels in subjects with diabetes mellitus after 6 months. Increased blood glucose levels inhibits the entry of lymphocytes in gingival tissue. Elevated blood glucose levels inhibits T lymphocyte motility.

28 FUTURE PERSPECTIVES

Knowledge completion amongst Pakistani dentists regarding research on oral health and diabetes.

Rule out a possible Hawthorne effect on blood glucose levels after periodontal treatment including qualitative and quantitative research.

Further research on the presence of T-cell subpopulation in gingival tissue in subjects with high blood glucose level.

29 ACKNOWLEGEMENTS

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