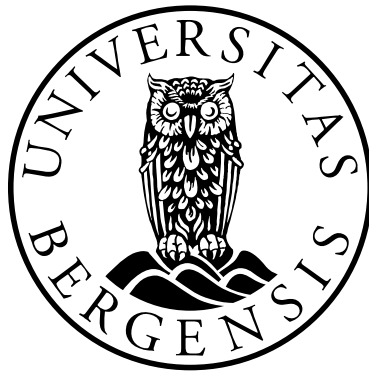


Impact of type 2 diabetes and periodontal disease on oral status of Sudanese adults

Clinical, microbial and immune-inflammatory aspects

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SCIENTIFIC ENVIRONMENT

This project was conducted during the years (2012-2015) under the supervision of Dr. Odont, Specialist in Periodontology, Salah Osman Ibrahim as a main supervisor, Professor Kamal Mustafa, Professor Anne Nordrehaug Åstrøm, Post doc. Shaza Bushra Idris as co-supervisors, and Dr. Mutaz Faisal Ahmed as an external mentor. The scientific activities took place at the Department of Clinical Dentistry and the Centre for International Health at the University of Bergen.

ABBREVIATIONS

AGEs	Advanced glycation end products
CI	Confidence interval
DMFT	Decayed, missed and filled teeth
ELISA	Enzyme linked immunosorbent assay
FGF	Fibroblast growth factor
GCF	Gingival crevicular fluid
G-CSF	Granulocyte-colony stimulating factor
GIP	Gastric inhibitory polypeptide
GLP	Glucagon-like peptide
GM-CSF	Granulocyte monocyte-colony stimulating factor
HbA1c	Glycated haemoglobin
IDF	International diabetes federation
IL	Interleukin
INF	Interferon
IP	Interferon inducible protein
MCP	Monocyte chemo-attractive protein
MIP	Macrophage inflammatory protein
MMPs	Matrix metalloproteinases
(M)PAMPs	Microbe - or Pathogen - associated molecular patterns
NF- κ B	Nuclear factor- κ B
OHRQoL	Oral health related quality of life
OIDP	Oral impact on daily performance
OPG	Osteoprotegerin
OR	Odds ratio
PAI	Plasminogen activator inhibitor
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor

PRRs	Pattern recognition receptors
RAGE	Receptor for advanced glycation end products
RANKL	Receptor activator of nuclear factor- κ B ligand
RANTES	Regulated upon activation, normally T-expressed, and presumably secreted
ROS	Reactive oxygen species
T2D	Type 2 diabetes
Th-cells	T-helper cells
TIMPs	Tissue inhibitors of metalloproteinases
TLR	Toll-like receptors
TNF	Tumor necrosis factor
T-reg	T-regulatory cells
VEGF	Vascular endothelial growth factor

SUMMARY

Diabetes is a major global public health challenge, afflicting 380 million people worldwide. The onset is insidious and progression is associated with irreversible medical complications, including oral diseases. Little is known about the oral health of patients with diabetes in developing countries such as The Sudan, which is currently experiencing an alarming increase in diabetes cases.

The overall aim of this thesis was to evaluate oral health indicators in Sudanese adults with type 2 diabetes (T2D) and to investigate the impact of T2D and chronic periodontitis on biomarkers of inflammation and glucose regulation in gingival crevicular fluid (GCF) and saliva.

The subjects comprised 157 T2D cases and 304 controls without diabetes, 461 in all. Participants were interviewed using a structured questionnaire on socio-demographics, lifestyle and oral health related quality of life (OHRQoL). The clinical examination comprised full mouth probing depths, bleeding on probing, dental plaque index, tooth mobility index, furcation involvement and coronal and root caries. In GCF samples, the levels of 10 glucoregulatory molecules and 27 inflammatory molecules were measured by bead-based multiplex assays. MMP-8, MMP-9, OPG and RANKL in whole saliva samples were quantified by ELISA. Subgingival plaque samples were analysed by conventional polymerase chain reaction (PCR), to assess the prevalence of six periodontal pathogens.

T2D patients had poorer periodontal parameters, more missing teeth and poorer OHRQoL than individuals without diabetes. Chronic periodontitis was associated with disturbed GCF levels of biomarkers related to the onset and medical complications of T2D. On the other hand, T2D was associated with a high Th-2/Th-1 cytokines ratio and disturbed levels of molecules involved in the anti-inflammatory and healing processes. T2D had no significant effect on either the prevalence of the investigated periodontal pathogens or the levels of salivary MMP-8, MMP-9 and OPG. Further research is warranted to identify disease markers which could form the basis of a test to alert the dentist to patients with undiagnosed T2D, or those at risk of developing the disease.

LIST OF PUBLICATIONS

This thesis is based on the following studies, referred to in the text by their Roman numerals:

- I. **Mohamed H.G.**, Idris S.B., Ahmed M.F., Bøe O.E., Mustafa K., Ibrahim S.O. and Åstrøm A.N. (2013) Association between Oral Health Status and Type 2 Diabetes Mellitus among Sudanese Adults: A Matched Case-Control Study. *PLoS One*; 8 (12): e82158.
- II. **Mohamed H.G.**, Idris S.B., Mustafa M., Ahmed M.F., Åstrøm A.N., Mustafa K., and Ibrahim S.O. (2015) Impact of Chronic Periodontitis on Levels of Glucoregulatory Biomarkers in Gingival Crevicular Fluid of Adults with and without Type 2 Diabetes. *PLoS One*; 10 (5): e0127660.
- III. **Mohamed H.G.**, Idris S.B., Ahmed M.F., Åstrøm A.N., Mustafa K., Ibrahim S.O., and Mustafa M. (2015) Influence of type 2 diabetes on local production of inflammatory molecules in adults with and without chronic periodontitis: a cross-sectional study. *BMC Oral Health*; 15 (1): 86.
- IV. **Mohamed H.G.**, Idris S.B., Mustafa M., Ahmed M.F., Åstrøm A.N., Mustafa K., and Ibrahim S.O. Influence of type 2 diabetes on prevalence of key periodontal pathogens, salivary matrix metalloproteinases and bone remodelling markers in Sudanese adults with and without chronic periodontitis. *Submitted for publication*

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1. INTRODUCTION

1.1 Diabetes

Diabetes mellitus is a metabolic disorder characterised by chronic hyperglycaemia and disturbed carbohydrate, fat and protein metabolism caused by defective insulin secretion, action, or both (1). Insulin is a hormone secreted by the β -cells of the pancreas. It allows the cells to utilise glucose and prevents conversion of glycogen (the stored form of glucose) to glucose in the liver. Thus the net effect of insulin is to reduce the level of circulating glucose. Disturbance to insulin function may occur either because β -cells are unable to produce sufficient insulin, or because tissue response to the secreted insulin is impaired. The end result is elevated blood glucose level (hyperglycaemia) (2). The classical symptoms of the disease are polyuria, polydipsia, polyphagia and blurred vision (3). If hyperglycaemia is not controlled, serious acute life-threatening complications, such as diabetic ketoacidosis and non-ketotic hyperosmolar syndrome, may ensue (3). Hyperglycaemia might also lead to chronic, irreversible tissue damage. Long-term complications include nephropathy, retinopathy, neuropathy, cardiovascular diseases, peripheral vascular diseases, delayed healing and periodontal diseases. The severity of the disease lies in the fact that it might remain undiagnosed for a long time, allowing hyperglycaemia to cause long-term tissue damage (3, 4).

Diabetes is a major public health concern, with 380 million people afflicted worldwide. It is reported that about 50% may be undiagnosed (5). Moreover, about 80% of patients with diabetes are from low- and middle-income countries (5). The African region is doubly burdened by disease: as well as the existing challenges such as malaria and HIV, the prevalence of non-communicable diseases is increasing (6). By 2030, it is expected that Africa will experience the largest proportional increase in adults with diabetes (7). In 2014, The Middle East and North Africa had the highest age-adjusted global prevalence of diabetes (about 11%) (5). In North African countries with limited

resources, such as The Sudan, diabetes and its medical complications impose a heavy economic burden and there is an urgent need for interventions to address this issue (8).

There is little research into diabetes in The Sudan. A search of PubMed using MeSH terms “diabetes” and “Sudan” revealed only 17 studies during the last 10 years. None included oral health aspects of diabetes (updated 28th July, 2015).

To date, the true prevalence of diabetes in The Sudan is unknown. In 1996, a study conducted among adults (≥ 25 years) in the northern part of the country reported a prevalence of 3.4% (9). In 1998, another study among the same age group and from the same geographical area reported a prevalence of 10% (10). According to a 2014 report by the International Diabetes Federation (IDF), the age-adjusted prevalence of diabetes in The Sudan is about 18% (5). This figure ranks The Sudan among countries with a high prevalence of the disease, not only in Africa, but also worldwide. Limited resources and challenging economic status in The Sudan have led to lack of satisfactory diabetic care and education, low rates of clinical attendance, a high prevalence of diabetes-related medical complications and poor quality of life (11, 12).

1.1.1 Diagnosis of diabetes

According to the American Diabetes Association (13), diagnosis is based on the following criteria:

- Fasting plasma glucose (no caloric intake for at least 8 h) of ≥ 126 mg/dL (7.0 mmol/L), OR
- Random plasma glucose (anytime of the day) of ≥ 200 mg/dL (11.1 mmol/L) AND the classical symptoms mentioned earlier, OR
- Plasma glucose of ≥ 200 mg/dL (11.1 mmol/L) after 2 h oral administration of 75 g anhydrous glucose dissolved in water.

According to a World Health Organisation (WHO) report from 2011 (14), glycated haemoglobin (HbA1c) of 6.5% (48 mmol/mol) can be used as a cut-off point to diagnose

diabetes, although HbA1c levels below this do not exclude the disease. HbA1c results may vary according to age, ethnicity and haemoglobin level. Therefore, these factors should be taken into consideration when using HbA1c as a diagnostic measure (13). HbA1c is also considered to be a good indicator of glycaemic control in patients with diabetes. It reflects the average blood glucose level within a range of 8 to 12 weeks, corresponding to the life span of circulating red blood cells (15).

There is a transient stage between normal glucose homeostasis and diabetes, in which individuals are at increased risk of developing the disease. This stage is referred to as pre-diabetes or impaired glucose tolerance. At this stage, fasting plasma glucose ranges between 100 mg/dL (5.6 mmol/L) and 125 mg/dL (6.9 mmol/L), the 2 h glucose tolerance test ranges between 140 mg/dL (7.8 mmol/L) and 199 mg/dL (11.0 mmol/L), and HbA1c from 5.7% (39 mmol/mol) to 6.4% (46 mmol/mol) (13). The pre-diabetic state is highly relevant in clinical dental practice. Oral manifestations may present as one of the early signs of the disease, hence the dental practitioner might recognise undiagnosed cases (16, 17).

1.1.2 Classification of diabetes

Categorisation of patients with diabetes into a specific type is sometimes challenging. Nevertheless, diabetes mellitus can be broadly classified as follows (13):

Type 1 diabetes

This type of diabetes affects 5-10% of patients with the disease; mostly children and young adults. It is characterised by absolute insulin deficiency due to cell-mediated autoimmune destruction of the β -cells of the pancreas (3). As the rate of destruction varies among patients, the age at which the disease becomes symptomatic also varies. Less commonly, the aetiology of type 1 diabetes might not be related to auto-immunity (idiopathic diabetes). This sub-class is characterised by strong genetic susceptibility. Most of the patients are either of African or Asian origin (3). Patients with type 1 diabetes may

also suffer from other auto-immune diseases and most require insulin therapy to survive (3).

Type 2 diabetes

Type 2 diabetes (T2D) is the most common type, affecting about 90% of patients with the disease. It usually affects adults and may remain undiagnosed for a long time. During the early stages of the disease, tissues become resistant to the secreted insulin. As a result, cells starve for glucose and produce signals that force the liver to release more glucose by hydrolysing the stored glycogen, and β -cells of the pancreas to secrete more insulin. Subsequently, hyperglycaemia occurs and the β -cells become less efficient over time, leading to relative insulin deficiency. Although the specific aetiology of T2D is unknown, obesity, high fat and sugar intake, physical inactivity and genetic predisposition have been implicated. T2D can be controlled by changing life style (dietary restriction of sugar and fat, weight loss and physical exercise). In addition, oral hypoglycaemic drugs might be used to enhance insulin secretion by β -cells and/or to reduce insulin resistance. Insulin therapy might be indicated for poorly controlled cases (3).

Gestational diabetes

This form of diabetes occurs during pregnancy. It is characterised by any degree of glucose intolerance that is recognised for the first time during the pregnancy period. In 2009, the International Association of the Diabetes and Pregnancy Study Groups modified the case definition by excluding high-risk women diagnosed with diabetes at their initial prenatal visit. This type of diabetes accounts for 7% of all pregnancies with about 200,000 cases per year (3). In some cases, the condition may persist after pregnancy. Associated risk factors for this type of diabetes include age, previous history of glucose intolerance, high blood glucose level and neonates who are large for gestational age (1, 3).

Other specified types of diabetes

These include less common forms of the disease such as monogenic diabetes syndromes, diseases of the exocrine pancreas and drug - or chemical - induced diabetes (13).

1.1.3 Oral manifestations of diabetes

There is abundant evidence that diabetes is associated with pathological changes in the oral cavity. These changes include mucosal ulceration, fungal infection, burning mouth syndrome, tooth loss, xerostomia, dental caries and periodontal disease (18). These manifestations are more pronounced among patients with uncontrolled blood glucose levels (19). It is important to note that these oral changes are not pathognomonic for diabetes (20, p187). Another important point is that pathological changes in the oral cavity are one of the early signs of diabetes (16). As diabetes may remain undiagnosed for a long time, dentists could be instrumental in facilitating early detection of diabetes. Early detection is the key factor for prevention of medical complications, as well as the economic burden related to management of the disease (21).



Figure 1. Clinical case of T2D patient with extensive periodontal tissue destruction and tooth loss.

1.2 Periodontal diseases

The periodontium is the apparatus that keeps the teeth in place. It consists of the gingiva, periodontal ligaments, cementum and alveolar bone. Together, the four components function as a one unit to support the teeth and maintain their function and stability (20, p9). Periodontal disease is an inflammatory condition that affects the above mentioned tissues that surround the teeth (22). It starts with the reversible form “gingivitis”. Page and Schroeder (23) classified the histological changes that occur during the course of periodontal disease. According to their classification, the disease starts as sub-clinical mild inflammation (initial lesion). Thereafter, the classical signs of gingivitis become noticeable and the gingiva becomes red, swollen and bleeds readily (early lesion). If the condition is not treated, it goes into a chronic stage (established lesion). As the disease progresses, bone tissue and periodontal ligaments become involved (advanced lesion) and the transition to periodontitis starts. Socransky *et al.*, (24) described the progression of the disease as intermittent *i.e.* periods of exacerbation followed by periods of remission, while Kinane (25) suggested that the process is continuous, with occasional episodes of exacerbation and remission.

1.2.1 Classification of periodontitis

Destructive periodontal diseases can be classified as follows (26):

- Chronic periodontitis
- Aggressive periodontitis
- Periodontitis as a manifestation of systemic diseases
- Necrotising ulcerative periodontitis
- Abscesses of the periodontium
- Combined periodontic-endodontic lesions

1.2.2 Chronic periodontitis

Unlike gingivitis, periodontitis is an irreversible condition, characterised by apical migration of epithelial attachment, accompanied by loss of supporting bone (27). This chronic inflammatory condition leads to pathological deepening of the gingival sulcus to form a periodontal pocket (28). If left untreated, tissue destruction may eventually lead to tooth loss (25). During the early stages of the disease, there is an increase in the vascular permeability and migration of the immune cells (mainly neutrophils) to the site of infection. As the disease progresses, lymphocytes and macrophages migrate to the affected site. If the condition remains untreated, destruction of the connective tissues and bone occurs and the junctional epithelium migrates apically along the root surface to form the periodontal pocket: at this point, plasma cells and lymphocytes become predominant (29).

Clinical presentation of the disease is determined by two major components: the periodontal pathogens and the host immune-inflammatory response (30, 31). Other factors implicated as potential mediators of these two determinants of the disease are age, gender, education, genetic background, smoking, life style and systemic conditions (32).

1.3 How diabetes affects chronic periodontitis

Diabetes and chronic periodontitis are both chronic inflammatory conditions. There are some commonalities of immune-inflammatory response throughout the course of both diseases. While this can partially explain the bi-directional relationship of the two diseases, it also reflects the complexity of the mechanisms by which one disease affects the other (33). The temporality of this bi-directional relationship was demonstrated in a study by Chiu *et al.*, (34) claiming that both diseases may share common latent traits and pathways.

The effect of diabetes on periodontal tissues has been thoroughly investigated in observational studies, demonstrating that diabetes is associated with exaggerated periodontal tissue destruction (35-39). Moreover, a 20-year follow-up study reported a

29% increased risk of periodontitis and a 22% increased risk of bone loss in patients with T2D (40). Another 5-year follow-up study demonstrated that periodontal tissue destruction is associated with poor glycaemic control ($\text{HbA1c} \geq 6.5\%$) (48 mmol/mol) with an OR of 2.9 (19).



Figure 2. Clinical case of T2D patient with chronic periodontitis.

1.3.1 Putative periodontal pathogens, “starting the battle”

Dental plaque is a structured, yellowish-greyish biofilm that accumulates on the hard surfaces of the oral cavity including teeth (41). With the gingival margin serving as an anatomical reference, dental plaque is located supra- or subgingivally. Subgingival plaque is a complex ecosystem containing vast numbers of micro-organisms (42). Some of those micro-organisms have been found to be associated with chronic periodontitis, such as *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Tannerella forsythia*, *Treponema denticola*, and *Prevotella intermedia* (43, 44). The periodontal pathogens are considered to be the initiating factors in the disease (45), challenging the periodontal tissues with metabolic waste products such as ammonia and hydrogen sulfide (20, p81).

Understanding the role of periodontal pathogens in the pathogenesis of chronic periodontitis has evolved over time, from the regarding dental plaque bacteria as the main

cause of the disease (46), to the theory of dysbiosis, whereby tissue destruction is caused by the host immune response, due to disturbance in the balance of relative quantities of existing bacteria in the dental biofilm. This implies that periodontal pathogens have an indirect role, changing the existing bacterial environment in the dental plaque from symbiotic to dysbiotic (47, 48). The pathogens able to play this role are called “keystone pathogens” (49). These keystone pathogens are essential, but cannot, on their own, cause a periodontal lesion (45).

1.3.2 Diabetes influences oral microbiota, “myth or truth?”

The impact of hyperglycaemia on the composition of dental plaque microbiota has been questioned for many years. Some studies report no significant differences in the bacterial composition of dental plaque between individuals with and without diabetes (39, 50). Moreover, the level of glycaemic control among patients with diabetes seems to have no significant effect on the microbial composition of dental plaque (51). In contrast, other studies have indicated significant differences in dental plaque bacteria between individuals with and without diabetes (52, 53). One of the suggested mechanisms that might lead to dissimilarities in microbial composition is the high glucose levels in saliva and gingival crevicular fluid (GCF) caused by hyperglycaemia, which in turn affects the level of carbohydrates in dental plaque. As a consequence, the biofilm environment will favour for the growth of the pathogens (53).

1.3.3 Host immune-inflammatory response, “losing the balance”

The response of the immune system to various stimuli is a fundamental process for maintenance of periodontal health. It involves a highly sophisticated and integrated series of events, starting with pathogen recognition, followed by activation of innate and adaptive immunity and concluding with healing and resolution (54). It is widely accepted that plaque-induced periodontal tissue destruction is the consequence of the host immune-inflammatory response to micro-organisms (31).

Inflammation is a process by which tissues react to stressful situations such as injury or infection. It is a protective mechanism intended to eliminate harmful stimuli and return the tissue to its homeostatic condition (55). The process involves recruitment of neutrophils, macrophages, activation of T- and B-lymphocytes, as well as activation of the complement system (29). The cross-talk between these cells is the responsibility of small soluble molecules called cytokines (56). Cytokines are secreted by immune cells such as neutrophils, macrophages, lymphocytes and mast cells, as well as non-immune cells such as fibroblasts and epithelial cells (57, 58). They can act in an autocrine, paracrine, endocrine or juxtacrine manner, and their action can be pleotropic (59). When binding to their receptors, they initiate an intra-cellular signalling cascade that eventually leads to up-regulation or down-regulation of specific genes. They thus function as a complex network, guiding the immune system towards an appropriate immune response, such as pro- or anti-inflammatory response, T-cell maturation and differentiation, B-cell class switching, etc (56).

The cells of the immune system respond to bacterial invasion through specific receptors called pattern recognition receptors (PRRs). The latter can recognise bacterial lipopolysaccharide, peptidoglycan, bacterial DNA, and double stranded RNA. These stimulators are collectively called microbe - or pathogen - associated molecular patterns (MAMPs or PAMPs). In periodontal tissues, toll-like receptors (TLRs) (one of the PRRs) on the surface of resident cells (such as epithelial cells, fibroblasts and neutrophils) recognise the PAMPs and initiate an innate immune response through activation of transcription factors such as nuclear factor- κ B (NF- κ B). Subsequently, the resident cells produce cytokines such as IL-1 β , IL-6 and TNF- α , and chemokines such as IL-8. As a result, the tissue undergoes vascular changes accompanied by recruitment of more inflammatory cells (neutrophils, macrophages and natural killer cells) to the affected site (29, 60).

If the innate immune response fails to eliminate the stimulus, bacterial antigen will be taken, processed and presented to the cells of the adaptive immune system by antigen-

presenting cells such as macrophages and dendritic cells, leading to activation of adaptive immunity (29). IL-12 and IL-4 enhance the development of Th-1 and Th-2 from naive Th-cells, respectively (61). The bridging between the innate and adaptive immune response is orchestrated by cytokines such as IL-12, IL-18 and INF- γ (62).

As the disease progresses, the tissue experiences continuous stress from the pro-inflammatory molecules produced by the immune cells, causing what is called para-inflammation. Although the condition might not be clinically detectable, dramatic changes might occur if the condition persists, leading to chronic inflammatory status (55). At this point, more neutrophils and macrophages will be attracted to the inflamed site by IL-8 and MCP-1, respectively (63). Like the other immune cells, neutrophils secrete inflammatory mediators such as IL-1 β , TNF- α , prostaglandins and matrix metalloproteinases (MMPs). Concurrently, Th-1 secretes IL-2 and INF- γ , Th-2 mediates humoral immunity and Th-17 secretes IL-17 (61, 64).

Following the establishment of the chronic inflammatory process, some of the inflammatory molecules such as MMPs and receptor activator of nuclear factor- κ B ligand (RANKL) will be up-regulated. These two molecules participate in the destruction of the non-mineralised and mineralised tissues of the periodontium, respectively (65, 66). MMPs are structurally related but genetically distinct zinc-dependent proteolytic enzymes produced by neutrophils, macrophages, fibroblasts and other resident cells (65). Apart from their role in normal physiological processes such as cell migration and tissue remodelling, they play a major role in extracellular matrix degradation (65, 67). Elevated MMPs have been associated with chronic periodontitis, particularly MMP-8 (collagenase-2), MMP-9 (gelatinase-B) and MMP-13 (collagenase-3) (68). The action of MMPs is modulated by serum inhibitors such as glycoprotein α_1 -antitrypsin and α_2 -macroglobulin, as well as tissue inhibitors of metalloproteinases (TIMPs) (20, p84). As a result of the non-mineralised tissue destruction, the inflammatory activity migrates apically towards the alveolar bone.

Bone remodelling is a dynamic process, involving a balanced system of bone turnover, whereby osteoclasts are responsible for bone resorption, and osteoblasts are responsible for bone deposition (69). Thus, if the balance shifts towards increased osteoclast activity, bone resorption occurs. Bone remodelling is highly regulated by the RANKL-RANK-osteoprotegerin (OPG) axis (70). RANKL is one of the TNF superfamily. When binding to its receptor (RANK), RANKL stimulates the maturation and activation of osteoclasts (66, 71). Pro-inflammatory cytokines such as TNF- α and IL-1 β enhance RANKL secretion by T- and B-cells (54, 72). On the other hand, OPG - a soluble decoy receptor - inhibits the differentiation of osteoclasts by competing with RANKL on its receptor (73). Therefore, the RANKL/OPG ratio is a good indicator of bone tissue destruction in periodontitis (70). Inflammatory mediators such as cytokines, chemokines and prostaglandins can enhance bone tissue destruction by increasing the RANKL/OPG ratio. They can also enhance bone destruction directly through a non-RANKL pathway, by activating osteoclasts through specific receptors (74). Given the fact that the immune system works in balance (*i.e.* pro-/anti-inflammatory cytokines, RANKL/OPG, MMPs/TIMP), an imbalance among the different elements of the immune system may determine the clinical presentation of periodontitis.

1.3.4 Effect of diabetes on host immune-inflammatory response

The exact mechanism(s) by which diabetes affects the host immune response is not fully elucidated (51). However, there is a general agreement that hyperglycaemia is the main cause of diabetic complications (3). The mechanisms proposed to explain the link between chronic hyperglycaemia and disturbed host immune-inflammatory response are discussed below.

1.3.4.1 Induction of inflammation

One of the proposed mechanisms by which hyperglycaemia could cause periodontal tissue damage is by oxidative stress. Hyperglycaemia affects the mitochondrial oxidation process which leads to accumulation of reactive oxygen species (ROS) such as oxygen

superoxide and hydrogen peroxide (75). When the anti-oxidant defence mechanisms fail to remove the ROS, oxidative stress occurs. ROS are considered as signalling molecules that can influence cellular proteins, lipids and DNA in high concentrations. In addition, these harmful substances can enhance the release of inflammatory mediators by activation of the intra-cellular signalling pathway NF- κ B (75, 76). Oxidative stress can also influence bone remodelling by enhancing osteoclast activity and inhibiting osteoblastogenesis (77).

Another mechanism by which hyperglycaemia can affect the host immune response is by accumulation of advanced glycation end products (AGEs). The process involves non-enzymatic irreversible glycation and oxidation of proteins and lipids (78). AGEs affect the tissue directly by provoking phenotypic and functional changes in collagen and other matrix molecules. They can also act through the receptor for advanced glycation end products (RAGE) (78). Moreover, hyperglycaemia can increase RAGE expression by epithelial cells (79). The binding of AGEs to their receptors triggers intra-cellular events that lead to up-regulation of pro-inflammatory cytokines, chemokines and cell adhesion molecules. Moreover, it is claimed that AGEs (as one of the TLR ligands) are associated with increased TLRs expression and impaired tissue repair in periodontal tissues (51, 80). Another indirect effect of AGEs is the enhancement of oxidative stress by increasing the production of ROS (81). It is also reported that blocking of the AGE-RAGE axis improves periodontal tissue integrity (82).

Neutrophils play a pivotal role in plaque-induced periodontal tissue destruction (83). Hyperglycaemia is reported to interfere with neutrophil rolling, chemotaxis and phagocytosis (4). It also increases the expression of leukocyte adhesion molecules (84). Moreover, neutrophil priming might be a consequence of increased levels of protein kinase-C as a result of hyperglycaemia (85). Under normal conditions, neutrophil apoptosis (programmed cell death) is delayed when interacting with bacteria. In a hyperglycaemic environment, however, neutrophil apoptosis is defective and this delaying effect is disrupted (86).

It is well documented in the scientific literature that apoptosis plays a critical role in diabetic complications, including periodontitis (87). Apoptosis is essential for maintaining tissue integrity by selective elimination of “bad” cells *i.e.* abnormal or aged cells. When the rate of apoptosis exceeds normal limits, there will be a lack of the matrix-producing cells essential for tissue healing and repair (88). Apoptosis can be provoked by accumulation of AGEs, oxidative stress and inflammatory mediators such as TNF- α (89). It is reported that hyperglycaemia enhances human periodontal ligament fibroblast apoptosis (90). Moreover, in animal models, diabetes is associated with fibroblast, osteoblast and osteocyte apoptosis in periodontal tissues (89, 91). These cells are the key players in bone coupling (69). In this context, there is ample clinical evidence that hyperglycaemia exacerbates periodontal bone tissue destruction by influencing the RANKL/OPG ratio (92, 93). It is also reported that in a rat model, hyperglycaemia negatively influences bone tissue turnover (94).

1.3.4.2 Resolution of inflammation

Resolution of inflammation is an active process involving the production of specific molecules through different pathways (95). The underlying mechanisms have been investigated in order to develop new therapeutic approaches to inflammatory diseases (96). Under normal conditions, the process involves termination of neutrophil recruitment, enhancement of neutrophil apoptosis and generation of functional decoys for chemokine receptors (95, 97). In addition, macrophage activity changes from production of pro-inflammatory cytokines to production of IL-10 and TGF- β instead (98). The macrophages also help with engulfing dead cells and tuning the sensitivity to TLRs (99). The above mechanisms are orchestrated by lipid molecules such as lipoxins and resolvins (100). Moreover, Th-2 produces IL-4, an anti-inflammatory cytokine that down-regulates MMPs and RANKL and promotes the secretion of their antagonists: TIMP and OPG, respectively (57). In addition, myeloid-derived suppressor cells and T-reg cells also have a role in counteracting the pro-inflammatory process and maintaining tissue homeostasis (95, 101).

The above-mentioned process is essential to resolve acute inflammation and prevent the condition from progressing to the chronic stage where tissue injury occurs. In mild cases, the condition is resolved by “regeneration”, whereby necrotic cells are replaced with new ones. If the tissue damage is extensive, the extracellular matrix will be damaged, limiting tissue regeneration. As a result, there will be deposition of fibroblast-mediated collagen and formation of granulation tissue. This process is called “tissue repair” or “scarring”(102).

In patients with diabetes, defective resolution of inflammation might be one of the mechanisms underlying the extensive periodontal tissue destruction these patients experience in comparison with systemically healthy individuals. The findings of an experimental study by Herrera *et al.*, (103) suggested impaired resolution pathways in T2D. A flawed reparative process was also reported in an animal study of T2D rats with periodontitis (104). Another study in diabetic mice reported failure of macrophages to clean dead cells from the diabetic wound site (efferocytosis) (105).

1.4 Periodontitis goes beyond the oral cavity

It is widely appreciated that the effect of chronic periodontitis extends beyond the periodontal tissues to have a systemic impact. Several studies have reported an association between periodontal disease and systemic conditions such as cardiovascular diseases, respiratory diseases, low birth-weight infants, rheumatoid arthritis and diabetes (83, 106).

The effect of periodontal disease on diabetes has been highlighted in several studies. The study of the First National Health and Nutrition Examination Survey (NHANES I) reported a two-fold increase in the odds of developing diabetes among individuals with periodontitis, compared to those without the disease (107). Moreover, the risk of developing diabetes was about 3.5 times higher for those with severe periodontitis (108). In a five-year follow-up study, periodontal disease was associated with deteriorating

glycaemic control (109). In contrast, a seven-year follow-up study from Japan indicated no significant association between periodontitis and the incidence of diabetes (110).

In the advanced stages of chronic periodontitis, oral bacteria, bacterial products and inflammatory mediators might escape the local environment into the systemic circulation through the ulcerated periodontal tissues, creating a chronic low-grade systemic inflammatory state (111). Almaghlouth *et al.*, (112) indicated an increase in systemic inflammatory molecules among individuals with periodontitis. Moreover, it was reported that non-surgical periodontal therapy significantly decreases the serum level of inflammatory molecules such as IL-6, TNF- α and C-reactive protein (112, 113).

Systemic up-regulation of certain pro-inflammatory molecules such as TNF- α , IL-1 β and IL-6 has been linked to insulin resistance and the aetiology of T2D (114). Under normal conditions, binding of insulin to its membrane receptor leads to phosphorylation and activation of the insulin receptor substrate. These changes facilitate the cellular import of glucose (115). The high levels of pro-inflammatory molecules activate the intracellular signalling pathway (NF- κ B) which eventually leads to structural changes in the insulin receptors (116). Moreover, up-regulated inflammatory molecules might also affect the activity of pancreatic β -cells through accumulation of ROS (117). Interestingly, one of the proposed mechanisms of the systemic impact of periodontitis on diabetes is by altering the composition of gut microbiota. Systemic inflammatory changes were observed after oral administration of *P.gingivalis* in mice, suggesting that oral bacteria can disturb the balanced microbial environment of the gut, which in turn induces systemic inflammation (118).

Among patients with diabetes, periodontitis is regarded as a predictor of non-oral complications (119). In longitudinal studies among Pima Indians - a population with a high prevalence of diabetes - periodontitis was associated with nephropathy, end-stage renal disease and cardio-renal mortality (120, 121).

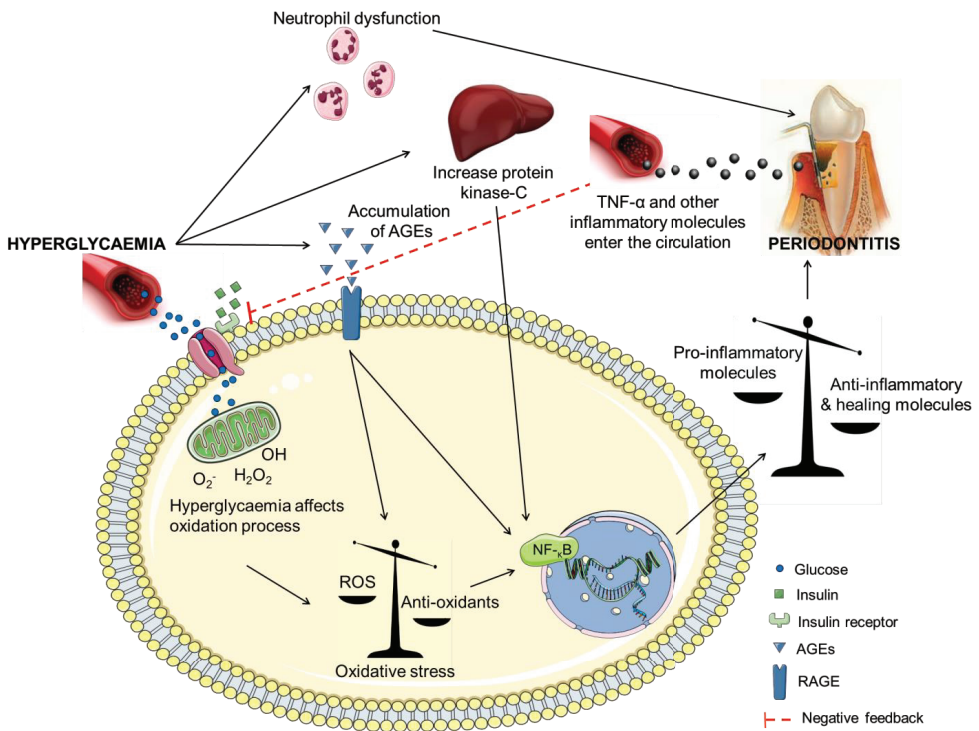


Figure 3. Schematic illustration of the proposed mechanisms explaining the bi-directional relationship between diabetes and chronic periodontitis. Hyperglycaemia increases reactive oxygen species (ROS), advanced glycation end products (AGEs) and protein kinase-C, which in turn causes intra-cellular events through activation of NF- κ B leading to up-regulation of pro-inflammatory molecules and/or down-regulation of anti-inflammatory, healing and regeneration molecules. Pro-inflammatory molecules on the other hand, escape to the systemic circulation through the inflamed periodontium, which might eventually lead to structural changes in the insulin receptors. The Figure is produced using Servier Medical Art.

1.5 Effect of periodontal therapy on diabetic status, “is it worth it?”

Management of periodontitis includes non-surgical mechanical removal of debris and calculus as a standard procedure, *i.e.* scaling and root planing. Adjuvant local or systemic antibiotic therapy might be indicated in some cases, and surgical intervention is sometimes indicated. With all these therapies, behavioural modification, oral hygiene instructions and follow-up are crucial to prevent recurrence (20, p409).

As in systemically healthy individuals, periodontal therapy in patients with T2D is associated with reduced systemic inflammation, reflected in the reduction of the systemic levels of TNF- α , C-reactive protein and other inflammatory mediators (122, 123). Nevertheless, the findings of studies of the effect of periodontal therapy on metabolic control among patients with diabetes are inconsistent. Two meta-analysis studies conducted in 2010 reported a significant HbA1c reduction of 0.40% (124, 125). Engebretson *et al.*, (126) in 2013 concluded with similar findings. Moreover, Nesse *et al.*, (127) reported a dose-response relationship between the inflamed surface area and HbA1c level among patients with T2D. On the other hand, some reports failed to confirm the association (128, 129). This inconsistency might be attributable to differences in the treatment protocol, *i.e.* surgical vs non-surgical periodontal therapy, and the use of antibiotics.

Whether periodontal therapy is effective in controlling glycaemic levels or not, a key factor in reducing the risk of medical complications related to diabetes is maintenance of blood glucose levels within the normal range. Improved glycaemic control over 10 years was shown to be associated with decreased risk of diabetes-related microvascular complications (130). It was also reported that each 1% (3 mmol/mol) reduction in HbA1c is associated with reduced diabetes-related microvascular complications by 37% (131).

1.6 Diabetes and dental caries

Dental caries is a major public health problem characterised by destruction (demineralisation) of the dental hard tissues by acidic by-products produced by bacterial biofilm as a result of fermentation of dietary carbohydrates (132). The suspected microbes responsible for dental caries are *Streptococcus mutans*, *Streptococcus sobrinus* and *Lactobacillus* (133).

Anatomically, dental caries can be classified into coronal and root caries. The lesion starts when demineralisation by microbial acids exceeds remineralisation (uptake of calcium, phosphate, and fluoride from food and saliva). During the early stages, the condition is reversible, but as the lesion progresses, the destruction of dental hard tissue becomes permanent and needs to be restored by dental material (132).

Data from epidemiological studies of the relationship between diabetes and dental caries are inconsistent. Higher caries experience in patients with diabetes is reported in some studies (134, 135), and not in others (136, 137). On the other hand, there is stronger evidence supporting higher prevalence of root caries in patients with diabetes (138).



Figure 4. Clinical case of T2D patient represents the exposed and decayed roots

1.7 Diabetes and oral health related quality of life

Inspired by the WHO definition of health “a complete state of physical, mental, and social well-being and not merely the absence of disease” (139), quality of life assessment covers crucial aspects of health, such as physical, mental and oral health (140). In addition to the generic measurements of quality of life, disease-specific measurements have been developed to improve patients’ responsiveness (141).

OHRQoL associates oral health with the other aspects of quality of life by linking the patient’s health experience to the clinical variables, and addressing the psycho-social consequences of oral diseases (142). Hence, both the psycho-social and clinical indicators are important for health promotion, disease prevention and allocation of resources (141). Moreover, it is important to consider both clinical and non-clinical variables, in order to gain a more comprehensive understanding of the processes that determine the influence of chronic disease on individuals’ daily life (143).

The OHRQoL of patients with diabetes is poorly documented. Improving quality of life of T2D patients is regarded as an important target for diabetes management (144). It adds another dimension to the “traditional” medical care of the disease. Moreover, improvement in oral health is reflected in general health, especially in patients with diabetes (145). Diabetes has an adverse effect on an individual’s quality of life (146). Patients with diabetes have a limited understanding of the detrimental effect of poor oral health on their general health (147). They also lack knowledge and awareness about the oral complications of diabetes (147, 148). Furthermore, it has been reported that the general oral health practices of patients with diabetes are poor (149). Neglect of oral health of patients with diabetes, at a professional as well as at patient level, seems to be a global issue, and is not limited to a specific geographical area (150). An important factor which might influence how patients with diabetes perceive oral health is that they are already burdened by other non-oral medical complications of the disease and are therefore, less likely to seek oral health care (147).

2. AIMS

The overall aim of this project was to study oral health indicators in Sudanese subjects with T2D and to investigate the impact of T2D and chronic periodontitis on biomarkers of inflammation and glucose regulation in gingival crevicular fluid (GCF) and saliva. It was hypothesised that T2D adversely influences oral health parameters, the expression of the related inflammatory molecules and the prevalence of periodontal pathogens. A further hypothesis to be tested was that chronic periodontitis exerts a systemic effect on glucose metabolism and this is reflected in the local expression, in GCF, of molecules that regulate glucose metabolism.

The specific objectives were:

- I. To compare clinical and subjective oral health indicators (periodontal parameters, dental caries and OHRQoL) in T2D subjects and in age- and gender-matched controls without the disease (*Study I*).
- II. To investigate the influence of chronic periodontitis on the levels of glucoregulatory biomarkers in GCF (*Study II*).
- III. To investigate the effect of T2D on the local expression of molecules involved in periodontal inflammation and healing in GCF (*Study III*).
- IV. To investigate the impact of T2D on the prevalence of putative periodontal pathogens in dental plaque, and on levels of salivary MMP-8, MMP-9, RANKL and OPG (*Study IV*).

3. MATERIALS AND METHODS

3.1 Study area and participants

3.1.1 Enrolment of patients with type 2 diabetes

The T2D patients were recruited from the dental clinic at Jaber Abol'ez Diabetes Centre in Khartoum city. It is one of the main public specialised referral hospitals for patients with diabetes in Khartoum state and the country, with a daily average of 250 patients visiting the centre (151). The study participants were enrolled between July and December 2012. One hundred and fifty seven T2D patients were invited to participate in the study (63 men and 94 women). Three patients resigned from participation due to time constraints. The mean age for T2D patients was 52.59 ± 10.50 years (range 24 to 70 years). Diabetes was diagnosed by specialist physicians at the centre according to the criteria of the American Diabetes Association (152). The eligibility criteria for enrolment were:

- Diagnosed with T2D more than one year ago.
- At least 10 remaining natural teeth.
- No medication with antibiotics or steroidal and/or non-steroidal anti-inflammatory agents over the past 3 weeks.
- No immunosuppressive chemotherapy, no current acute illness, no professional periodontal treatment during the last 6 months and no pregnancy or lactation.

To determine the level of glycaemic control, the T2D patients underwent an HbA1c test by boronate affinity chromatography (153) (well-controlled: $\text{HbA1c} \leq 8\%$ and poorly controlled: $\text{HbA1c} > 8\%$, $8\% = 64 \text{ mmol/mol}$).

3.1.2 Enrolment of subjects without diabetes

Three hundred and four subjects without diabetes were recruited from the outpatient dental clinic at the Khartoum Dental Teaching Hospital in Khartoum city (119 men and 185 women). One participant was diagnosed with diabetes and excluded after recruitment.

Khartoum Dental Teaching Hospital is the main public referral hospital in Khartoum state, providing dental services for the entire country. The mean age of participants without diabetes was 52.36 ± 10.50 years (range 24 to 70 years). With the exception of a diagnosis of diabetes, the same selection criteria as above were applied to recruitment of participants without diabetes. Subjects without diabetes were asked about signs and symptoms of diabetes and if suspected, they were referred for confirmation.

3.1.3 Allocation of participants in studies I-IV

- **Study I:** All the enrolled participants were included in this study (457 in total). Participants were grouped according to diabetic status: 154 patients with T2D and 303 without the disease. The two groups were individually matched according to age and gender, with a ratio of 2 controls for each case.
- **Study II:** A total of 152 participants were included in this study. Subjects were allocated into 4 groups according to diabetic and periodontal status; 54 with both T2D and chronic periodontitis, 24 with T2D, 30 with chronic periodontitis and 44 with neither T2D nor periodontitis.
- **Study III:** In this study, 108 individuals were included, representing three groups: 54 with both T2D and chronic periodontitis, 30 with chronic periodontitis and 24 with T2D.
- **Study IV:** There were 80 participants, stratified as follows: 31 with both T2D and chronic periodontitis, 29 with chronic periodontitis and 20 with T2D.

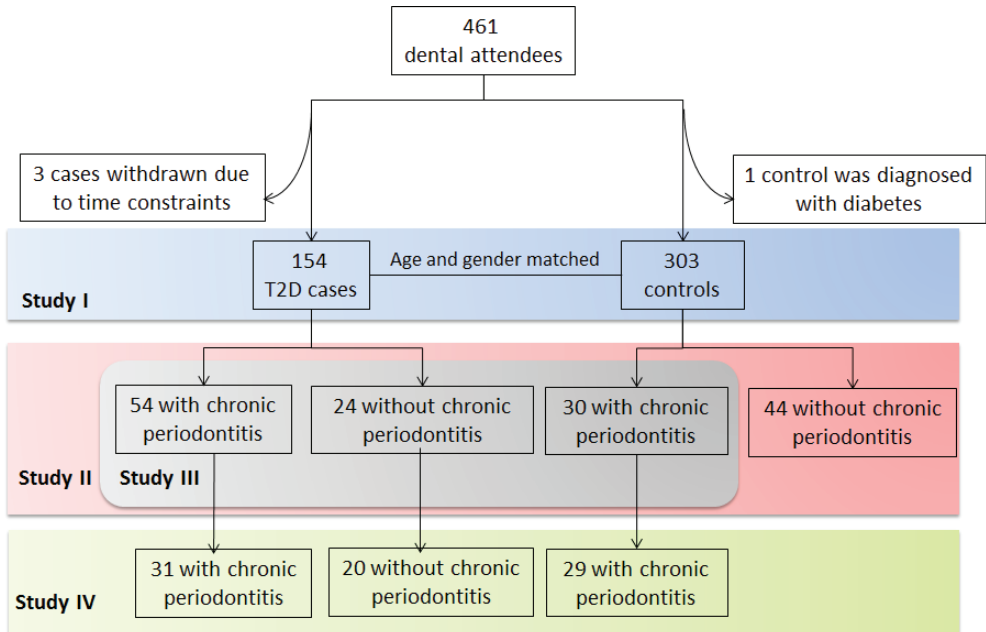


Figure 5. Flowchart of the studies comprising the thesis.

3.2 Questionnaire-guided interview

All participants were interviewed by trained research assistants (three in total) using a structured questionnaire. The questionnaire was designed to gather data about socio-demographic characteristics (age, gender, employment status and educational level), life style factors (alcohol consumption, tobacco use and consumption of sugary drinks), medical history, regularity of dental attendance and self-assessed oral health evaluation. In addition, the OHRQoL was assessed using the eight-item Oral Impact on Daily Performance inventory (OIDP) (154).

3.3 Clinical examination

After completion of the interview, the participants underwent clinical examination of all teeth (except 3rd molars) and soft tissues of the oral cavity. One examiner, the author, conducted all examinations. The examination comprised assessment of dental plaque [Silness and Loe Index (155)], tooth mobility index (156), bleeding on probing (yes/no), probing depth [measured from the gingival margin to the base of the periodontal pocket (mm) at four sites of each tooth (mesial, distal, buccal and lingual)], furcation involvement (157), root caries (yes/no) and DMFT Index (158).

3.4 Collection and laboratory analysis of biological samples

GCF, dental plaque and saliva samples were obtained from 309 participants (154 T2D patients and 155 controls).

3.4.1 Gingival crevicular fluid

Method of collection

GCF samples were collected using paper strips (PERIOPAPER[®] Gingival Fluid Collection Strips, Oraflow Inc., New York, USA). Four samples, representing the four quadrants, were obtained from each participant. The samples were immediately pooled, labelled and stored in liquid nitrogen.

Protein extraction and quantification

Protein was extracted from the GCF samples by Tween buffer, and quantified using a commercially available kit (Pierce[™] BCA Protein Assay Kit, ThermoScientific, Rockford, USA). Absorbance was measured at 560 nm on a plate reader (FLUOstar OPTIMA- BMG Labtech, Germany). Total protein per sample (4 strips) was calculated in micrograms (μg).

Multiplex assays

Bead-based multiplex assays were used to detect 10 glucoregulatory molecules (*Study II*) (Appendix A) and 27 inflammatory molecules (*Study III*) (Appendix B) in the GCF samples (Bio-Plex Human Cytokine Assay, Bio-Rad Inc., Hercules, CA, USA) (159).

3.4.2 Subgingival plaque

Method of collection

Subgingival plaque samples were collected on sterile endodontic paper points ISO (International Organisation for Standardisation) size 40. The samples were taken from the same sites as the GCF samples. The samples were immediately pooled, labelled and stored in liquid nitrogen.

DNA purification

DNA was purified using a commercially available kit, according to the manufacturer's instructions (MasterPure DNA purification kit, Epicentre Biotechnologies, Madison, Wisconsin). The amount of DNA was quantified for each sample by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Polymerase chain reaction

After DNA purification, conventional polymerase chain reaction (PCR) was performed under standard conditions, to investigate the prevalence of *P.gingivalis*, *T.forsythia*, *P.intermedia*, *T.denticola*, *C.rectus*, and *A.actinomycetemcomitans*. The PCR products were loaded and separated by agarose gel electrophoresis (2.2% FlashGel DNA system, Lonza, Walkersville, MD). The stained DNA bands were visualised by ultraviolet light.

3.4.3 Whole saliva

Method of collection

Unstimulated whole saliva was collected by instructing the participants to rinse their mouths with water, tilt their heads downwards and expectorate into a sterile container for 5 minutes. The samples were then aliquoted and immediately stored in liquid nitrogen (160).

Enzyme linked immunosorbent assay

The concentrations of MMP-8, MMP-9, OPG (Sigma-Aldrich, St. Louis, MO, USA) and RANKL (MyBiosource, CA, USA., and PeproTech EC, London, UK) were determined using enzyme linked immunosorbent assays (ELISA) according to the manufacturers' instructions. Optical densities were determined using a microplate reader (FLUOstar OPTIMA- BMG Labtech, Germany).

3.5 Ethical considerations

Ethical clearance was obtained from The Ministry of Health in The Sudan and The Norwegian Research Ethics Committee at The University of Bergen (2012/1470/REK Vest). Participation was confirmed by written informed consent (Appendix C), and the steps involved in the clinical examination and sampling procedures were explained to each participant. Participants were also informed that their participation was voluntary and that they had the right to withdraw at any time. All participants were provided with verbal and written oral hygiene instructions, informed of their dental diagnosis and referred for appropriate dental treatment if needed.

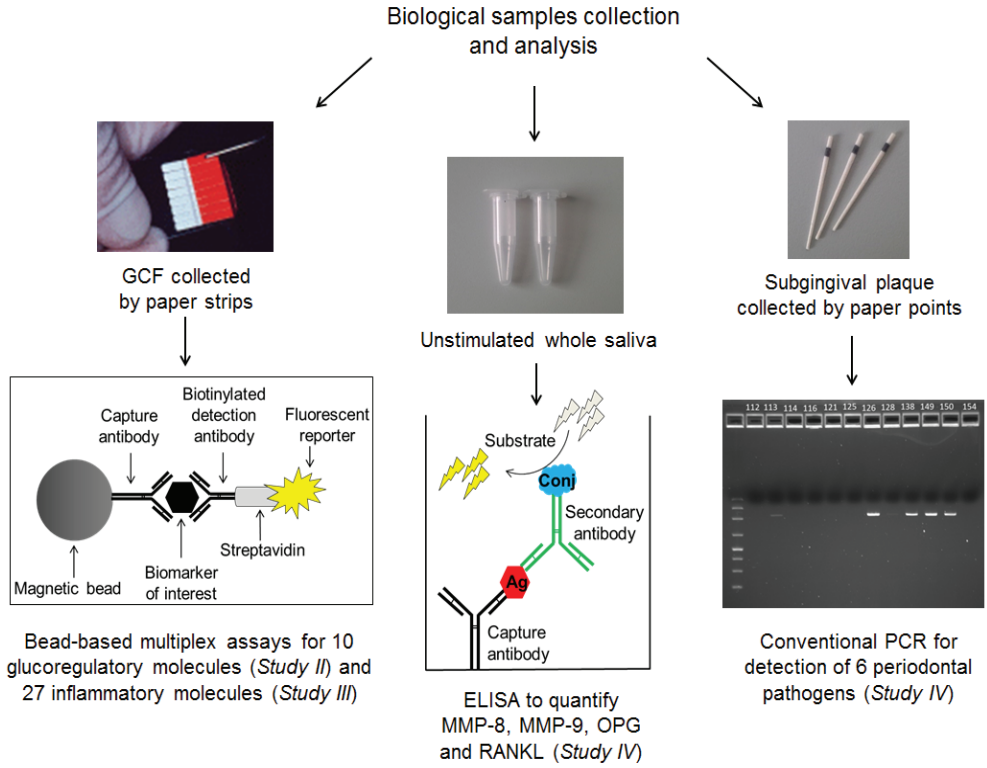


Figure 6. Methods used for biological samples collection and laboratory analysis

4. METHODOLOGICAL CONSIDERATIONS

4.1 Study design and participants

This series of studies is the first time to present the oral health status of Sudanese adults with T2D. The project was therefore designed to record as much information as possible about this section of the Sudanese population, to serve as a firm foundation for further research and interventions. The recruitment of both T2D patients and controls was hospital-based. Both hospitals are part of the public healthcare system, providing services covered by government health insurance. Thus, these two hospitals provide healthcare to a wide sector of the residents of Khartoum state. As both cases and controls were hospital-based, there might be some selection bias, because the participants were seeking healthcare at the time of recruitment, implying that they probably have more severe oral diseases and higher treatment needs than subjects of population-based studies, recruited from the general population (161).

The sample size calculation was performed according to the first objective of this project (*Study I*). It was not feasible to process all the biological samples obtained from the participants. Therefore, the sample size was reduced for *Studies II-IV* in order to be comparable with other scientific studies on the same topic. Moreover, participants without T2D and chronic periodontitis were excluded from *Studies III* and *IV* because all the study participants were dental attendees. Therefore, other dental problems might influence the results of these two studies (local expressions of different inflammatory molecules) (162); while in *Study II*, most of the investigated molecules are systemically produced, thus the influence of the local oral environment on their level of expression is minimal. For each study, the allocation of participants and the statistical analyses were planned in accordance with the specific objective.

OIDP is one of the inventories for assessing OHRQoL. It quantifies the potential impact of oral problems on 8 daily tasks (141, 154). The Arabic version of the OIDP inventory has been validated and applied in The Sudan to a sample of 12 year-old school-

children and to adults with mucocutaneous diseases (163, 164). The OIDP has also been applied in other low-income countries such as Tanzania and Uganda (165, 166).

Both pocket depth and bleeding on probing were used to define cases of chronic periodontitis. These clinical parameters reflect the current activity of the disease, which was the focus of this project (167, 168). Bleeding on probing is regarded as a sign of active host inflammatory response. Its absence indicates periodontal stability (169). Both pocket depth and bleeding on probing are highly correlated with dental plaque microbiota and inflammatory status (170).

The intra-examiner reliability of the solo examiner HGM was assessed by Cohen's kappa (κ) to estimate coefficients of agreement of dichotomous judgments in two different sessions (171). For this purpose, within 2 weeks, the oral examination was repeated for 20 randomly selected participants (172), and (κ) was calculated for tooth mobility (0.74), root caries (0.80), periodontal diagnosis (0.88) and dental caries (1.00).

The formula used for calculating (κ) is:

$$\kappa = \frac{P_o - P_c}{1 - P_c}$$

Where P_o is the proportion of agreement of the units, and P_c is the proportion of units for which agreement is expected by chance. The κ value ranges from 0 to 1 (1 = perfect agreement).

4.2 Biological sampling techniques and laboratory analysis

4.2.1 Gingival crevicular fluid

GCF is a complex fluid containing molecules derived from serum, cells of the periodontal tissue, immune cells and micro-organisms. Some of these molecules can be used as indicators of periodontal disease progression and the healing process (173). GCF can be collected by gingival washing, capillary tubes or absorbent paper strips (174). In *Studies II* and *III*, GCF was collected by paper strips. This method is non-invasive, but time consuming and technique sensitive (175). Guentsch *et al.*, (176) compared the three main GCF sampling techniques: paper strips, paper points and washing method. They concluded that collecting GCF with paper strips is the method of choice for detection of most biomarkers in immunologic studies. As the availability of GCF for sampling varies from tooth site to site, hence the collected GCF samples should be standardised. One means of standardisation is measuring the GCF volume. Weighting of strips before and after the GCF sampling is also suggested, though this requires very sensitive scales, able to detect such a minor change in strip weight (174). An alternative approach is to standardise the time of collection (175). In *Studies II* and *III*, GCF collection time was standardised (30s) and the results were reported as the total amount in the timed sample.

Although ELISA is considered as the gold standard for measuring inflammatory molecules, the multiplex assay is a powerful tool that offers some advantages over ELISA (177). It is a high throughput technology, able to detect up to 100 different analytes in a relatively small sample volume (159, 178). Moreover, both antigens and capture antibodies are freely suspended inside the reaction wells of the multiplex plate, while in ELISA, the capture antibodies are attached to the bottom of the well. Multiplex assays are therefore claimed to be at least as sensitive as ELISA (178). By assaying a relatively wide range of both glucoregulatory and inflammatory molecules, application of the multiplex technique in *Studies II* and *III* revealed a global picture of the inflammatory process involved in both diabetes and periodontal disease.

4.2.2 Subgingival plaque

Several techniques are available for sampling dental plaque. Paper points and curettes are widely used in practice (179). It is claimed that a curette samples the entire pocket, while a paper point collects the outer layer, which contains more pathogens (180). In contrast, a recent study reported higher bacterial DNA in curette samples (181). Jervoe-Storm *et al.*, (182) in a comparative study of samples collected by paper points and curettes, reported quite similar compositions of plaque microbiota. In the present study, subgingival plaque samples were collected on sterile paper points, ISO size 40. The use of paper points was more convenient in the field. It was less invasive for the patient and it was possible to transfer the samples immediately to transport vials. One disadvantage is that paper points might absorb moisture from GCF (183). To ensure a dry field, the subgingival plaque was sampled after collecting GCF from the sampling sites. In *Study IV*, plaque samples were obtained from 4 sites, one in each quadrant. This sampling strategy provides a representative, whole-mouth sample of subgingival microbiota. Moreover, sampling 4 sites minimises false negative results associated with sampling only one or two sites (184). As the focus of interest was at the individual level rather than site-specific, the pooled design was adopted for both GCF and dental plaque samples (60, 185).

In general, microbial analysis of dental plaque falls into one of the following methods: (i) polymerase chain reaction (PCR) *i.e.* conventional and quantitative PCR, (ii) DNA-DNA hybridisation methods, which include *in situ* hybridisation, checkerboard hybridisation and oligonucleotide microarrays, and (iii) sequencing techniques (186). PCR is a powerful tool for gene amplification in simplified and automated fashion developed in 1985 by Kary Mullis and associates (187). In *Study IV*, conventional PCR was used to determine the prevalence (presence or absence) of the bacteria under investigation.

4.2.3 Whole saliva

Saliva is produced mainly by the three major salivary glands; the parotid, submandibular and sublingual. Saliva can be sampled in the resting or stimulated state and may comprise whole or gland-specific saliva. Saliva also contains secretions from the minor salivary glands, GCF, desquamated epithelial cells, micro-organisms, immune cells, food remnants and circulating blood (188). Several biomarkers related to the pathogenic process of periodontal disease can be identified by saliva proteomic analysis (189). In *Study IV*, unstimulated (resting) saliva samples were collected. For detection of disease markers, resting whole saliva is preferred to stimulated saliva. It is also reported that substances used to stimulate salivary flow influence the pH and make saliva more watery, which might result in dilution of the protein content (190).

5. SUMMARY OF RESULTS AND GENERAL DISCUSSION

5.1 Type 2 diabetes patients have poorer oral health indicators than controls

The results of *Study I* reveal that T2D patients have a higher prevalence of tooth mobility (OR: 5.90, 95% CI: 2.26-15.39), furcation involvement (OR: 2.96, 95% CI: 1.36-6.45) and periodontal pocket ≥ 4 mm (OR: 4.07, 95% CI: 1.74-9.49). These findings are in accordance with those of several earlier studies reporting compromised periodontal parameters among patients with T2D (36, 38, 39). Moreover, a four-year follow-up study by Timonen *et al.*, (191) indicated an association between impaired glucose metabolism (insulin resistance and β -cell function) and periodontal pocket formation among subjects without diabetes. They reported that impaired glucose metabolism weakly predicted periodontal pocket formation over the study period.

Tooth loss is a serious dental problem which can have a negative impact on a person's quality of life (192). *Study I* demonstrated that T2D patients suffered from more tooth loss (≤ 21 remaining teeth) than their controls (OR: 4.31, 95% CI: 1.57-11.76). This is in agreement with other reports (35, 40). Moreover, Costa *et al.*, (19) have found an association between the level of glycaemic control and the number of standing teeth. Kaur *et al.*, (193) reported an adjusted OR of 1.93 (95% CI: 1.37-2.71) that those with type 1 diabetes have more missing teeth, while their results for those with T2D were not statistically significant. The non-significant results were attributed to the effect of age as a confounding factor. However, in *Study I*, after controlling for potential confounders including age, the results remained statistically significant.

The adjusted analysis of *Study I* revealed no significant difference between T2D cases and controls for DMFT or root caries (OR: 0.38, 95% CI: 0.09-1.66) and (OR: 1.65, 95% CI: 0.84-3.26), respectively. A search of the scientific literature revealed conflicting findings about an association between T2D and dental caries. The lack of consensus can be attributed to the fact that many factors confound the association. For example, T2D patients are usually under strict dietary control, with low sugar intake. In addition,

xerostomia is an acknowledged complication of diabetes, and this increases susceptibility to caries (135). One of the suggested explanations for high prevalence of caries in T2D patients is the high glucose level in saliva and GCF, which increases the activity of saccharolytic bacteria. These bacteria produce organic acids that cause demineralisation of enamel (194). The association between T2D and root caries is reported more frequently (138). Hintao *et al.*, (195) reported a significant association between root caries and T2D, while the association with coronal caries was not significant. In *Study I*, the sub-group analysis among T2D patients indicated an association between duration of the disease and DMFT, with higher DMFT in those diagnosed with T2D more than 10 years ago (OR: 2.94, 95% CI: 1.24-6.94). Similar findings were reported elsewhere (196). It has also been reported that duration of diabetes adversely influences the periodontal parameters (197), however, the results of *Study I* disclosed no significant association between duration of diabetes and periodontal parameters.

In general, utilisation of dental services by T2D patients was low in *Study I*, as only 7.8% of the T2D patients reported that they were regular dental attenders. A low rate of dental attendance (10.8%) was also reported among Indian patients with T2D (198). Poor dental attendance might be attributable to financial restraints, dental anxiety or ignorance (199). It might also be due to the fact that oral diseases are not a priority for T2D patients, especially those who have serious non-oral medical complications (147).

Studying OHRQoL adds another dimension to the objective measurements of dental problems such as periodontitis and dental caries, by taking into account the patients' perceptions of their oral health status. Inclusion of OHRQoL assessment contributes to our understanding of the impact of the disease on the patient's functional and psychosocial well-being (141). The results of *Study I* indicated that T2D impacts OIDP - as a measure of OHRQoL - with an OR of 3.46 (95% CI: 1.61-7.42). Another study among adults with diabetes in the USA reported that poor health-related quality of life was associated with deterioration of oral health and lack of dental care (200). In contrast, Irani *et al.*, (201) investigated OHRQoL in patients with and without T2D using the oral health

impact profile (OHIP-49) and concluded that periodontal diseases (gingivitis and periodontitis), but not T2D, were associated with poorer OHRQoL. Moreover, two qualitative studies, one in Ghana (202) and the other in the USA (203) investigated contextual factors related to diabetes and oral health care and concluded that patients with T2D did not perceive a correlation between diabetes and oral diseases to be a complication of diabetes.

Study I is the first to report OHRQoL among adults with T2D in The Sudan. OHRQoL has been investigated in Sudanese 12 year-old school attendees (163) and in Sudanese adults with mucocutaneous diseases (164). A study by Abdelgadir *et al.*, (11) investigated health-related quality of life in Sudanese patients with diabetes who had undergone lower limb amputation, concluding that lower limb amputation negatively affected the quality of life of patients bearing the disease.

The results of *Study I* prompted more questions about why patients with T2D have poorer periodontal parameters and whether chronic periodontitis has any impact on glucose metabolism. Within the limits of the study design and logistics, these questions were addressed in *Studies II-IV*.

5.2 Chronic periodontitis influences gingival crevicular fluid levels of glucoregulatory biomarkers

C-peptide, insulin and insulin-stimulating hormones

Study II revealed that the levels of insulin-stimulating hormones (GIP and GLP-1) were higher in T2D patients with chronic periodontitis than in those without periodontitis. In subjects without diabetes, the differences between those with periodontitis and those without were not statistically significant. Both GIP and GLP-1 are incretin hormones which reduce blood glucose levels by stimulating insulin secretion in response to food intake (204). Recently, these hormones have been intensively investigated for their therapeutic potential in treatment of high blood glucose (205). Although there was no

statistically significant difference in GCF levels of insulin between the two groups of patients with T2D (with and without chronic periodontitis), the high levels of GIP and GLP-1 in T2D patients with chronic periodontitis might be due to negative feedback from exogenous insulin, as the percentage of subjects on insulin therapy was low (29.6%) in patients with chronic periodontitis compared to those without periodontitis (50.0%). In this context, C-peptide is considered to be a better indicator of β -cell activity than insulin, because its level is not affected by exogenous insulin (206). The results for C-peptide were not statistically significant among patients with T2D. On the other hand, in participants without T2D, those with chronic periodontitis had lower levels of C-peptide than those without periodontitis. This might reflect lower β -cell activity in patients with chronic periodontitis than in their control group. Animal studies also indicate that periodontal disease adversely influences insulin metabolism and provokes insulin resistance (207, 208)

Glucagon

In *Study II*, GCF levels of glucagon were higher in T2D patients with chronic periodontitis than in their controls (T2D patients without periodontitis). Glucagon is an important hormone secreted by the pancreatic α -cell. It increases the blood glucose level by enhancing glycogenolysis (glycogen breakdown) and gluconeogenesis (synthesis of glucose from non-carbohydrate molecules) in the liver (209). Therefore, the high level of glucagon in T2D patients with chronic periodontitis might reflect the adverse systemic effect of chronic periodontitis on metabolic control in those patients.

Anti-diabetic hormones (ghrelin and leptin)

Ghrelin and leptin are both anti-diabetic hormones. Leptin improves peripheral insulin resistance (210), while ghrelin enhances insulin secretion, although recent evidence questions the role of ghrelin in insulin secretion (211). In *Study II*, the results for leptin were not statistically significant. In individuals without T2D, expression of ghrelin was lower in periodontitis patients than in their controls. In general, low systemic levels of

both hormones are reported in patients with T2D (212, 213). Moreover, Yilmaz *et al.*, (214) reported elevated plasma ghrelin levels in individuals with chronic periodontitis compared to those without periodontitis. Ohta *et al.*, (215) were the first to detect ghrelin in the GCF of 12 healthy volunteers. To the best of our knowledge, there are no data about GCF ghrelin levels in patients with T2D and periodontitis.

An experimental study indicated that leptin negatively influences the regenerative capacity of human periodontal ligament cells (216). Another study reported that serum leptin levels are higher in patients with chronic periodontitis than in those without the disease (217), while Ay *et al.*, (218) reported no variations in serum leptin levels associated with periodontal status. Furthermore, it was reported that leptin is expressed in lower amounts in gingival tissues of chronic periodontitis patients with T2D than in those without diabetes (219).

Plasminogen activator inhibitor-1

In *Study II*, there was a tendency towards increased plasminogen activator inhibitor-1 (PAI-1) levels in T2D patients with chronic periodontitis compared to those without periodontitis. The opposite trend was observed in individuals without T2D. Neither of these results reached the level of significance. PAI-1 is a known marker for cardiovascular complications, especially among patients with diabetes (220, 221). Bizzarro *et al.*, (222) reported higher levels of plasma PAI-1 in subjects with chronic periodontitis than in healthy controls. Moreover, a recent *in vitro* study found that *P.gingivalis* induces the expression of PAI-1 by human gingival fibroblasts (223).

Resistin and visfatin

The name resistin is derived from its action “insulin resistance”. It is claimed that the main source of resistin in humans is macrophages rather than adipocytes (224). The levels of resistin were above the detection limit in *Study II*. Some studies have reported up-regulated GCF levels of resistin in patients with T2D and chronic periodontitis compared

to healthy controls (225, 226). Moreover, a review article by Devanoorkar *et al.*, (227) indicated that chronic periodontitis is associated with increased resistin levels. In contrast, another study found that periodontal status does not influence serum resistin levels (228).

Visfatin is known to mimic insulin. Its expression is induced by hyperglycaemia (229). Visfatin is also considered as a pro-inflammatory molecule which enhances the expression of IL-1 β , TNF- α and IL-6 (230). In addition, it up-regulates the expression of MMP-1 and MCP-1 genes (231). It might also influence periodontal healing and regeneration (232). The results of *Study II* show a weakly positive correlation between visfatin levels and periodontal pocket depth. Elevated levels of visfatin have been reported in the serum, saliva and GCF of patients with chronic periodontitis (233, 234). It was found to be expressed in higher amounts in the gingival tissues of chronic periodontitis patients with T2D than in those without diabetes (219).

5.3 Type 2 diabetes enhances Th-2 response and adversely influences local expression of anti-inflammatory and healing molecules

Pro-inflammatory cytokines and chemokines

Among the pro-inflammatory cytokines investigated in *Study III*, IL-1 β levels were higher in T2D patients with chronic periodontitis than in those with T2D alone and chronic periodontitis alone, but the differences were not statistically significant. The levels of TNF- α were significantly higher in individuals with chronic periodontitis than in T2D patients with chronic periodontitis. It is claimed that there is insufficient evidence to support the effect of diabetes on TNF- α expression (51). There is, however, stronger evidence to support an association between diabetes-mediated periodontal tissue destruction and IL-1 β (235).

IL-6 is a pleotropic cytokine with both pro- and anti-inflammatory effects. It is involved in the activation of osteoclasts and Th-17 cells (236, 237). In contrast, IL-6 also induces the production of IL-1ra, thus contributing to the anti-inflammatory process

(238). In *Study III*, IL-6 levels were lower in T2D patients with chronic periodontitis than in those with T2D without periodontitis. A meta-analysis study reported no significant difference in IL-6 levels between periodontally diseased patients with and without T2D (235).

Our data showed significantly lower levels of IL-7 in T2D patients with chronic periodontitis than in the other groups. In contrast, Duarte *et al.*, (239) observed higher GCF levels of IL-7 in patients with uncontrolled T2D than in healthy controls. IL-7 is a key cytokine in naive T-cell homeostasis and also enhances Th-1 response (240, 241). Therefore, the present results suggest a disturbance of IL-7 mediated Th-1 response in T2D patients with chronic periodontitis.

In *Study III*, some of the investigated chemokines such as IL-8 and MIP-1 β were up-regulated in T2D patients with chronic periodontitis, while others such as MIP-1 α and RANTES were down-regulated. Longo *et al.*, (242) studied serum IL-8 in T2D patients with chronic periodontitis and reported no significant difference in relation to diabetic or periodontal conditions. Moreover, it was reported that the levels of IP-10, RANTES and MCP-1 correlate negatively with increased periodontal tissue inflammation (243). IP-10 works specifically as Th-1 activator, thus enhancing the production of INF- γ (244). The adjusted analysis of *Study III* indicated a non-significant trend towards decreased IP-10 levels in T2D patients with chronic periodontitis compared to the other groups, which might explain the low INF- γ level (Th-1 cytokine) in this group.

T-helper cells

Cells of the adaptive immunity play a pivotal role in inflammatory-mediated periodontal tissue destruction (57). Th-1 secretes IL-2 and INF- γ , while Th-2 secretes IL-4, IL-5, IL-6, IL-10 and IL-13 (64). In *Study III*, chronic periodontitis patients with T2D had significantly lower levels of INF- γ than those without diabetes. INF- γ is associated with up-regulation of other pro-inflammatory cytokines and chemokines (57). On the other

hand, INF- γ was found to have a protective role in bone tissue destruction, exerting an anti-osteoclastogenic effect (245).

It is suggested that the Th-2/Th-1 cytokines ratio could determine the progression of chronic periodontitis (244). In *Study III*, T2D patients had a higher Th-2/Th-1 ratio than those without T2D (regardless of periodontal status). Shifting the activity of Th-cells from Th-1 to Th-2 reflects an enhanced humoral immune response and progression of periodontal tissue destruction in T2D patients via Th-2/B-cell axis (61, 246). However, others have identified Th-1 as the predominant response in periodontal tissue destruction (247).

In *Study III*, levels of Th-17 cytokine (IL-17) were found to be low in T2D patients (with and without chronic periodontitis) compared to chronic periodontitis subjects without diabetes. However, the results did not reach significance. Low IL-17 level is associated with reduced neutrophil activity and increased bacterial dissemination (248). Duarte *et al.*, (249) reported high IL-17 gene expression in gingival biopsies obtained from chronic periodontitis patients regardless of their diabetic status. In contrast, Zhao *et al.*, (250) indicated that T2D patients had higher plasma IL-17 levels than their healthy counterparts.

Anti-inflammatory and healing molecules

T2D is reported to down-regulate IL-4, a cytokine involved in modulation of inflammation as well as in the healing process (57, 251). The present results demonstrated that chronic periodontitis patients with T2D had lower levels of IL-4 compared to those without diabetes. Ribeiro *et al.*, (92) reported similar findings in T2D patients compared with systemically healthy subjects with comparable periodontal conditions. In *Study III*, subjects with chronic periodontitis had significantly lower levels of IL-10 than T2D patients without periodontitis. The interpretation of IL-10 results is complicated by the fact that it has both pro- and anti-inflammatory effects. A part from its immune-regulatory

effect [inhibition of osteoclastogenesis (252)], IL-10 is involved in stimulation of adaptive immunity (253).

In *Study III*, the GCF levels of VEGF were higher in patients with T2D than in chronic periodontitis subjects without diabetes. Similar trends are reported elsewhere (254). This observation might be attributable to the fact that oxidative stress induces the VEGF signalling in patients with T2D (255). In addition, the data demonstrated that T2D is associated with down-regulation of FGF and PDGF. These molecules are involved in periodontal tissue regeneration, angiogenesis and the healing process (256). Therefore, disturbance to the levels of molecules involved in the anti-inflammatory and healing processes might be one of the mechanisms by which T2D adversely influences periodontal health in our patients.

After investigating the local expression of molecules involved in the host inflammatory response, the next step was to explore whether T2D has any influence on the prevalence of putative periodontal pathogens in dental plaque, or markers of periodontal tissue destruction in saliva.

5.4 Type 2 diabetes has no significant effect on either prevalence of periodontal pathogens or salivary MMP-8, MMP-9 and OPG

The results of *Study IV* demonstrated that the investigated bacteria were more prevalent in individuals with chronic periodontitis (regardless of diabetic status) than in T2D patients without periodontitis, suggesting that T2D does not have a significant influence on the prevalence of the microbes under investigation.

The prevalence of *P.gingivalis* was highest in the subjects with chronic periodontitis (81.5%), followed by T2D patients with chronic periodontitis (59.3%) and T2D patients without periodontitis (55.0%) ($P > 0.05$). The results for both *P.intermedia* and *T.denticola* showed similar tendencies. All plaque samples obtained from chronic periodontitis patients were positive for *T.forsthysia*, compared to 90% in samples of T2D

patients without periodontitis. *C.rectus* scored positive in plaque samples of all the study participants. In contrast, the prevalence of *A.actinomycescomitans* was generally low: 7.4% in T2D patients with chronic periodontitis, 11.1% in subjects with chronic periodontitis; in the T2D patients without periodontitis, all the samples were negative to *A.actinomycescomitans*. Using quantitative PCR, Field *et al.*, (50) reported similar findings, that the quantities of *A.actinomycescomitans*, *F.nucleatum* and *P.gingivalis* vary according to periodontal disease status and that T2D does not influence sub-gingival microbiota. Moreover, Li *et al.*, (257) reported no significant differences in *P.gingivalis* between chronic periodontitis patients with T2D and those without diabetes. They also reported lower levels of *P.intermedia* in T2D patients with chronic periodontitis. Their results are in agreement with the present findings for *P.gingivalis* and *P.intermedia*. In contrast, other studies report significant variations in microbial composition of subgingival plaque between individuals with and without T2D (52, 53). Furthermore, a recent study by Demmer *et al.*, (258) reported that increased levels of periodontal pathogens (*A.actinomycescomitans*, *P.gingivalis*, *T.denticola* and *T.forsythia*) were associated with two- to three-fold higher prevalences of prediabetes.

The present data did not show any significant association between the prevalence of the periodontal pathogens investigated and the level of glycaemic control. As part of the Third National Health and Nutrition Examination Survey (NHANES III), Merchant *et al.*, (259) investigated the levels of serum antibodies of putative periodontal pathogens in adults. They concluded that hyperglycaemia is associated with high antibody titres against periodontal micro-organisms. Moreover, an interventional study from Japan reported that the response of T2D patients with decreased HbA1c was better than that of T2D patients with increased HbA1c in terms of the prevalence of *P.gingivalis* after periodontal treatment (260). In contrast, another study reported no significant difference in periodontal pathogens between patients with and without T2D after periodontal treatment (261). It was suggested that the virulence of *P.gingivalis* varies according to its fimbriae, the hair-like extensions from the bacterial wall, and it was claimed that type II fimbriae

are associated with diabetes-mediated chronic periodontitis (262). This observation was confirmed by others (260).

In *Study IV*, comparisons among the study groups failed to disclose any statistically significant differences in the concentrations of salivary MMP-8, MMP-9 or OPG. Nonetheless, there was a tendency towards increased salivary MMP-8 in subjects with chronic periodontitis (regardless of diabetic status) compared to T2D patients without periodontitis ($P > 0.05$). These results are in accordance with those of Costa *et al.*, (263). They reported no significant differences in salivary MMP-8 between patients with T2D alone, subjects with chronic periodontitis alone, and patients with both diseases. Another study reported comparable levels of salivary MMP-8 and MMP-9 in subjects with and without T2D (264). In contrast, Rathnayake *et al.*, (265) observed higher levels of salivary MMP-8 in T2D patients than in systemically healthy controls. Moreover, it is reported that MMP-8 and MMP-9 protein expression in the periodontal tissues of patients with T2D and chronic periodontitis is higher than in those with chronic periodontitis alone (266).

The present results showed a non-significant trend towards increased OPG concentrations in patients with chronic periodontitis (regardless of diabetic status) compared to T2D patients without periodontitis. This can be explained by the fact that OPG concentration tends to be up-regulated in response to high osteoclastogenic activity (267). Conversely, a previous study observed significantly higher concentrations of salivary OPG in patients with T2D than in systemically healthy individuals with chronic periodontitis (263). Santos *et al.*, (93) conducted a longitudinal study investigating RANKL and OPG in the GCF of well- and poorly controlled T2D patients before and after periodontal treatment. Throughout the study, the concentrations of RANKL and RANKL/OPG ratios were significantly higher in poorly controlled than in well-controlled T2D patients. In contrast, OPG concentrations did not differ significantly between the two groups at any time point. In *Study IV*, the concentrations of RANKL were below the detection limit, although two different commercial kits were used for detection. Frodge *et*

al., (268) also reported difficulty detecting RANKL in whole saliva. The reason might be that RANKL can be found as membrane-bound or soluble forms (71).

One of the limitations of this project is the case definition of chronic periodontitis. Periodontal pocket depth and bleeding on probing were both used to define cases of chronic periodontitis, as clinical attachment loss data were not available. As a result, the effect of T2D on the study outcomes might be underestimated (269). Nevertheless, it was reported that both periodontal pocket depth and bleeding on probing reflect current disease status and are strongly related to local inflammatory activity, whereas clinical attachment loss reflects past disease experience (167, 270, 271). In order to exclude undiagnosed cases with diabetes, participants in the control group were asked about the classical symptoms of diabetes to exclude the disease. Confirmation by laboratory tests for this group was not achievable in the field. Therefore, there might be some cases of undiagnosed or pre-diabetes in the control group and this would underestimate the differences in the outcome variables between those with T2D and those without the disease. Another point to be considered is the sampling of GCF. Measuring GCF volume was not possible during the data collection in Khartoum. In the multivariate analysis to control for the potential effect of variability of GCF volume on the results, total GCF protein was used as a surrogate measure of GCF volume (272).

6. CONCLUSIONS

The results confirm that patients with T2D have poorer OHRQoL and less favourable periodontal parameters than individuals without the disease. This seems to be related more to disturbed host immune-inflammatory response than to dissimilarities in the periodontal pathogens in dental plaque. Moreover, the deterioration in periodontal parameters influences local expression of molecules involved in glucose metabolism in the GCF of patients with and without T2D.

The following conclusions can be drawn from the studies on which this thesis is based:

- Patients with T2D present with more periodontal pockets (≥ 4 mm), tooth mobility, missing teeth, furcation involvement and OIDP than individuals without the disease.
- Long-term T2D patients (> 10 years) have a higher DMFT than those who have had the disease for less than 10 years.
- Poorly controlled T2D patients have a higher tooth mobility index than those with good glycaemic control.
- Chronic periodontitis is associated with disturbed GCF levels of biomarkers related to the onset as well as to medical complications of T2D.
- T2D and chronic periodontitis may adversely influence the GCF levels of inflammatory molecules, not only independently, but also synergistically.
- T2D is associated with a high Th-2/Th-1 cytokines ratio, reflected in the GCF levels of the cytokines that they produce.
- T2D adversely influences the local expression of molecules involved in the anti-inflammatory and healing processes.
- T2D has no significant effect on either the prevalence of the investigated periodontal pathogens, or the levels of salivary MMP-8, MMP-9 and OPG.

7. FUTURE PERSPECTIVES

There is ample evidence to support the bi-directional relationship between diabetes and chronic periodontitis. Several mechanisms that might explain this complex relationship are highlighted, but the puzzle of the role of the immune system and the factors which modulate its action have yet to be solved.

To date, there is no ideal biomarker which can be applied as a point-of-care test to assess risk, to diagnose, monitor progression, or predict the prognosis of either diabetes or chronic periodontitis. Research over the last decade, has focused on the interaction between different inflammatory molecules within different pathways, for better understanding of the complex inflammatory process that governs the pathophysiology of both diseases. Further research is warranted to identify disease markers which could be used by dentists to predict risk, diagnose and monitor T2D.

There is emerging evidence of the importance of the anti-inflammatory response as an active process in controlling the progression of chronic inflammatory diseases. Anti-inflammatory molecules, such as lipoxins and resolvins, which modulate the host defence mechanisms, are showing good potential as therapeutic agents (95, 102). To date, subantimicrobial dose doxycycline (SDD) is the only drug to have been approved by the United States Food and Drug Administration for modulating the host response by antagonising the action of MMPs (273). Yet, the therapeutic challenges require a sophisticated biological approach to develop an efficient therapy for chronic inflammatory conditions including chronic periodontitis and diabetes.

The development of a multidisciplinary approach by the medical and dental professions for the management of chronic diseases, including diabetes, warrants further attention. In this context, in countries such as The Sudan, where diabetes is a rapidly escalating burden on the healthcare system, there is an urgent need to bridge the current gap between physicians and dentists.

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9. APPENDICES

Appendix A. The source and action of the glucoregulatory molecules investigated in *Study II*

Inflammatory molecule (alternative names)	Production cells	Action	References
C-peptide (Connecting peptide)	β -cells	Links the A and B chains of insulin	Hoekstra <i>et al.</i> , 1982 (274)
Insulin	β -cells	Anti-inflammatory effect, stimulates glucose uptake and lipid synthesis, inhibits lipolysis, proteolysis, glycogenolysis and gluconeogenesis	Mao <i>et al.</i> , 2009 (275) Sonksen and Sonksen, 2000 (2)
GIP (Gastric inhibitory polypeptide, Glucose-dependent insulinotropic peptide)	Gastrointestinal track in response to food intake	Glucose dependent potentiation of insulin secretion	Fujita <i>et al.</i> , 2010 (204)
GLP-1 (Glucagon-like peptide-1)	Gastrointestinal track in response to food intake	Stimulates insulin secretion and inhibits glucagon secretion	Larsson <i>et al.</i> , 1997 (276)
Glucagon	α -cells	Gluconeogenesis, increases hepatic glucose production	D'Alessio, 2011 (277) Lin and Accili, 2011 (278)
Ghrelin (Hunger hormone)	Oxyntic mucosa of the stomach	Stimulates food intake, energy homeostasis and anti-inflammatory activity	Ohta <i>et al.</i> , 2011 (215)
Leptin (Satiety hormone)	Adipocytes	Control of appetite, pro-inflammatory actions	Fantuzzi, 2005 (224)
PAI-1 (Plasminogen activator inhibitor-1, Endothelial plasminogen activator inhibitor, Serpin E1)	Hepatocytes, vascular smooth muscle cells, endothelial cells, fibroblasts and adipocytes	Promotes thrombosis and fibrosis	Lyon and Hsueh, 2003 (220)
Visfatin (Pre-B-cell colony-enhancing factor-1, Nicotinamide phosphoribosyltransferase)	Adipocytes	Pro-inflammatory role, activates insulin receptor, insulin-mimetic actions	Fantuzzi, 2005 (224) Adeghate, 2008 (229)
Resistin (Tissue-specific secretory factor, C/EBP-epsilon-regulated myeloid-specific secreted cysteine-rich protein)	Macrophages, adipocytes	Induces insulin resistance	Fantuzzi, 2005 (224)

Appendix B. The source and action of the inflammatory molecules investigated in *Study III*

Inflammatory molecule (alternative names)	Production cells	Action	References
IL-1β (IL-1F2, Catabolin)	Monocytes, macrophages, neutrophils, fibroblasts, epithelial cells	Activation of innate immunity, regulation of adaptive immunity (T-cells and myeloid cells), stimulation of connective tissue turnover	Preshaw and Taylor, 2011 (31) Janeway <i>et al.</i> , 2001 (279)
IL-1ra (Interleukin-1 receptor antagonist)	Monocytes, macrophages, neutrophils, fibroblasts, epithelial cells	Antagonises IL-1 β and IL-1 α	Preshaw and Taylor, 2011 (31)
IL-2 (T-cell growth factor, Lymphokine)	T-cells	T-cells and natural killer cells proliferation, maintaining T-cytotoxic, directing the development and function of T-reg cells	Gaffen and Liu, 2004 (280)
IL-4 (BCGF-1, BSF-1)	T-cells, mast cells	Activation of B-cells, suppression of Th-1 cells	Janeway <i>et al.</i> , 2001 (279)
IL-5 (EDF, TRF)	T-cells, mast cells	Differentiation of eosinophil and B-cells	Janeway <i>et al.</i> , 2001 (279)
IL-6 (HGF, HSF, BSF-2)	T-cells, macrophages, endothelial cells	T- and B-cells growth and differentiation, increases MCP-1, adhesion molecules and IL-1ra	Kishimoto, 2006 (281) Preshaw <i>et al.</i> , 2007 (62) Janeway <i>et al.</i> , 2001 (279)
IL-7 (Lymphopoietin-1, Pre B-cells factor)	Non-marrow derived stromal and epithelial cells	T- and B-cells development	Fry and Mackall, 2002 (241)
IL-8 (CXCL-8)	Endothelial cells, neutrophils	Attracts neutrophils, basophil and T-cells	Janeway <i>et al.</i> , 2001 (279)
IL-9 (T-cell growth factor p40)	T-cells	Enhances mast cells and Th-2 activity	Goswami and Kaplan, 2011 (282)
IL-10 (B-TCGF, CSIF, TGIF)	T-cells (mainly T-reg), macrophages,	Suppresses innate immunity and cytokines, activates B-cells, counteracts macrophages function	Garlet, 2010 (57)
IL-12 (TcMF-1, CLMF, TSF)	B-cells, macrophages	Activates natural killer cells, enhances Th-1 and INF- γ	Preshaw and Taylor, 2011 (31)
IL-13 (P600)	T-cells	Inhibits macrophages and Th-1, enhances B-cells proliferation	Janeway <i>et al.</i> , 2001 (279)
IL-15	Monocytes, macrophages, dendritic cells	T-cells and natural killer cells activation and proliferation	Fehniger and Caligiuri, 2001 (283)
IL-17 (CTLA-8)	Th-17	Induces the production of pro-inflammatory cytokines and chemokines	Gaffen and Hajishengallis, 2008 (61)

Inflammatory molecule (alternative names)	Production cells	Action	References
Eotaxin (CCL-11, Eosinophil chemotactic protein)	INF- γ stimulated endothelial cells, TNF- α stimulated monocytes	Attracts eosinophils	Janeway <i>et al.</i> , 2001 (279)
Basic FGF (Basic fibroblast growth factor, HBGF-2)	Neutrophils, macrophages, fibroblasts, mast cells, epithelial cells	Wound healing and angiogenesis	Sonmez and Castelnuovo, 2014 (284)
G-CSF (Granulocyte-colony stimulating factor, CSF-3)	Fibroblasts, monocytes, endothelial cells	Neutrophil proliferation and maturation	Root and Dale, 1999 (285)
GM-CSF (Granulocyte monocyte-colony stimulating factor, CSF-2)	Fibroblasts, monocytes, endothelial cells, T-cells	Neutrophil, macrophages and eosinophils proliferation and maturation	Root and Dale, 1999 (285)
INF-γ (Interferon- γ)	T-cells, natural killer cells	Activates macrophages, suppresses Th-2	Janeway <i>et al.</i> , 2001 (279)
IP-10 (Interferon inducible protein-10, CXCL-10, crg-2)	Monocytes, lymphocytes, keratinocytes and endothelial cells in response to IFN- γ	Activates Th-1	Gemmell and Seymour, 2004 (244) Cassatella <i>et al.</i> , 1997 (286)
MCP-1 (Monocyte chemo-attractive protein-1, CCL-2)	Macrophages, fibroblasts, epithelial cells, endothelial cells	Attracts T-cells, monocytes basophils, natural killer and dendritic cells	Takahashi <i>et al.</i> , 2009 (287)
MIP-1α (Macrophage inflammatory protein-1 α , CCL-3)	Macrophages, dendritic cells, lymphocytes	Attracts macrophages, T-cytotoxic and natural killer cells	Maurer and von Stebut, 2004 (288)
MIP-1β (Macrophage inflammatory protein-1 β , CCL-4)	Macrophages, dendritic cells, lymphocytes	Attracts macrophages, T-cytotoxic and natural killer cells	Maurer and von Stebut, 2004 (288)
PDGF-BB (Platelet-derived growth factor subunit B)	Activated platelets, macrophages, endothelial cells	Attracts neutrophils, macrophages and fibroblast, wound healing and angiogenesis	Barrientos <i>et al.</i> , 2014 (256)
RANTES (Regulated upon activation, normally T-expressed, and presumably secreted, CCL-5)	Neutrophils, macrophages, fibroblasts, mast cells, epithelial cells	Attracts memory T-cells, monocytes, basophils and eosinophils	Preshaw and Taylor, 2011 (31)
TNF-α (Tumor necrosis factor- α , Cachectin)	Macrophages, natural killer cells, T-cells, neutrophils, fibroblasts, B-cells	Vasodilatation, increases vascular permeability, enhances intra-cellular adhesion molecules	Preshaw and Taylor, 2011 (31)
VEGF (Vascular endothelial growth factor, Vascular permeability factor)	Macrophages, platelets	Increases vascular permeability and angiogenesis	Yanagita <i>et al.</i> , 2014 (289)

Appendix C: Consent forms**Informed Consent****Permission Code: KS/MH/AA/AB**

We are inviting you to participate in this application. Participation is entirely voluntary. Please read each sentence below and think about your choice. After reading each sentence, select 'Agree' or 'Disagree' and sign your name on the consent. If you have any questions, please talk to our medical service staff or research assistants, or call our research review board at the Federal Ministry of Health phone number (+24983769928).

Title of this Application: Association between oral health status and diabetes mellitus (D.M.) among Sudanese patients with type 2 diabetes.

Principle Investigator: Hasaan Mohamed

Telephone No.: (+249912225656)

Sponsors: University of Bergen- Norway

(+249123025656)

Purpose:

Aim of this large-scale study is to investigate the effect of type 2 diabetes mellitus on oral health among individuals with type 2 D.M.

The purpose and procedures have been explained to the participants.

Do you agree to participate in this study?

- Agree
 Disagree

I agree to participate in this research entitled “: Association between oral health status and diabetes mellitus (D.M.) among Sudanese patients with type 2 diabetes”, which is being conducted by principle investigator: Hasaan Mohamed and phone number: (+249912225656) (+249123025656) where he can be contacted. I understand that this participation is entirely voluntary; I can withdraw my consent at any time and have the results of the participation returned to me, removed from the experimental records, or destroyed. I have read and understand the potential risks and side-effects of the sampling.

Signature of participant: _____ Date:

If you are an agent of the participant,

Please sign your name: _____ Date:

Interviewer's name: _____ Date:

* Arabic translation of the informed consent

إقرار مطلع عليه

رمز البحث: وخ/وص/اع/اب

ندعوكم للمشاركة بهذا البحث. المشاركة تطوعية تماماً. الرجاء قراءة كل جملة و التفكير في الخيارات. بعد قراءة كل جملة اختار (أوافق) أو (لا أوافق) ثم قم بالتوقيع في نهاية الإقرار. إذا كان لديك أي استفسار الرجاء التحدث إلى كوادرن الطبية أو الباحثين المساعدين أو الاتصال بهيئة مراجعة البحوث بوزارة الصحة الولائية تلفون: (+٢٤٩٨٣٧٦٩٩٢٨).

عنوان البحث: العلاقة بين صحة الفم ومرض السكري بين المرضى السودانيين المصابين بداء السكري من النوع ٢.

الباحث الرئيسي: حسان قاسم سعد محمد تلفون(+٢٤٩٩١٢٢٢٥٦٥٦) (+٢٤٩١٢٣٠٢٥٦٥٦)

المتكفل بالبحث: جامعة بيرقن- النرويج.

الهدف:

الغرض من هذه الدراسة هو دراسة تأثير داء السكري من النوع ٢ في صحة الفم لدى الأفراد المصابين بمرض السكري من النوع ٢.

الهدف و الطريقة تم شرحها للمشاركين بالبحث.

هل توافق على المشاركة بهذه الدراسة ؟

١. أوافق

٢. لا أوافق

أوافق على المشاركة بهذا البحث (العلاقة بين صحة الفم ومرض السكري بين المرضى السودانيين المصابين بداء السكري من النوع الثاني)، الباحث الرئيسي: حسان قاسم سعد محمد, رقم تلفونه للاتصال به: (+٢٤٩٩١٢٢٢٥٦٥٦) (+٢٤٩١٢٣٠٢٥٦٥٦), أتفهم أن هذه المشاركة هي اختيارية تماماً, أستطيع التخلّص من إقراري وقتما أشاء كما أستطيع الاطلاع على نتائج المشاركة و تمحى من السجلات و تدمر وقتما أشاء. لقد قرأت و فهمت المخاطر من أخذ العينة

توقيع المشترك: التاريخ:

إذا كنت وكيل المشترك,

الرجاء التوقيع: التاريخ:

اسم الباحث: التاريخ:

Appendix D. Questionnaire

Serial No./ID.
Interview date
Interviewer

/ /

1-Personal information

Interviewee: _____

Hospital: _____

Tel. number: _____

Address: _____

1. Gender:	<input type="checkbox"/> 1. Male	<input type="checkbox"/> 2. Female
2. Age:	_____	
3. Marital status:	<input type="checkbox"/> 1. Married	<input type="checkbox"/> 2. Not married
4. Education:	<input type="checkbox"/> 1. Illiterate	<input type="checkbox"/> 5. High secondary school
	<input type="checkbox"/> 2. Literate	<input type="checkbox"/> 6. College, university
	<input type="checkbox"/> 3. Primary school	<input type="checkbox"/> 7. Graduate institute
	<input type="checkbox"/> 4. Middle secondary school	
5. Employment:	<input type="checkbox"/> 1. Unemployed	<input type="checkbox"/> 2. Student
	Unemployed, but is	<input type="checkbox"/> 3. Housewife
		<input type="checkbox"/> 4. Retired
	<input type="checkbox"/> 5. Employed	Your occupation type: _____
		Title: _____
6. Where were you born?	_____	
7. Tribe:	_____	
8. Current weight:	_____ kg	
9. Current Height:	_____ cm	

2-Dietary Habits and physical activity:**1-What is the frequency of your consumption of the following food per week?**

- a- Milk b- Red Meat c- Chicken
- d- Fish e- Eggs f- Fresh Green Salad
- g- Fresh Fruits h- Fresh Fruit Juice i- Cakes & Deserts
- j- Natural honey K- bread

2- What is the type of food you eat the most (or your most frequent main dish) in the week?

1. Kiswa, Gurrasa or Bourages (Sorghum , Wheat , Maze, ...) 2. Meat.
3. Beans with bread. 4. Cooked vegetables. 5. Others. _____

3- What is the frequency of your consumption of sugary drinks per day?*Example: soft drinks, beverages with added sugar, sweet soup. 1 cup \cong 250 ml.

0. None 1. \leq 1 Cup 2. 2-3 Cups 3. \geq 4 Cups

4- Do you eat regular meals every day? (Breakfast, lunch and dinner).

0. No 1. Yes

5- Frequency of physical activity?

0. Never 1. Once / week 2. 2-3 times/week 3. > 3 times/week

6- For how long do you exercise?

0. Never 1. < 15 min. 2. 15 - 29 min. 3. 30 min to 1 hr. 4. > 1 hr.

3-Medical History:**1- Whether you have the following diseases diagnosed by clinical doctors?**

No.	Disease	0= No, 1= Yes, 2= Unknown	Comments (site or more specification, etc. ...)
(1)	Diabetes		Duration: _____
(2)	Hypertension		
(3)	Cancers		Type: _____
(4)	Hyperlipidaemia		
(5)	Asthma		
(6)	Heart disease		
(7)	Liver disease		
(8)	Kidney disease		
(9)	Gout		
(10)	Skin warts? If yes, where?		
(11)*	Sexually transmitted disease?		Which: _____
(12)	Others		

*(11) Syphilis; Gonorrhea; Condyloma/Warts; HIV/AIDS?

2- Family history of diabetes:

0. No 1. yes, If yes which side? _____

3- Type of diabetes medication used:

1. Dietary control only 2. Oral hypoglycaemic 3. Insulin
 4. Both (Oral hypoglycaemic and insulin)

4-Part A. Alcohol use:**1. Do you ever habitually drink alcohol (Defined as at least 1 time per week)?**

- 0.No
 1. Yes, only less than twice in life time.
 2. Past drinker; when did you start to drink? _____ years old; when did you stop to drink? _____ years old
 3. Current drinker; when did you start to drink? _____ years old

2. When you drink, what types of alcoholic beverages you drink?

Illustration:

Large bottle (1 L)

Medium bottle (500 ml.)

Small bottle (250 ml)

Cup (150ml)

Shot (25 ml)

 a. Marissa (Sorghum): <6%

Days / week

 1 2 3 4 5 6 7**Average daily consumption** 1.Large bottle (1 L) ____Units 2.Medium bottle (500 ml) ____Units 3.Small bottle (250 ml) ____Units 4.Cup (150ml) ____Units 5.Shot (25 ml) ____Units b. Aragi: (dates, Sorghum , etc) <40%

Days / week

 1 2 3 4 5 6 7**Average daily consumption** 1.Large bottle (1 L) ____Units 2.Medium bottle (500 ml) ____Units 3.Small bottle (250 ml) ____Units 4.Cup (150ml) ____Units 5.Shot (25 ml) ____Units c. Imported alcohol: ≥40% (Whisky, Vodka, Gin, Others)

Days / week

 1 2 3 4 5 6 7**Average daily consumption** 1.Large bottle (1 L) ____Units 2.Medium bottle (500 ml) ____Units 3.Small bottle (250 ml) ____Units 4.Cup (150ml) ____Units 5.Shot (25 ml) ____Units d. Others: _____

Days / week

 1 2 3 4 5 6 7**Average daily consumption** 1.Large bottle (1 L) ____Units 2.Medium bottle (500 ml) ____Units 3.Small bottle (250 ml) ____Units 4.Cup (150ml) ____Units 5.Shot (25 ml) ____Units

4-Part B. Toombak (smokeless tobacco) use:**1. Do you ever habitually use *Toombak* (Defined as at least 1 Saffa per week)?**

0. No
1. Yes, only less than twice in life time.
2. Past user; when did you start dipping? _____ years old; when did you stop dipping? _____ years old
3. Current dipper; when did you start dipping? _____ years old

2. What is the frequency of your usage?


Days / week _____ How many times per day _____ How long (min/saffa) _____

4-Part C. Smoking:**1. Do you ever habitually smoke cigarettes? (Defined as at least 1 cigarette per week)**


0. No
1. Yes, only less than twice in a life time.
2. Past smoker; when did you start to smoke? _____ years old; when did you stop to smoke? _____ years old
3. Current smoker; when did you start to smoke? _____ years old

2. When you smoke, what types of tobacco you smoke? (More than one answer is available):


Heavy cigarettes

	Days / week			Average daily consumption (cigarettes/day)
	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	_____ Cigarettes
	<input type="checkbox"/> 4	<input type="checkbox"/> 5	<input type="checkbox"/> 6	
	<input type="checkbox"/> 7			


Light cigarettes

	Days / week			Average daily consumption (cigarettes/day)
	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	_____ Cigarettes
	<input type="checkbox"/> 4	<input type="checkbox"/> 5	<input type="checkbox"/> 6	
	<input type="checkbox"/> 7			

Pipes:

 <i>Ceci n'est pas une pipe.</i>	Days / week			Average daily consumption (/day)
	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	_____ Times
	<input type="checkbox"/> 4	<input type="checkbox"/> 5	<input type="checkbox"/> 6	
	<input type="checkbox"/> 7			

Shisha:

	Days / week			Average daily consumption (/day)
	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	_____ Times
	<input type="checkbox"/> 4	<input type="checkbox"/> 5	<input type="checkbox"/> 6	
	<input type="checkbox"/> 7			

Others:

	Days / week			Average daily consumption (/day)
	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	_____ Times
	<input type="checkbox"/> 4	<input type="checkbox"/> 5	<input type="checkbox"/> 6	
	<input type="checkbox"/> 7			

5. Self-assessed oral health evaluation:

1. Tooth brushing (times/day)?	<input type="checkbox"/> 0. Never	<input type="checkbox"/> 1. 1	<input type="checkbox"/> 2. 2	<input type="checkbox"/> 3. ≥ 2
2. Which instrument you use for brushing?	<input type="checkbox"/> 0. Tooth brush.	<input type="checkbox"/> 1. Muswaak.	<input type="checkbox"/> 2. Others _____	
3. Which material you use for brushing?	<input type="checkbox"/> 1. Tooth paste.	<input type="checkbox"/> 2. Others _____		
4. Do you have gum bleeding when washing/brushing teeth?	<input type="checkbox"/> 0. No.	<input type="checkbox"/> 2. Yes.		
5. Do you use mouth wash?	<input type="checkbox"/> 0. No.	<input type="checkbox"/> 1. Yes		
6. Have you noticed any visible lesion in your mouth recently?	<input type="checkbox"/> 0. No.	<input type="checkbox"/> 1. Yes		
7. Do you Wear a denture? If yes, since when?	<input type="checkbox"/> 0. No	<input type="checkbox"/> 1. Yes: Since _____		
8. If last answer is yes, what is your denture type?	<input type="checkbox"/> 1. Partial.	<input type="checkbox"/> 2. Complete.		
9. Dental check-ups?	<input type="checkbox"/> 0. Never	<input type="checkbox"/> 1. >5 years	<input type="checkbox"/> 2. 2-5 years	
	<input type="checkbox"/> 3. Every year or less			
10. Do you feel a change in the smell of your mouth	<input type="checkbox"/> 0. No	<input type="checkbox"/> 1. Yes		
11. Change in taste?	<input type="checkbox"/> 0. No	<input type="checkbox"/> 1. Yes		
12. History of dry mouth?	<input type="checkbox"/> 0. No	<input type="checkbox"/> 1. Yes		

OIDP

خلال الستة شهور الاخيرة , هل عانيت من مشكلة في فمك وأسنانك أو أسنانك سببت لك بعض الصعوبات في الاتي :-

أ- اكل ومضغ الطعام :

1/ اطلاقا	2/ اقل من مرة في الشهر
3/ مرة او مرتين في الشهر	4/ مرة او مرتين في الاسبوع
	5/ دائما تقريبا يوميا

ب- التحدث والتعبير عن نفسك بوضوح :

1/ اطلاقا	2/ اقل من مرة في الشهر
3/ مرة او مرتين في الشهر	4/ مرة او مرتين في الاسبوع
	5/ دائما تقريبا يوميا

ت- نظافة أسنانك:

1/ اطلاقا	2/ اقل من مرة في الشهر
3/ مرة او مرتين في الشهر	4/ مرة او مرتين في الاسبوع
	5/ دائما تقريبا يوميا

ث- النوم والاسترخاء:

1/ اطلاقا	2/ اقل من مرة في الشهر
3/ مرة او مرتين في الشهر	4/ مرة او مرتين في الاسبوع
	5/ دائما تقريبا يوميا

ج- الحفاظ علي الحالة النفسية:

1/ اطلاقا	2/ اقل من مرة في الشهر
3/ مرة او مرتين في الشهر	4/ مرة او مرتين في الاسبوع
	5/ دائما تقريبا يوميا

ح- القيام بمعظم الواجبات والاعمال:

1/ اطلاقا	2/ اقل من مرة في الشهر
3/ مرة او مرتين في الشهر	4/ مرة او مرتين في الاسبوع
	5/ دائما تقريبا يوميا

خ- التعامل مع الناس بارتياح:

1/ اطلاقا	2/ اقل من مرة في الشهر
3/ مرة او مرتين في الشهر	4/ مرة او مرتين في الاسبوع
	5/ دائما تقريبا يوميا

د- الابتسام واطهار الاسنان:

1/ اطلاقا	2/ اقل من مرة في الشهر
3/ مرة او مرتين في الشهر	4/ مرة او مرتين في الاسبوع
	5/ دائما تقريبا يوميا

Appendix E. Examination sheet

Index

Date / /

Name:

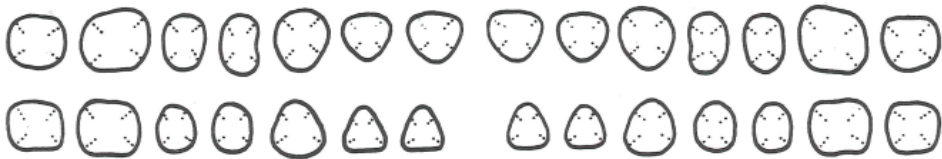
Age:

Sex:

General examination:

	17	16	15	14	13	12	11	21	22	23	24	25	26	27
Mucogingival problems														
Plaque index														
Calculus														
Recession														
Mobility														
Root caries														
	47	46	45	44	43	42	41	31	32	33	34	35	36	37
Mucogingival problems														
Plaque index														
Calculus														
Recession														
Mobility														
Root caries														

Specific examination:



Periodontal pocket: blue (mm)

Bleeding on probing: (red dot)

Furcation involvement:



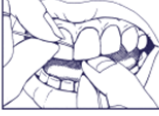



Furcation involvement: I = II = III = (red)

Dental caries (DMFT):

17	16	15	14	13	12	11	21	22	23	24	25	26	27
47	46	45	44	43	42	41	31	32	33	34	35	36	37

Appendix F. Oral hygiene instructions card

إرشادات عامة للمحافظة على صحة الفم و الأسنان	
<ul style="list-style-type: none"> • تسوس الأسنان وأمراض اللثة من الأمراض الشائعة لدى البالغين • تنظيف الأسنان و اللثة يجب أن يكون مرتين يومياً (صباحاً و مساءً) • يجب استخدام فرشاة أسنان ذات رأس مضغوط وشعيرات ناعمة • يجب استعمال معجون أسنان مع الفلورايد • يجب استعمال خيط تنظيف الأسنان مرة واحدة يومياً لتنظيف المناطق التي لا تستطيع الفرشاه الوصول إليها 	
<p>خذ ١٨ انش من خيط تنظيف الأسنان و لفه حول الاصبع الأوسط لكل يد</p>	 <p>ضع شعيرات الفرشاه على خط اللثة بزواوية ٤٥ درجة من الأسنان. اضغط بلطف واستخدم اهتزازات قصيرة للأمام والخلف ثم باتجاه الأسنان</p> 
<p>امسك نصف إنش من الخيط بإحكام بين السبابة و الإبهام، انزله برفق بين الأسنان حتى يلامس اللثة</p>	 <p>ضع الفرشاه عمودياً لتنظيف المنطقة الداخلية من الأسنان الأمامية العلوية و السفلية</p> 
<p>لف الخيط على شكل "C" حول السن، حرك الخيط برفق للأعلى و الأسفل. أخرج الخيط بلطف و كرر العملية مع كل الأسنان</p>	 <p>ابدأ بالأسنان العلوية، نظف السطح الخارجي، الداخلي ثم سطح المضغ، كرر نفس العملية مع الأسنان السفلية و تأكد من تنظيف كل الأسنان</p> 
<p>تذكر.... أن الأسنان خلقت لتعيش معنا مدى الحياة !</p>	<p>نظف سطح اللسان فقد يحتوي على كمية كبيرة من البكتيريا</p> 

Adapted from: Washington State Department of Social & Health Services, Division of Developmental Disabilities. Oral hygiene guidelines: Preventing dental disease in people with special health care needs. Olympia, WA: The Department; 2000.

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11. STUDIES I-IV

Study I

Association between Oral Health Status and Type 2 Diabetes Mellitus among Sudanese Adults: A Matched Case-Control Study

Association between Oral Health Status and Type 2 Diabetes Mellitus among Sudanese Adults: A Matched Case-Control Study

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Abstract

Aim: The purpose of this study was to compare the clinical and subjective oral health indicators of type 2 diabetic patients (T2DM) with age and gender matched non-diabetic controls. A second aim was to identify clinical and subjective oral health indicators that discriminate between well-controlled and poorly controlled T2DM patients as well as between patients with long and short duration of the disease.

Methods: A total of 457 individuals participated in the study (154 T2DM cases and 303 non-diabetic controls). The T2DM group was sub-divided according to metabolic control [(well-controlled: glycosylated haemoglobin test \leq 8%), (poorly controlled: glycosylated haemoglobin test $>$ 8%)] and according to duration of T2DM [(long duration: $>$ 10 years), (short duration: \leq 10 years)]. Participants were interviewed using a structured questionnaire including socio-demographics, lifestyle and oral health related quality of life factors. The clinical examination comprised full mouth probing depths, plaque index, tooth mobility index, furcation involvement and coronal and root surface caries.

Results: The T2DM patients presented with more probing depths \geq 4mm, furcation involvement, tooth mobility, missing teeth, and oral impacts on daily performance (OIDP). The corresponding adjusted odds ratios and their 95% confidence intervals were 4.07 (1.74–9.49), 2.96 (1.36–6.45), 5.90 (2.26–15.39), 0.23 (0.08–0.63) and 3.46 (1.61–7.42), respectively. Moreover, the odds ratio was 2.60 (1.21–5.55) for the poorly controlled T2DM patients to have high levels of mobility index and 2.94 (1.24–6.94) for those with long duration of T2DM to have high decayed, missed and filled teeth (DMFT) values.

Conclusion: This study revealed that chronic periodontitis, tooth mobility, furcation involvement and OIDP were more prevalent among T2DM patients compared to their non-diabetic controls.

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Introduction

Diabetes Mellitus (DM) is a metabolic disorder characterized by chronic hyperglycaemia and disturbances of carbohydrate, fat and protein metabolism [1]. Type 1 DM is most common in children and adolescents, whereas type 2 DM (T2DM) affects adults. T2DM constitutes about 90–95% of all patients having the disease [2]. Patients with T2DM usually have insulin resistance which alters the utilization of endogenously produced insulin at the target cells. During the early stage of the disease, insulin production is increased resulting in hyperinsulinemia. However, as the condition progresses, the production of insulin decreases leading to insulin deficiency [2]. Whereas both type 1 DM and T2DM have a genetic predisposition, the aetiology of T2DM is also related to life style factors such as high fat and sugar intake, physical inactivity and obesity [2]. Worldwide; 346 million people suffer from DM [3] and this disease is ranked as the ninth most common disorder

amassing a 68% increase from 1990 to 2010 [4]. Between the years 2010 and 2030, the number of adults with DM in developing countries is expected to increase by 70%, most extensively in Africa [5]. In the Sudan, the prevalence of DM is increasing to epidemic proportions, affecting about 14% of a total population of 31 million [6].

Poorly controlled DM has been associated with increased susceptibility to oral infections including periodontal disease [7–10]. Periodontal disease is characterized by loss of connective tissue and bone support, which eventually might lead to tooth loss. Previous studies have suggested that periodontal infection and DM have a two-way relationship [10,11]. Løe, [12] stated that periodontal disease is the sixth most common complication of DM, whereas Lalla et al., [10] reported that DM is the strongest risk factor for periodontal infection compared to the other systemic conditions such as hypertension. Moreover, it has been demon-

strated that individuals with periodontal pocket $\geq 6\text{mm}$ are 3.5 times more likely to develop T2DM than those having periodontal pocket $< 6\text{mm}$ [13]. By now there is strong evidence suggesting that the prevalence and severity of periodontal disease are higher among T2DM patients when compared with non-diabetic individuals [8,9,14–18]. Few studies have reported on no difference in periodontal disease between individuals with and without T2DM [19,20]. Whereas some studies have reported worse periodontal condition among poorly controlled T2DM patients [8,9], others have disconfirmed an association between periodontal disease and metabolic control [21,22].

Few studies have examined the situation of dental caries among T2DM patients. It has been demonstrated that the number of decayed, missed and filled teeth (DMFT) is higher among individuals with than without T2DM [18,23,24]. Moreover, Leung et al., [25] have found the risk of dental caries to be twice as high in T2DM patients compared to healthy controls. Other studies have disconfirmed such an association [26–28]. Compared to coronal caries; a relationship between DM and root surface caries has been more obvious [10]. Thus, individuals with T2DM have higher prevalence of root surface caries compared to non-diabetic individuals [26,29]. This evidence is equivocal as some studies have found no difference in root surface caries between individuals with and without T2DM [28,30].

The overall contribution of oral diseases to quality of life among T2DM patients has not been frequently investigated. According to the literature, oral health-related quality of life (OHRQoL) did not discriminate between individuals with and without DM [31,32]. It has also been reported that patients with both types of DM have limited awareness of the possible health consequences of poor oral health [32]. OHRQoL measures have been tested in various populations to supplement clinical indicators of oral diseases [33]. One promising measure is the oral impact of daily performance inventory (OIDP) [34]. This measure is based on the WHO classification of impairments, disabilities and handicaps [35] and Locker's theoretical framework [36]. Since its development, the OIDP has shown to be reliable and valid in population based studies as well as in studies of patients with specific disorders [37–39]. An Arabic version of the OIDP inventory has been validated and used in the Sudan among 12 years old school children as well as among adult dental attendees with and without mucocutaneous diseases [40–42]. The OIDP has also been applied in other low-income countries such as Tanzania and Uganda [38,39].

In order to control DM in the Sudan, it is important to bridge the gap between physicians and dentists and to increase the involvement of the dental profession in the secondary prevention of this disease. Information about oral manifestations of T2DM should be made easily available to raise the awareness and knowledge of the importance of dental care for T2DM patients. To our knowledge, there is no published data available documenting the oral diseases and self-reported oral health situation among adult diabetic patients in the Sudan. The purpose of this study was to examine the clinical and subjective oral health indicators among T2DM patients receiving ongoing treatment in an outpatient specialist clinic and to compare the observations with age and gender matched non-diabetic controls taken from the same population. It was hypothesized that T2DM patients were more likely to have oral disease and oral impacts on daily performances than non-diabetic controls. A second aim was to - within the T2DM patients- identify clinical and subjective oral health indicators that discriminate between well-controlled and poorly controlled T2DM patients as well as between patients with long and short duration of the disease. It was hypothesized that

poorly controlled T2DM patients had more oral disease and more oral impacts on their daily performances than their well-controlled counterparts. Moreover, it was hypothesized that patients with long duration of T2DM had poorer oral health than their counterparts having short duration of the disease.

Materials and Methods

Study design and participants

This study was designed as a gender and age matched case-control study with a ratio of 2 controls per 1 case. Ethical clearance was obtained from the Ministry of Health in the Sudan and from the Norwegian Research Ethical Committee at the University of Bergen (2012/1470/REK vest). Written informed consent was obtained from each participant. The objectives, steps of oral clinical examination and sampling procedures were explained for the participants. All participants were informed about their dental diagnosis and referred for appropriate dental treatment as needed.

Sample size was calculated to be 450 using Openepi version 3.01 with a power of 90%, alpha level of 0.05, ratio of controls to cases of 2, percentage of exposed controls of 50% and an odds ratio (OR) of 2 as a minimum difference between groups to be detected. From July 2012 to December 2012, 157 cases previously diagnosed with T2DM (63 males and 94 females) who attended for dental treatment at Jaber Abo'ez Diabetes Center were invited to participate in the study. A total of 304 non-diabetic controls (119 male and 185 female) were recruited from the outpatient dental clinic at the Khartoum Dental Teaching Hospital. Illegibility criteria for enrolment of the cases were (i), being diagnosed with T2DM for more than one year and attending a specialized diabetes clinic (ii), having at least 10 remaining natural teeth (iii), no antibiotic, no steroidal and/or non-steroidal anti-inflammatory medication used during the last 3 weeks (iv), not treated with immunosuppressive chemotherapy, no current acute illness, no professional periodontal treatment received during the last 6 months and no pregnancy or lactation. The non-diabetic controls were selected according to the same criteria except for being diagnosed with DM. At the time of enrollment; glycosylated haemoglobin test (HbA1c) was undertaken for the T2DM cases to determine the level of glycemic control (well-controlled: HbA1c $\leq 8\%$ and poorly controlled: HbA1c $> 8\%$) [19]. The test was performed at the laboratory of Jaber Abo'ez Diabetes Center using a commercial kit (LabonaCheck™ A1c analyzer). Individuals in the control group were asked about signs and symptoms of DM and if suspected, they were referred to Khartoum Teaching Hospital for confirmation.

Interview

All participants were interviewed by a trained research assistant using a standardized structured questionnaire. The interview schedule was constructed in English, but participants were interviewed in Arabic and some illustrations were used to help the participants to understand the questions. *Socio-demographics* were assessed in terms of age, gender (male, female), educational level and employment status. Educational level was originally measured as (0 = illiterate, 1 = literate, 2 = primary school, 3 = middle school, 4 = high school, 5 = college, 6 = post-graduation studies) and was recoded into illiterate = 1 (including the original category 0) and literate = 2 (including the categories 1–6). Employment status was measured as (0 = unemployed, 1 = student, 2 = housewife, 3 = retired, 4 = employed), then recoded into unemployed = 1 (including the original categories 0–3) and employed = 2 (including the original category 4). *Lifestyle factors*

were assessed in terms of alcohol consumption (yes/no), smoking (yes/no), hypertension (yes/no), regular dental attendance (yes/no) and sugary drinks consumed per day (0 = no, 1 = 1 cup or less, 2 = 2 to 3 cups, 3 = 4 cups or more), recoded into no = 1 (including the original category 0) and yes = 2 (including the categories 1–3). The *self-reported main explanatory variables* were assessed in terms of history of dry mouth (yes/no) and OHRQoL which was assessed using the eight items OIDP frequency inventory; 'During the past 6 months, how often have problems with your mouth and teeth caused you any difficulty with: *eating and chewing food; speaking and pronouncing clearly; cleaning teeth; sleeping and relaxing; smiling and showing teeth without embarrassment; maintaining usual emotional state; carrying out major work and social role; and enjoying contact with people*'. Each item was assessed using a 5-point scale: (1) Never affected; (2) Less than once a month; (3) Once or twice a month; (4) Once or twice a week; (5) Every, or nearly every day. An additive sum score (OIDP ADD) was constructed from the 8 items as originally scored (1–5) (range 8–40). Secondly, each OIDP frequency item was dichotomized, yielding the categories: 0 = never affected (including the original category 1), 1 = affected (including the original categories 2–5). Simple count scores (SC) were created for the OIDP by adding the eight dichotomized variables. For the purpose of cross-tabulation and logistic regression analysis, the OIDP SC scores (0–8) was dichotomized as 0 = no daily performance affected and 1 = at least one daily performance affected. The distribution of the OIDP SC scores supported this cut-off point.

Oral clinical examination

In preparation for the clinical examination, the main single investigator (HGM) was trained and calibrated to perform oral examination and differential diagnosis for dental caries, periodontal disease and other oral disorders at the Department of Clinical Dentistry-University of Bergen, Norway under the supervision of the team's principal investigator (SOL).

For each participant, clinical examination of all teeth (except 3rd molars) and soft tissues of the oral cavity was performed immediately after completion of the interview. Tools used for the examination were (N22) Color Coded Probe 2-4-6-8-10-12 mm markings, (NAB2) Color Coded Nabors Furcation Probe 3-6-9-12 mm markings, curette, mirror, probe, tweezers and cotton rolls. For *plaque index* (PI) [43] and *mobility index* [44], one value was recorded for each tooth (range 0-3). The sum of values of all teeth examined was divided by the number of teeth examined to give the individual scores. PI and mobility index were recoded into low = 1 and high = 2 using the median as a cutoff point for the sub-group analysis. Another dichotomous variable was created for mobility index (mobility index = 0, mobility index \geq 1). *Probing depth* (PD) was measured from the gingival margin to the base of the periodontal pocket (mm) at four sites of each tooth (mesial, distal, buccal and lingual). Individual scores were expressed as percentage of sites with PD of \geq 4mm. Individuals were diagnosed as having chronic periodontitis if there was at least one site with PD of \geq 4mm. *Furcation involvement* (FI) [45] was recorded as grade I (\leq 3mm), grade II ($>$ 3mm) and grade III (through and through). A dichotomous variable for FI was created (FI \leq grade I, FI \geq grade II). *Root surface caries* was recorded as (yes/no) and expressed as percentage of teeth with root caries for each individual. *Coronal caries* was measured using DMFT index [46]. Individual scores were calculated as sum of decayed, missed and filled teeth. DMFT was dichotomized into (DMFT = 0, DMFT \geq 1). Another cutoff (low: DMFT < median, high: DMFT \geq median) was used for the sub-group analysis.

Statistical analysis

Statistical Package for Social Sciences (SPSS) version 21 was used to analyze the data. The Kappa test was performed to assess the intra-examiner reliability. Chi-square and independent sample T tests were used to assess the differences in categorical and continuous variables between the cases and controls and to identify possible confounding variables. The Cox regression procedure was used to fit conditional logistic regression models since each case was paired with 2 controls. Adjusted odds ratios (ORs) and their 95% confidence intervals (CI) were calculated with T2DM status (cases/controls) as the outcome variable and periodontitis, dental caries and OHRQoL as main explanatory variables whilst adjusted for a number of possible confounding variables (employment status, educational level, consumption of sugary drinks, smoking, hypertension, PI and regular dental attendance). The selection of independents included in the Cox regression model was based on the theoretical relevance and statistical significant relationship with T2DM in the bivariate analysis. Sub-group analysis was performed within the T2DM group to identify the clinical and self-reported oral health indicators that discriminated between the well-controlled (HbA1c \leq 8%) and poorly controlled (HbA1c $>$ 8%) patients and between patients with long ($>$ 10 years) and short (\leq 10 years) duration of the disease. Two binary logistic regression models were constructed to assess the relationship between mobility of teeth and level of metabolic control and the relationship between DMFT and duration of T2DM. Age, gender and frequency of regular dental attendance were adjusted for during the analyses. *P* values less than 0.05 were considered statistically significant.

Results

Of the 461 individuals recruited for the study, three participants from the T2DM group did not continue with the interview due to time constraints. One participant from the control group was diagnosed with DM and excluded after the recruitment. The mean duration of T2DM was (9 \pm 7.4) years. A total of 31.2% of the cases had experience with the diagnosis for more than 10 years, 70% were poorly controlled and 50% had a family history of DM. The oral examination was repeated for 20 participants randomly selected within 2 weeks. Kappa values (*k*) were 0.74 for tooth mobility, 0.80 for root surface caries, 0.88 for periodontal diagnosis (PD \geq 4mm.) and 1.00 for dental caries.

As shown in Table 1, the mean age for both the cases and controls was (52 \pm 10.5) years and 39% of both groups were men. A total of 34.4% versus 13.9% ($P < 0.001$) of the cases and controls reported any oral impact (OIDP $>$ 0). The two groups differed with respect to chewing problems (22.7% versus 10.9%, $P < 0.001$) and sleeping problems (15.6% versus 5.0%, $P < 0.001$) (data not shown). Chewing, sleeping, cleaning teeth and smiling were the impacts most frequently mentioned among the cases, whereas chewing and sleeping were those most frequently mentioned among the controls. Reported dry mouth was more common in the cases than the controls (37.7% versus 10.9%, $P < 0.001$) (data not shown). Hypertension was also more common in the cases than the controls (31.8% versus 14.9%, $P < 0.001$). Visible dental plaque, furcation involvement, tooth mobility, chronic periodontitis, root surface caries and less than 21 remaining teeth were all more frequently observed in the cases group ($P < 0.05$).

The Cox regression analysis, adjusting for possible confounding variables, revealed statistically significant covariates of T2DM in terms of tooth mobility (OR = 9.63, 95% CI: 4.29–21.58), furcation involvement (OR = 5.23, 95% CI: 2.79–9.80), chronic periodontitis (OR = 3.97, 95% CI: 2.08–7.59), root surface caries,

Table 1. Distribution of confounders and main explanatory variables according to T2DM status (n = 457).

Confounding factors	Categories	Cases (n = 154)	Controls (n = 303)
Age, mean (SD)		52.6 (10.5)	52.4 (10.5)
Gender, % (n)	Male	39.0 (60)	39.3 (119)
	Female	61.0 (94)	60.7 (184)
Education, % (n)	Illiterate	26.6 (41)	30.7 (93)
	Literate	73.4 (113)	69.3 (210)
Employment status, % (n)	Unemployed	64.9 (100)	63.4 (192)
	Employed	35.1 (54)	36.6 (111)
Consumption of sugary drinks per day, % (n)	No	83.8 (129)	67.7 (205)
	Yes	16.2 (25)	32.3 (98)**
Smoking, % (n)	No	87.0 (134)	81.5 (247)
	Yes	13.0 (20)	18.5 (56)
Hypertension, % (n)	No	68.2 (105)	85.1 (258)
	Yes	31.8 (49)	14.9 (45)**
Regular dental attendance, % (n)	No	92.2 (142)	96.4 (292)
	Yes	7.8 (12)	3.6 (11)
Plaque index, % (n)	Low (< median)	24.0 (37)	63.0 (191)
	High (\geq median)	76.0 (117)	37.0 (112)**
Main explanatory variables			
Number of present teeth, % (n)	\leq 21 teeth	26.0 (40)	9.6 (29)
	> 21 teeth	74.0 (114)	90.4 (274)**
Tooth mobility, % (n)	No	8.4 (13)	46.9 (142)
	Yes	91.6 (141)	53.1 (161)**
Furcation involvement, % (n)	No	55.8 (86)	87.8 (266)
	Yes	44.2 (68)	12.2 (37)**
Chronic periodontitis, % (n)	No	13.6 (21)	45.5 (138)
	Yes	86.4 (133)	54.5 (165)**
Dental caries (DMFT), % (n)	DMFT = 0	5.2 (8)	4.3 (13)
	DMFT > 0	94.8 (146)	95.7 (290)
Root surface caries, % (n)	No	47.4 (73)	60.4 (183)
	Yes	52.6 (81)	39.6 (120)*
OHRQoL, % (n)	OIDP = 0	65.6 (101)	86.1 (261)
	OIDP > 0	34.4 (53)	13.9 (42)**

* $P < 0.05$.** $P < 0.01$.

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(OR = 1.80, 95% CI: 1.07–3.02), OIDP (OR = 3.85, 95% CI: 2.11–7.01), and having more than 21 remaining teeth (OR = 0.34, 95% CI: 0.17–0.68). When all the main explanatory variables were mutually adjusted for, the statistically significant covariates of T2DM were tooth mobility, furcation involvement, chronic periodontitis, OIDP and having more than 21 remaining teeth. The corresponding ORs were 5.90 (95% CI: 2.26–15.39), 2.96 (95% CI: 1.36–6.45), 4.07 (95% CI: 1.74–9.49), 3.46 (95% CI: 1.61–7.42) and 0.23 (95% CI: 0.08–0.63), respectively (Table 2).

Table 3 depicts the differences in socio-demographic characteristics and clinical indicators between the well- and poorly controlled T2DM patients and between patients with long and short duration of the disease. The mean age among short- and long term T2DM cases was (50.72 \pm 10.78) and (56.69 \pm 8.61) years respectively, ($P < 0.001$). The well- and poorly controlled T2DM groups consisted of 46.7% and 68.6% women ($P < 0.05$). Regular dental attendance was reported by 3.8% of the short duration

cases versus 16.7% of the long duration cases, ($P < 0.05$). High mobility index was less prevalent in the well- than the poorly controlled T2DM cases (37.8% versus 59.0%, $P < 0.05$), and high median DMFT index was less frequently found in the short- compared with the long duration group (57.5% versus 75.0%, $P < 0.05$). More than 21 remaining teeth were more frequently observed in patients with short- than long duration of T2DM (79.2% versus 62.5%, $P < 0.05$).

The multiple logistic regression analyses adjusting for the confounding effect of age, gender and dental attendance, revealed that compared to the short duration-T2DM cases, T2DM cases with long duration were more likely to have DMFT (i.e. above the median) with OR of 2.94 (95% CI: 1.24–6.94) (Table 4). Compared to the well-controlled T2DM cases, the poorly controlled counterparts were more likely to be above the median mobility index with OR of 2.60 (95% CI: 1.21–5.55) (Table 5).

Table 2. All main explanatory variables according to T2DM status adjusted for possible confounding variables, (n = 447).

Variables	n	Adjusted OR (95% CI) ^a	Adjusted OR (95% CI) ^b
Number of present teeth			
≤21 teeth	66	1	1
> 21 teeth	381	0.34 (0.17–0.68)**	0.23 (0.08–0.63)**
Tooth mobility			
No	153	1	1
Yes	294	9.63 (4.29–21.58)**	5.90 (2.26–15.39)**
Furcation involvement			
No	344	1	1
Yes	103	5.23 (2.79–9.80)**	2.96 (1.36–6.45)**
Chronic periodontitis			
No	157	1	1
Yes	290	3.97 (2.08–7.59)**	4.07 (1.74–9.49)**
Dental caries (DMFT)			
DMFT = 0	21	1	1
DMFT > 0	426	0.82 (0.28–2.37)	0.38 (0.09–1.66)
Root surface caries			
No	252	1	1
Yes	195	1.80 (1.07–3.02)*	1.65 (0.84–3.26)
OHRQoL			
OIDP = 0	353	1	1
OIDP > 0	94	3.85 (2.11–7.01)**	3.46 (1.61–7.42)**

P* < 0.05.*P* < 0.01.

a) Adjusted for employment status, educational level, consumption of sugary drinks, hypertension, smoking, plaque index and regular dental attendance.

b) Adjusted for employment status, educational level, consumption of sugary drinks, hypertension, smoking, plaque index, regular dental attendance and other main explanatory variables.

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Discussion

The present study confirmed the hypothesis that clinical indicators of periodontal disease, furcation involvement, mobility of teeth and number of teeth present as well as the OIDP discriminated between T2DM patients and their non-diabetic controls. The hypothesis that poorly controlled and long duration-T2DM patients presented with more oral disease and OIDP compared with their well-controlled and short duration counterparts was partly confirmed in this study. Thus, mobility index and dental caries were the only clinical indicators that discriminated significantly between the sub-groups of T2DM cases.

Before discussing the findings further, it is appropriate to consider the limitations and strengths of the study. As both the cases and controls were attending oral health care services for dental treatment, they probably presented with more severe oral diseases and treatment needs compared to their non-dental attendee counterparts. It would have been an advantage to identify mild oral problems representing early signs of T2DM so that the dentist could contribute with referrals of potentially diseased patients. However, the present findings were restricted to subjects already diagnosed with T2DM. Subjects in the control group were asked about signs and symptoms of T2DM. A more appropriate screening of unidentified individuals with T2DM among the controls as suggested by Borrell et al., [47] would have contributed to the internal validity of the results. Although the control group may represent dental attendees in the general Khartoum population, it is possible that the recruitment procedure

of the diabetic patients introduced a bias since a convenience sample attending the Jaber Abol'ez Diabetes Center was utilized [48]. Nevertheless, the socioeconomic status did not differ between the cases and controls in this study. The purpose was to identify covariates of T2DM rather than to estimate disease prevalence in the Sudanese population in general.

Recall bias is of major concern when a case-control design is utilized. One advantage of this study includes the verification of information gathered from the study participants by comparing with medical records from the registry, thus reducing the risk of recall- and social desirability bias. The use of the OIDP index with a long history of validation in various contexts and the availability of novel clinical measurements are further strengths. Moreover, many potential confounding factors were considered in the multivariable analysis [49]. This was considered necessary as periodontal disease, dental caries and T2DM are multifactorial diseases.

Consistent with previous studies, the T2DM patients presented with more visible dental plaque, more missing teeth and were more likely to suffer from chronic periodontitis compared with the non-diabetic controls [17,19]. Although the T2DM patients presented with limited sugary drink consumption and low frequency of smoking, PI was significantly higher among the cases than the controls. Presence of more dental plaque and poorer oral hygiene among diabetic compared to non-diabetic subjects have been reported in a number of studies and might be attributed to DM patients having higher levels of glucose in gingival crevicular fluid (GCF) and saliva [8,19]. The prevalence of poor oral hygiene

Table 3. Clinical and self-reported oral health indicators by subgroups of T2DM cases according to glycemic control and duration of diabetes.

Confounding factors	Well-controlled	Poorly controlled	Short duration:	Long duration:
	DM: HbA1c ≤8	DM: HbA1c > 8	DM ≤10 years	DM > 10 years
	(n = 45)	(n = 105)	(n = 106)	(n = 48)
Age, mean (SD)	53.84 (9.34)	52.13 (10.90)	50.72 (10.78)	56.69 (8.61)**
Gender, % (n)				
Male	53.3 (24)	31.4 (33)	37.7 (40)	41.7 (20)
Female	46.7 (21)	68.6 (72)*	62.3 (66)	58.3 (28)
Education, % (n)				
Illiterate	22.2 (10)	29.5 (31)	29.2 (31)	20.8 (10)
Literate	77.8 (35)	70.5 (74)	70.8 (75)	79.2 (38)
Employment status, % (n)				
Unemployed	51.1 (23)	71.4 (75)	61.3 (65)	72.9 (35)
Employed	48.9 (22)	28.6 (30)*	38.7 (41)	27.1 (13)
Regular dental attendance, % (n)				
No	93.3 (42)	91.4 (96)	96.2 (102)	83.3 (40)
Yes	6.7 (3)	8.6 (9)	3.8 (4)	16.7 (8)*
Plaque index, mean (SD)	1.62 (0.41)	1.64 (0.33)	1.62 (0.34)	1.65 (0.37)
Main explanatory variables				
Tooth mobility, % (n)				
Low (< median)	62.2 (28)	41.0 (43)	50.9 (54)	41.7 (20)
High (≥median)	37.8 (17)	59.0 (62)*	49.1 (52)	58.3 (28)
Chronic periodontitis, % (n)				
No	15.6 (7)	12.4 (13)	11.3 (12)	18.8 (9)
Yes	84.4 (38)	87.6 (92)	88.7 (94)	81.2 (39)
Furcation involvement, % (n)				
No	64.4 (29)	51.4 (54)	57.5 (61)	52.1 (25)
Yes	35.6 (16)	48.6 (51)	42.5 (45)	47.9 (23)
Root surface caries, % (n)				
No	51.1 (23)	45.7 (48)	52.8 (56)	35.4 (17)
Yes	48.9 (22)	54.3 (57)	47.2 (50)	64.6 (31)
Dental caries (DMFT), % (n)				
Low (< median)	42.2 (19)	34.3 (36)	42.5 (45)	25.0 (12)
High (≥median)	57.8 (26)	65.7 (69)	57.5 (61)	75.0 (36)*
Number of present teeth, % (n)				
≤21 teeth	31.1 (14)	22.9 (24)	20.8 (22)	37.5 (18)
> 21 teeth	68.9 (31)	77.1 (81)	79.2 (84)	62.5 (30)*
OHRQoL, % (n)				
OIDP=0	73.3 (33)	63.8 (67)	69.8 (74)	56.2 (27)
OIDP>0	26.7 (12)	36.2 (38)	30.2 (32)	43.8 (21)

*P<0.05.

**P<0.01.

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among dental attendees from the general population in Khartoum has been reported to be about 39% [50]. This figure is consistent with that of 37% observed in the control group of the present study, suggesting that this group mirrors the background population as well. More missing teeth among the T2DM patients has been reported to be a characteristic of a population with poor oral hygiene [18]. Corresponding difference has not been disclosed in populations with good oral hygiene [51].

Since the relationship between T2DM and periodontal disease have been investigated in few countries [52], this study emanating from sub-Saharan Africa provides a valuable contribution to the literature. Notably, the prevalence of chronic periodontitis as defined by pocket depths rather than clinical attachment level might have been underestimated [53]. Moreover, the lack of a generally accepted case definition throughout studies impedes comparison of prevalence figures between them [54]. In addition

Table 4. DMFT regressed on duration of T2DM; adjusted for age, gender and regular dental attendance.

Variables	n	OR (95% CI)
Age	153	0.99 (0.95–1.02)
Gender		
Male	59	1
Female	94	3.45 (1.67–7.14)**
Regular dental attendance		
No	141	1
Yes	12	0.57 (0.15–2.18)
Duration of DM		
DM ≤10 years	105	1
DM > 10 years	48	2.94 (1.24–6.94)*

*P<0.05.
 **P<0.01.
 -2 Log likelihood = 183.536, Cox & Snell R²=0.114, Nagelkerke R²=0.155.
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to chronic periodontitis per se, clinical signs such as tooth mobility and furcation involvement were more frequent among the cases compared to their non-diabetic controls. These results coincide with findings from previous studies [16,18]. Although the mechanism by which DM affects periodontal tissues is not fully understood [55], it has been suggested that the magnitude of inflammatory response of periodontal tissues to oral micro-flora is exaggerated in DM patients. Moreover, advanced glycation end products (AGEs) are formed when excess glucose comes in contact with specific proteins which triggers series of pro-inflammatory reactions [56,57].

Although the T2DM patients in this study presented with more visible dental plaque and more missing teeth than non-diabetic subjects, there was no difference with respect to DMFT status. In this aspect, the present findings corroborate with those reported by other researchers [26,51,58]. A lack of a significant relationship between DM and dental caries has been attributed to confounding factors such as xerostomia, periodontal disease and a strict carbohydrate diet [59]. Nevertheless, a recent study by Jawed et al., [23] reported higher levels of DMFT among individuals with than without T2DM. An important finding in this study is that both the cases and controls presented with high prevalence of DMFT (95%), reflecting an urgent need for treatment and preventive oral health care programs. Whereas Hintao et al., [26] found a significant higher prevalence of root surface caries among T2DM patients compared to non-diabetic controls, the present finding suggesting absence of such an association corroborates those reported by Collin et al., [28] and Lin et al., [30]. The importance of measuring psychological and social impacts of oral diseases as a supplement to clinical indicators was pointed out by Cohen and Jago [33]. In this study, the T2DM patients were almost 3 times more likely than their non-diabetic controls to have at least one oral impact on their daily performances. This is at odds with the results of other studies suggesting that oral problems might not be a priority in DM subjects who are burdened with health complications in general [31,32].

The majority (70%) of the T2DM patients investigated in this study were poorly controlled. This agrees with recent data from other studies and illustrates the difficulties in maintaining good glycemic control [60]. In the present study, the poorly controlled

Table 5. Mobility index regressed on the level of glycemic control; adjusted for age and gender.

Variables	n	OR (95% CI)
Age	149	1.05 (1.01–1.08)**
Gender		
Male	56	1
Female	93	1.32 (0.63–2.78)
Glycemic control		
Well-controlled DM	45	1
Poorly controlled DM	104	2.60 (1.21–5.55)*

*P<0.05.
 **P<0.01.
 -2 Log likelihood = 191.545, Cox & Snell R²=0.093, Nagelkerke R²=0.124.
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cases were 2.6 times more likely to present with high mobility index (Table 5). This is consistent with other studies reporting a significant worse periodontal condition among poorly controlled DM patients compared to patients with good metabolic control [7–10].

Duration of DM might play an important role when the relation between DM and oral diseases is investigated [61]. The level of coronal caries was significantly higher in the long- compared to the short duration-T2DM group. This finding is in agreement with the normal pathogenesis of dental caries as “time” is an important factor for the development of the disease [62]. Moreover, the long duration-T2DM group had fewer teeth, which corroborates with the study of Santos et al., [21] in which the number of remaining teeth was negatively correlated with HbA1c levels.

Conclusion

This study revealed that chronic periodontitis, tooth mobility, furcation involvement and OIDP were more prevalent among T2DM patients than their non-diabetic matched controls. Although pocket depths as a measure of chronic periodontitis did not associate with the level of glycemic control or the duration of T2DM, there was an association in the expected direction with tooth mobility and dental caries. The present findings have implications for both DM and oral health care provision. For future prevention and management, it is important to know whether periodontitis plays a role in the development and control of DM and its complications [63]. Further large scale prospective studies are needed to investigate the effect of treatment of periodontitis on the management and control of T2DM.

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Author Contributions

Conceived and designed the experiments: HGM MFA SOI ANÀ. Performed the experiments: HGM SBI. Analyzed the data: OEB ANÀ. HGM. Contributed reagents/materials/analysis tools: SOI HGM KM. Wrote the paper: HGM ANÀ SOL.

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Study II

**Impact of Chronic Periodontitis on Levels of Glucoregulatory
Biomarkers in Gingival Crevicular Fluid of Adults with and
without Type 2 Diabetes**

II

RESEARCH ARTICLE

Impact of Chronic Periodontitis on Levels of Glucoregulatory Biomarkers in Gingival Crevicular Fluid of Adults with and without Type 2 Diabetes

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Abstract

The relationship between diabetes and periodontal disease is bidirectional, but information about the effect of chronic periodontitis on the levels of the glucoregulatory biomarkers locally in gingival crevicular fluid (GCF) is limited. The aim of this study was to compare the levels of 10 glucoregulatory biomarkers in GCF, firstly in subjects with type 2 diabetes (T2DM) presenting with and without chronic periodontitis and secondly, in subjects without diabetes, with and without chronic periodontitis. The material comprised a total of 152 subjects, stratified as: 54 with T2DM and chronic periodontitis (G1), 24 with T2DM (G2), 30 with chronic periodontitis (G3) and 44 without T2DM or periodontitis (G4). The levels of the biomarkers were measured using multiplex biometric immunoassays. Periodontal pocket depths were recorded in mm. Subsets G1 and G2 and subsets G3 and G4 were compared independently. Among T2DM subjects, GIP, GLP-1 and glucagon were significantly up-regulated in G1 compared to G2. Moreover, there were no statistical differences between the two groups regarding C-peptide, insulin, ghrelin, leptin and PAI-1. Comparisons among individuals without T2DM revealed significantly lower amounts of C-peptide and ghrelin in G3 than in G4. The number of sites with pocket depth ≥ 4 mm correlated negatively with C-peptide (Spearman's correlation co-efficient: -0.240, $P < 0.01$) and positively with GIP and visfatin (Spearman's correlation co-efficient: 0.255 and 0.241, respectively, $P < 0.01$). The results demonstrate that chronic periodontitis adversely influences the GCF levels of glucoregulatory biomarkers, as it is associated with disturbed levels of biomarkers related to the onset of T2DM and its medical complications.

Introduction

Diabetes mellitus represents a heterogeneous group of metabolic disorders associated with disturbances of carbohydrate, fat and protein metabolism. There are two major classes of the disease: Type 1 and Type 2. Type 2 diabetes (T2DM) is characterized by elevated blood glucose levels caused by increased production of glucose in the liver and increased peripheral insulin resistance, which might eventually lead to a reduction in insulin secretion [1]. In sub-Saharan Africa, T2DM is becoming increasingly prevalent, presenting a major public health burden in countries with scarce resources [2]. In The Sudan, the prevalence is increasing to epidemic proportions, affecting about 18 percent of the population [3]. Of particular concern is the fact that the onset is insidious and the condition may remain undiagnosed for a long time. In this context, the dental profession has a potentially important role and the condition may first be identified during a dental examination [4].

Periodontal disease is one of the most common diseases affecting humans [5]. Chronic periodontitis is an inflammatory condition characterized by apical migration of junctional epithelium, leading to formation of deep periodontal pockets around the teeth due to destruction of periodontal tissue fibers and the supporting bone [6]. Periodontal pockets may host highly virulent microorganisms and if left untreated; the host inflammatory-immune response progresses leading to tissue destruction [7]. For many years, chronic periodontitis was regarded as a local inflammatory condition, but recent research confirms that the condition has a systemic impact as the total surface area of the ulcerated periodontal pockets may be in the range of 8 to 20 cm², approximately the size of the palm of an adult hand [8]. Recently, more attention is directed towards the systemic effect of chronic periodontitis including its role in the aetiology of T2DM as well as its impact on the metabolic control and medical complications associated with patients bearing the disease.

The scientific literature however, shows lack of consensus about the risk of onset of T2DM in subjects with chronic periodontitis. A review by Sima *et al.*, [9] cited two longitudinal studies. The first study reported an odds ratio between 1.5 and 2.1 for patients with high periodontal index scores or tooth loss at baseline to develop diabetes [10], while the second one found no association between periodontal disease and the incidence of diabetes [11].

Gingival crevicular fluid (GCF) is a serum transudate found in the gingival sulcus. It contains not only connective tissue degradation products but also inflammatory molecules derived from circulating blood [12]. The levels of biomarkers in the GCF are potentially important as predictors of disease progression [7]. Few studies have investigated the levels of diabetes-related biomarkers in GCF such as ghrelin [13], leptin [14], and visfatin [15]. In the present study, the multiplex biometric immunoassay technique was applied in order to determine the amounts of 10 diabetes-related protein biomarkers known to be involved in the regulation of glucose metabolism, namely: *C-peptide*, *ghrelin*, *gastric inhibitory polypeptide* (GIP), *glucagon-like peptide-1* (GLP-1), *glucagon*, *insulin*, *leptin*, *plasminogen activator inhibitor-1* (PAI-1), *resistin* and *visfatin*.

The aim of the study was to investigate the influence of chronic periodontitis on the levels of glucoregulatory biomarkers in the GCF, by comparing the levels of the above biomarkers in subjects with T2DM, with and without chronic periodontitis, and in subjects without diabetes, with and without chronic periodontitis. We tested the hypothesis that in patients with T2DM, chronic periodontitis interferes with the regulation of glucose metabolism and that this effect is reflected in the relative quantities of the corresponding glucoregulatory biomarkers in the GCF, compared to the levels in subjects with T2DM who do not have chronic periodontitis. The second hypothesis tested was that in subjects without T2DM, chronic periodontitis is associated with increased GCF levels of biomarkers known for their association with the onset of T2DM.

Materials and Methods

Subjects and study design

The sample population comprised a total of 152 participants, 78 with T2DM and 74 without T2DM, representing a randomly selected subset from 461 participants recruited for a previous study by Mohamed *et al.* [16]. The T2DM subjects were stratified according to periodontal status, into two groups: 54 with chronic periodontitis (G1) and 24 without periodontitis (G2). Subjects without T2DM were also stratified according to periodontal status, into two groups: 30 with chronic periodontitis (G3) and 44 without periodontitis (G4). The T2DM subjects were recruited from The Jaber Abol'ez Diabetes Center in Khartoum-Sudan. T2DM was diagnosed by specialist physicians at the center according to the criteria of The American Diabetes Association [17]. Briefly, diagnosis of T2DM was based on one of the following: fasting plasma glucose of ≥ 126 mg/dl (≥ 7.0 mmol/L), random plasma glucose of ≥ 200 mg/dl (≥ 11.1 mmol/L), or plasma glucose of ≥ 200 mg/dl (≥ 11.1 mmol/L) after 75 g oral glucose tolerance test. A glycated haemoglobin test (HbA1c) was undertaken at the center's laboratory, using a commercially available kit (LabonaCheck A1c analyzer). Subjects without T2DM were recruited from the out-patient dental clinic at the Khartoum Dental Teaching Hospital. Recruitment of the study participants and the eligibility criteria for enrolment have been described in detail elsewhere [16]. Briefly, the criteria for enrolment were (i), being diagnosed with T2DM for more than one year and attending a specialized diabetes clinic for the T2DM patients- (ii), having at least for 10 remaining teeth (iii), no antibiotic, no steroidal and/or non-steroidal anti-inflammatory medication used during the last 3 weeks (iv), not treated with immunosuppressive chemotherapy, no current acute illness, no professional periodontal treatment received during the last 6 months and no ongoing pregnancy or lactation.

The study protocol was approved by the Ministry of Health in the Sudan and by the Norwegian Research Ethics Committee at the University of Bergen (2012/1470/REK Vest). Participation was confirmed by written informed consent and the steps of the clinical examination and sampling procedures were explained to each participant. All participants were provided with oral and written oral hygiene instructions, informed of their dental diagnosis and referred for appropriate dental treatment if needed.

Clinical examination

Periodontal examination included all teeth except 3rd molars. All participants were examined by a single, calibrated examiner (HGM) using a color-coded periodontal probe (N22, 2-4-6-8-10-12 mm markings), a color-coded Nabors furcation probe (NAB2, 3-6-9-12 mm markings), curette, mirror, probe, tweezers and cotton rolls. Dental plaque was recorded according to the Silness and Loe Index [18]. Periodontal pocket depths (PD) were measured from the gingival margin to the base of the periodontal pocket (mm) at four sites on each tooth (mesial, distal, buccal and lingual). Subjects with at least two sites with PD of ≥ 4 mm (not on the same tooth) with bleeding on probing were diagnosed as having chronic periodontitis [19,20]. It was reported that both periodontal pocket depth and bleeding on probing reflect the current disease status and are strongly related to the local inflammatory activity [21,22].

GCF sampling

Before the clinical examination, GCF samples were collected on the same day, using paper strips (PERIOPAPER Gingival Fluid Collection Strips, Oraflow Inc, New York, USA). Four samples, representing the four quadrants, were collected from each participant. Samples were collected from the mesiobuccal site of the sulcus/pocket. The 1st molar or, if missing, the 2nd

molar, 2nd premolar or 1st premolar was sampled respectively. If the target teeth were missing in one quadrant, then no GCF sample was collected from that quadrant. After removing the supra-gingival biofilm with sterile cotton pellets, the sites were dried and isolated with cotton rolls to avoid saliva contamination. To collect the GCF, the paper strips were inserted 2 mm into the sulcus/pocket and left in place for 30 seconds. Strips that were visually assessed as contaminated with blood or saliva were discarded. The four strips were immediately pooled in one tube, labeled and stored in liquid nitrogen until analysed.

Protein extraction and quantification

For protein extraction, Tween buffer was added to each GCF sample (4 strips). The tubes were kept in a shaker for 30 minutes and then centrifuged for 10 minutes at 4°C and 1400 rpm. The extracted protein was quantified using a commercially available kit (Pierce BCA Protein Assay Kit, ThermoFisher Scientific, Rockford, USA) following the manufacturer's instructions. Absorbance was measured at 560 nm on a plate reader (FLUOstar OPTIMA- BMG Labtech, Germany) and the total protein per sample (4 strips) was calculated in micrograms (μg).

Multiplex biomarkers assay

Multiplex biometric immunoassay containing fluorescent dyed magnetic beads conjugated with monoclonal antibodies specific for the targeted 10 protein biomarkers was used according to the manufacturer's instructions (Bio-Plex Human Cytokine Assay; Bio-Rad Inc., Hercules, CA, USA) [23]. Following protein extraction and purification, GCF samples were diluted to a ratio of 1:4 and incubated with the magnetic beads. After a series of washes with Bio-Plex Pro wash station, biotinylated detection antibody was added to create a sandwich complex. Thereafter, Streptavidin-Phycoerythrin conjugate was added as a fluorescent indicator. A range of (352290–0.97) pg/ml recombinant cytokines was used to establish the standard curves. Biomarkers quantities were determined using a multiplex array reader powered by Luminex-200 System. The amounts were calculated using Bio-Plex Manager software and reported as picograms per 30 seconds (pg/30 s) [24].

Statistical analysis

Comparisons were made between the T2DM groups (G1 and G2) and between the groups without T2DM (G3 and G4) independently. Distributions of the continuous variables among each group were assessed using Kolmogorov-Smirnov test. Independent sample-T test, Mann-Whitney U test, chi-square test and Fisher's Exact test were used to investigate the differences in the distribution of demographic data between the study groups. Quantities of the detected biomarkers were compared between the study groups using Mann-Whitney U test. Logistic regression analysis adjusting for age, gender, dental plaque and total protein was applied to test the relationship between periodontal status and the quantities of biomarkers. Spearman's correlation coefficients were calculated to assess the correlation between the number of sites with PD \geq 4 mm and the amount of each biomarker. Stata 13 (Stata Corp. 2013, Stata Statistical Software: Release 13. College Station, TX: StataCorp LP) was used for statistical analysis. P values less than 0.05 were considered statistically significant.

Results

As presented in Table 1, the mean ages of the T2DM patients in G1 and G2 were 54.76 ± 1.38 and 50.79 ± 2.09 ($P > 0.05$), respectively. The age ranges were 24–70 and 33–70 years, respectively. There were no significant intergroup differences between G1 and G2 in the demographic

Table 1. Demographic characteristics and clinical indicators of the study groups.

Variable	Type 2 diabetes		P value	No diabetes		P value
	Chronic periodontitis (G1), n = 54	No periodontitis (G2), n = 24		Chronic periodontitis (G3), n = 30	No periodontitis (G4), n = 44	
Age, mean (SE)	54.76 (1.38)	50.79 (2.09)	0.12 ^a	55.37 (1.77)	47.15 (1.56)	<0.01 ^a
Gender, % (n)))		
Male	42.60 (23)	29.20 (7)	0.38 ^b	60.00 (18)	34.10 (15)	0.05 ^b
Female	57.40 (31)	70.80 (14)		40.00 (12)	65.90 (29)	
Education, % (n)						
Literate	70.37 (38)	79.17(19)	0.42 ^b	66.67 (20)	77.27 (34)	0.31 ^b
Illiterate	29.63 (16)	20.83 (5)		33.33 (10)	22.73 (10)	
Employment, % (n)						
Employed	37.04 (20)	29.17 (7)	0.50 ^b	56.67 (17)	36.36 (16)	0.09 ^b
Unemployed	62.96 (34)	70.83 (17)		43.33 (13)	63.64 (28)	
Smoking, % (n)))		
Yes	13.00 (7)	12.50 (3)	1.00 ^c	26.70 (8)	13.60 (6)	0.27 ^b
No	87.00 (47)	87.50 (21)		73.30 (22)	86.40 (38)	
Plaque index, mean (SE)	1.66 (0.05)	1.49 (0.05)	0.05 ^a	1.49 (0.06)	1.28 (0.06)	0.03 ^a
Pocket depth, % (n)						
4–5 mm	59.30 (32)	0.00 (0)	—	70.00 (21)	0.00 (0)	—
≥ 6 mm	40.70 (22)	0.00 (0)		30.00 (9)	0.00 (0)	
Total protein -µg, mean (SE)	82.78 (7.53)	77.53 (9.06)	0.92 ^d	84.23 (15.25)	86.50 (9.51)	0.25 ^d
Duration of T2DM, mean (SE)	8.44 (0.83)	9.67 (1.70)	0.61 ^d	—	—	—
T2DM medication used, % (n)))		
Dilatory control	0.00 (0)	12.50 (3)	<0.01 ^c	—	—	—
Oral-hypoglycemic	66.70 (36)	33.30 (8)		—	—	—
Insulin	29.60 (16)	50.00 (12)		—	—	—
Both	3.70 (2)	4.20 (1)		—	—	—
HbA1c %, mean (SE)	9.17 (0.24)	9.25 (0.49)	0.89 ^a	—	—	—

^a Independent sample-T test.

^b Chi-square test.

^c Fisher's Exact test.

^d Mann-Whitney U test.

n: sample size, SE: standard error of the mean, T2DM: type 2 diabetes, HbA1c: glycated haemoglobin.

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and clinical indicators. Oral hypoglycemic drugs were the most common drugs for treatment of diabetes in G1 (66.7%) and insulin in G2 (50.0%). In subjects without T2DM, the mean ages of G3 and G4 were 55.37 ± 1.77 and 47.15 ± 1.56 ($P < 0.01$), respectively. The age ranges were 38–70 and 24–65 years, respectively. Plaque index was the only clinical indicator that differed significantly between G3 and G4. Among those with chronic periodontitis, the PD ranged from 4 to 10 mm in G1, and from 4 to 6 mm in G3, while participants without chronic periodontitis (G2 and G4) had a normal gingival sulcus ranged from 1–3 mm.

Table 2. Amounts of the detected glucoregulatory biomarkers (pg/30 s).

Biomarker, mean (SE)	Type 2 diabetes			No diabetes		
	Chronic periodontitis (G1), n = 54	No periodontitis (G2), n = 24	P value ^a	Chronic periodontitis (G3), n = 30	No periodontitis (G4), n = 44	P value ^a
C-peptide	26.22 (0.87)	25.27 (1.55)	0.82	35.15 (1.91)	42.98 (2.52)	0.10
Insulin	24.36 (1.38)	20.02 (1.94)	0.12	25.59 (1.51)	30.92 (2.28)	0.17
GIP	164.21 (3.35)	144.64 (4.54)	<0.01	141.45 (3.76)	151.27 (3.14)	0.07
GLP-1	210.99 (5.27)	183.40 (7.65)	<0.01	188.63 (7.42)	203.02 (6.52)	0.30
Glucagon	261.77 (8.06)	223.82 (10.72)	0.01	242.91 (9.40)	250.17 (7.29)	0.60
Ghrelin	303.63 (15.82)	239.00 (19.34)	0.04	303.20 (19.00)	374.22 (21.91)	0.03
Leptin	85.85 (5.52)	64.78 (5.49)	0.05	84.46 (6.36)	99.57 (6.66)	0.14
PAI-1	1027.19 (155.80)	901.53 (260.49)	0.32	512.54 (86.75)	619.04 (110.98)	0.18
Visfatin	6411.37 (471.25)	5976.73 (1201.05)	0.13	7013.91 (978.87)	5194.03 (414.78)	0.26

^a Mann-Whitney U test.

n: sample size, SE: standard error of the mean, GIP: gastric inhibitory polypeptide, GLP-1: glucagon-like peptide-1, PAI-1: plasminogen activator inhibitor-1.

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Of the 10 glucoregulatory biomarkers investigated in this study, resistin was excluded from the analysis, as it was not detected in 60% of the GCF samples. The mean quantities of the detected diabetes-related biomarkers among the study groups are presented in Table 2.

After adjustment for potential confounders, GIP, GLP-1 and glucagon were significantly higher in G1 than in G2. Moreover, C-peptide, insulin, ghrelin, leptin and PAI-1 were insignificantly higher in G1 than in G2 (Table 3). In contrast, in subjects without T2DM, the levels of C-peptide and ghrelin were significantly higher in G4 than in G3. All the other biomarkers under investigation were insignificantly higher in G4 than in G3, except visfatin which was insignificantly higher in G3 (Table 3).

As the number of diseased sites increased (PD ≥ 4 mm), the amount of C-peptide decreased significantly (Spearman’s correlation co-efficient: -0.240). In contrast, the amounts of both GIP

Table 3. Logistic regression analysis with periodontal status as dependent variable (yes/no).

Biomarker	Type 2 diabetes			No diabetes		
	Coefficient (SE)	OR (95% CI)	P value ^a	Coefficient (SE)	OR (95% CI)	P value ^a
C-peptide	0.008 (0.041)	1.008 (0.931–1.092)	0.85	-0.053 (0.023)	0.948 (0.907–0.992)	0.02
Insulin	0.060 (0.033)	1.062 (0.996–1.133)	0.07	-0.049 (0.027)	0.952 (0.902–1.005)	0.07
GIP	0.035 (0.014)	1.035 (1.008–1.064)	0.01	-0.028 (0.016)	0.972 (0.942–1.003)	0.08
GLP-1	0.022 (0.010)	1.022 (1.003–1.042)	0.02	-0.009 (0.007)	0.991 (0.977–1.006)	0.12
Glucagon	0.013 (0.006)	1.013 (1.001–1.025)	0.03	-0.003 (0.006)	0.997 (0.986–1.008)	0.60
Ghrelin	0.005 (0.003)	1.005 (0.999–1.010)	0.11	-0.006 (0.003)	0.994 (0.989–0.999)	0.02
Leptin	0.016 (0.010)	1.016 (0.997–1.036)	0.09	-0.015 (0.008)	0.985 (0.970–1.001)	0.06
PAI-1	0.006 (0.024)	1.006 (0.959–1.054)	0.82	-0.079 (0.058)	0.924 (0.825–1.035)	0.17
Visfatin	-0.001 (0.006)	0.999 (0.986–1.011)	0.85	0.009 (0.008)	1.009 (0.993–1.025)	0.27

^a Adjusted for age, gender, plaque index and total GCF protein.

OR: adjusted odds ratio, CI: confidence interval, SE: standard error, GIP: gastric inhibitory polypeptide, GLP-1: glucagon-like peptide-1, PAI-1: plasminogen activator inhibitor-1.

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Table 4. Correlations between number of sites with PD \geq 4mm and the amounts of the detected biomarkers.

Biomarker	Correlation coefficient ^a	P value
C-peptide	-0.240	<0.01
Insulin	-0.005	0.95
GIP	0.255	<0.01
GLP-1	0.131	0.11
Glucagon	0.153	0.06
Ghrelin	-0.017	0.84
Leptin	0.030	0.72
PAI-1	0.081	0.32
Visfatin	0.241	<0.01

^a Spearman's correlation.

GLP: gastric inhibitory polypeptide, **GLP-1**: glucagon-like peptide-1, **PAI-1**: plasminogen activator inhibitor-1.

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and visfatin increased significantly as the number of diseased sites increased (Spearman's correlation co-efficient: 0.255 and 0.241, respectively). We also observed a weakly positive correlation between the number of sites with PD \geq 4 mm and the amounts of both GLP-1 and glucagon (Spearman's correlation co-efficient: 0.131 and 0.153, respectively) (Table 4).

Discussion

There is ample evidence from the scientific literature that the relationship between diabetes and chronic periodontitis is bidirectional [25,26]. However, the focus of this report was to investigate the impact of chronic periodontitis on levels of glucoregulatory biomarkers in the GCF. In the present study, chronic periodontitis adversely influenced the levels of glucoregulatory biomarkers in the GCF, indicating that it might be associated with the onset of T2DM and the risk of diabetes related medical complications in subjects with the disease. Multiplex technology has been applied in a few studies, to investigate diabetes-related biomarkers in patients with systemic conditions such as obesity [27] and gestational diabetes [28]. In the present study, this high-throughput technology provided a highly accurate analysis of biological samples, given that it requires less sample volume and is able to detect different proteins within a broad range of concentrations [29]. When tested on the same analytics, multiplex assays are reported to compare favorably with ELISA with respect to sensitivity, accuracy, and reproducibility [30].

C-peptide is a polypeptide secreted from the secretory granules of β -cells during the conversion of pro-insulin to insulin [31]. C-peptide and insulin are released into the circulation in comparable amounts, but C-peptide is a more reliable indicator of β -cell activity, as it has a longer half-life and is not affected by insulin-specific antibodies [32]. To date, there are no published data on the relationship between periodontal disease and C-peptide levels in adults with T2DM. However, Merchant *et al.*, [33] investigated such an association in a sample comprising young subjects with both type 1 and 2 diabetes and concluded that periodontal disease was associated with lower fasting C-peptide. We observed the same trend among subjects without T2DM, as the level of C-peptide was lower among subjects with periodontitis (G3) than in those without periodontitis (G4). Moreover, the amount of C-peptide was negatively correlated with the number of diseased sites with PD \geq 4 mm, suggesting that chronic periodontitis might be associated with disturbed β -cells activity. In the present study, comparisons of insulin

among the different study groups showed similar trends, but there was no correlation between insulin and the severity of periodontal disease.

GLP-1 and GIP are insulin-stimulating hormones secreted from the gastrointestinal system after food intake [34]. Patients with T2DM do not generally exhibit reduced GLP-1 secretion [35]. In contrast, results from studies comparing GIP levels in T2DM patients and systemically healthy controls were inconclusive [34]. In the present study, both GIP and GLP-1 were found to be significantly higher in G1 than in G2. This observation may be attributable to the fact that 50% of patients in G2 were under insulin therapy, compared to 29.6% in G1. Thus, exogenous insulin might have down-regulated the insulin-stimulating hormones by negative feedback. Moreover, the levels of GIP and GLP-1 were lower in G3 than in G4. Despite the statistical insignificance, these findings support the hypothesis that chronic periodontitis is associated with disturbed GCF levels of hormones responsible for regulating the blood glucose levels among individuals without T2DM.

Glucagon is a hormone secreted by α -cells in response to food intake and hypoglycemia [36]. It regulates blood glucose levels and counteracts the effect of insulin by increasing blood glucose levels. In T2DM, the feed-back mechanism which controls glucagon secretion in response to blood glucose level seems to malfunction [37]. Thus, glucagon remains inappropriately elevated in hyperglycemia at comparable levels of blood glucose. Our results indicated no statistical difference in glucagon between G3 and G4. In subjects with T2DM however, the amount of glucagon was higher in G1 than in G2. This finding suggests that in T2DM subjects, chronic periodontitis may exert a systemic effect by its association with increased glucagon secretion.

Ghrelin is a peptide hormone secreted from the oral epithelium and fibroblasts. It has a major role in regulating neutrophil-mediated innate immune response [13]. Low ghrelin concentration is associated with T2DM [38]. The amount of ghrelin in G3 was significantly lower than in G4. Yilmaz *et al.*, [39] reported inconclusive results in a study investigating the level of plasma ghrelin in systemically healthy subjects with and without periodontitis. In the present study, the opposite trend was observed in the T2DM groups, as those with chronic periodontitis had higher levels of ghrelin than those without periodontitis. One explanation is that there might be a synergistic systemic effect of both T2DM and chronic periodontitis, as high ghrelin levels have been reported in other chronic inflammatory diseases such as Crohn's disease and inflammatory bowel disease [40].

Leptin is a hormone secreted by adipose tissues. It exerts an anti-diabetic effect by reducing insulin resistance [41]. The leptin detected in GCF probably diffuses from the microvasculature to the gingival tissues, as there are no adipocytes within the gingival tissue [14]. Tatti *et al.*, [42] have reported higher leptin concentration in systemically healthy individuals than in those with T2DM. In contrast, earlier investigations comparing Sudanese adults with and without diabetes have reported elevated plasma leptin levels in subjects with diabetes [43]. The present findings with respect to subjects without T2DM are consistent with earlier reports of higher levels of leptin in periodontally healthy subjects than in those with periodontal disease [14,44]. This has been attributed to the fact that during inflammation, the amount of leptin decreases because the increased vascular permeability allows leptin to escape from the gingival tissue [44]. In this context, it is noteworthy that detection of high local leptin levels in T2DM patients with periodontal disease is regarded as one of the indicators of cardiovascular complications [45]. PAI-1 is a serine protease inhibitor secreted by endothelial cells, fibroblasts, liver and adipose tissue [46]. Increased PAI-1 concentration is reported to be associated with venous thrombosis, pulmonary embolism and the aetiology of T2DM [47,48]. In the present study, the results of PAI-1 were insignificant. Visfatin is an adipokine secreted mainly by visceral adipose tissue [49]. It is also secreted by neutrophils in response to pathogens and acts as a pro-inflammatory cytokine which

stimulates monocytes to produce inflammatory mediators [50]. In the present study, there was a significant positive correlation between PD and visfatin, a finding which is in concordance with a previous report by Pradeep *et al* [15].

It was not possible to measure the GCF volume. Instead, we used total GCF protein as a surrogate measure of the GCF volume in the multivariate analysis as an attempt to control for the potential effect of variability of GCF volume on our results [51]. Data on the study participant's height and weight were unavailable. Thus, we were not able to calculate the body mass index (BMI) which is a proxy variable reflecting the amount of adipose tissue for each participant. This might affect our findings, as some of the biomarkers studied are secreted by adipocytes such as leptin, PAI-1 and visfatin.

Within the limitations of the study, our data imply that chronic periodontitis is associated with disturbance of the local expressions of biomarkers related to the onset of T2DM and its medical complications in GCF. Large-scale longitudinal studies are required to confirm the association between chronic periodontitis and the onset of T2DM and its medical complications.

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Author Contributions

Conceived and designed the experiments: HGM MFA ANÅ SOI. Performed the experiments: HGM MM SBI. Analyzed the data: HGM ANÅ. Contributed reagents/materials/analysis tools: KM. Wrote the paper: HGM SOI MM ANÅ KM SBI MFA.

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Study III

Influence of type 2 diabetes on local production of inflammatory molecules in adults with and without chronic periodontitis: a cross-sectional study

RESEARCH ARTICLE

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Influence of type 2 diabetes on local production of inflammatory molecules in adults with and without chronic periodontitis: a cross-sectional study

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Abstract

Background: Pathological changes in periodontal tissues are mediated by the interaction between microorganisms and the host immune-inflammatory response. Hyperglycemia may interfere with this process. The aim of this study was to compare the levels of 27 inflammatory molecules in the gingival crevicular fluid (GCF) of patients with type 2 diabetes, with and without chronic periodontitis, and of chronic periodontitis subjects without diabetes. A putative correlation between glycated haemoglobin (HbA1c) and levels of the inflammatory molecules was also investigated.

Methods: The study population comprised a total of 108 individuals, stratified into: 54 with type 2 diabetes and chronic periodontitis (DM + CP), 30 with chronic periodontitis (CP) and 24 with type 2 diabetes (DM). Participants were interviewed with the aid of structured questionnaire. Periodontal parameters (dental plaque, bleeding on probing and periodontal pocket depth) were recorded. The GCF levels of the 27 inflammatory molecules were measured using multiplex micro-bead immunoassay. A glycated haemoglobin (HbA1c) test was performed for patients with diabetes by boronate affinity chromatography.

Results: After adjustment for potential confounders, the DM + CP group had higher levels of IL-8 and MIP-1 β , and lower levels of TNF- α , IL-4, INF- γ , RANTES and IL-7 compared to the CP group. Moreover, the DM + CP group had lower levels of IL-6, IL-7 and G-CSF compared to the DM group. The DM group had higher levels of IL-10, VEGF, and G-CSF compared to the CP group. The levels of MIP-1 α and FGF were lower in diabetes patients (regardless of their periodontal status) than in chronic periodontitis subjects without diabetes. Diabetes patients (DM + CP and DM) had higher Th-2/Th-1 ratio compared to the CP group. HbA1c correlated positively with the pro-inflammatory cytokines (Pearson correlation coefficient = 0.27, P value: 0.02).

Conclusion: Type 2 diabetes and chronic periodontitis may influence the GCF levels of inflammatory molecules synergistically as well as independently. Type 2 diabetes was associated with high Th-2/Th-1 ratio, and modulated the local expression of molecules involved in the anti-inflammatory and healing processes.

Keywords: Diabetes mellitus, Chronic periodontitis, Inflammation, Gingival crevicular fluid, Cytokines

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Background

Diabetes mellitus represents a heterogeneous group of metabolic disorders in which elevated blood glucose levels result in disturbance of carbohydrate, fat and protein metabolism [1]. The most common form is type 2 diabetes [2]. Diabetes is a major public health concern with 380 million people suffering from the disease worldwide, and about 80 % of the patients are from low- and middle-income countries [3]. It is expected that Africa will take the lead in terms of the largest proportional increase in adults with diabetes by 2030 [4]. Prevalence of diabetes in The Sudan, as in many other low-income countries, is increasing to epidemic proportions [5]. In 2014, the prevalence of the disease in The Sudan was about 18 % [3], which ranks The Sudan among countries with high prevalence of diabetes in Africa and the world. It also reflects the change in life style and the urbanization movement of the population.

Chronic hyperglycemia is associated with irreversible complications such as nephropathy, retinopathy, neuropathy, cardiovascular diseases, peripheral vascular diseases, delayed healing and periodontal diseases [6]. Periodontal diseases, including the reversible form (gingivitis), are highly prevalent and affect up to 90 % of adults worldwide [7]. Chronic periodontitis is characterized by apical migration of the epithelial attachment accompanied by loss of connective tissue and alveolar bone [8]. These changes are mediated by the interaction between pathogens and the host immune-inflammatory response [9]. Although periodontal pathogens are considered as the initiative factor of the disease, [10] tissue destruction in chronic periodontitis is the consequence of the host response to those pathogens [11].

The exact mechanism by which diabetes affects periodontal tissues is not fully elucidated [12]. An altered immune-inflammatory response to bacterial pathogens has been suggested [11]. Hyperglycemia can affect periodontal tissues by increasing oxidative stress as a result of the imbalance between reactive oxygen species and antioxidants, which may eventually lead to accumulation of Advanced Glycation End products (AGE) [13]. The binding of AGE to their receptors (RAGE) triggers intracellular events that enhance the production of pro-inflammatory cytokines, chemokines and cell adhesion molecules [14]. Hyperglycemia can be assessed by measuring the concentration of glycated haemoglobin (HbA1c), which reflects mean glucose levels over the previous 8–12 weeks [15].

One means of investigating the local inflammatory status of the oral cavity is by analysis of gingival crevicular fluid (GCF), a non-invasive approach for assessing the presence or absence of various inflammatory molecules [16]. GCF is a transudate or an inflammatory exudate that can be collected from the gingival crevice surrounding the

teeth. It contains components of circulating blood, local tissues and most importantly, host-derived inflammatory molecules [16, 17].

Most of the studies of GCF inflammatory molecules in individuals with diabetes have generally been based on small subject samples and investigation of a limited number of inflammatory molecules, with inconclusive results [12]. Multiplex analysis of inflammatory molecules, whereby a large number of inflammatory molecules can be investigated at the same time, would facilitate understanding of the inflammatory process involved in both diabetes and periodontal diseases.

The aim of this study was to investigate the effect of type 2 diabetes on the local expression of inflammatory molecules involved in periodontal inflammation and healing by comparing GCF levels of 27 inflammatory molecules in patients with type 2 diabetes, with and without chronic periodontitis, and in chronic periodontitis subjects without diabetes. A putative correlation between HbA1c and the molecules under investigation was also explored. We tested the hypothesis that type 2 diabetes adversely influences the local expression of the inflammatory molecules under investigation.

Methods

Study design and participants

In all, 108 individuals were enrolled in the study, representing a randomly selected subset from 461 participants recruited for a previous study by Mohamed et al. [18]. The subjects were stratified into three groups: 54 with type 2 diabetes and chronic periodontitis (DM + CP), 30 with chronic periodontitis (CP) and 24 with type 2 diabetes (DM). The study participants were aged 24–70 years. Diabetes patients were recruited from The Jaber Abol'ez Diabetes Center in Khartoum-Sudan. Diabetes was diagnosed by specialist physicians at the center according to the criteria of The American Diabetes Association [19]. Whole blood samples obtained from patients with diabetes were analysed for HbA1c by boronate affinity chromatography using a commercially available kit (LabonaCheck[™] A1c analyzer) [20]. The CP group was recruited from the out-patient dental clinic at the Khartoum Dental Teaching Hospital. Recruitment of study participants and eligibility criteria for enrolment have been described earlier [18]. Briefly, criteria for enrolment were (i), being diagnosed with type 2 diabetes for more than one year and attending a specialized diabetes clinic -for patients with diabetes- (ii), having at least 10 remaining teeth (iii), no antibiotic, no steroidal and/or non-steroidal anti-inflammatory medication used during the last 3 weeks (iv), not treated with immunosuppressive chemotherapy, no current acute illness, no professional periodontal treatment received during the last 6 months and no ongoing pregnancy or lactation.

Demographic data were obtained from the study participants by means of a structured questionnaire.

The study protocol was approved by The Ministry of Health in The Sudan and The Norwegian Research Ethics Committee at The University of Bergen (2012/1470/REK Vest). The study participants were enrolled between July and December 2012. Written informed consent was obtained from each participant after the project objectives, the steps in the oral clinical examination and the sampling procedures had been explained. The participants were informed of their dental diagnosis and referred for appropriate dental treatment if indicated.

Clinical periodontal examination

All clinical assessments, group allocations and sampling-site selection were undertaken by a single, calibrated examiner (HGM). The periodontal examination included all teeth except the 3rd molars using a color-coded periodontal probe (N22, 2-4-6-8-10-12 mm markings), a color-coded Nabors furcation probe (NAB2, 3-6-9-12 mm markings), curette, mirror, probe, tweezers and cotton rolls. The clinical examination comprised dental plaque assessment using the Silness and Loe Index [21], bleeding on probing (BoP), scored as present or absent, and probing depth (measured from the gingival margin to the base of the periodontal pocket in millimeters) at four sites on each tooth (mesial, distal, buccal and lingual). Participants were diagnosed as having chronic periodontitis if they had at least two sites with bleeding pockets of ≥ 4 mm (not on the same tooth) [22, 23]. The oral examination was repeated for 20 participants randomly selected within 2 weeks. Intra-examiner reliability was assessed by Cohen's kappa coefficient [24]. Kappa value (κ) was 0.88 for chronic periodontitis (yes/no).

GCF sampling

GCF samples were collected using perio-paper strips, (PERIOPAPER® Gingival Fluid Collection Strips, Oraflow Inc., New York, USA). Four samples, each representing a quadrant, were collected from each participant. The strip was inserted into the mesiobuccal site of the sulcus/pocket of the 1st molar. If missing, the 2nd molar, 2nd premolar or 1st premolar was sampled, respectively. Quadrants with no posterior teeth were excluded from the sampling. After the supra-gingival biofilm had been removed with sterile cotton pellets, the sites were dried and isolated with cotton rolls. The paper strips were inserted 2 mm into the sulcus/pocket and left in place for 30 s. Strips that were visually assessed as contaminated with blood or saliva were discarded. The 4 strips were immediately pooled in one tube, labeled and stored in liquid nitrogen for further analysis.

Protein extraction and quantification

For protein extraction, Tween buffer (230 μ l) was added to each of the tubes containing the 4 strips. The tubes were shaken for 30 min and then centrifuged for 10 min at 4 °C and 1400 rpm. The extracted protein was quantified using a commercially available kit and following the manufacturer's instructions (Pierce™ BCA Protein Assay Kit, Thermo scientific, Rockford, USA). Absorbance was measured at 560 nm on a plate reader (FLUOstar OPTIMA- BMG Labtech, Germany). Total protein per sample (4 strips) was calculated in micrograms (μ g).

Analysis and grouping of inflammatory molecules

Following protein extraction, GCF samples (20 μ l each) were processed by multiplex immunoassay containing fluorescent dyed microspheres conjugated with monoclonal antibody specific for 27 inflammatory molecules (Bio-Plex Human Cytokine Assay; Bio-Rad Inc., Hercules, CA, USA) [25]. The following molecules were investigated: *IL-1 β* , *IL-1ra*, *IL-2*, *IL-4*, *IL-5*, *IL-6*, *IL-7*, *IL-8*, *IL-9*, *IL-10*, *IL-12*, *IL-13*, *IL-15*, *IL-17*, *Eotaxin*, *Basic Fibroblast Growth Factor* (FGF), *Granulocyte Colony Stimulating Factor* (G-CSF), *Granulocyte-Monocyte Colony Stimulating Factor* (GM-CSF), *Interferon- γ* (INF- γ), *Interferon Inducible Protein-10* (IP-10), *Monocyte Chemo-attractive Protein-1* (MCP-1), *Macrophage Inflammatory Protein-1 α* (MIP-1 α), *Macrophage Inflammatory Protein-1 β* (MIP-1 β), *Platelet-Derived Growth Factor* (PDGF), *Regulated Upon Activation, Normally T-Expressed, and Presumably Secreted* (RANTES), *Tumor Necrosis Factor- α* (TNF- α) and *Vascular Endothelial Growth Factor* (VEGF). Samples were diluted 1:4 (50 μ l in total) and incubated with coupled beads. Complexes were washed, incubated with detection antibody and thereafter, with Streptavidin-Phycoerythrin. A range of 105876 – 0.29 pg/ml recombinant cytokines was used to establish the standard curves. The levels of the inflammatory molecules were measured on a multiplex array reader (Bio-Plex Workstation from Bio-Rad Laboratories). The final quantities were calculated using software provided by the manufacturer and were reported as picograms per 30 s (pg/30 s) [26].

Based on the biological effect of each molecule, the molecules under investigation were grouped as: pro-inflammatory cytokines (IL-1 β , IL-6, IL-9, IL-12 and TNF- α), anti-inflammatory cytokines (IL-4 and IL-10), chemokines (IL-8, IP-10, MCP-1, MIP-1 α , MIP-1 β and RANTES) and T-helper 2/T-helper 1 ratio (Th-2/Th-1) (IL-4, IL-6, IL-9, IL-10/INF- γ , IL-2).

Statistical analysis

Inter-group differences in demographic and clinical data were assessed using chi-square and Fisher's exact test for categorical variables, one way analysis

of variance (ANOVA) with post-hoc (Sidak) adjustment for multiple comparisons for normally distributed continuous variables, and Kruskal-Wallis and Mann-Whitney test for skewed data. Since the distribution of the levels of the studied molecules is skewed, the natural logarithm links were calculated and used to detect the differences between the study groups, and one way analysis of variance (ANOVA) with post-hoc (Sidak) was conducted. Generalized linear models (GLM) with Gaussian family and log function were used to adjust for the potentially confounding effect of age, gender, smoking status, dental plaque, BoP and total protein on the outcome (molecule quantities). A possible correlation between HbA1c and the inflammatory status was investigated using Pearson correlation. Stata 13 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP.) was used for data analysis. P values less than 0.05 were considered statistically significant.

Results

The demographic characteristics and clinical parameters of the study groups are presented in Table 1. BoP was the only clinical parameter that differed significantly between the groups. It was higher in the DM + CP group than in the CP and DM groups. The following inflammatory molecules, which were not detected in more than 30 % of the GCF samples, were excluded from the analysis: (IL-1ra, IL-5, IL-13, IL-15 and eotaxin). The unadjusted means of the quantities of the detected molecules across the study groups are presented in Table 2. After adjustment for potential confounders (age, gender, smoking status, BoP, dental plaque index and total protein), the DM + CP group had higher levels of IL-8 and MIP-1 β , and lower levels of TNF- α , IL-4, INF- γ , RANTES and IL-7 compared to the CP group. Moreover, the DM + CP group had lower levels of IL-6, IL-7 and G-CSF than the DM group. The DM group had higher levels of IL-10, VEGF, and G-CSF than the CP group. Both diabetes groups (DM + CP and DM) had lower levels of MIP-1 α and FGF compared to chronic periodontitis subjects without diabetes (CP) (Table 3).

The Th-2/Th-1 ratio was significantly higher in the diabetes groups (DM + CP and DM) than in the CP group (Fig. 1d). A weak positive correlation was observed between HbA1c and the levels of the pro-inflammatory cytokines (Pearson correlation coefficient: 0.27, P value: 0.02) (Fig. 2), while the correlation between HbA1c and the anti-inflammatory cytokines was not statistically significant (Pearson correlation coefficient: -0.11, P value: 0.33) (Fig. 3).

Table 1 Distribution of socio-demographic and clinical indicators by study groups

Variable	DM + CP (n = 54)	CP (n = 30)	DM (n = 24)
Age, mean (SE) ¹	54.76 (1.37)	55.37 (1.83)	50.79 (2.05)
Gender, % (n) ²			
Male	42.59 (23)	60.00 (18)	29.17 (7)
Female	57.41 (31)	40.00 (12)	70.83 (17)
Education, % (n) ²			
Illiterate	29.63 (16)	33.33 (10)	20.83 (5)
Literate	70.37 (38)	66.67 (20)	79.17 (19)
Employment, % (n) ²			
Unemployed	62.96 (34)	43.33 (13)	70.83 (17)
Employed	37.04 (20)	56.67 (17)	29.17 (7)
Smoking, % (n) ³			
Yes	12.96 (7)	26.67 (8)	12.50 (3)
No	87.04 (47)	73.33 (22)	87.50 (21)
Hypertension, % (n) ²			
Yes	31.48 (17)	20.00 (6)	29.17 (7)
No	68.52 (37)	80.00 (24)	70.83 (17)
Regular dental attendance, % (n) ³			
Yes	3.70 (2)	10.00 (3)	8.33 (2)
No	96.30 (52)	90.00 (27)	91.67 (22)
Duration of diabetes, mean (SE) ⁴	8.44 (0.83)	—	9.67 (1.70)
HbA1c %, mean (SE) ⁵	9.17 (0.24)	—	9.25 (0.49)
Plaque index, mean (SE) ¹	1.66 (0.05)	1.40 (0.06)	1.47 (0.07)
Percentage of teeth with BoP, mean (SE) ¹	58.88 (2.85)a	28.97 (3.82)b**	32.51 (4.28)b**
Pocket depth, % (n) ²			
4-5 mm	59.30 (32)	70.00 (21)	0.00 (0)
\geq 6 mm	40.70 (22)	30.00 (9)	0.00 (0)
Pocket depth, mean (SE) ⁴	4.16 (0.09)	4.25 (0.12)	—
Total protein - μ g, mean (SE) ⁶	82.78 (7.53)	84.23 (15.25)	77.84 (9.06)

Different letters indicate statistically significant differences

¹one-way ANOVA

²chi-square test

³Fisher's exact test

⁴Mann-Whitney U test

⁵independent sample T test

⁶Kruskal-Wallis test

**P < 0.01

Discussion

Pro-inflammatory cytokines induce inflammatory response to various stimuli such as bacterial lipopolysaccharides [27]. In patients with diabetes, IL-1 β is regarded as one of the key cytokines in inflammatory periodontal tissue destruction [28]. In the present study, the level of IL-1 β was highest in the DM + CP group,

Table 2 Levels of the detected inflammatory molecules among the study groups (pg/30s)

Inflammatory molecule, mean (SE)	DM + CP (n = 54)	CP (n = 30)	DM (n = 24)
IL-1β	350.50 (31.18)	260.31 (41.45)	261.74 (46.34)
IL-6	9.01 (0.83)a	11.12 (1.11)ab	12.67 (1.27)b*
IL-9	6.91 (0.57)	9.64 (0.76)	8.57 (0.87)
IL-12	13.93 (1.07)	16.44 (1.43)	17.13 (1.63)
TNF-α	17.70 (1.84)a	31.57 (2.47)b**	22.05 (2.82)ab
IL-4	0.83 (0.06)a	1.26 (0.09)b**	0.92 (0.10)ab
IL-10	27.74 (1.43)ab	23.68 (1.90)a	33.02 (2.17)b**
IL-2	8.01 (0.98)a*	8.93 (1.30)a*	12.68 (1.48)b
INF-γ	29.55 (2.61)a**	47.50 (3.50)b	33.89 (3.92)a*
IL-8	633.87 (48.22)	501.96 (62.25)	507.65 (69.60)
IP-10	19.32 (2.30)a	24.60 (3.01)ab	29.13 (3.43)b*
MCP-1	6.10 (0.87)a	10.73 (1.11)b**	7.88 (1.24)ab
MIP-1α	3.14 (0.27)a*	4.52 (0.36)b	2.98 (0.40)a*
MIP-1β	23.53 (2.64)	21.37 (3.54)	26.03 (3.95)
RANTES	32.93 (2.77)	45.64 (3.72)	40.12 (4.16)
FGF	76.91 (4.62)a**	105.16 (6.20)b	76.34 (6.93)a*
PDGF	13.05 (0.93)a	17.22 (1.24)b*	15.45 (1.39)ab
VEGF	212.01 (11.75)	188.50 (15.77)	222.83 (17.63)
IL-7	2.63 (0.31)a	4.85 (0.42)b**	4.24 (0.47)b**
G-CSF	122.73 (12.48)a	131.02 (16.75)ab	196.73 (19.13)b**
GM-CSF	621.28 (21.82)	558.27 (29.28)	555.30 (33.44)
IL-17	72.04 (3.71)a	246.03 (62.90)b**	79.98 (6.68)ab

Different letters indicate statistically significant differences using one-way ANOVA for natural log. links of the amounts of inflammatory molecules
*P < 0.05
**P < 0.01

albeit not statistically significant. A recent meta-analysis reported that type 2 diabetes patients with chronic periodontitis were found to have significantly higher GCF levels of IL-1β than their systemically healthy counterparts [29]. In contrast, others have failed to confirm an association between levels of IL-1β and diabetes in individuals with periodontal disease [30, 31]. These inconsistencies might be attributed to the difference in HbA1c levels and the duration of diabetes. Contradictory results were reported from studies of TNF-α levels in oral fluids among patients with diabetes and chronic periodontitis [12]. In the present study, the levels of both TNF-α and IL-7 were lower in the DM + CP group than in the CP group. In contrast, other studies have reported higher GCF levels of TNF-α and IL-7 in patients with type 2 diabetes than in systemically healthy individuals [29, 32]. In this context, it is of interest to note that the levels of pro-inflammatory cytokines and chemokines in the DM group were comparable with those in the CP group, reflecting a local active inflammatory process in the DM

Table 3 Adjusted means of the levels of inflammatory molecules (pg/30s)

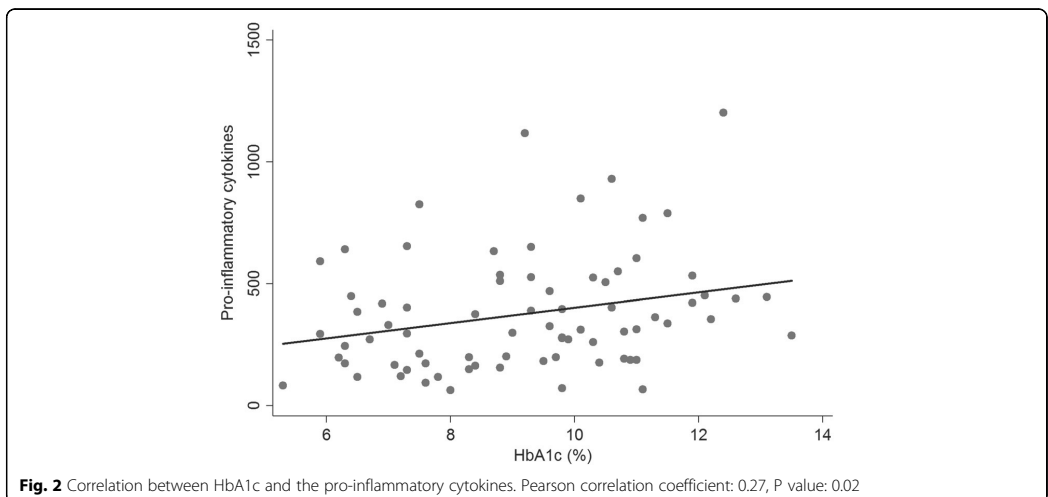
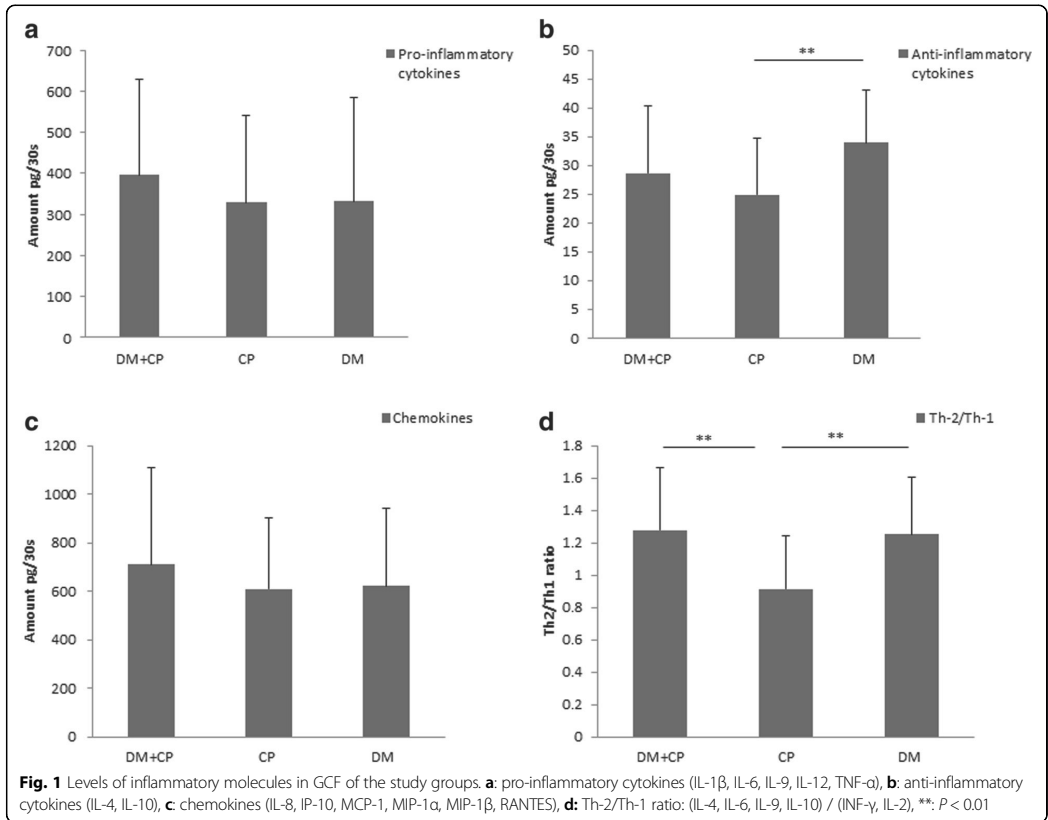
Inflammatory molecule, mean (SE)	DM + CP (n = 54)	CP (n = 30)	DM (n = 24)
IL-6	9.01 (0.94)a	11.11 (1.30)ab	14.01 (1.50)b*
TNF-α	17.68 (2.06)a	31.64 (3.16)b**	23.13 (3.20)ab
IL-4	0.84 (0.07)a	1.25 (0.11)b*	0.96 (0.11)ab
IL-10	28.82 (1.66)ab	22.46 (1.99)a	33.72 (2.42)b**
INF-γ	30.62 (2.99)a	45.77 (4.29)b*	36.88 (4.62)ab
IL-8	699.48 (53.44)a	464.17 (57.03)b*	503.04 (71.04)ab
MIP-1α	3.05 (0.32)a*	4.79 (0.47)b	2.99 (0.45)a*
MIP-1β	28.71 (3.05)a	16.84 (2.86)b*	22.37 (3.40)ab
RANTES	31.94 (3.14)a	48.01 (4.70)b*	41.16 (4.79)ab
FGF	71.66 (5.17)a**	113.28 (7.90)b	80.86 (7.93)a**
VEGF	215.04 (12.69)ab	177.52 (16.40)a	255.82 (19.39)b**
IL-7	2.81 (0.35)a	4.47 (0.51)b*	4.78 (0.56)b*
G-CSF	131.71 (14.41)a*	116.98 (17.79)a**	206.60 (21.25)b

Different letters indicate statistically significant differences using GLM with Gaussian family and log. function adjusting for age, gender, smoking status, BoP, dental plaque index and total protein
*P < 0.05
**P < 0.01

group (Fig. 1a–1c). A weak positive correlation was observed between HbA1c and the levels of pro-inflammatory cytokines. Another study reported a significant positive correlation between IL-1β levels in GCF and HbA1c [33].

Studying the effect of IL-6 and IL-10, particularly in cross-sectional studies, is complicated by the fact that both are multifunctional cytokines (i.e. pro- and anti-inflammatory) [34, 35]. IL-6 is involved in activation of osteoclasts and Th-17 cells [36]. In contrast, it also induces the production of IL-1ra, thus contributes to the anti-inflammatory process [37]. There is no evidence to support an association between increased levels of IL-6 and destructive periodontal disease among individuals with hyperglycemia [38]. However, Javed et al., [39] reported that up-regulation of IL-6 together with IL-1α could be associated with diabetes related periodontal tissue destruction. In the present study, the levels of the anti-inflammatory cytokine IL-4 were lower in the DM + CP group than in the CP group. It has been reported that IL-4 is down-regulated in patients with type 2 diabetes [40]. In addition, in an in-vivo animal model for ligament healing, IL-4 was found to contribute to the proliferative phase of healing [41].

Chemokines are small peptides that recruit immune cells from the circulation to the tissues as needed [42]. Most investigations of the role of chemokines have focused on IL-8 [12]. In the present study, IL-8 was up-regulated in the DM + CP group compared to the CP



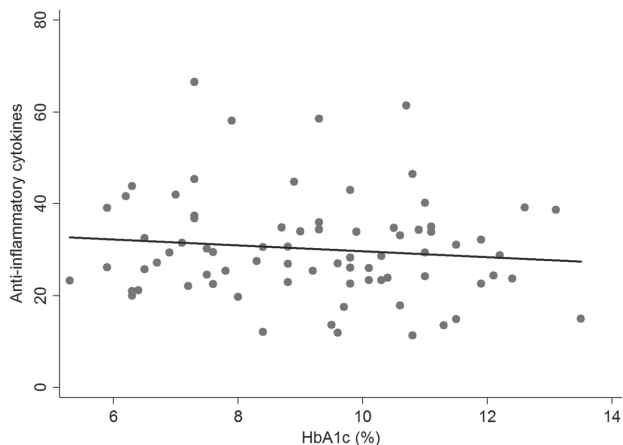


Fig. 3 Correlation between HbA1c and the anti-inflammatory cytokines. Pearson correlation coefficient: -0.11, P value: 0.33

group. The same trend has been observed in an investigation of serum levels of IL-8 [43]. MIP-1 α is a potent chemokine that attracts macrophages, T-cytotoxic and natural killer cells [44]. It was down-regulated in both diabetes groups (DM + CP and DM) compared to the CP group. In contrast, Duarte et al., [32] reported higher levels of MIP-1 α in patients with poorly controlled type 2 diabetes compared to systemically healthy controls. The present study demonstrated lower levels of RANTES in the DM + CP group compared to the CP group. It was reported that levels of RANTES negatively correlate with increased inflammation [45].

Type 2 diabetes might adversely affect periodontal health by down-regulating molecules involved in periodontal tissue regeneration and healing process such as FGF and PDGF [46, 47]. FGF was detected in lower amounts in the diabetes groups (DM + CP and DM) than in the CP group. Moreover, the levels of VEGF were higher in the DM group compared to the CP group. This observation might be explained by the fact that oxidative stress induces the VEGF signaling in patients with type 2 diabetes [48]. Moreover, our VEGF results corroborate with a previous study which reported a non-significant trend towards increased VEGF in GCF of patients with type 2 diabetes and chronic periodontitis compared to systemically healthy individuals with chronic periodontitis [49]. In contrast, Guneri et al., [50] concluded that GCF levels of VEGF were elevated in subjects with chronic periodontitis regardless of their diabetic status.

According to the cytokines they produce, Th-cells are divided into Th-1, Th-2, Th-17 and T-regulatory cells. Th-1 secretes IL-2 and INF- γ , while Th-2 secretes IL-4,

IL-6, IL-9, IL-10 and IL-13 [51, 52]. Only limited information is available about the role of Th-cells in type 2 diabetes patients with periodontitis [12]. Th-2/Th-1 ratio was higher in both diabetes groups (DM + CP and DM) than in the CP group, indicating enhanced humoral response and progression of periodontal disease among patients with type 2 diabetes via Th-2/B-cell axis [53, 54].

In the present study, the low levels of some of the pro-inflammatory molecules detected in type 2 diabetes patients compared to those without diabetes can be explained by the fact that some of the patients with diabetes were receiving insulin therapy, which might affect the local expression of inflammatory molecules [43, 55]. Moreover, the more pronounced clinical signs of periodontal disease in individuals with type 2 diabetes can be attributed to the disturbance in the balance between the molecules involved in active inflammatory process on the one hand, and the molecules involved in controlling inflammation, healing and regeneration of periodontal tissues on the other hand [56, 57]. Up to date, there is no ideal biomarker that can be nominated for disease detection or progression. Therefore, the interest has been shifted towards considering combinations of various host responses [11, 58]. In the present study, the GCF volume was not measured. Therefore, total GCF protein was used as a surrogate measure of the GCF volume in the multivariate analysis to control for the potential effect of variability of GCF volume on our results [59]. In addition, pocket depth and bleeding on probing were both used to define cases with periodontitis, as clinical attachment loss data were not available. Consequently, the effect of type 2 diabetes on the study outcome might be underestimated [60]. Nevertheless, it was reported that both periodontal pocket depth and

BoP reflect the current disease status and are strongly related to the local inflammatory activity compared to clinical attachment loss, which reflects past disease experience [61–63].

Conclusions

Type 2 diabetes and chronic periodontitis may adversely influence the GCF levels of inflammatory molecules synergistically as well as independently. Moreover, type 2 diabetes was associated with high Th-2/Th-1 ratio, and adversely influenced the local expression of molecules involved in the anti-inflammatory and healing processes. Further prospective studies are warranted to produce sufficient evidence to support the application of specific GCF biomarkers for prediction and prognosis of periodontal disease among patients with diabetes.

Abbreviations

GCF: Gingival crevicular fluid; HbA1c: Glycated haemoglobin; AGE: Advanced glycation end products; BoP: Bleeding on probing; IL: Interleukin; FGF: Basic fibroblast growth factor; G-CSF: Granulocyte colony stimulating factor; GM-CSF: Granulocyte-macrophage colony stimulating factor; INF- γ : Interferon- γ ; IP-10: Interferon inducible protein-10; MCP-1: Monocyte chemo-attractive protein-1; MIP: Macrophage inflammatory protein; PDGF: Platelet-derived growth factor; RANTES: Regulated upon activation, normally T-expressed, and presumably secreted; TNF- α : Tumor necrosis factor- α ; VEGF: Vascular endothelial growth factor; Th: T-helper; ANOVA: Analysis of variance; GLM: Generalized linear model.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

This study was designed by HGM, MFA, ANÅ and SOI. KM Contributed to the materials and analysis tools and to the discussion. HGM performed the laboratory experiments under the supervision of MM and SBI. HGM and ANÅ analysed the data. HGM wrote the manuscript which was edited by SOI, MM, ANÅ, KM, SBI and MFA. All authors read and approved the final manuscript.

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Study IV

Influence of type 2 diabetes on prevalence of key periodontal pathogens, salivary matrix metalloproteinases and bone remodeling markers in Sudanese adults with and without chronic periodontitis

Influence of type 2 diabetes on prevalence of key periodontal pathogens, salivary matrix metalloproteinases and bone remodeling markers in Sudanese adults with and without chronic periodontitis

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Abstract

Objectives: This study compared the occurrence of periodontal pathogens in plaque samples obtained from patients with type 2 diabetes, with and without chronic periodontitis, and from chronic periodontitis subjects without diabetes. Additionally, levels of salivary MMP-8, MMP-9, RANKL and OPG were compared across the study groups.

Methods: The study enrolled 31 patients with type 2 diabetes and chronic periodontitis (DM+CP), 29 with chronic periodontitis only (CP) and 20 with type 2 diabetes only (DM). Questionnaire-guided interviews were conducted and dental plaque index, bleeding on probing and periodontal pocket depth were recorded. Polymerase chain reaction (PCR) was utilized to determine the prevalence of the periodontal pathogens. The levels of salivary MMP-8, MMP-9, RANKL and OPG were determined by enzyme linked immunosorbent assay (ELISA).

Results: The CP group had the highest prevalence of *P.gingivalis* (81.5%), followed by the DM+CP (59.3%) and DM (55.0%) groups ($P > 0.05$). Similar trends were observed for *P.intermedia* and *T.denticola*. The prevalence of *T.forsthysia* was 100% in both periodontitis groups (DM+CP and CP) compared to 90% in the DM group. All plaque samples in the DM group were negative for *A.actinomycetemcomitans*, while the prevalence in the DM+CP and CP groups was 7.4% and 11.1%, respectively. There were no significant differences between the groups regarding the concentrations of MMP-8, MMP-9 or OPG, while the concentrations of RANKL were below the detection limit.

Conclusions: Our data show that type 2 diabetes has no significant influence on either the prevalence of the investigated periodontal pathogens, or the levels of salivary MMP-8, MMP-9 and OPG.

Introduction

Chronic periodontitis is an inflammatory condition that affects oral tissues surrounding the teeth. It is characterized by destruction of periodontal connective tissues and tooth-supporting bone accompanied by apical migration of the epithelial attachment, potentially leading to tooth loss (1). This process is guided by the host immune-inflammatory reaction in response to putative pathogenic bacteria that are embedded in dental plaque (2). Dental plaque is a biofilm attached to the tooth surface and harbors a complex microbiological community (3), among which, some have been found to be highly associated with chronic periodontitis such as *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Tannerella forsythia*, *Treponema denticola*, *Campylobacter rectus* and *Prevotella intermedia* (4, 5).

It is now widely accepted that chronic periodontitis is one of the classical complications of diabetes (6). There is, however, contradictory evidence about the effect of type 2 diabetes on dental plaque microbiota. Some studies have reported significant differences in the bacterial composition of dental plaque between individuals with and without type 2 diabetes (7, 8), while others failed to detect any difference (9, 10).

One of the suggested mechanisms by which hyperglycemia might influence chronic periodontitis is by interfering with the host immune-inflammatory response (11). As part of the host response to bacterial challenge, resident and chemo-attracted immune cells secrete a group of zinc-dependent endopeptidase enzymes, collectively known as matrix metalloproteinases (MMPs). These enzymes are responsible for most of the extracellular matrix degradation in both healthy and diseased tissues (12). MMP-8 (collagenase-2) and MMP-9 (gelatinase-B) are the most common MMPs involved in periodontal tissue destruction (13). Most of the MMPs detected in saliva are secreted by polymorphonuclear leukocytes (14). The action of MMPs is opposed by

tissue inhibitors of metalloproteinases (TIMPs), thus the imbalance between both enzymes can shape periodontal disease progression (12, 15).

A balance between osteoblasts and osteoclasts maintains the integrity of bone tissues (16). Accordingly, bone resorption occurs if the balance is shifted towards increased osteoclast activity. Osteoclasts are activated by the osteoclast differentiation factor called receptor activator of nuclear factor- κ B ligand (RANKL) (17). It is mainly secreted by activated T- and B-cells (18). The action of RANKL can be blocked by osteoprotegerin (OPG), a soluble decoy receptor that competes with RANKL by binding to its receptor (RANK) on the surface of pre-osteoclasts and osteoclasts (19). RANKL/OPG ratio has been reported to be a good surrogate marker for periodontitis-induced bone destruction (20). Further, it has been reported that type 2 diabetes is associated with higher gingival crevicular fluid (GCF) levels of RANKL (21). Moreover, a positive correlation between RANKL/OPG ratios in GCF and glycated hemoglobin (HbA1c) has been also observed (22).

Saliva is a promising point-of-care diagnostic tool because it can be utilized in chair-side detection of disease markers, as it contains a vast number of locally as well as systemically expressed proteins (23). In addition, it can be easily collected with minimum invasiveness and cost. The combined analysis of periodontitis related salivary markers and bacterial pathogens is a powerful approach for disease detection and follow-up (24).

The aim of this study was to investigate the influence of type 2 diabetes on the prevalence of six putative periodontal pathogens in subgingival plaque samples obtained from patients with and without chronic periodontitis. The impact of type 2 diabetes on levels of salivary MMP-8, MMP-9, RANKL and OPG was also investigated. We tested the hypothesis that type 2 diabetes adversely influences the prevalence of the periodontal pathogens investigated and the levels of salivary MMP-8, MMP-9, RANKL and OPG.

Materials and methods

Study design and participants

In total, 80 individuals were included in this descriptive cross-sectional study representing a randomly selected subset from 461 participants recruited for a previous study by Mohamed *et al.* (25). The subjects were stratified into three groups: 31 with type 2 diabetes and chronic periodontitis (DM+CP), 29 with chronic periodontitis (CP) and 20 with type 2 diabetes (DM). The study participants were enrolled between July and December 2012. Patients with type 2 diabetes were enrolled from Jaber Abol'ez Diabetes Center in Khartoum-Sudan. Diabetes was diagnosed by specialist physicians at the center according to the criteria of the American Diabetes Association (26). An HbA1c test was performed for patients with type 2 diabetes to determine the level of glycemic control (well-controlled: HbA1c \leq 8% and poorly controlled: HbA1c $>$ 8%) using a commercially available kit (LabonaCheck™ A1c analyzer). Participants in the CP group were recruited from the outpatient dental clinic at the Khartoum Dental Teaching Hospital. Eligibility criteria for participation were (i), being diagnosed with type 2 diabetes for more than one year -for patients with diabetes - (ii), having at least 10 remaining teeth (iii), no antibiotic, no steroid and/or non-steroidal anti-inflammatory medication used during the last 3 weeks (iv), no immunosuppressive chemotherapy, no current acute illness, no professional periodontal treatment received during the last 6 months and no ongoing pregnancy or lactation. Questionnaire-guided interviews were conducted for all participants after enrolment (25). Ethnicity was categorized into Afro-Arab and African tribes (27).

The study protocol was approved by The Ministry of Health in The Sudan and The Norwegian Research Ethics Committee at The University of Bergen (2012/1470/REK Vest). Written informed consents were obtained from all participants and the steps of the oral clinical

examination and the sampling procedures were explained. The participants were informed of their dental diagnosis and referred for appropriate dental treatment if indicated.

Clinical examination

The clinical examination was performed by a single examiner (HGM). The examination included all teeth except the 3rd molars using a color-coded periodontal probe (N22, 2-4-6-8-10-12 mm markings), a color-coded Nabors furcation probe (NAB2, 3-6-9-12 mm markings), curette, mirror, probe, tweezers and cotton rolls. Dental plaque was assessed using the Silness and Loe Index (28). Bleeding on probing (BoP) was recorded as present or absent, and probing depths were scored as mm (from the gingival margin to the base of the periodontal pocket) at four sites per tooth (mesial, distal, buccal and lingual). Participants were diagnosed as having chronic periodontitis if they had at least two sites with bleeding pockets of ≥ 4 mm (not on the same tooth) (29).

Subgingival plaque samples

Four microbial samples were obtained from each participant, representing the four quadrants. The samples were collected from the 1st molar. If missing, the 2nd molar, 2nd premolar or 1st premolar was sampled, respectively. Quadrants with no posterior teeth were excluded from the sampling. After removing the supra-gingival biofilm with sterile cotton pellets, the selected sites were dried and isolated with cotton rolls. A sterile paper point ISO (International Organization for Standardization) size 40 was inserted in the sulcus/pocket. Thereafter, the four paper points were pooled into one tube, labeled and stored in liquid nitrogen for further analysis.

Saliva samples

Unstimulated whole saliva samples were collected from all the study participants before the clinical examination. Participants were asked to refrain from eating or drinking for at least one hour prior to saliva collection. Donors, sitting comfortably in an upright position with their heads tilted slightly downwards, were instructed to allow saliva to collect in their mouths before gently expectorating into a sterile 10 ml tube for 5 minutes. The samples were then aliquoted and immediately stored in liquid nitrogen for further analysis.

DNA purification

Bacterial DNA was extracted and purified using the MasterPure DNA purification kit according to the manufacturer's instructions (Epicentre Biotechnologies, Madison, Wisconsin). Briefly, the pooled samples were suspended in 300 µl TE buffer and incubated overnight with 1 µl lysozyme solution at 37 °C. Next day, tissue and cell lysis solution, protease and RNase were added. Thereafter, protein precipitation reagent was added and the mixture was centrifuged. The supernatant was collected, mixed with isopropanol and then centrifuged. Finally the DNA pellet was washed with ethanol and re-suspended in 25 µl TE buffer. The amount of DNA was measured for each sample by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Polymerase chain reaction

After DNA purification, conventional polymerase chain reaction (PCR) was performed under standard conditions to investigate the prevalence of *P.gingivalis*, *T.forsythia*, *P.intermedia*, *T.denticola*, *C.rectus*, and *A.actinomycetemcomitans*. A set of specific primers were used for the PCR (30): *P.gingivalis*, sense, 5'-AGGCAGCTTGCCATACTGCGG-3' and antisense, 5'-ACTGTTAGCAACTACCGATGT-3'; *T.forsythia*, sense, 5'-GCGTATGTAACCTGCCCCGA-3'

and antisense, 5'-TGCTTCAGTGTTCAGTTATACCT-3'; *C.rectus*, sense, 5'-TTTCG-GAGCG TAAACTCCTTTTC-3' and antisense, 5'-TTTCTGCAAGCAGACACTCTT-3'; *P.intermedia*, sense, 5'-TTTGTGGGGAGTAAAGCGGG-3' and antisense, 5'-TCAACAT-CTCTGTATCCT GCGT-3'; *T.denticola*, sense, 5'-TAATACCGAATGTGCTCATTTACAT-3' and antisense, 5'-TCAAAGAAGCATTCCCTCTTCTTCTTA-3'; and *A.actinomycetemcomitans*, sense, 5'-AAA CCCATCTCTGAGTTCTTCTTC-3' and antisense, 5'-ATGCCAACTTGACGTAAAT-3'.

The final reaction volume (50 µl) consisted of 100 µM dNTP, 0.4 µM of each primer, 1.75 mM MgCl₂, 1 U of AmpliTaq Gold[®] DNA Polymerase (Applied Biosystems, Foster City, CA, USA) and 50 ng of extracted DNA. The target genes were amplified in a thermocycler (GeneAmp PCR system 9700, Applied Biosystems, Foster City, CA, USA) as follows: one cycle at 95 °C for 10 minutes, 40 cycles at 95 °C for 30 seconds for denaturation, 62 °C for 30 seconds for annealing, 72 °C for 1 minute for extension, and a final extension of 72 °C for 10 minutes. The annealing temperature was adjusted for the *A.actinomycetemcomitans* to 60 °C. Negative control (master mix without DNA template) was included in each PCR run. The PCR products were loaded and separated by agarose gel electrophoresis (2.2% FlashGel DNA system; Lonza, Walkersville, MD). The stained DNA bands were visualized by ultraviolet light and the data were reported as present or absent (Figure 1).

Enzyme linked immunosorbent assay

Frozen saliva samples were thawed, centrifuged at 14000 rpm for 15 minutes and the supernatants were collected. The levels of MMP-8, MMP-9, OPG (Sigma-Aldrich, St. Louis, MO, USA) and RANKL (MyBiosource, CA, USA. and PeproTech EC, London, UK) were determined using enzyme linked immunosorbent assays (ELISA) according to the manufacturers'

instructions. Optical densities were determined using a microplate reader (FLUOstar OPTIMA-BMG Labtech, Germany). The final concentrations are presented in pg/ml.

Statistical analysis

Potential differences in demographic and clinical indicators between the study groups were tested using chi-square and Fisher's exact test for categorical variables, independent sample T test and one-way ANOVA for normally distributed variables, and Mann-Whitney U test and Kruskal-Wallis test for skewed data. Frequencies of detection of the microbes under investigation are expressed in (%), and chi-square test or Fisher's exact test was used to examine the statistical differences between the study groups. The concentrations of the detected salivary molecules were compared by Kruskal-Wallis test. Adjustment for the potentially confounding effect of age, gender, smoking status and ethnicity was done by logistic regression analysis for the prevalence of bacteria, and by generalized linear models (GLM) with Gaussian family and log function for concentrations of salivary markers. Stata 13 (Stata Corp. 2013, Stata Statistical Software: Release 13. College Station, TX: StataCorp LP) was used for statistical analysis. P values less than 0.05 were considered statistically significant, and were adjusted for multiple comparisons when indicated.

Results

The distributions of the demographic and clinical indicators across the study groups are presented in Table 1. The age of the study participants ranged from 24 to 70 years. In general, the clinical periodontal parameters (dental plaque index, BoP and periodontal pocket depth ≥ 6 mm) differed significantly between the study groups; with the DM+CP group having the highest scores.

The CP group had the highest prevalence of *P.gingivalis* (81.5%), followed by DM+CP (59.3%) and DM (55.0%) ($P > 0.05$). The same trend was observed for *P.intermedia* and

T.denticola. The prevalence of *T.forsthysia* was 100% in both periodontitis groups (DM+CP and CP) compared to 90% in the DM group. *C.rectus* was detected in plaque samples of all the study participants. All plaque samples in the DM group were negative for *A.actinomycetemcomitans* while the prevalence in the DM+CP and CP groups was 7.4% and 11.1%, respectively (Table 2).

Comparisons of salivary MMP-8, MMP-9 and OPG across that study groups were not statistically significant (Table 3). Nonetheless, there was a trend towards increased concentration of MMP-8 in the chronic periodontitis groups (DM+CP and CP) compared to the DM group. In addition, the levels of the molecules under investigation did not differ significantly between well-controlled- and poorly controlled type 2 diabetes patients (Figure 2). The concentrations of RANKL were below the detection limit in all saliva samples.

Regardless of the diabetic status, MMP-8 levels remained higher in subjects with positive *P.gingivalis* and *P.intermedia* scores than in those with negative scores, albeit not statistically significance (Figure 3). The levels of MMP-9 followed the same pattern as MMP-8 for *P.gingivalis* results. On the other hand, subjects without diabetes had similar MMP-9 level comparing those with positive, and negative *P.intermedia* scores (Figure 4). In patients with type 2 diabetes, the concentration of OPG did not discriminate between those with and without *P.gingivalis* or *P.intermedia* in their plaque samples, while for those without diabetes, individuals with positive *P.gingivalis* and *P.intermedia* scores tend to have higher concentrations of OPG (Figure 5).

Discussion

Periodontal pathogens are considered as the triggering factor of the disease (31). Recently, it was suggested that periodontal tissue destruction is mediated by the host inflammatory response when the balance in the relative quantities of the existing bacteria in dental plaque is disturbed (32, 33).

The pathogens that are responsible for this imbalance are called “keystone pathogens” (34). In the present study, most of the investigated microbes were more prevalent in individuals with chronic periodontitis (DM+CP and CP) compared to the DM group. In addition, the effect of type 2 diabetes on the prevalence of the investigated bacteria was not significant. Similar findings were reported by others using different methodological approaches (10, 35). Field *et al.*, (10) reported that *P.gingivalis* is significantly higher in chronic periodontitis patients with and without type 2 diabetes than in type 2 diabetes patients without periodontitis, which is in line with our findings, although the difference in our patients was not statistically significant. Moreover, our results demonstrated that 30% of the plaque samples obtained from participants with chronic periodontitis scored negative for *P.gingivalis* (data not shown). Although *P.gingivalis* is one of the keystone pathogens in chronic periodontitis, it is not always detectable in periodontally diseased sites (36).

In the present study, the prevalence of *A.actinomycescomitans* was relatively low in all the study groups. Elabdeen *et al.*, (37) investigated subgingival microorganisms in Sudanese patients (with an age range of 13-30 years) with aggressive periodontitis using DNA-DNA hybridization (checkerboard) technique. They reported relatively low levels of *P.gingivalis* and *A.actinomycescomitans* in aggressive periodontitis patients as well as in healthy controls. Additionally, studies among patients with chronic periodontitis from Italy (38), Thailand (39) and Japan (40) reported low prevalence of *A.actinomycescomitans*. In that regard, a study from China demonstrated a low prevalence of *P.intermedia* in patients with type 2 diabetes and chronic periodontitis when compared to systemically healthy individuals with chronic periodontitis (41). The same trend was observed in the present study. It is noteworthy that periodontal microbiota may vary according to the geographical areas, which makes comparisons in that regard between studies from different geographical backgrounds rather difficult (4).

The role of MMP-8 and MMP-9 in periodontal tissue destruction is well established (12). In the present study, as in a study from another group, (42) there were no significant differences in the levels of salivary MMP-8 and MMP-9 between the study groups. Another study reported no significant difference comparing MMP-8 in GCF between individuals with and without type 2 diabetes (43), while others reported higher MMP-8 in saliva of patients with type 2 diabetes than in subject without the disease (44, 45). Moreover, Javed *et al.*, (46) investigated the level of salivary MMP-8 among pre-diabetic patients. They concluded that MMP-8 levels did not differ significantly between chronic periodontitis subjects with and without pre-diabetes.

In the present study, there was a trend towards increased OPG levels in both the DM+CP and CP groups compared to the DM group. This can be attributed to the fact that OPG levels increases in response to increased osteoclastogenesis (47). Moreover, our data demonstrate that the level of glycemic control did not affect the concentrations of molecules under investigation. A follow-up study investigating the GCF levels of RANKL and OPG over 6 months found no difference in OPG concentrations between well- and poorly controlled type 2 diabetes patients at any time point, while RANKL remained significantly higher in the poorly-controlled group at all the study time points (22).

The association between the systemic levels of OPG and the non-oral medical complications of type 2 diabetes is well documented (48, 49). In contrast, information about its levels in oral fluids of type 2 diabetes patients and its association with periodontal disease is scarce. Costa *et al.*, (44) reported elevated levels of salivary OPG in patients with type 2 diabetes compared to systemically healthy subjects with chronic periodontitis. Another study among patients with type 1 diabetes concluded that those with diabetes had higher plasma concentration of OPG and lower RANKL/OPG ratio compared to individuals without diabetes (50). In contrast,

an experimental study among rats demonstrated that type 1 diabetes influences periodontal bone tissues by decreasing OPG and increasing the RANKL/OPG ratio (51).

In the present study, a qualitative PCR method was used, as the aim was to investigate the prevalence (presence or absence) of the bacteria under investigation. It is simple, rapid and with comparable sensitivity to other PCR methods (52). Moreover, the four plaque samples obtained from each participant were pooled. It has been reported that pooling design counteracts the inter-subject variations and does not adversely affect gene analysis when compared to non-pooled design (53). In the present study, periodontal pocket depth and bleeding on probing were used to define cases with chronic periodontitis. Both periodontal parameters reflect current disease status, and have been reported to be associated with local inflammatory activity as well as ecological changes at sample sites (54, 55).

Within the limitations of the present study, our data suggest that type 2 diabetes has no significant influence on the prevalence of the investigated periodontal pathogens. Moreover, we were unable to detect a significant difference in levels of salivary MMP-8, MMP-9 and OPG between individuals with and without type 2 diabetes. These findings suggest that the impaired periodontal parameters in seen patients with type 2 diabetes might be due to disturbed host immune-inflammatory response to the periodontal pathogens rather than disturbed bacterial composition of dental plaque as suggested in an earlier study among the same study population (56). Large follow-up studies are needed to trace the potential effect of type 2 diabetes on bacterial composition of dental plaque and on bone remodeling markers in oral fluids.

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Table 1. Distribution of socio-demographic and clinical indicators

Variable	DM+CP (n= 31)	CP (n= 29)	DM (n= 20)
Age, mean (SE) ¹	53.55 (1.82)	52.03 (1.34)	51.15 (2.39)
Gender, % (n) ²			
Male	48.40 (15)	48.30 (14)	30.00 (6)
Female	51.60 (16)	51.70 (15)	70.00 (14)
Ethnicity, % (n) ³			
Afro-Arab	93.55 (29)	82.76 (24)	63.16 (12)
African	6.45 (2)	17.24 (5)	36.84 (7)
Education, % (n) ²			
Illiterate	25.80 (8)	34.50 (10)	25.00 (5)
Literate	74.20 (23)	65.50 (19)	75.00 (15)
Employment, % (n) ²			
Unemployed	51.60 (16)	51.70 (15)	75.00 (15)
Employed	48.40 (15)	48.30 (14)	25.00 (5)
Smoking, % (n) ³			
Yes	16.10 (5)	31.00 (9)	10.00 (2)
No	83.90 (26)	69.00 (20)	90.00 (18)
Hypertension, % (n) ²			
Yes	29.00 (9)	17.20 (5)	30.00 (6)
No	71.00 (22)	82.80 (24)	70.00 (14)
Regular dental attendance, % (n) ³			
Yes	0.00 (0)	6.90 (2)	10.00 (2)
No	100.00 (31)	93.10 (27)	90.00 (18)
Duration of diabetes- years, mean (SE) ⁴	8.40 (1.09)	-----	10.50 (1.99)
HbA1c %, mean (SE) ⁵	9.17 (0.34)	-----	9.37 (0.52)
Plaque Index, mean (SE) ¹	1.68 (0.07)a	1.42 (0.06)b*	1.47 (0.06)ab
Percentage of teeth with BoP, mean (SE) ⁶	56.95 (3.71)a	22.21 (2.50)b**	29.50 (3.99)b**
Pocket depth, mean (SE) ⁴	4.18 (0.05)	4.25 (0.09)	-----
Pocket depth, % (n) ³			
4-5 mm	58.10 (18)	86.20 (25)*	-----
≥ 6 mm	41.90 (13)	13.80 (4)	-----

Different letters indicate statistically significant differences

1 one-way ANOVA

2 chi-square test

3 Fisher's exact test

4 Mann-Whitney U test

5 independent sample T test

6 Kruskal-Wallis test

* P < 0.05

** P < 0.01

Table 2. Prevalence of periodontal pathogens detected by PCR (*n* = 74)

Bacteria, % (n)		DM+CP (n= 27)	CP (n= 27)	DM (n= 20)
P.gingialis ¹	+	59.3 (16)	81.5 (22)	55.0 (11)
	-	40.7 (11)	18.5 (5)	45.0 (9)
P.intermedia ¹	+	44.4 (12)b*	77.8 (21)a	30.0 (6)b**
	-	55.6 (15)	22.2 (6)	70.0 (14)
T.forsthysia ²	+	100.0 (27)	100.0 (26)	90.0 (18)
	-	0.0 (0)	0.0 (0)	10.0 (2)
T.denticola ²	+	88.9 (24)	100.0 (27)	70.0 (14)
	-	11.1 (3)	0.0 (0)	30.0 (6)
C.rectus ²	+	100.0 (27)	100.0 (27)	100.0 (20)
	-	0.0 (0)	0.0 (0)	0.0 (0)
A.actinomycetemcomitans ²	+	7.4 (2)	11.1 (3)	0.0 (0)
	-	92.6 (25)	88.9 (24)	100.0 (20)

Different letters indicate statistically significant differences, adjusting for age, gender, smoking status and ethnicity

1 chi-square test

2 Fisher's Exact test

* *P* < 0.05

** *P* < 0.01

Table 3. Concentrations of the detected inflammatory molecules by ELISA ($n = 80$)

Inflammatory molecule, mean (SE)	DM+CP (n= 31)	CP (n= 29)	DM (n= 20)
MMP-8	1256.27 (166.65)	1305.30 (193.85)	923.35 (194.74)
MMP-9	16188.39 (1303.30)	14147.76 (1274.33)	11378.75 (1401.43)
OPG	33.51 (7.11)	39.91 (10.02)	23.53 (4.26)

No significant differences between the study groups (Kruskal-Wallis test), adjusting for age, gender, smoking status and ethnicity

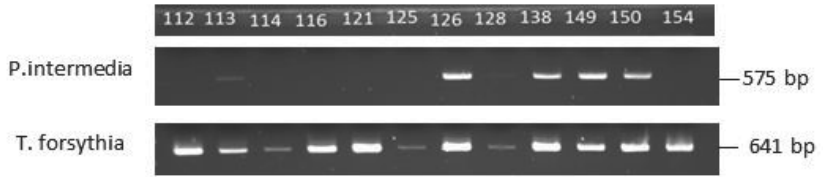


Figure 1. Representative results of electrophoresis of PCR products from dental plaque samples obtained from patients (112 to 154) using *P. intermedia* and *T. forsythia* specific primers

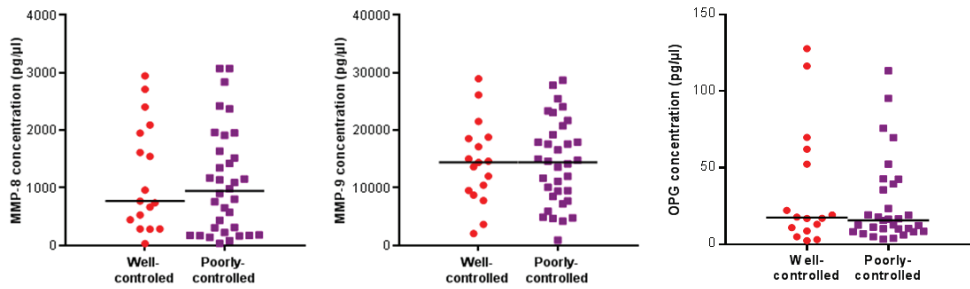


Figure 2. Distribution of salivary MMP-8, MMP-9 and OPG concentrations (pg/ml) in well- and poorly-controlled type 2 diabetes patients.

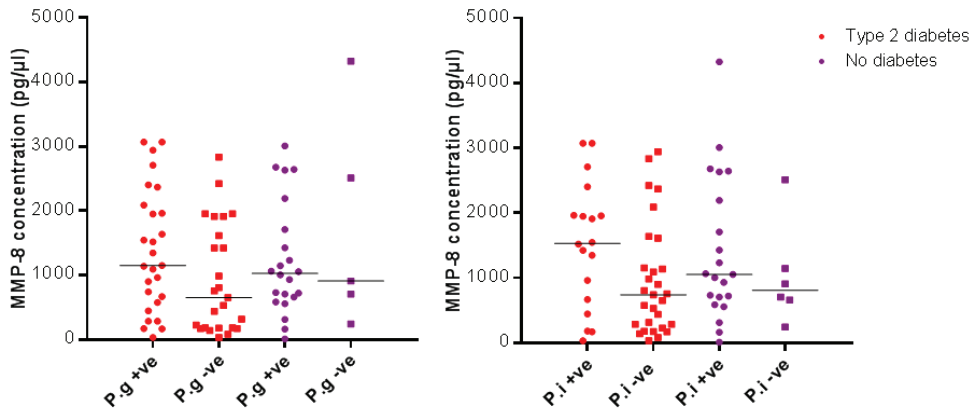


Figure 3. Distribution of salivary MMP-8 concentrations (pg/ml) according to the prevalence of *P.gingivalis* (P.g) and *P.intermedia* (P.i) in subjects with and without type 2 diabetes.

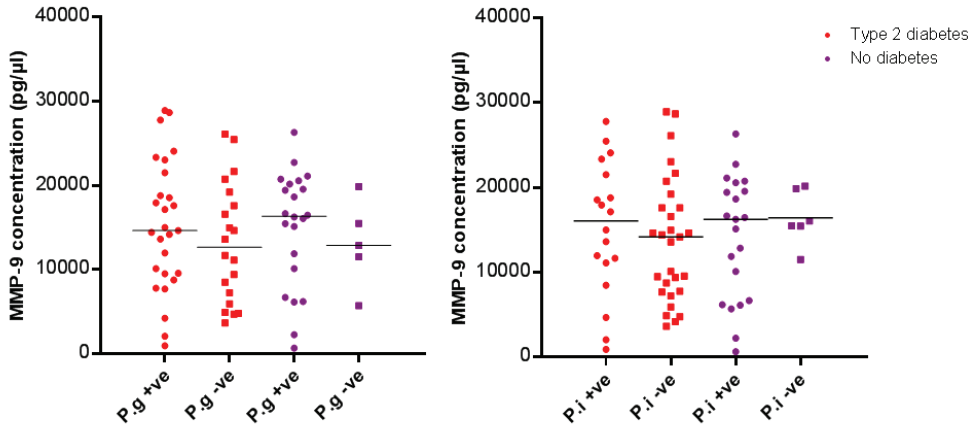


Figure 4. Distribution of salivary MMP-9 concentrations (pg/ml) according to the prevalence of *P.gingivalis* (P.g) and *P.intermedia* (P.i) in subjects with and without type 2 diabetes.

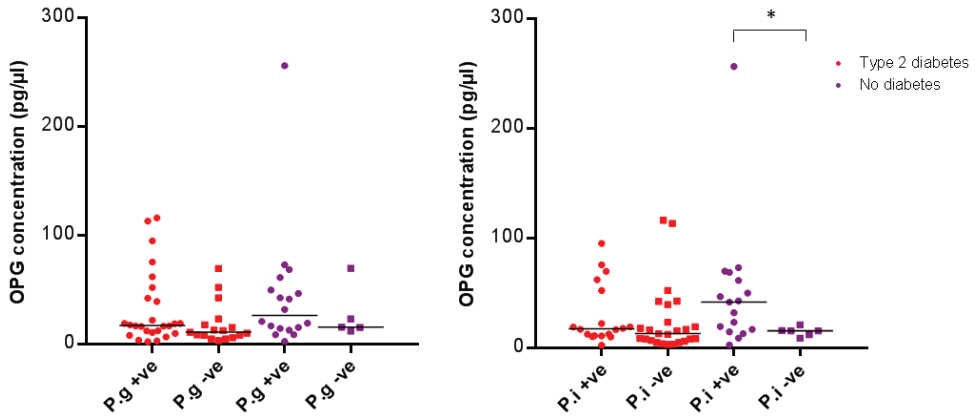


Figure 5. Distribution of salivary OPG concentrations (pg/ml) according to the prevalence of *P.gingivalis* (P.g) and *P.intermedia* (P.i) in subjects with and without type 2 diabetes.

