Type 2 diabetes in Sri Lanka : Genetic epidemiology and periodontal association.

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REFERENCE

Type 2 **Diabetes in** Sri Lanka: **Genetic Epidemiology and** Periodontal **Association**

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Abstract

Prevalence rates of type 2 diabetes and impaired fasting glyceamia (IFG) in Sri Lanka are high and an increasing number of people are succumbing to disease. Identifying people at risk of developing complications is a healthcare priority of the county to prevent morbidity and mortality. Type 2 diabetes is familiarly aggregated and maternal influence for disease transmission has been observed in European countries but not in South India. The severity of periodontitis is reported to be high when diabetes control decreases but data relating to periodontitis as a complication of diabetes is not present in this population. Periodontal associated genotype (PAG), presence of allele 2 of both {*IL1A (+4845)* & *IL1B (+3954)*}) is positively associated with periodontal disease in some populations but ruled out in other populations. Heightened levels of TNF-α level have been observed in both patients with type 2 diabetes and periodontitis.

This study aimed to determine familial aggregation of type 2 diabetes and parental influence of disease transmission in the Sri Lankan population. The project also aimed to investigate periodontal status in diabetic patients and the effect of glycaemic control on periodontal status. The final aim of the project was to investigate the role of cytokine polymorphisms PAG and *TNFA (-308)* in association with periodontitis in people with type 2 diabetes.

Family history data for one thousand patients was collected and analyzed. Subjects with established diabetes were recruited to the periodontal study (n=285) with an age and sex matched control population (n=72). All subjects underwent both periodontal and general health examination. Their periodontal parameters and metabolic parameters were measured including blood pressure, TG and LDL values. Patients were genotyped by PCR/RFLP method.

The results of the study indicated that 59.4% of the diabetic subjects had at least one affected first degree relative in the family. It was also observed that 15.6% of mothers transfer the disease compared to (12.5%) of fathers ($p<0.001$). When both parents had diabetes the early age of onset of diabetes is observed among offspring compared to offspring of maternal, paternal and no parental history of diabetes respectively ($p<0.05$). 16.67% (12/72) of the control patients were identified as IFG and 66.67% (8/12) of this group were diagnosed with periodontitis. Systolic BP, diastolic blood pressure and LDL values were significantly lower in patients than controls ($P<0.05$). Triglyceride levels were significantly higher in patients with diabetes than controls ($p=0.001$). A higher number of diabetic subjects were affected with chronic periodontitis (33.3%) compared to the non diabetic population (21.7%) ($p=0.077$). People with diabetes had significantly higher mean recession, percent bleeding on probing (BOP), maximum Probing depth (PD), PD > 4mm, PD >5mm and maximum loss of attachment (LOA) scores than the non diabetic population ($p<0.01$). Glycaemic control had no significant effect on periodontal status ($p>0.05$). Subjects with periodontitis were significantly older than those with gingivitis or periodontally healthy ($p<0.01$). Only FBG values were significantly higher in subjects with chronic periodontitis compared to periodontally healthy subjects ($p<0.01$). Population frequency analysis of allele 1 vs. allele 2 were 0.9 & 0.1 for *IL1B (+3954),* 0.74 and 0.26 for *IL1A (+4845)* and 0.91 and 0.09 for *TNFA (-308)* respectively. The *TNFA (-308)* polymorphism deviates from the frequencies observed in North British Caucasians but similar to that observed in Taiwan and Japan. PAG had no significant effect on periodontal status in diabetes and controls ($p>0.05$). The percentage of PAG (12.5%) is low compared to Caucasians but higher compared to Chinese population. *TNFA (-308)* allele distribution had no significant effect on periodontal status. *TNFA (-308)* genotypes were significantly correlated with HDL.

The results of the present study indicate that type 2 diabetes is familiarly aggregated in the Sri Lankan diabetic population and maternal excess is observed in the transmission pattern. There is a trend towards periodontital status to be higher in the diabetic population. Severity of periodontitis is higher in people with diabetes than those without and there is an indication that periodontitis may induce hyperglycaemia. PAG distribution shows population variation and the *TNFA (-308)* polymorphism may be associated with diabetes.

Publications

Papers published

1. Periodontitis : A complication of type 2 diabetes in Sri Lankans. **Nimali T De Silva,** Philip M. Preshaw, John J. Taylor, Shanthalal D Jayaratne, Peter A Heasman, Devaka J.S. Fernando, Diabetes Research and Clinical Practice, 74, 2006, 209-210.
2. Excess maternal transmission and familial aggregation of Type II Diabetes Mellitus in Sri Lanka. **De Silva SNT**, Weerasuriya N, De Alwis NMW, Fernando DJS. Diabetic Research and Clinical Practice, 58, 2002, 173-177
3. Genetics of Type II Diabetes. **De Silva N,** Fernando DJS. Ceylon Collage of Physicians Journal; Editorial; 2001.33(2) 66-70.
4. Ethical Aspects of Genetic Studies **De Silva N**, Fernando DJS, Ceylon Collage of Physicians Journal Editorial; 2001.34 12-18.
5. Unraveling Genetic of complex diseases. **De Silva N**, Fernando DJS, Jayaratne, SL, Taylor J.J. Ceylon Collage of Physicians Journal, 2004.
6. Compromised periodontal status in urban Sri Lankan adults with type 2 diabetes. Philip M. Preshaw, **Nimali De Silva,** Giles I. McCracken, Devaka Fernando, Caroline F. Dalton, Nick Steen, Peter A. Heasman.(Submitted Journal of Clinical Periodontology)

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Abbreviations

ADA	American Diabetes Association
AGEs	Advanced glycation end products
ANOVA	Analysis of Variance
Anti-GAD	Anti-glutamic acid decarboxylase
ASP	Affected Sib Pair
ATP III	Adult treatment Panel III
BMI	Body mass index
BOP	Bleeding on probing
BRCA1	Breast Cancer 1
BRCA2	Breast Cancer 2
CAL	Clinical Attachment Loss
CEJ	Cemento Enamel Junction
CHD	Coronary heart disease
CRP	C reactive proteins
DAG	Diacylglycerol
DCCT	Diabetes Control and Complication Trial
DGC	Diabetes with good glycaemic control
DHAP	Dihyroxy acetone phosphate
DMC	Diabetes with moderate glycaemic control
DNA	Deoxyribose Nucleic Acid
DPC	Diabetes with poor glycaemic control
EDTA	Ethylenediaminetetraacetic acid
FBG	Fasting blood glucose
FFA	Free fatty acids
FGM	Free gingival margin
GABA	Gamma-amino butyric acid
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GCF	Gingival Crevicular Fluid
GFAT	Glutamine fructose-6-phosphate amidotransferase
GR	Gingival Recession
H_2O_2	Hydrogen peroxide
HbA1c	Glycated Haemoglobin A1c
HDL	High density lipoprotein
HLA	Human Leukocyte Antigen
HNF1α	Hepatocyte nuclear factor 1α
HNF4α	Hepatocyte nuclear factor 4α
IBS	Identity by State
IDF	International Diabetes Federation

IFG	Impaired fasting glycaemia
IGT	Impaired glucose tolerance
IL-1β	Interleukin 1-β
IL-10	Interleukin 10
IL-6	Interleukin 6
IRS	Insulin receptor
KDa	Kilodalton
LADA	Latent autoimmune diabetes in adults
LDL	Low Density Lipoprotein
LDL	low density lipoprotein
LTA	Lymphotoxin Alpha
LOA	Loss of attachment
LPS	Lipopolysaccharides
Maximum LOA	Maximum loss of attachment
Maximum PD	Maximum Probing Depth
Mean LOA	Mean Loss of Attachment
Mean PD	Mean probing depth
Mean Rec	Mean Recession
MGF	Maternal grandfather
MGM	Maternal grandmother
MMPs	Matrix metalloproteinases
MnSOD	mitochondrial isoform of the enzyme superoxide dismutase
MODY	Maturity onset of diabetes of the young
mtDNA	Mitochondrial DNA
NCEP	National Cholesterol Education Program
NDC	Non diabetic control group
NF-kB	nuclear factor-kB
NHANES III	National Health and Nutrition Examination survey
OXPHOS	oxidative phosphorylation
PAG	periodontal associated genotype
PAI-1	Plasminogen activator inhibitor-1
PARP	Poly (ADP-ribose) polymerase
PCR	Polymerase Chain Reaction
PGE_2	Prostaglandin E_2
PGF	Paternal grand father
PGM	Paternal Grand mother
PKC	Protein Kinase C
PMN	Polymorphonuclear
PPARγ	peroxisome proliferators activated receptor gamma

RAGE	AGE receptors for Advanced Glycation End products
RFLP	Restriction fragment length polymorphism
ROS	Reactive Oxygen Species
SNP	Single nucleotide polymorphism
SPSS 16	Statistical Package for the Social Sciences
TCA	Tri Carboxylic Acid
TCF7L2	Transcriptional factor 7
TDT	Transmission Disequilibrium Tests Statistics
TGF-β1	expression of transforming growth factor
TNF-α	Tumor Necrosis Factor- α
TOC	Cholesterol
TPS probe	True pressure sensitive
TRG	Triglycerides
UKPDS	UK Prospective Diabetes Study
UN	United Nations
VLDL	very low density lipoprotein
WHO	World Health Organisation

1.1. Diabetes mellitus: a brief overview

Diabetes Mellitus is a common metabolic disorder of multiple aetiology characterised by hyperglycaemia. According to recent estimated measures this disease has afflicted 246 million people throughout the world in the year 2007 and the projected disease prevalence in year 2025 is 380 million representing 7.1% of the world adult population (UN resolution 61/225 2007). This spreading epidemic causes a major public health problem and has a huge economic impact. The effects of diabetes mellitus are long term. Prolonged hyperglycaemia causes failure and damage to various organs in the body (Alberti & Zimmet 1998). It is associated with reduced life expectancy, significant morbidity due to specific diabetes related microvascular complications, increased risk of macrovascular complications (ischaemic heart disease, stroke and peripheral vascular disease), and diminished quality of life.

The disease is known to prevail from antiquity. The first written record of the disease dates back to ancient Egypt in 1550 B.C. George Moritz Ebers discovered the papyrus named after him in 1862 in a Thebes cave, which describes the polyuric state of diabetes. Aretaeus of Cappadocia assigned the epithet diabetes to the disease in second century AD referring to the excessive passage of urine. The existence of two forms of diabetes, one clearly affecting thin people who did not survive and the other affecting older fatter people was first reported in Sanskrit Literature by two Indian Physicians, Susruta and Charuka. Accurate clinical features, gangrene and collapse of sexual function and the sweet taste of the urine of the diabetic patients came to light in the 10th century. Thomas Willis rediscovered the presence of sweet taste of urine in the 17th century. Mellitus, a Latin term meaning as sweet as honey was used to distinguish the disease from other polyuric states and some important thoughts for the involvement of pancreas with the disease were put forward in 18th century. Claude Bernard (1813-1878) discovered the association of the central nervous system in the balance of blood glucose concentration and storage of glucose in the liver in the form of glycogen during the progression of understanding of the disease in the 19th century (Conti 2001). Coma associated with diabetes, retinosis in glycosuria patients, the glucose lowering property of the pancreas, and the discovery of islet cells in the pancreas by Paul Langerhans were some other important discoveries during the latter 19th century (Egeler et al. 1994). The most important breakthrough was made in 1921 with

the discovery of insulin by the Toronto team (Macleod, Banting, Best and Collip) (Jarrett 1991).

The principal feature of diabetes is hyperglycaemia, which may result due to disturbances in carbohydrate, fat and protein metabolism. The endocrine and metabolic systems of the body collectively play a major role in glucose homeostasis. Glucose is consumed by the peripheral tissues to produce energy. In abundance it is stored as glycogen in the liver and excess is converted to lipid via lipogenesis. When the metabolic fuels are insufficient, the liver converts stored glycogen to glucose to overcome the deficit and if this is not sufficient, it will produce glucose via gluconeogenesis. All these metabolic pathways are regulated by specific enzymes that are coded by different genes but the tissue uptake and utilisation of glucose is controlled by the hormone insulin secreted by pancreatic beta cells (Bergman 2007).

Insulin is a major anabolic hormone. It regulates glycogenolysis, gluconeogenesis, lipogenesis and glycolysis. In the absence of, or with defects in insulin secretion, mutated insulin or with an insulin resistance state, the normal tissue uptake of glucose diminishes and plasma level of glucose rises. This signals to the metabolic system a glucose reducing state, converting the entire anabolic system to a catabolic state. The ultimate result is the production of glucose in the liver via gluconeogenesis further increasing blood glucose levels. If one of the enzymes is defective in one of the metabolic pathways of carbohydrate metabolism, it may lead to disturbances of glucose homeostasis. Enzymes are products of genes. Therefore, different genotypes can result in a common phenotype of glucose homeostasis. Thus, the disease is heterogeneous and complex in etiology. Certain clear monogenic forms of diabetes have shown defects in different genes, peroxisome proliferator activated receptor gamma (PPARγ) (Altshuler et al, 2000), Hepatocyte nuclear factor 1α (HNF1α) and 4α (HNF4α) (Hegele et al, 1999), Insulin receptor (Huxtable et al, 2000), Calpain 10 gene (Tsuchiya 2006), Transcriptional factor 7 (TCF7L2) (Grant et al. 2006) and mitochondrial genome (Poulton et al, 2002) appear to influence type 2 diabetes.

1.2 Classification and diagnosis of diabetes

The present classification system was based on the clinical stages and aetiological types of diabetes mellitus (Report of WHO, 2006). Based on the clinical stages, the early stages are classified firstly as impaired glucose regulation in which two distinct states have been identified, impaired glucose tolerance (IGT) and impaired fasting glycaemia (IFG). These stages are encountered during the progression of the disease and an individual subject may move from one stage to another in either direction, either towards normoglycaemia or towards diabetes mellitus. A fasting venous plasma glucose concentration <110mg/dl has been considered as normoglycaemia. Individuals with impaired glucose regulation (either IGT or IFG) are at greater risk of developing micro and macrovascular complications (Table 1.1).

Diabetes is considered to be present when sequential fasting plasma glucose levels are ≥126mg/dl (7 mmol/l) or 2-hour post glucose loading plasma levels are ≥ 200mg/dl (11.1mmol/l). Individuals with impaired fasting glucose (IFG) demonstrate fasting plasma levels of 110-126mg/dl (6.1 - 6.9 mmol/l), while those with impaired glucose tolerance (IGT) display 2-hour post glucose loading plasma levels between 140mg/dl and 200mg/dl. (Alberti & Zimmet 1998 & report of WHO 2006). Diagnosis of diabetes by impaired fasting glucose or an oral glucose tolerance test requires that the patient be fasting. Recently, consensus was generated regarding screening and diagnosis of diabetes with particular reference to the use of hemoglobin A1c (HbA1c). HbA1c is a form of hemoglobin used primarily to identify the average plasma glucose concentration over prolonged periods of time. It is formed in a non-enzymatic pathway by hemoglobin's normal exposure to high plasma levels of glucose. There are several advantages of using Hb1Ac for screening and diagnosis over currently established diagnostic methods as patients do not need to be fasting, HbA1c values more accurately reflect the longer term glycaemia than plasma glucose, and laboratory methods are standardized and reliable. Errors, if any, can be minimized by confirming the diagnosis with a plasma glucose specific test. At present HbA1c ≥6.1% confirmed by plasma or oral glucose tolerance test can be used to confirm the diagnosis of diabetes (Saudek et al. 2008).

Table 1.1 WHO diagnostic criteria for diabetes diagnosis

	Glucose concentration {mmol l^{-1} ($mgdl^{-1}$)}		
	whole blood		Plasma
Diabetes	Venous	Capillary	Venous
Fasting or	≥6.1(≥110)	≥6.1(≥110)	≥7.0 (≥126)
2hours post glucose load or both	≥10.0 (≥180)	≥11.1(≥200)	≥11.1 (≥200)
Impaired Glucose Tolerance (IGT)			
Fasting if measured	<6.1 (110)	<6.1(<110)	<7.0 (<126)
And 2hours glucose load	≥6.7 (≥120) and <10.0 (<180)	≥7.8 (≥140) and <11.1(<200)	≥7.8 (≥140) and <11.1 (200)
Impaired fasting glycaemia (IFG)			
Fasting	≥5.6 (≥100) and <6.1 (<110)	≥5.6 (≥100) and <6.1 (<100)	≥.1(≥110) and <7.0 (< 126)
2 hours if measured*	<6.7 (<120)	<7.8 (<140)	<7.8 (<140)

*Venous plasma glucose- 2 hours after ingestion of 75g oral glucose load.

Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (1997 & Report of WHO/IDF consultation 2006).

1.2.1 Type 1 diabetes

In type 1 diabetes, major contributory factors to the predisposition of the disease are genetic and environmental factors which are poorly understood. Autoimmune antibodies destroy the β cells in the pancreas resulting in an absolute deficiency of insulin (Jarrett 1991). The presence of anti-glutamic acid decarboxylase (anti-GAD) islet cell or insulin antibodies that cause β-cell destruction have been observed in some patients with type 1 diabetes. GAD is an intracellular enzyme required for the synthesis of neurotransmitter gamma-amino butyric acid (GABA). GAD65 is primarily expressed in pancreatic β cells (Lernmark 1996). In some patients there is no evidence for an autoimmune process, and their diabetes falls into the category of idiopathic type 1 diabetes mellitus. The rate of destruction of β cells is rapid in children but slower in adults. Adult onset of type 1 diabetes is also referred to as latent autoimmune diabetes in adults (LADA) (Pozzilli & Di Mario 2001).

There are seven symptoms listed by the American Diabetes Association (ADA) in association with type 1diabetes. These are thirst, polyuria, weight loss, increased fatigue and tiredness with minor symptoms like muscular cramps, irritability and blurred vision due to osmotic changes in the lens. (Nathaniel et al. 2007). In addition, skin infections, penile or vulval pruritis due to Candidiasis is common. Nausea, vomiting and drowsiness usually denote impending ketoacidosis and possible coma. Microvascular complications are extremely rare. The duration of symptoms is short, usually only 2-3 weeks or less (Alberti & Zimmet 1998).

Clinical stages of the disease are diagnosed according to the WHO criteria mentioned above (Table 1.1). Apart from the clinical features, the presence of islet cell auto-antibodies, antibodies to insulin and antibodies to (GAD65) confirm type 1 diabetes (Ylihärsilä et al. 1999).

Patients with type 1 diabetes require insulin for survival or to prevent ketoacidosis, coma and death (Alberti & Zimmet 1998). After commencing insulin treatment many patients enjoy excellent glycaemic control with only small doses of insulin (sometimes referred to as the honeymoon period). This improvement corresponds to temporary improvements in β cell function caused by lowering of high glucose levels, which directly impair β cell function and insulin release. However, continued autoimmune destruction of the remaining β cells may reach a point that cannot be compensated for by insulin. As hyperglycaemia itself may damage remaining β cells, intensive blood glucose control is required immediately after diagnosis. Islet cell transplantation offers the potential to improve glycaemic control (Shapiro et al. 2006).

1.2.2 Type 2 diabetes mellitus

The disease is characterised by insulin resistance and impaired insulin secretion (Valsania & Micossi 1994) and both are present at the time of clinical manifestation. The question of which defect occurs first was debated for a long period. Recent studies provide evidence that a phase of hyperinsulinaemia is preceded by the development of type 2 diabetes, thus insulin resistance is the possible initiator for the etiology. During this stage, hepatic glucose output is normal (Gorden 1997). Insulin resistance primarily affects the peripheral utilisation of glucose mainly by the muscle (Granner & O'Brien 1992). Increased glucose

levels in the blood signal to the pancreatic β cells an insulin reduced state, which leads to further secretion of insulin. Finally, the β cells reach an 'exhausted' state where they are no longer capable of producing insulin. Thus, impaired insulin secretion results, ultimately leading to glucose imbalance. The disease usually remains undiagnosed for several years. Hyperglycaemia may be present but it alone is not severe enough to provoke the symptoms. By the time of diagnosis, hyperglycaemia has often caused significant damage to the organs and these patients are at a risk of developing micro- and macrovascular complications (Alberti & Zimmet 1998).

The classical symptoms of type 2 diabetes are those of hyperglycaemia, thirst, polyuria and fatigue. Weight loss and ketoacidosis are not frequent (Jerrett 1991). Ketoacidosis may be associated with the stress of another illness such as infection (Nathaniel et al. 2007).

Patients are initially treated with a normocaloric diet excluding refined carbohydrates; if this control is still inadequate a sulphonylurea drug is usually added (Mooradian 1996). These drugs act by stimulating insulin release from pancreatic β cells in response to a glucose load and promote insulin uptake in body tissues. Short acting sulphonylurea drugs are tolbutamide, tolazamide and acetohexamide which are maximally effective for up to 24 hours, while long active agents such as chloropropamide and glipizide are effective for up to 36 hours (Physicians' Desk Reference 1998). Repaglinide is an ultra-short-acting agent with rapid onset and is usually taken immediately prior to each meal and has a decreased risk of hypoglycaemia (Melander 1996). Obesity promotes insulin resistance, and insulin sensitivity can be improved by weight reduction and increased physical activity. Obese patients are initially treated with a weight-reducing diet (also sugar free), with metformin often used as second line drug treatment (Jerrett 1991). This is thought to act by stimulating glucose uptake by skeletal muscle and decreasing hepatic glucose output. It also causes anorexia and reduces glucose absorption thereby reducing weight. In decreased insulin production, injectable insulin is available in multiple forms from bovine, porcine and human made by recombinant DNA technology (Mealey 1998).

1.2.3 Other forms of diabetes

Maturity onset of diabetes of the young (MODY)

This is a less common early onset form of diabetes, and the disease has a clear autosomal dominant inheritance pattern. However, a variation in different genes to this form has been observed in different families (Froguel & Velho 1999). Therefore MODY is a genetically heterogeneous monogenic form of type 2 diabetes, characterised by early onset, usually before 25 years of age (Fajans 1989). Approximately 5% of the patients with type 2 diabetes have this form.

Gestational hyperglycaemia and diabetes.

Gestational diabetes is carbohydrate intolerance resulting in hyperglycaemia of variable severity with onset or first recognition during pregnancy (Moore 2005). Pregnant women who meet the WHO standards for diabetes diagnosis following an oral glucose tolerance test after an overnight fast fall into this category.

Diabetes Insipidus

This results from a deficiency in the pituitary hormone vasopressin or resistance for this hormone by the kidney. The decreased production of an action of vasopressin results in excessive urine production and polyuria but does not have any effect on blood glucose levels (Position paper 2000).

1.3 Genetics of complex diseases

Many complex diseases such as type 2 diabetes, obesity and periodontitis are regarded as resulting from the interaction between genes, proteins and the environment. Therefore, improved understanding of genetic and environmental contributions to complex diseases may ultimately shift the focus of medicine from the disease itself to the genetic characteristics of individuals, and to their experiences, habits and environmental conditions. A focus on the genetic, developmental and environmental components of diseases and their unique combinations in a given individual could potentially facilitate more effective methods of prevention. Once the genetic basis for susceptibility to a complex disease is established, the healthcare profession could target and monitor potential sufferers, help them to avoid the environmental factors that can provoke disease and encourage early self-detection of signs and symptoms.

The basis of genetic variation

Variations in DNA have arisen during evolution as a consequence of mutations, and new mutations arise in single individuals in somatic cells or in the germline. If a germline mutation does not impair an individual's ability to have offspring who can transmit the mutation, it can become established in the population, and such mutations are frequently called polymorphisms (Greek polymorphos, which means multiform) (Chakravarti 1999, Strachan & Read 1999). In genetics, the term "polymorphism" is used to indicate that a particular position in the DNA has more than one form in the population (Strachan & Read 1999). The term "allele" is used to refer to a particular form occurring at a given position, and the position is called a "locus." A genotype is a description of the two alleles at a particular locus. A distinction has been made between "variations" and "polymorphisms" in that a locus is only called polymorphic if the most common variant occurs in $< 99\%$ of the population and consequently all other variants occur with a total frequency of $\geq 1\%$. The term "mutation" refers to a rare variant that is the primary cause of a clinical phenotype or disease, whereas the term "polymorphism" is used to denote a variant present in the population in a relatively high frequency. Polymorphism itself is not sufficient to cause a disease, although it may contribute to susceptibility to a disease or variation in functional properties of a protein (Romero et al. 2002).

There are different types of variations in DNA. The simplest is a single nucleotide polymorphism (SNP). This is a variation in the identity of a single nucleotide at a particular site in the genome (Chakravarti 1999, Weiss & Terwilliger 2000, Fallin et al. 2001, Tabor et al. 2002). SNPs are classified according to their position in or around genes into coding and noncoding polymorphisms (Chakravarti 1999). Noncoding polymorphisms may occur in the promoter region of the gene, within introns, 5′- and 3′- untranslated regions (regions of the gene that are present in the mRNA but do not code for protein), and intergenic regions (Strachan &Read 1999). Noncoding polymorphisms do not alter the base pair sequence in the part of the DNA that codes for the protein but may alter the rate of transcription, the processing, or the stability of the mRNA. They may change the amount of protein generated by translation. Coding SNPs are classified into synonymous and nonsynonymous (Chakravarti 1999, Weiss & Terwilliger 2000, Fallin et al. 2001, Tabor et al. 2002). Synonymous polymorphisms change the codon into another that codes for the same amino acid. Therefore, there is no change in the structure of the protein.

Nonsynonymous SNPs change the codon to one specifying for a different amino acid and therefore may change protein structure. In addition there are more complex type of DNA variations, microsatellite repeats and minisatellite repeats which consist of repeated units of nucleotides of variable lengths (Strachan & Read 1999).

1.3.1 DNA markers for genetic mapping

To identify polymorphisms in the genome by genetic mapping DNA markers are required. Minisatellites and microsatellites DNA markers are used to identify polymorphisms in the genome. They are highly polymorphic and microsatellites are preferred as a marker since they are evenly spaced across the genome and PCR has made mapping using microsatellites relatively quick and easy. Mapping genes with SNPs has also become easy using high throughput methodologies such as DNA microarrays. There could be 3 genotypes for a single SNP among individuals in a given population. A SNP which generates a restriction fragment length polymorphism (RFLP) can be easily typed by PCR. A sequence including the variable restriction site is amplified; the PCR product is incubated with the appropriate restriction enzyme and then run on a gel to analyse the digestion products.

1.3.2 Mapping genes in complex diseases

There are several main approaches that have been used in the search for the genes involved in complex diseases

Candidate gene approach

In the candidate gene approach, genes with a known or proposed function with potential to influence the disease phenotype are investigated for a direct role in the disease. Here, a candidate gene encodes a protein of known function which, if mutated, could account in part for the pathogenesis of the disorder. The success of this approach depends on knowing enough about the possible molecular pathogenesis of the disorder to make an educated guess as to candidates. These approaches can be applied without any prior knowledge of the biological basis of the disease using genome-wide studies, combined with the candidate gene approach and comparative analyses using animal models of disease. The most successful application of the candidate gene approach to mapping complex diseases in humans has been with the HLA region. Genes in the HLA region have been implicated in the aetiology of over 200 diseases (Svejgaard 1996, Thorsby 1997). These include:

complex autoimmune diseases such as type 1 diabetes, rheumatoid arthritis and multiple sclerosis; cancers such as Hodgkin's disease; infectious diseases such as malaria, tuberculosis and AIDS; and other diseases such as narcolepsy.

Parametric linkage analysis

Parametric linkage analysis depends on following the inheritance of genetic markers in extended pedigrees to look for cosegregation of marker alleles in affected individuals, under a defined model of inheritance. The logic is that if a gene somewhere in the genome is responsible for the disease, affected family members are expected to have inherited the same disease-predisposing allele at the locus, and markers that lie physically near this disease gene will be transmitted with the disease allele (Risch 2000). This requires a precise genetic model, detailing the mode of transmission, gene frequencies, penetrance, dominance, and genetic heterogeneity. This is successful in mapping genes for Mendelian diseases. In the context of complex diseases this method is successful in a subset of cases where alleles are segregating in a Mendelian fashion. *(BRCA1* and *BRCA 2* genes)

Non-parametric analysis

This method does not require a specific genetic model. These methods ignore unaffected people and look for alleles or chromosomal segments that are shared by affected individuals. Shared segment methods can be used in whole populations, within nuclear families and within known extended families.

Population association studies

Association studies are usually carried out in populations and based on the assumption that affected individuals carry a particular marker allele more frequently than unaffected members of the control group (tests for co occurrence of marker and disease) (Khoury et al. 1993, Cardon & Bell 2001). Association may also be due to linkage disequilibrium which can occur because an ancestral mutation responsible for the disease in a population occurred on a chromosome carrying the associated allele at a closely linked site. Linkage disequilibrium is a population association between a marker and a disease allele (Khoury et al. 1993). The most commonly applied strategies for association studies are case control study designs (Cardon & Bell 2001). The advantage of this approach is that cases are readily available, can be efficiently genotyped and compared with control populations.

However, the results of case control studies are often inconsistent and this can be attributed to number of factors including clinical heterogeneity (associations are observed with certain subsets of the disease), genetic heterogeneity (it should be expected to find ethnic differences in association studies) and study design (small sample size, poor quality control of genotyping data and inappropriate selection of controls which are not ethnically matched will lead to spurious associations).

Family based association studies

A study design unique to genetic epidemiology is the "family-based association" design (Risch & Merikangas 1996, Collins-Schramm et al. 2002, Cardon & Bell 2001). Genetic association methods developed have focused on the need to account for population stratification. Family-based association studies use relatives of cases as controls, such as unaffected siblings or the non-transmitted alleles from parents.

Affected sibpair analysis (ASP)

This is usually based on affected sib pairs (ASP). Identity by state (IBS) alleles look the same and may have the same DNA sequence but are not derived from a known common ancestor. Identity by descent alleles (IBD) are demonstrably copies of the same ancestral (usually parental) allele. A pair of siblings are expected to share 0, 1 or 2 parental haplotypes with percentage frequency of 25%, 50% and 25%. If the diseased siblings carry a mutant allele at a particular locus and if the disease is dominant they will share at least one parental haplotype and if the disease is recessive they will share both haplotypes (Strachan & Read 1999).

Patients and parents (Trio Model)

A commonly used family based association study is heterozygous parents and affected cases using transmission disequilibrium test statistics (TDT) (Dahlman et al. 2002). The test examines the transmission of potential disease alleles from a parent who is heterozygous for the marker to an affected offspring. It is a test of association only in the presence of linkage, and because family members act as controls, spurious associations due to population differences do not arise. The original test uses a single affected offspring and both parents. A number of modifications to the original TDT have been proposed that allow

parents and an unaffected sibling to be analysed, making maximum use of incomplete nuclear family data.

1.4 Genetic basis of diabetes

Twin studies in type 2 diabetes have repeatedly shown that the concordance rate (the parameter that is usually measured in twins which describes the percentage proportion of identical twins to suffer from the same disease) in monozygotic twins is higher than that of the dizygotic twins, which ranges from 34% to 58% in monozygotic twins and 10% to 16% in dizygotic twins (Newman et al.1987, Kaprio et al.1992). According to the fundamental twin theory, monozygotic twins are genetically identical (Neale & Cardon 1990), and therefore if the compared disease rate in monozygotic twins is significantly higher than in dizygotic twins, this indicates that genetic factors are important in the disease etiology. If the rates are approximately equal this would infer that environmental factors predominate. Twin studies in Finland and Denmark, which were based on National Twin Registries (Hawkes, 1997) support the theory of additive genetic factors and shared and unshared environment factors, which may be the cause of type 2 diabetes (Kaprio et al. 1992).

Further evidence supporting a genetic basis for type 2 diabetes is provided by the fact that prevalence of diabetes varies in different ethnic backgrounds. It is believed that the unique genetic composition of each population may interact differently with environment factors for the development of the disease. The highest prevalence (48%) of type 2 diabetes of any population in the world is observed in the Pima Indians of Arizona (King & Rewers 1993, Pratley 1998). A high prevalence of 30.2% was observed among Nauruans (Diabetes Atlas 2003) and 30% of aborigines in Australia when compared to 4% in the general population (Australian Institute of Health and Welfare 2003)

The prevalence of diabetes in the USA is 6.3% (National Diabetes Statistics fact Sheet NIDDK 2003) and 5.1% in the British population (Joint Health Survey Unit 2008). In the USA 8.7% of the people over 20 have diabetes, which is approximately 18 million people and an estimated 5.2 million people in the USA have undiagnosed type 2 diabetes (National Diabetes Statistics fact Sheet NIDDK 2003). The prevalence of type 2 diabetes is 11.4% of all non Hispanic Black and 8.2% for Hispanic Latino Americans over 20 years of age in the US (National Diabetes Statistics fact Sheet NIDDK 2003). 3% of women and 4% of men

were reported to have type 2 diabetes in Canada (Statistics Canada, National Population Health Survey, Health Canada, 1996/97) A low prevalence of 2% is seen in Iceland and 2.7% in China (Diabetes Atlas 2003). Recent findings suggest that the prevalence of type 2 diabetes in Sri Lanka is 14.2% in women and 13.5% in men (Wijewardena et al. 2005). Impaired glucose tolerance has risen from 5.07% in 1992 to 14.2% in 2005 in the adults between 35-65 years (Fernando 1992, Wijewardena et al. 2005). A similar cross sectional study conducted in Sri Lankans ≥ 20 years of age revealed that the age-sex standardized prevalence of diabetes is 10.3% males and 10.9% for females. This study also highlighted that 36% of the people with diabetes were previously undiagnosed. The prevalence of pre-diabetes (IFG, IGT and IFG+IGT) was 11.5%. This study forecasts the prevalence of diabetes, pre-diabetes and overall dysglycaemia for the year 2030 would be 13.9%, 13.1% and 26.2% respectively (Katulanda et al. 2008).

The prevalence of diabetes can increase as members of populations move from one geographical area to another. For example, the prevalence of diabetes has been observed to increase in Japanese as they moved from Hiroshima to Hawaii, and then from Hawaii to Seattle (Gorden 1997). As another example, the prevalence of diabetes in South Asian immigrants to UK was reported to be 20-30% in the age 40-70 years age group in 1991 (Jarrett 1991), whereas the prevalence of type 2 diabetes in the same ethnic groups in Singapore was 7.5% for Chinese and 14.7% for Malaysians at the same point in time (King & Rewers 1993).

A study in people of African descent, who reside in geographically diverse environments, provided an opportunity to examine the effects of environment and genetic factors which contribute to the predisposition of the disease (Osei et al. 1993). The populations examined were glucose tolerant first degree relatives of African American and Nigerian type 2 diabetic patients living in US and healthy control subjects living in their native countries. It was clearly observed that geographical differences exist in insulin secretion (the mean fasting serum insulin levels were significantly greater in African American relatives than controls 16.0 ± 3.0 vs 6.3 ± 1.4 mU/L) and possibly also in metabolism in African immigrants living in the industrialized world compared to native Africans (Osei et al.

1993). These data provide evidence for variation in the interactions between genetics and different environmental conditions.

1.4.1 Impact of lifestyle on diabetes prevalence

Life style clearly has a profound effect on the prevalence of diabetes. For example, prevalence increases as people move from a rural environment to an urban environment. This has been attributed to the changes of traditional lifestyle from rural to westernized lifestyle in urban areas, such as increased food intake and reduced physical activity. Evidence supporting this was found in rural and urban Polynesians of Western Samoa, in which populations the prevalence of diabetes was 3.6% and 10.0% respectively (Permutt 1990). In rural communities in Mexico the prevalence of type 2 diabetes in 1990 was 0.9 - 3.0% in contrast to urban Mexican populations in which the estimate was 6.7 - 8.7% (Permutt 1990). The prevalence of type 2 diabetes in rural communities in Sri Lanka was only 2.5% in 1990 (Illangasekera et al. 1994), but this had risen to 8.5% by 2000 (Illungasekera et al. 2004) and 8.7% in 2006 (Katulanda et al. 2008). The prevalence rates in Sri Lankan urban communities have risen from13.9 % in 2005 (Wijewardena et al. 2005) to 16.4% in 2006 (Katulanda et al. 2008). The absence of type 2 diabetes mellitus in the traditional indigenous communities from Durango, Mexico, Tepehuano, Huichol and Mexicanero tribe members who do not have racial admixture and a minimal Western influence on lifestyle suggests that this disease is rare in traditional indigenous communities. It is assumed that this group of people in Mexico may be associated with less exposure to environmental risk factors or genetic susceptibility (Guerrero-Romero et al. 1997). According to King and Rewers prevalence of diabetes among certain ethnic groups in rural and urban areas is presented in figure 1.1. (King & Rewers 1993).

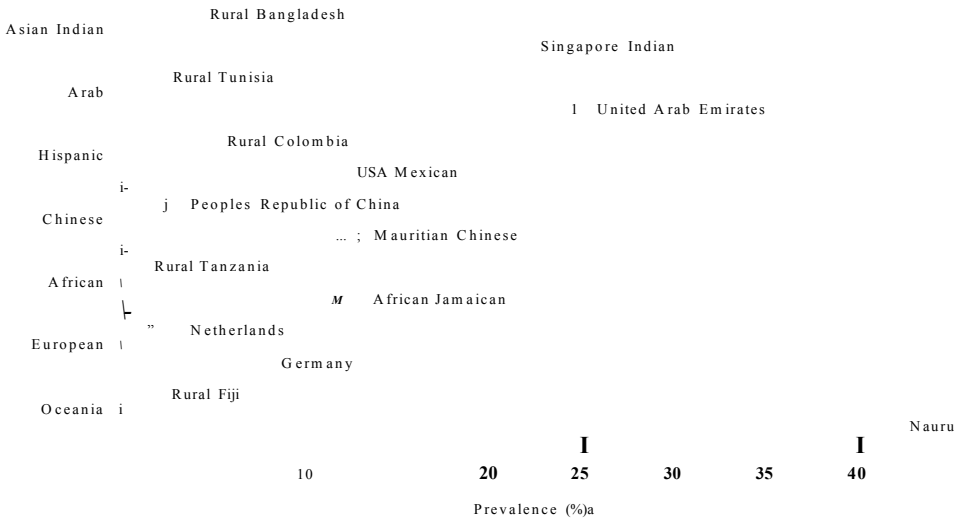


Figure 1.1. Prevalence of type 2 diabetes within the same or similar ethnic groups living under different geographical regions and environments

Figure 1.1 highlights the large range of type 2 diabetes prevalence even within the same or similar ethnic groups when living under different geographical and environmental conditions (King & Rewers 1993 adapted from Diabetes Atlas 2003).

1.4.2 Thrifty genotype and thrifty phenotype hypotheses

James Neel, in 1962, first suggested the concept of the thrifty genotype (Neel 1962). Based on his observations in Pima Indians, he assumed that so-called “thrifty genes” which increase susceptibility to diabetes, might have provided some form of evolutionary advantage for survival during the periods of starvation which regularly confronted our ancestors. Such a genotype would permit for storage of energy in the form of lipid during times of plenty to be utilised for survival in times of starvation. However, although this would provide an evolutionary advantage to our hunter-gatherer ancestors, in modern day times of plenty, and in a more sedentary society, this genotype instead increases susceptibility to diabetes (Turner et al. 1993). As a very rapidly occurring and recent example of this, during the last 100 years Nauruans in the South Pacific have developed extreme obesity, insulin resistance and type 2 diabetes as a result of rapid introduction of Western lifestyles (Turner et al. 1993). The mechanism of action of thrifty genotype in different ethnic groups has been suggested. They may operate via selective tissue insulin resistance.

In addition to the thrifty genotype hypothesis, the thrifty phenotype hypothesis has also been proposed. This suggests that during gestation and the early postnatal period of life an individual becomes programmed for nutritional thrift in order to adapt to and survive in an environment of limited resources and poor nutrition (Barker et al. 2002, Wells 2007). This hypothesis raises questions regarding the extent to which the associations between nutrition, birth weight and adult health outcomes such as type 2 diabetes can be explained by common genetic factors or whether they result from foetal programming by the early environment (Hales et al. 1991, Philips 2004). Once established, this acquired metabolic phenotype is maintained throughout the lifetime of the individual and does not change. Thus, type 2 diabetes and related symptoms arise if the metabolic program is set to "thrift" during early life and does not "match" the Westernised environment that an individual may encounter later in life. An example in support of this hypothesis is a study conducted in the Netherlands ("Dutch Hunger Winter Study") that found a significantly higher incidence of obesity in a cohort of young adults whose mothers had been exposed to famine during their first two trimesters in 1944-1945 (Ravelli et al. 1976). The intrauterine environment appears to be a strong determinant of the body fat mass of an individual later in life (Wells et al. 2007). These findings led to the conclusion that the future functioning of hypothalmic centers regulating food intake is influenced by the amount of calorie intake at crucial times during development supporting early foetal programming (Wells et al. 2007).

1.5. Thrifty epigenotype hypothesis

A recently established thrifty epigenotype hypothesis states that the metabolic thrift, which is the capacity for efficient acquisition, storage and utilization of energy, is a complex trait. It further states that the environmentally responsive gene network of this trait is subjected to genetic canalization. According to this mechanism polygenic traits such as type 2 diabetes are insensitive to allelic variations which are buffered and protected against these changes (Stoger 2008). Furthermore, this model predicts most of the polymorphisms identified in candidate genes play a minor role in aetiology in type 2 diabetes and obesity. Moreover, the hypothesis states that disease susceptibility is mainly due to epigenetic variations which are heritable variations that occur in the DNA structure (Methylation of CpG islands and modification of histone proteins) without that information being encoded in the nucleotide sequence of the gene (Bernstein et al. 2007).

1.5.1 Genetic information about diabetes based on family studies

The positive family history observed in patients with type 2 diabetes also provides evidence of shared environmental and genetic factors among relatives. Familial aggregation of type 2 diabetes is observed in Japan (Ryoko et al. 1994), India (Ramachandran et al.1988, Viswanathan et al.1985), Tunisia (Arfa et al. 2007) and the USA (Valdez et al. 2007). It has been indicated that people with a family history of diabetes have a two to six times risk of developing type 2 diabetes than those without (Harrison et al. 2003). Moreover, some European populations have shown excess of mothers' influence in the disease transmission (Arfa et al. 2007, Ramachandran et al. 1988, Alcolado & Alcolado 1991, Scott et al. 1992, Thomas et al. 1994, Ryoko et al. 1994, Gonzalez-Ortiz et al. 1997) but not in other populations (Viswanathan et al. 1996, Osei 1999). Possible ethnic and racial differences have been suggested to explain the absence of excess maternal influence in some communities (Viswanathan et al. 1996). These aspects are discussed in further detail in chapter 2.

1.6 Type 2 diabetes mellitus and mechanisms of tissue damage

Type 2 diabetes is associated with a wide range of complications that increase morbidity and mortality in affected individuals. These complications result from abnormal regulation of glucose metabolism. Long term, systemic complications of diabetes may include microvascular disease, which may lead to devastating complications of diabetic retinopathy, potentially leading to loss of vision, and diabetic nephropathy leading to progressive renal dysfunction. In the macrovasculature, accelerated and aggressive atherosclerosis portends the development of premature cardiovascular and cerebrovascular events. Peripheral neuropathy and vascular disease leads to loss of limbs. In addition, there is increasing evidence that diabetes increases the susceptibility to progressive periodontal disease (Kannel et al. 1979, Terry 2000).

1.6.1 Hyperglycaemia induces tissue damage in diabetes

There are many mechanisms by which hyperglycaemia may induce diabetes complications. Many of these mechanisms are linked by the overproduction of superoxide by the mitochondrial electron transport chain. In cells exposed to hyperglycaemia, there is increased production of reactive oxygen species (ROS). In diabetes, cells have elevated intra-cellular glucose levels and thus more glucose is oxidised in the TCA cycle which

pushes more NADH and $FADH_2$ into the electron transport chain. As a result, the potential across the mitochondrial membrane increases until a critical threshold is reached. At this point, electron transfer inside the mitochondrial complex III is blocked, causing electrons to back up to coenzyme Q which donates electrons one at a time to molecular oxygen, thereby generating superoxide. The mitochondrial isoform of the enzyme superoxide dismutase (MnSOD) degrades this free radical to hydrogen peroxide (H_2O_2) which is then converted to H_2O and O_2 by other enzymes. Hyperglycaemia-induced mitochondrial superoxide production activates damaging pathways inside a cell by activating initially poly (ADP-ribose) polymerase (PARP) due to strand breaks in nuclear DNA and this inhibits glyceraldehyde 3 phosphate dehydrogenase (GAPDH) activity of glycolysis. As a result, glycolytic intermediates accumulate inside a cell (Brownlee 2005).

In the hyperglycaemic state, glucose accumulates in the cell. Cells utilize intracellular glucose by the polyol pathway via the enzyme aldose reductase (Lee & Chung 1999). Under homeostatic, normoglycaemic conditions, this enzyme has a low affinity for glucose and processes little substrate. However, in hyperglycaemia the production of sorbitol increases, which is later oxidised to fructose. In this process, the enzyme consumes the cofactor NADPH which is a critical cofactor required in the regeneration of the antioxidant, glutathione. Thus, due to a lack of glutathione (which is a critical in removing reactive oxygen intermediates from cells), oxidative stress develops inside cells (Brownlee 2005). In support of this, in certain populations, recognized polymorphisms in the aldose reductase gene have been associated with increased prevalence of diabetes complications (Heesom et al. 1997).

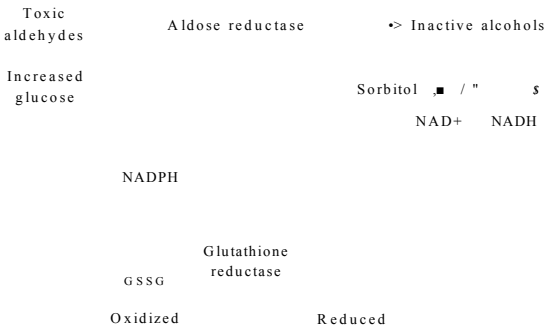


Figure 1.2 Influx of glucose through polyol pathway

Figure 1.2 shows under hyperglycaemic conditions increases glucose flux through polyol pathway resulting in reduced levels of glutathione and increased oxidative stress. (Brownlee 2001).

1.6.2 The role of AGEs (advanced glycation end products) in tissue damage

A second mechanism by which glucose is utilized in cells is through production of advanced glycation end products (AGEs). AGEs appear to damage cells by three mechanisms. Firstly, inside endothelial cells they tend to modify intracellular proteins, most importantly the proteins involved in the regulation of gene transcription (Giardino et al. 1994). Secondly, these AGE precursors can diffuse out from the cells and modify extracellular matrix molecules nearby (McLellan et al. 1994). This changes the signaling between the matrix and the cell and causes cellular dysfunction. Thirdly, AGE precursors diffuse out and modify circulating proteins in the blood. Interaction of these modified products with AGE receptors (RAGE) induces multiple effects in the target cells (Vlassara et al. 2002) (Figure 1.3).

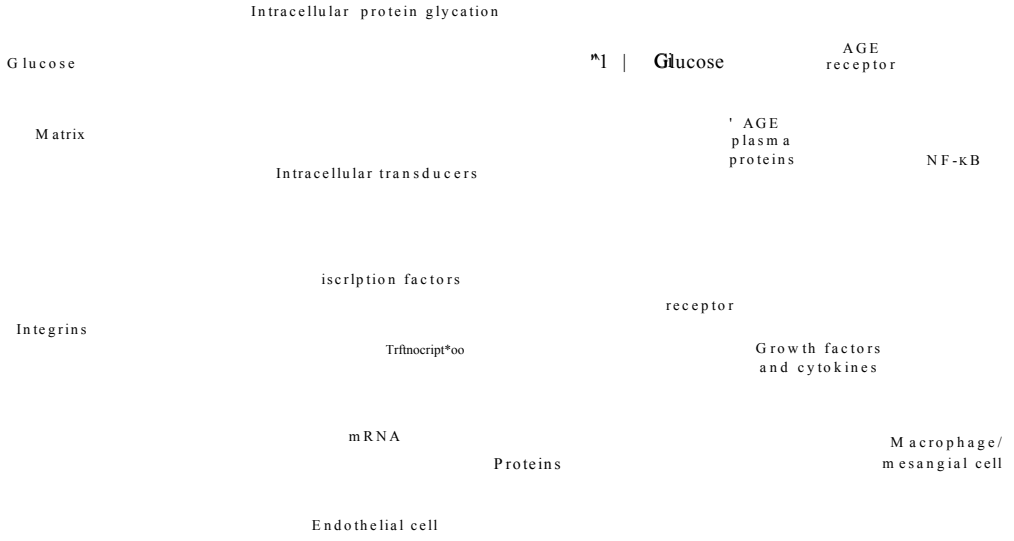


Figure 1.3 Effect of AGE precursors on nuclear factors and transcription factors, extracellular matrix and macrophages that causes pathological consequences (Brownlee 2001).

Interaction of AGEs with RAGE leads to various effects including a reduction of enzymatic activity, damage to nucleic acids, impaired degradation of proteins (mainly collagen), and an increase in collagen cross-linking, resulting in the thickening of the basement membrane. This leads to abnormal barrier function and integrity, which impairs oxygen diffusion, waste elimination, leukocyte migration and the diffusion of immune factors such as immunoglobulins and complement proteins. As a result of induction of cytotoxic pathways inside cells, ROS are generated leading to altered cellular function and activation of proinflammatory pathways inside the cell (Lalla et al. 2001), usually via nuclear factor-kB (NF-kB), an oxidative stress marker (Schmidt et al. 1994). These alterations in cellular phenotype result in an environment in which proinflammatory events favour the development and sustained progression of vascular lesions. Some studies indicate that susceptibility to diabetic complications may be influenced by genetic factors because environmental influences do not appear to explain all the variation in presentation of vascular disorders. Sequence variants within the RAGE gene may influence development of complications by altering the AGE-RAGE interaction.

1.6.3 PKC activation and the hexosamine pathway

A third mechanism by which cellular activities can be altered in hyperglycaemic cells is through activation of the PKC pathway. When glyceraldehyde 3 phosphate dehydrogenase (GAPDH) is inhibited by PARP, the level of dihydroxy acetone phosphate (DHAP) level inside the cell increases. This leads to increased levels of diacylglycerol (DAG) which activates the protein kinase C pathway inside the cell which has implications on vascular changes such as blood flow abnormalities (vasoconstriction increases), vascular and capillary occlusions and proinflammatory gene expression via activating NF-kB (Koya & King 1998).

A further mechanism by which hyperglycaemia mediates tissue damage is via the hexosamine pathway, in which fructose-6-phosphate (an intermediate of glycolysis) is converted to glucosamine-6-phosphate by glutamine fructose-6-phosphate amidotransferase (GFAT). Glucosamine-6-phosphate is then converted to UDP-N-acetylglucosamine which interacts with serine and threonine residues of transcription factors. Modification of the transcriptional factor Sp-I in this way results in increased expression of transforming growth factor (TGF-β1) and Plasminogen activator inhibitor-1 (PAI-1) resulting in damage to diabetic vessel walls.

1.7 Type 2 diabetes as an inflammatory disease

Pickup and Crook first proposed the concept that type 2 diabetes may be an inflammatory condition characterized by elevated concentrations of acute phase inflammatory reactants in the plasma such as sialic acid and proinflammatory cytokines such as TNF-α and IL-6 (Pickup & Crook 1998). A major source of these mediators in type 2 diabetes may be the adipocytes, and they are also produced by AGE-stimulated leucocytes (Hotamisligil et al. 1995, Vlassara & Palace 2002).

Hyperglycaemia results in inflammation and cellular oxidative stress by activation of NF-kB inducing proinflammatory pathways. Hyperglycaemia is now considered as a pro-inflammatory state. In support of this, *ex vivo* experiments on human monocytes have demonstrated increased levels of IL-6 and TNF-α production when cells are exposed to increasing concentrations of glucose (Morohoshi et al. 1995, Morohoshi et al. 1996).

Adipokines secreted by adipose tissue also contribute to inflammation. There is a wide range of molecules synthesized by adipose tissues (referred to collectively as adipokines). Some of these molecules serve as key mediators of energy management and metabolic homeostasis. However, in obesity, enlarging adipocytes begin to produce chemotactic signals (increased chemokine secretion) leading to macrophage recruitment and activation. Adipocytes constitutively release TNF-α in obese animals and humans (adipose and muscles) and a significant correlation between BMI and plasma TNF-α concentrations has been observed (Tsigos et al. 1999).

IL-6 and TNF-α both play a direct role in inflammation and immunity and are both produced by adipocytes. Other adipokines such as leptin, adiponectin, resistin and visfatin have interesting immunobiological properties (Preshaw et al. 2007). Leptin levels are upregulated and adiponectin levels are down regulated in type 2 diabetes (Dullaart et al. 2008). Both leptin and adiponectin are implicated in inflammation acting as pro-inflammatory and anti-inflammatory mediators, respectively (Preshaw et al. 2007). Leptin is also believed to contribute to diabetes pathogenesis by inhibition of insulin secretion and action (Girard 1997)

In addition to its pro-inflammatory role, TNF-α also contributes to insulin resistance. The action of insulin is mediated via its receptor, which belongs to the receptor tyrosine kinase family. Among a large number of intracellular substrates used by these receptors, six belong to the family of insulin receptor substrate (IRS) proteins (IRS-1 – 6). Insulin stimulated IRS protein activation is a crucial event in mediating insulin action. However, this step in insulin receptor signaling is defective in most cases of systemic insulin resistance both in experimental models and humans. The effect of TNF-α on insulin resistance is related to inhibition of serine phosphorylation of IRS-1 and IRS-2 by TNF-α. (Grossi & Genco 1998, Valverde et al. 1998).

Wound healing is delayed in diabetes, and this is also linked to pro-inflammatory events. Wound healing occurs in phases (inflammatory, proliferative, maturation) in which PMNs play a key role in the inflammatory phase to remove foreign materials and cell debris, followed by protein synthesis for tissue repair. Inadequate insulin impairs protein synthesis,

specifically collagen formation in the maturation phase. Increased collagenase in diabetes also contributes to delayed wound repair. Glycosylation of existing collagen (i.e. formation of AGEs) at the wound margins results in reduced solubility and delayed remodelling and increased collagenase activity readily degrades newly synthesized (less completely cross-linked) collagen at the wound site, further delaying the healing process (Lien et al. 1984).

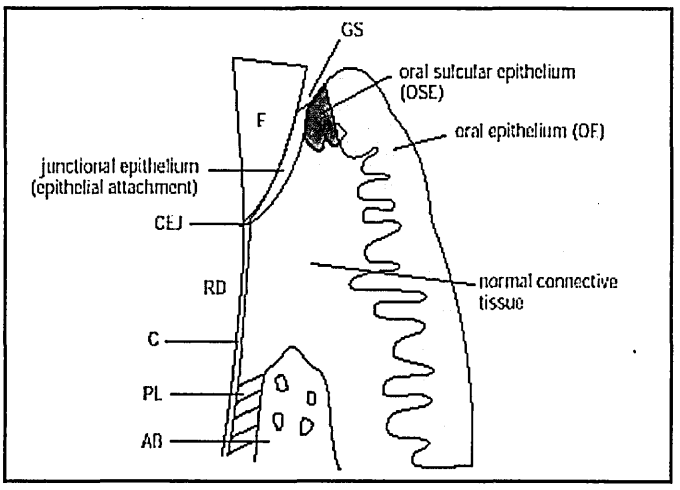
AGEs also contribute to inflammation and micro-chemotaxis experiments have demonstrated that movement of macrophages is arrested when AGEs are immobilized on the surface. AGEs, via interaction with macrophage RAGE may result in migration of macrophages toward an inflammatory focus, resulting in chronic macrophage activation. (McMullen et al. 1981, Lien et al. 1984).

1.8 Periodontal disease

Periodontitis is an inflammatory disease of the periodontium characterised by destruction of periodontal attachment tissues that can ultimately lead to exfoliation of teeth. The two most common periodontal diseases are gingivitis and chronic periodontitis.

In health, the gingiva is a band of specialized keratinized tissue mucosa closely adapted to the tooth. The junctional epithelium is the tissue that joins the soft tissues of the gingiva to the tooth and acts as a 'seal' between the oral environment and the underlying supporting tissues. The periodontal ligament, which consists of collagenous fiber bundles, is the connective tissue structure that attaches the roots of the teeth to the alveolar bone (figure 1.4). The gingival sulcus is normally 0.5-2.5mm in depth.

There is continuity of the oral epithelium, the sulcular epithelium and junctional epithelium. The connective tissue is comprised of well ordered densely packed collagen fiber bundles, and even in clinically healthy tissues, inflammatory cells such as neutrophils are usually present (though in low numbers) adjacent to the junctional epithelium (Preshaw 2004).



Caption

E Enamel
RD - Root dentin
AB - Alveolar Bone
CEJ - Cemento Enamel Junction
PL- Periodontal Ligament
C- Cementum
GS- Gingival Sulcus

Figure 1.4. A longitudinal section through dento-gingival part of a healthy tooth and its periodontium (Gillet et al, 1990)

Gingivitis is inflammation of the gingiva, and is the first stage of inflammatory changes in the gingival tissues. Gingivitis results from accumulation of dental plaque, a biofilm of many hundreds of bacterial species that produces a variety of waste products and proinflammatory antigens that penetrate the junctional epithelium and invoke an inflammatory response in the tissues. As a result of this, there is increased infiltration of the tissues by defense cells, breakdown of collagen fiber bundles, increased vascular permeability and vasodilatation, and the clinical signs of gingivitis thus become apparent. The clinical signs and symptoms include erythema, oedema, and bleeding on brushing and probing. Bleeding may be induced when the tissue at the depth of the gingival sulcus is gently manipulated by probing. It is a sign of inflammation which occurs as a result of ulcerated pocket epithelia. (Fermin 2002). Bleeding on probing is considered as a poor predictor of periodontitis. However, absence of bleeding on probing is a strong predictor of periodontal health (Lang et al. 1990) and has become a standard diagnostic test for identifying sites at risk for disease progression (Lang & Tonetti 1996). Treatment at this stage (removal of plaque and improved oral hygiene) will resolve the inflammation.

Periodontitis is extension of the gingival inflammation to involve the periodontal ligament and the alveolar bone (Scully 2004). As the junctional epithelium breaks down in response to the inflammatory challenge, it migrates apically along the root surface following destruction of the underlying connective tissue attachment. The pathologically deepened sulcus now constitutes a periodontal pocket. Periodontal pocket formation is one of the

most important clinical signs of periodontitis. A true pocket results from the apical migration of the junctional epithelium following loss of connective tissue attachment to the root surface. As the pocket deepens, the subgingival plaque biofilm proliferates down the root surface, providing a continued challenge, and propagating the inflammatory response. Inflammation extends apically and laterally, leading to increased collagen breakdown, further recruitment of inflammatory cells, and osteoclastic resorption of alveolar bone. Breakdown of the attachment apparatus results in tooth mobility. Clinical features include oedema, erythema, bleeding upon probing, and loss of clinical attachment and alveolar bone loss. Additional features include gingival recession and enlargement, root furcation exposure, increased tooth mobility, drifting and eventually exfoliation of teeth. The condition is usually painless but may be associated with bleeding, halitosis, and tooth mobility. The condition is diagnosed by clinical and radiographic examination. Severity can be characterized by measuring the depth of the pocket to determine the amount of clinical attachment loss (Wiebe & Putnins 2000). Radiographs are used to assess resorption of the alveolar bone (Figures 1.5 & 1.6).

Chronic periodontitis can be considered localized (<30% of sites involved) or generalized (>30% of sites involved). Severity is determined by the amount of clinical attachment loss (CAL) (Slight =1-2mm, moderate = 3-4mm, severe >= 5mm) (Wiebe & Putnins 2000). Although both gingivitis and periodontitis are inflammatory conditions, they are usually regarded as different disease entities. That is to say, while gingivitis is the precursor to periodontitis, not all gingivitis progresses to periodontitis (Lehner 2003). Gingivitis is a relatively simpler local inflammatory response to the accumulation of the plaque biofilm, and is so common as to be almost considered normal. However, periodontitis is much more complex, and the multifactorial model for the aetiology of periodontitis incorporates the role of personal, environmental and systemic factors along with the presence of the plaque biofilm and subsequent inflammatory responses to explain varying susceptibility to periodontitis (Clarke & Hirsch 1995, Haffajee & Socransky 1994). Most important risk factors for plaque associated gingivitis and periodontitis are ineffective oral hygiene, cigarette smoking and age (Pihlstrom et al. 2005). In addition, epidemiological studies, a number of case reports, cross sectional studies and a few longitudinal studies have shown that diabetes is a major risk factor for periodontitis (Mealy & Moitz 2003, Southerland et al. 2006).

(A) Healthy periodontium

(B) radiographic status of healthy periodontium

Figure 1.5 (A) healthy periodontium and (B) radiographic photograph of a healthy periodontium

(A) Clinical status of Periodontitis

(B) Radiographic photograph illustrating status of periodontitis

Figure 1.6 (A) clinical status of a patient with chronic periodontitis and (B) radiographic photograph illustrating alveolar bone loss in the same patient with periodontitis

1.9 Evidence for association between type 2 diabetes and periodontitis

Table 1.2. summarizes a number of studies conducted to identify associations between type 2 diabetes and periodontitis over the last two decades. Most of the earlier studies were conducted in the USA in Pima Indians, a population with a uniquely high prevalence of diabetes (Knowler et al. 1978). In one of these studies, 3219 subjects from this community were examined, and attachment loss and radiographic alveolar bone loss quantified. This study identified that subjects with diabetes had a higher prevalence of periodontitis than those without, irrespective of their age (Shlossman et al. 1990). Further studies conducted by Emrich *et al.* on a subset of dentate individuals in this population (n=1342) showed that diabetes mellitus increased the risk for periodontal disease by 2.8x when periodontal disease was defined by clinical attachment loss and 3.4x when defined by radiographic bone loss. This increased risk for periodontitis could not be explained on the basis of age, sex, oral hygiene or other oral measures (Emrich et al. 1991). In another study conducted in the same population, a significant association between diabetes mellitus and periodontitis was identified, and diabetes increased the risk for periodontal disease 2-fold independent of the confounding effect of age and local factors such as dental plaque and calculus. This study reported age and gender adjusted incidence estimates of periodontitis in subjects with type 2 diabetes to be 2.6x compared to those without type 2 diabetes (Nelson et al. 1990).

In addition to these studies conducted in the Pima Indians of Arizona, studies in Yugoslavia (Bacic et al. 1988), Minnesota, Brazil (Novaes et al. 1996), Sweden (Sandberg et al. 2000), Mauritius (Morton et al.1995), Hispanic Americans (Novak et al.2008), Australia (Cutler et al. 1999), France (Mattout et al. 2006), South Africa (Peck et al. 2006), China (Lu and Young 2004 & Hao et al.2007) Japan (Marugame et al.2003) and Jordan (Khader et al. 2008) collectively provide evidence that type 2 diabetes is associated with increased prevalence, severity and incidence of periodontitis.

Similar to type 2 diabetes, positive associations between type 1 diabetes and periodontitis have also been reported by some studies while others have reported no significant associations (Kjellman et al. 1970, Goteiner et al. 1986, Harrison & Brown 1987). Many of these studies were performed in young type 1 subjects in whom periodontitis may have not yet developed (Little et al. 1997). A definite relationship between type 1 diabetes and

periodontitis has been shown in studies of adult populations (Firali 1997). Furthermore, a recent review by Mealy and co-workers after analysing over 200 publications in relation to these two conditions concluded that diabetes (both type 1 and type 2) increases the risk for, and severity of, periodontal disease (Mealy et al. 2006).

Table 12 Summary of selected studies in literature on periodontal disease in association with type 2 diabetes mellitus

Table 1.2. Continued

Table 12 Continued

1.10 Periodontal disease, insulin resistance and dyslipidemia

To date molecules that are known to induce insulin resistance in humans are TNF-α, and free fatty acids (Boden et al. 1994). IL-6 is also reported to cause insulin resistance (Lagathu et al. 2003, Rotter et al. 2003) by selectively suppressing insulin action in hepatocytes (Klover et al. 2003, Senn et al. 2002). In people with diabetes, upon periodontal infection, tissue resident macrophages and Kupffer cells express increased amounts of IL-6 leading to stimulation of hepatocytes. This results in increased synthesis and secretion of acute phase proteins such as C reactive proteins (Gabay & Kushner 1999).

Activated Kupffer cells also express higher amounts of tumour necrosis factor alpha (TNF-α) resulting in increased insulin resistance in the liver. Patients with severe periodontitis exhibit increased IL-6 and C reactive protein levels, as compared with systemically and orally healthy controls (Loos et al. 2000). Additionally these inflammatory markers as well as TNF-α a levels decline with successful periodontal treatment (D'Aiuto et al. 2004, Iwamoto 2001, Iwamoto 2003). All these data support the fact that severe periodontal disease causes insulin resistance (Nishimura et al. 2001). The most probable target organ influenced by periodontal infection is hepatocytes in the liver as it is well known that CRP is produced by hepatocytes (Gabay & Kushner 1999). Circulating TNF-α may also cause insulin resistance in muscle cells (Ciaraldi 1998) and adipocytes (Szalkowski 1995).

Chronic inflammation and infection may also influence lipid metabolism (Khovidhunkit 2000). Infections by gram negative bacteria has been suggested to cause abnormal lipid profiles including increased concentrations of both very low density lipoproteins (LDL) and LDL cholesterol in rats (Lanza-Jacoby et al.1992) It has been suggested that the changes in the plasma lipoprotein composition might be attributed to altered hepatic synthesis, peripheral metabolism or hepatic uptake of lipoproteins and their remnants, indicating that hepatic function may play a key role in abnormal, infection induced lipid profiles (Lanza-Jacoby et al. 1992). Endotoxin itself also causes hypertriglyceridemia in an animal model (Gouni et al. 1993).

An association between periodontal disease and abnormal levels of triglycerides, LDL, cholesterol and total cholesterol in subjects with severe periodontitis has been reported (Katz et al. 2002). In addition a recent report indicated that intensive periodontal

treatment resulted in decreased total LDL cholesterol levels as well as a C - reactive protein value suggesting that periodontal infection and/or inflammation up-regulates the LDL-cholesterol level (D'Aiuto et al. 2005).

1.11 Two-way relationship between diabetes and periodontal disease

Diabetes is a major risk factor for periodontitis, and there is strong evidence to suggest that the incidence and severity of periodontitis is influenced by the presence or absence of diabetes (Bacic et al. 1988), the level of diabetes control (Tervenon & oliver 1993, Tervonen & Karjalainen, 1997), and diabetes duration (Hugoson et al. 1989). The data regarding the effect of diabetes mellitus on the oral cavity suggest that oral tissues are adversely affected similarly to other body systems, by uncontrolled diabetes mellitus (Bartolucci & Parks 1981, Seppala et al. 1997). In addition, animal studies in diabetic rats provide evidence that periodontal disease has a negative impact on metabolic control in diabetes (Andersen et al. 2006). Periodontal disease has been described as the sixth complication of diabetes mellitus (Loe 1993), and a variety of mechanisms link the two diseases, as described below.

PMN leucocytes are the primary defensive cells in the periodontium. Although some defects of PMN function are of genetic origin (Bisanda et al. 1982), patients with diabetes demonstrate impaired PMN chemotaxis, adherence and phagocyte function due to interaction of AGE with cell surface receptor RAGE. It has been observed that diabetic patients with severe periodontitis have depressed PMN chemotaxis compared to those with mild periodontitis or non-diabetic subjects with severe or mild periodontitis (Manouchehr-Pour et al. 1981). Furthermore, apoptosis of PMNs is delayed in diabetes, leading to prolonged retention of PMNs in the periodontal tissues, which likely results in increased tissue destruction as a result of extra-cellular release of destructive enzymes (Preshaw et al. 1999). An abnormal monocyte inflammatory response to lipopolysaccharides (LPS) challenge has also been shown to result in an exaggerated secretion of inflammatory mediators such as IL-1β in patients with diabetes (Offenbacher 1993, Salvi et al. 1997). Complicating matters further increased AGE deposition increases collagen cross linking in people with diabetes, leading to reduced flexibility of vessel walls and narrowing of the lumen. This impairs leukocyte migration into the tissues. An altered inflammatory response is probably the key link in the so-called bidirectional relationship between periodontitis and diabetes. The assumption underlying this concept is that changes in immunologically active molecules as a result

of diabetes disturb the finely balanced cytokine network within the periodontium, leading to increased tissue damage. Increased levels of inflammatory markers such as CRP and proinflammatory cytokines have been observed in systemic circulation in both periodontal and type 2 diabetes (Engebretson et al. 2007, Salzberg et al. 2006). In diabetes, binding of macrophage RAGE to AGE leads to an hyperinflammatory phenotype resulting in the upregulation of cytokine expression. With increased secretion of IL-6 and TNF-α there is an induction of oxidative stress (Schmidt et al. 1994). Macrophages that are already activated and primed by binding to AGE via RAGE demonstrate a magnified inflammatory response when challenged with LPS from periodontal organisms leading to greater destruction of periodontal connective tissue and increased periodontitis severity (Grossi & Genco 1998).

In addition to an effect of diabetes in periodontitis, it is likely that periodontitis has a negative impact on glycaemic control. It is already well recognised that acute infections cause increased insulin resistance. In periodontitis, inflammation results in local production of TNF-α and other inflammatory mediators, which could induce insulin resistance and poorer diabetes control. There is increasing evidence from controlled studies to indicate that severe oral infection of any type, including periodontitis may increase insulin resistance (Grossi and Genco 1998).

In support of this concept, significantly higher cytokine levels have been found in the gingival crevicular fluid (GCF) of diabetics compared with non-diabetics, and in those who suffer from periodontitis (Oliver & Tervonen 1993). Furthermore some studies have demonstrated a significant correlation of GCF IL-1β levels with level of glycaemic control. Patients with HbA1c >8% had significantly higher levels of GCF IL-1β levels than those with HbA1c < 8% (Engebretson et al. 2004). Since people with diabetes already have impaired immune defense mechanisms, they are more susceptible to infection and those infections are more severe when compared to people with normal glucose tolerance (Mealey 1999). There is evidence for decreased insulin-mediated glucose uptake by skeletal muscles if bacterial infections are present (position paper 1996). Infection-induced insulin resistance further complicates and increases hyperglycaemia, enhancing AGE formation, which in turn intensifies cytokine levels (via activation of macrophages via RAGE), contributing to a 'vicious circle' that is difficult to break and increases susceptibility to diabetic complications (Donahue 2001). A proposed bidirectional model whereby chronic periodontitis could magnify elevated

cytokines response associated with diabetes and its contribution to the overall burden of systemic inflammation is presented in figure 1.7.

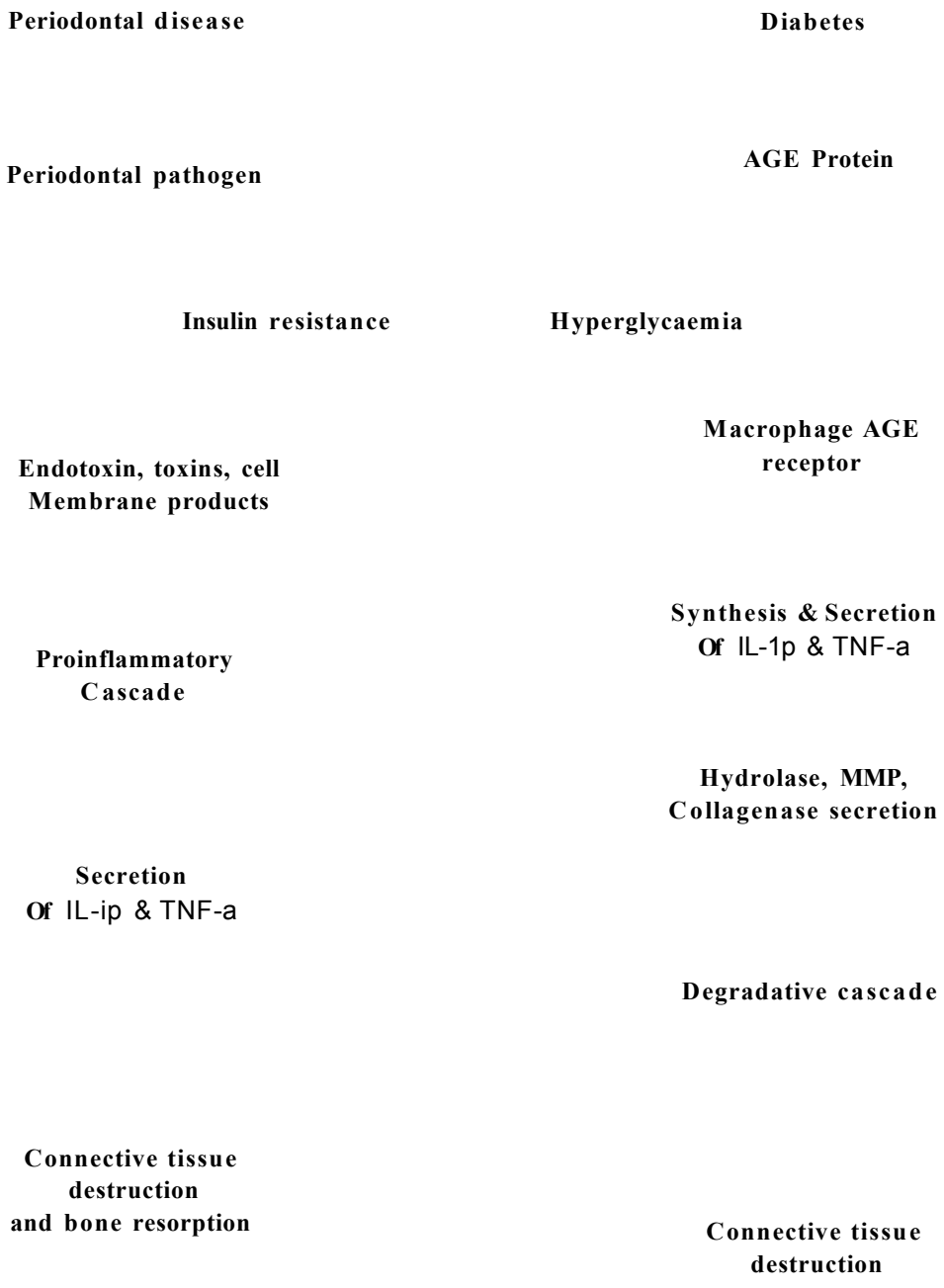


Figure 1.7 Proposed two way relationship between type 2 diabetes and periodontitis (Grossi & Genco 1998)

In patients with diabetes, hyperglycaemia induces formation of AGEs. Interaction of AGEs with blood monocytes/macrophages causes the secretion of IL-If, IL-6 and TNF-a. As a consequence a tissue degradative cascade is initiated. Host defense responses to periodontal infection proinflammatory cytokines are produced which will further intensify tissue breakdown.

1.12 Cytokines and periodontitis

Cytokines are signaling proteins that have an extensive role in cellular communication. They are effective in very low concentrations, produced transiently, act locally and are often self regulatory. They act on their target cells by binding to specific receptors and initiate intracellular second messengers resulting in phenotypic changes in the cell via altered gene regulation (Birkedal-Hansen 1993). Individual cytokines do not act in isolation; they act as a flexible complex network bringing together elements of both adaptive and innate immunity in the defense against infection and disease (Banyer et al. 2000).

Cytokines play a key role in the immune response in periodontal disease (Gemmell et al. 2000, Landi et al. 1997 & Okada & Murakami 1998). In response to periodontopathic bacteria, a variety of cell types in the periodontium including macrophages and B cells have been shown to secrete cytokines importantly IL-1β and TNF-α (Seymour et al. 2001). These proinflammatory cytokines have a key role in the initiation, regulation and perpetuation of the innate response in the periodontium (Alexander et al.1994, Birkdel-Hansen 1993, Darveau 2000 & Gemmell et al. 2000). This results in vascular changes and the migration of effector cells into the periodontium with appropriate immune response aimed at containing and eradicating periodontal pathogens. However, inappropriate cytokine responses can lead to inflammation and concomitant tissue damage which is mainly due to host inability to regulate the immune response. Other key inflammatory mediators include prostaglandins (e.g. prostaglandin E_2, PGE_2) and matrix metalloproteinases (MMPs) which can induce bone and connective tissue degradation (Kornman et al. 2000). The nature of the host inflammatory response is governed by genetic factors as well as environmental and local risk factors for the disease (Seymour and Taylor 2004). It is now recognized that the host immune-inflammatory response is the critical determinant in defining disease susceptibility and inter-individual variations observed in susceptibility to periodontal disease (Kornman 1997).

1.12.1 Genetic polymorphism in IL-1 and TNF cytokine genes

Duff and coworkers have revealed that many cytokine genes harbour genetic polymorphisms. This genetic polymorphism may directly influence complex common diseases via a direct effect on gene function (Duff 2006). Inter-individual genomic variations are mainly encoded by SNPs.

SNPs in *IL1A* and *IL1B and TNFA*

SNPs exists at *IL1A (-889)* position and *IL1A (+4845)* and in *IL1B* gene *IL1B (-31)*, *IL1B (-511)* and *IL1B (+3954)* positions (Bailly et al 1993). Inter individual variation in cytokine production by peripheral blood mononuclear cells has been observed (Enders et al. 1988, Molvig et al. 1988, Enders et al. 1989, Jacob et al. 1990, Danis et al. 1995). In particular the three genotypes of *IL1B* at *(+3954)* are associated with different levels of IL-1β production (Pociot et al. 1992). Elevated Gingival Crevicular Fluid (GCF) concentrations of IL-1β have been observed in the shallow sites of patients carrying risk allele (allele 2) (Engebretson et al. 1999). Monocytes from individuals homozygous for the *IL1B (+3953)* allele 2 produce four-fold more IL-1β and heterozygous cells produce approximately two fold more IL-1β than cells from individuals homozygous for allele 1 (Pociot et al. 1992).

IL1-α protein is reported to be present at very low level in healthy GCF. In addition to *IL1A (+4845)*, (Gubler et al. 1989) another polymorphism was detected at *(-889)* in the promoter region. Polymorphism analysis of *IL1A (-889)* variant with IL1-α levels in GCF implicated that in the diseased population there was at least a four fold increase in the mean level of IL-1α protein in patients who carried one or two copies of the allele 2 of the *IL1A (-889)* gene variant when compared with those who were homozygous for allele 1(Shirodaria et al. 2000).

Several polymorphisms within the *TNFA* gene have been characterised (Wilson et al.1992 & Alfonso & Richiardi 1994) and have been studied in association with periodontitis and type 2 diabetes. Wilson *et al* identified the genetic G/A polymorphism at the 308 nucleotide upstream of the transcriptional start site. This polymorphism affects the consensus sequence for a binding site of the transcriptional factor AP-2 (Abraham & Kroeger 1999). The rare allele of *TNFA (-308)* promoter polymorphism has been connected with a higher transcriptional activity (transcriptional factors

preferentially binds to rare allele) (Wilson et al. 1997) and leads to a two or four fold increase in transcriptional activity when stimulated with lipopolysaccharides (LPS) (Braun et al. 1996, Kroeger et al. 2000). It has also been observed that LPS stimulated TNF-α secretion from blood cells (neutrophils) is higher in individuals carrying homologous or heterologous allele 2 than subjects carrying homologous *TNFA* allele 1 (Galbraith et al. 1998). Therefore in response to a given bacterial challenge these individuals may produce more tissue destructive TNF-α levels than those who carry wild type allele 1 (Folwaczny et al. 2004). Consistent with this, individuals who are homozygous for –308 A (allele 2) have higher circulating TNF-α than homozygotes for the –308 G (allele I) (Bouma et al. 1996).

1.12.2 Inter-individual variations in cytokine response in periodontal disease

A heritable component to both susceptibility and progression or severity of periodontal disease has been reported (Hodge & Michalowicz 2001, Baker & Roopenian 2002, Schenkein 2002). A continuum of variation in the development and progression of periodontitis has been observed in different individuals. Inter-individual variation in periodontal microflora and the immune inflammatory response to the presence of subgingival plaque (Offenbacher 1996), cytokine synthesis and secretion to standard stimuli (Molvig et al. 1988, Enders et al. 1989) and cytokine polymorphisms have been observed.

The concept of population dichotomy of high and normal responders with respect to IL1 secretion has been reported (Endres et al. 1989). To a standard stimulus, high responders will produce elevated quantities of inflammatory mediators and destructive cytokines. As a result, for a given bacterial challenge, this group of individuals is more susceptible to periodontitis than normal responders who produce minimal levels of inflammatory mediators (Position paper 1996).

1.12.3 Association of cytokine gene polymorphism and periodontal disease

Several recent reports provide evidence that cytokine gene polymorphism may influence the severity of chronic periodontitis (Kornman et al. 1997, Galbraith et al. 1998, Gore et al. 1998). A study by Gore and coworkers also reported that allele 2 of the *IL1B (+3954)* genotype was significantly increased among patients with chronic periodontitis (Gore et al. 1998). An investigation of IL-1 genotype and periodontal disease in a diabetic population has revealed an association of *IL1B (-511)* genotype with

periodontal disease in African American patients. The same study reports a borderline significant association between *IL1B (+3954)* and periodontal disease in Caribbean periodontal patients (Guzman et al. 2003). No significant difference was found between early onset periodontitis and either *IL1B (+3954)* polymorphism or *IL1A (-889)* polymorphism in European White Caucasian population (Hodge et al. 2001).

Gore et al found no significant association of *IL1A (-889)* polymorphism with chronic periodontitis (Gore et al. 1998). In order to address the question of how cytokine (IL-1) polymorphism may affect the cytokine profile with patients with chronic periodontitis, Havemose-Poulsen *et al* investigated genotypes of *IL1A (-889)*, *IL1A (+4845)*, *IL1B (-511)* and *IL1B (+3954)*. It has been found that allele 2 of *IL1B (+3954)* is associated with lower levels of IL1-α, IL-6, IL-10 and TNF-α levels (Havemose-Poulsen et al. 2007). Recently conducted meta- analysis of this polymorphism has indicated a moderate positive association between the *IL1A (-889)* variant and chronic periodontitis in Caucasians (Nikolopoulos et al. 2008).

1.12.4 The role of periodontal associated genotype (PAG) and pathogenesis of periodontal disease

Kornman et al. observed a significant association of aggressive periodontitis in non smokers, when considered together with the presence of at least one copy of the rare allele of both *IL1A (-889)* and *IL1B (+3954)* polymorphism (Kornman et al. 1997). This genotype was named as the periodontal associated genotype (PAG). The currently used two SNP positions in PAG definition are *IL1B (+3954)* and *IL1A (+4845)*. Papapanou and coworkers also discovered the association of PAG with severity of periodontal disease as measured by attachment loss (Papapanou et al. 2001). However, research so far conducted in PAG in association with susceptibility to periodontitis has generated conflicting results. A Meta analysis performed considering 13 studies published on composite genotype concluded that the rare allele of *IL1A (-889)* and rare allele of *IL1B (+3954)* positive genotype has significant association with chronic periodontitis. This analysis has revealed that the positive genotype is more frequent among periodontitis cases compared with controls, even though the estimates were marginally insignificant. However, Caucasians carrying the positive genotype (*IL1A (-899)* and *IL1B (+3954)* loci) had a statistically significant elevated risk of developing chronic periodontitis compared with the separate effect of each SNP. It also documents an elevated risk for *IL1B (+3954)* allele 2 carriers particularly in Asian patients (Nikolopoulos et al. 2008)

based on the observation of a study conducted in India to access the SNPs at *IL1A (+4845)* and *IL1B (+3954).* This study found associations of the IL-1 genotype as a risk factor for severe chronic periodontitis (Agrawel et al. 2006). Moreover, the IL-1β levels in shallow periodontal pockets have been reported to be higher in PAG positive patients compared to PAG negative patients (Engebretson et al. 1999). Another longitudinal study has observed that PAG is associated with the extent of tooth loss (McGuire et al.1999) but some other studies were unable to repeat the results (Cattabriga et al. 2001 & Ehmke et al. 1999).

1.12.5 Smoking and IL-1 genotype in association with periodonttis

Smoking is considered as an increased risk factor for periodontitis (Tomar & Asma 2000). Smokers demonstrate more severe periodontitis and are resistant to periodontal therapy (Kinane & Chestnutt 2000). It is also known that smoking elevates serum LDL/TRG levels (Vincelj et al. 1997). Therefore smoking could obscure the polymorphism related increase in risk. In a study of patients with aggressive periodontitis, Parkhill and co-workers suggest that an *IL1B (+3954)* genotype in combination with smoking and a combined *IL1B (+3954)* and *IL1RA* genotype are risk factors for aggressive periodontitis and support a role for genetic and environmental factors in susceptibility to aggressive periodontitis (Parkhill et al. 2000). Kornman in his study found that severity of disease was correlated in non-smokers with chronic periodontitis and suggested that non-smoking PAG positive subjects had an approximately 7 times greater chance of having severe periodontitis than those who were PAG-negative (Kornman et al. 1997). Contradicting these results Meisel and coworkers reported smokers bearing PAG positive genotype (*IL1A (-889)* and *IL1B (+3954)*) have an increase risk of periodontitis in a study conducted in Germany (Meisel et al. 2004). It has also been demonstrated that heavy smoking and positive genotype act as synergistic risk factors for earlier tooth loss (Meisel et al. 2002). However a study conducted by Laine et al. found PAG was not significantly associated in either smokers with chronic periodontits or non smokers with chronic periodontitis (Laine et al. 2001).

The role of smoking in the pathogenesis of periodontal disease is uncertain, One possible mechanisms may include altered subgingival microflora (Zambon et al. 1996), vasoconstriction of gingival microcirculation (Clarke et al. 1981) and decreased immune cell function and impaired inflammatory response (Johnson et al. 1990). *In*

vitro studies have addressed the potential alteration of cytokine production in smoke exposed macrophages. There are variable reports of suppressed or unaltered IL-1β secretion from macrophages in smokers (Soliman & Twigg 1992, Sauty et al. 1994). It has been suggested that smoking may not actually affect the synthesis of IL-1β but may suppress its release from cells (Soliman & Twigg 1992). Furthermore, Berzweig and colleagues observed that nicotine significantly down regulates IL-1β secretion by gingival mononuclear cells (Bernzweig et al. 1998). It has been suggested that allele 1 of the *IL1B (+3954)* SNP may have a synergistic immunosuppressive effect with smoking, thereby increasing susceptibility to aggressive periodontitis.

1.12.6 TNF- α and Periodontitis

TNF-α has a strong potential to increase bone resorption and is involved in the degradation of connective tissue. Therefore, it was assumed that individuals susceptible to periodontitis might be related to genetically determined differences in the TNF-α production and polymorphisms (Shapira et al. 2001, Craandijk 2002). Although the biological significance of these findings are not yet fully established it was proposed that this genotype could be a prognostic marker in periodontitis.

A Meta analysis of cytokine SNPs in association with periodontitis has revealed that 15 studies thus far have examined the *TNFA (-308)* polymorphism, the majority carried out in Caucasian populations (Nikolopoulos et al. 2008). The role of *TNFA (-308)* SNP was not found to be associated with disease severity in a study of 134 patients with chronic periodontitis (Kornman et al. 1997). Furthermore, Galbraith et al. studied 32 patients with chronic periodontitis but found no significant association with three separate SNPs (including the -308 SNP) in the promoter region of the *TNFA* gene (Galbraith et al. 1998). No significant disease associations were found in a study of four *TNFA* promoter SNPs in 90 chronic periodontitis patients (Craandijik et al. 2002) and *TNFA* microsatellite polymorphisms and aggressive periodontitis in a study of 91 patients (Kinane et al. 1999). There was no significant association between *TNFA* genotype and aggressive periodontitis in a Japanese population (Endo et al. 2001). A lack of association between *TNFA (-308)* promoter polymorphism and periodontal disease has been demonstrated in Germany (Folwaczny et al. 2004) and a Czech population (Fassmann, 2003). In summary all data indicates the lack of association between the *TNFA* alleles and susceptibility to chronic periodontitis. In contrast Kornman and di Giovine reported a higher *TNFA (-308)* allele 2 frequency in Caucasian patients with

chronic periodontitis than in healthy individuals. This difference was correlated with the severity of the disease. (Kornman et al. 1997, Kornman & di Giovine 1998). Similarly Galbraith et al. demonstrated that the *TNFA* allele 2 is a risk factor for the severity of adult chronic periodontitis (Galbraith et al. 1999) as its presence is detected in the most severe cases of the disease (Galbraith et al. 1998). More recently Lin et al demonstrated an increased frequency for the *TNFA* allele 2 in Chinese patients with chronic periodontitis (Lin et al. 2003).

1.12.7 Composite polymorphism of *IL1B (+3954)* and *TNFA (-308)*

Lin et al. have studied the correlation of these two polymorphisms and the genetic susceptibility to moderate or advance adult periodontitis. They revealed a relationship of *IL1B (+3954)* and *TNFA (-308)* positive genotype to disease status while adjusting for other potential confounders. The frequency of *IL1B (+3954)* (28.23%) and *TNFA (-308)* (29.03%) allele 2 positive genotype in patients with moderate-to-advanced aggressive periodontitis showed a statistically significant increase compared to the control group frequency of 13.95% *IL1B (+3954)* and 11.05% *TNFA (-308)* respectively (Lin et al. 2003). This study concluded that *IL1B (+3954)* and *TNFA (-308)* allele 2 positive genotype may increase the odds of having moderate-to-advanced aggressive periodontitis (Lin et al. 2003).

1.13 Summary

Type 2 diabetes and periodontal disease both are common complex diseases in which genetic factors and environment factors contribute to disease pathogenesis. High prevalence of periodontal disease has been observed in patients with type 2 diabetes and severity of the disease increases as the diabetes control decreases. Conversely, periodontal disease may have a negative impact on glycaemic control in people with diabetes. Both diseases are inflammatory conditions, and a multiplicity of excessive and dysregulated inflammatory processes are likely to link the pathogenesis of the two diseases. In Sri Lanka, type 2 diabetes prevalence rates are moderately high and the genetic basis for the disease has not been previously investigated. Furthermore, prevalence estimates do not exist for periodontitis in the diabetic population and further, the association of cytokine gene polymorphisms in relation to type 2 diabetes or periodontitis were not assessed in this population.

1.14 Aims and objectives of the study

This study was initiated with broad aims of establishing a database of patients with type 2 diabetes to study genetic epidemiology and provide background to the genetic basis of the disease and to study the relationship between diabetes and newly recognised diabetes related complication periodontitis. Constructing a DNA repository is one of the secondary objectives in order to conduct population association genetic research in related to diabetes.

There are several lines of evidence to indicate that type 2 diabetes is familiarly aggregated and there may be parental influence for the transmission pattern of the disease where mothers seems to play a predominant role. Therefore the initial studies investigated the family history of diabetes in patients with diabetes to see whether the disease is familiarly aggregated in the Sri Lankan population. This component of the study also investigated whether possible influence for the transmission pattern does exist from the maternal side in Sri Lankan population. It also evaluated whether the parental diabetes could influence the onset of diabetes in the offspring.

Generally tissues of people with diabetes are affected due to long term exposure to hyperglycaemia. As a consequence the immune system of these people is compromised and they are prone to more infections than those who have not developed diabetes. Periodontitis, a chronic complex disease is more common in patients with diabetes than normal healthy controls. Although the exact molecular mechanisms that link the two diseases are not known yet it is believed that periodontitis worsens the underlying metabolic imbalance in diabetes, and poorly controlled diabetes aggravates the severity of periodontitis. In this study it was hypothesized that periodontal disease is more prevalent in the type 2 diabetic population than the control population in Sri Lanka. In addition the severity of periodontal disease was expected to be higher in patients with diabetes than those without as measured by probing pocket depth. This study also aimed to investigate the effect of level of glycaemic control (as measured by glycosylated hemoglobin) with disease severity (as measured by probing pocket depth).

Kornman 1997 found that the presence of recessive alleles of both *IL1A -889, IL1B+3954* (PAG) is associated with periodontal disease and may be a risk factor in never smokers. It was suggested that SNPs within the IL-1 cluster increase the genetic risk of periodontal pathogenesis and population variation of PAG percentage and

association with periodontitis was observed. Therefore, one of the hypotheses to be tested is that PAG status may be a risk factor in the Sri Lankan population too.

Secretory TNF-α levels are high in patients with type 2 diabetes. Also it induces insulin resistance via interacting with the insulin receptor substrate. Inducing insulin resistance will lead to abnormal lipid metabolism. Therefore it was hypothesized that *TNF-(308)* promoter sequence SNP aggravates pathogenesis of diabetes by impairing the metabolic conditions of the affected individual. It was assumed that *TNF-(308)* genotypes may be influencing changes in blood chemical parameters, exposing them to more diabetes complications and therefore people with periodontitis with polymorphic allele may have more severe periodontal destruction than those without.

Chapter 2

Genetic epidemiology of type 2 diabetes in Sri Lanka

2.1 Introduction

The precise causative factors for type 2 diabetes remain obscure. Twin studies, family studies, population studies, some genetic admixture studies and certain monogenic forms of the disease provide evidence that genetic and environmental factors together contribute to the predisposition of the disease. If a single gene was the mode of inheritance, then we would expect a Mendelian pattern of inheritance of type 2 diabetes with complete penetrance. But, for diabetes, except for certain maturity onset diabetes in the young there is no clear Mendelian pattern of inheritance observed. Type 2 diabetes is therefore considered a multigenic disorder with a threshold in which individual genetic factors contribute additively with a major locus and relatively minor influencing loci. Environmental factors together with the genetic factors are thought to contribute additively and the disease develops when the susceptibility threshold is exceeded. Hence, type 2 diabetes develops through a progression of stages and manifests late in life rather than at birth (Granner & O'Brien 1992).

2.1.1 Evidence for familial aggregation of type 2 diabetes

In addition to the evidence for a general genetic susceptibility to type 2 diabetes as described in Chapter 1, increased risk of diabetes with increasing familial aggregation was first proposed by Steinberg (Steinberg 1959). In Japan, a reported positive family history increases risk for diabetes. In 1982, the reported familial aggregation was 43-49% for type 2 diabetes (Ryoko et al. 1994). In another study in India, the probability of diabetes developing in a person with only one diabetic parent was estimated at 36%, while the estimate increased to 54% if there was one diabetic parent and the other parent had a relative with diabetes (Ramachandran et al. 1988). The cumulative risk of developing diabetes by the age of 70 years increases from 41.3% - 64.2% by this additional influence of familial aggregation (Viswanathan et al. 1985). If both parents have diabetes there is an increased prevalence of type 2 diabetes in their offspring. The highest prevalence rate for diabetes among offspring of conjugal parents is reported in India (50%) and a further 12% had impaired glucose tolerance. Thus, 62% of offspring had abnormal glucose tolerance (Ramachandran et al. 1988). A recent study conducted in Tunisia reported that familial aggregation of diabetes was prominent among first degree relatives (70%) of the diabetic probands (Arfa et al. 2007).

2.1.2 Evidence of Maternal Influence on diabetes susceptibility

Recent studies on the parental history of type 2 diabetes provide evidence of maternal influence in the transmission pattern (Arfa et al. 2007, Ramachandran et al. 1988, Alcolado & Alcolado 1991, Scott et al. 1992, Thomas et al. 1994, Ryoko et al. 1994, Gonzalez-Ortiz et al. 1997). A sex-specific parental effect was observed on insulin and HDL concentrations (Grrop et al. 1996) and a sex-specific environmental and genetic effect transmitted differently between sexes was suggested by Mitchell and co workers in the USA (Mitchell et al. 1993). Evidence for a possible maternal influence on predisposition to gestational diabetes has been observed and this finding confirms that vulnerability to gestational diabetes is increased by exposure to an abnormal environment during intrauterine development (Martin et al. 1985).These results indicate that maternal influence may play a major role in the environmental and genetic predisposition to type 2 diabetes. Gonzalez-Ortiz and colleagues have studied the maternal effect of insulin sensitivity and metabolic profile in healthy young Mexicans and found that family history of maternal side increases both systolic blood pressure and serum uric acid levels in the probands without modification in their insulin sensitivity. Probands of this study had a tendency for higher triglyceride concentrations than controls (Gonzalez-Ortiz et al. 1999).

Mitchell tested the hypothesis of excess maternal influence for transmission of diabetes (Mitchell et al. 1995). His findings suggested that there is no evidence for parental differences in the transmission, if both parents and their offspring were actually tested for diabetes. He concluded that this apparent excess could be attributed to reporting bias. Absence of excess maternal transmission was observed by a family study in South India (Viswanathan et al. 1996) and the study of Kwame Osei (Osei 1999). Possible ethnic and racial differences have been suggested to explain the absence of excess maternal influence in some communities (Viswanathan et al. 1996).

If a maternal influence exists on susceptibility to type 2 diabetes, then it must be questioned whether there is a plausible biologic rationale to explain this. For example, 13 essential oxidative phosphorylation (OXPHOS) proteins are synthesised within the mitochondria from the maternally inherited mitochondrial DNA (mtDNA). Specific mtDNA deletions can cause maternally-inherited diabetes often associated with deafness (Scott et al. 1992). Subtle genetic variation of the mtDNA might contribute to the risk of developing the disorder by interacting with other genetic and environmental

factors. A study conducted in the UK with 897 cases and 1010 population matched controls showed that European mtDNA haplogroups are unlikely to play major role in the risk of developing the disorder (Chinnery et al. 2007). A recently conducted study in Sri Lanka has revealed the low prevalence of the mt3243A>G mutation, which is related to deafness in association with diabetes, was 0.9% in young adult onset patients with diabetes (Katulanda et al. 2008).

According to novel epigenetic hypothesis, heritable modifications in the DNA structure can be induced by environmental factors (Jablonka & Lamb 2005). Epigenetic variations seem to determine specific genes transcriptional potential ranges from high expression to complete inactivity (Bird 2002). Evidence for epigenetic variations is observed from animal studies (Cropley et al. 2006). When obese yellow pregnant mice were fed with extra vitamins they give birth to standard brown, lean and healthy babies as a result of interaction of vitamins with the foetus which turns off the yellow allele. The addition of methyl groups to the DNA backbone is used on some genes to distinguish between the gene copy inherited from the father and that inherited from the mother. In this situation, known as 'imprinting' the marks both distinguish the gene copies and tell the cell which copy to use to make proteins. In contrast to Mendel's laws of inheritance imprinted genes depend on which parent it was inherited from. For some imprinted genes, the cell only uses the copy of the mother to make proteins and for others only that from the father. Imprinted genes are expressed only from one allele in a strict parent of origin manner (Surani 1998).

Nutrients, toxins, behaviour or environmental exposures of any sort can silence or activate a gene without altering its genetic code in any way by adding methylation markers on DNA structure (Bernstein et al. 2007). Thus anything that influences methylation patterns during development can change the animal or person lifetime. Epidemiological data from Sweden provide evidence of transgenerational effects from the paternal line. A grandfather who was well nourished before puberty may transmit a four times higher than normal risk of type 2 diabetes to his grandchildren. This study also showed that paternal grandfather's food supply during mid childhood was linked to the mortality risk ratio of grandsons, but not grand daughters. The study suggested that in humans a one off environmental event could influence phenotype for more than one generation in a sex specific way (Kaati et al. 2002, Pembrey et al. 2006). However, the molecular basis of this transgenerational effect is not yet fully understood.

2.1.3 Family History and early onset of type 2 diabetes

A stronger family history of diabetes associated with an earlier age at diagnosis of diabetes was observed in India (Vishwanathan et al. 1995). Similar findings were observed in Japan that younger onset of type 2 diabetes has a strong hereditary background. In patients with younger age of onset a high prevalence of diabetes was observed among parents (Hagura et al. 1994).

In summary, mutations in mitochondrial genome and haplotypes may have a direct influence and association with maternally inherited diabetes. There is considerable evidence to suggest that maternal nutrition imbalance and metabolic disturbances during critical times of development may have a persistent effect on health of the offspring and may even be transmitted to the next generation. Genetic imprinting and effect of epigenetic markers provide evidence that transmission of polygenic traits such as diabetes occurs in a sex specific manner. Recent evidence suggests that the paternal diet and behaviour may also affect health of offspring. Epigenetics may contribute profoundly to the observed familial aggregation of the disease in different populations. Thus it can be assumed that intrauterine environment and early foetal programming based on epigenetic markers and genetic imprinting onto maternal DNA may have a direct collective influence to transmit disease predominantly in a maternal excess way. Earlier onset of diabetes among offspring of conjugal diabetes parents may be as a result of stronger effect of bilineal inheritance of both environment and genetic factors.

2.1.4 Chapter aims and objectives

This part of the study was designed to

1. construct a database with family histories of patients with type 2 diabetes with a view of drawing samples for genetic research in relation to type 2 diabetes.
2. investigate the familial aggregation of type 2 diabetes in Sri Lanka and to determine the effect of parental history in relation to development of type 2 diabetes.
3. investigate whether the existence of an excess parental transmission pattern in patients with type 2 diabetes is present in Sri Lanka
4. create a DNA bank for patients with type 2 diabetes for the Sri Lankan population

2.2 Methods

2.2.1 Patient identification and data collection for family history database

Primary-care (general) practitioners in defined suburbs in the greater Colombo area of Sri Jayawardenapura, Maharagama, Dehiwela, Mount Lavinia, Rathmalana, Moratuwa, Nugegoda, Pannipitiya and Piliyandala were invited to participate in the study by referring all newly diagnosed diabetic patients to a specialist diabetic clinic. The clinic was held on three days of the week. All the patients who attended the clinic had clinical data recorded in standardised forms and stored in a computerized database. These data included their name, address, diabetic status, date of birth, and age of onset and clinical data on glycaemic control and complications (Weerasuriya et al. 1998).

Figure 2.1 Map that indicates the local area from where patients were recruited

One thousand index patients from the above database were given a questionnaire to complete relating to their family history of diabetes. Details of family history were recorded for five generations. The questionnaire contained information on patient's index number, contact numbers, name, address, gender, and diabetic status of parents, grand parents, siblings, children and grandchildren. Their living or non-living status, total number of siblings, children and grand children were recorded. The clinical database was scanned for the age of diagnosis and date of birth. The generational model of the questionnaire is shown in figure 2.2. Incomplete questionnaires were supplemented by a postal questionnaire. A separate family history database was

compiled with the information provided. The records were checked to exclude related patients. The responses to the questionnaire were requested in the form of "yes/no" or "don't know". If the "don't know" responses would have been considered as "no" this would result in misclassification of the family history results and would reduce the power of the study. Also samples obtained from such a database would not reflect the true heritability of diabetes in Sri Lankan community. Hence, the probands with "do not know" responses were considered separately. The created family history database provide a useful resource to select subjects for different study designs such as patient parent trio studies, sibpair analysis or case control studies. SPSS 13 statistical software package was used for data analysis. Descriptive statistics were used to find means and percentages. Paternal and maternal groups were compared for differences by cross tabulating to compute *yj* test statistics.

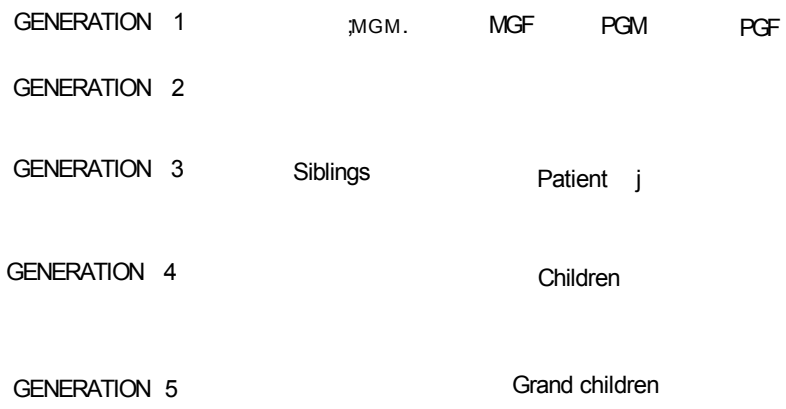


Figure 2.2. General format for data collection for family history database.

Our contact point in this study was patient at generation 3

(M- Mother, MGM - Maternal Grand mother, MGF - Maternal grandfather, F- Father, PGF- Paternal grandfather, PGM - Paternal Grand mother)

2.3 Results

2.3.1 Classification of family histories based on parental data provided

Complete family history data were obtained for one thousand indexed subjects. The sample (n=1000) included 502 males and 498 females. The mean (sd) age of onset of the diabetes was 47.0(12) years and mean duration of diabetes was 9.2 (07) years. 370 (37%) subjects (187 male) reported that at least one parent had diabetes. 469 (46.9%) subjects reported no parents with diabetes, while 161 (16.1%) were not aware of the diabetes status of at least one parent. From the total sample, 59.4% of probands had at

least one affected relative. The probands were classified as maternal (diabetes in the mother only), paternal (diabetes in the father only), both, and neither and unknown (Table 2.1).

2.3.2 Parental diabetic status

Among the 370 probands with a parental history of diabetes and where both parents' diabetes status was known and only one was affected, diabetes was more common among mothers (n=156) than fathers (n=125, $p< 0.001$). Both parents of 5.4% of subjects had diabetes but 3.5% were unaware whether or not the second parent had diabetes.

The diabetes status of either the mother or father was unknown in 196 (19.6%) of probands. Among the 145 probands who were not aware of the mother's diabetes status, the mother was not alive in 121 (83.3%) of cases, was living in 14 (9.7%) of cases, while in 10 (6.9%), it could not be determined if the mother was alive or not, owing either to illegible records or missing data. In 151 probands who did not know whether their fathers had diabetes, the father was dead in 136 (90.1%) of cases, living in 5 (3.3%) of cases and dead/living status was unclear in 10 (6.6%).

Table 2.1 Diabetes status of parents of 1000 subjects with type 2 diabetes

Mother	Father			
	Yes	No	Unknown	All
Yes	54 (5.4%)	156 (15.6%)	20 (2.0%)	230 (23.0%)
	Both	Maternal*	Unknown	
No	125 (12.5%)	469 (46.9%)	31(3.1%)	625 (62.5%)
	Paternal*	Neither	Unknown	
Unknown	15 (1.5%)	30 (3.0%)	100 (10.0%)	145 (14.5%)
	Unknown	Unknown	Unknown	
All	194 (19.4%)	655 (65.5%)	151 (15.1%)	1000 (100%)

*$p< 0.001$ * parental diabetes status was significantly different in mothers compared to fathers*
Number (%) Unknown = at least one parent of uncertain diabetes status

2.3.3 Diabetes status of Grandparents

A positive history of diabetes in maternal grandparents was more frequent in probands with maternal than paternal diabetes ($p<0.001$) (Table 2.2). In the maternal diabetes group, 36/156 (23%) grandmothers and 31/156 (20%) grandfathers had diabetes. In the paternal diabetes group, 11/125 (9%) grandmothers and 37/125 (30%) grandfathers had diabetes. However, the diabetes status of maternal grandparents was unknown in 89/156 (57%) of all probands and the diabetes status of paternal grandparents was not clear in 77/125 (53.0%) (Table 2.2).

2.3.4 Siblings and children

Among probands in the both-parents diabetic group, the frequency of affected siblings was higher than in other groups 33/54 (61%). The frequency of diabetes among the siblings were 84/156 (54%) in maternal group and 53/125 (42%) in the paternal group ($p<0.001$). When considering the children, the number affected {7/156 (5%)} was higher in the maternal group than in the paternal group, 2/125 (2%) ($p<0.001$) respectively. Among probands who had siblings with type 2 diabetes, 290/333 (88%) of those siblings were living. Among those who had children with type 2 diabetes, 43/45 (96%) of those children were living (Table 2.3). Of the 1000 probands, 330 (33%) had one affected sibling, 159 (16%) had two affected siblings, 73 (7%) had three affected siblings and 31 (3%) had four affected siblings. Among the probands with a maternal history of diabetes, parents were living in 28/156 (18%) of cases, mothers only were living in 41/156 (26.3%) of cases and fathers only were living in 11/156 (7.1%) of cases. In probands with a paternal history of diabetes, both parents were alive in 23/125 (18%) of cases, mothers only were alive in 46/125 (37%) of cases and fathers only in 5/125 (3%) of cases (table 2.3). Overall, there were 145 probands with both parents alive (trios).

2.3.5 Age of onset of diabetes in both parental diabetes groups

The mean (sd) age of onset of diabetes was earlier in the both-parents diabetic group 41(±11.0) year and mean (sd) duration of the condition 11(±6.0) year was longer than in no parental diabetes group 47.7(±11.9)] ($p<0.05$) (Table 2.4).

Table 2.2 Diabetes status of grandparents of probands grouped by parental diabetes status

Grandparent	Parental status of diabetes					
	Maternal	Paternal	Both	Unknown	Neither	All
Maternal grandmother	33 (21.2%)*	6 (4.8%)*	12 (22.2%)	1 (0.5%)	10 (2.1%)	62 (6.2%)
Maternal Grandfather	27 (17.3%)	22 (17.6%)	13 (24.1%)	1 (0.5%)	7 (1.5%)	70 (7.0%)
Paternal grandmother	3 (1.9%)	5 (4.0%)	4 (7.4%)	1 (0.5%)	8 (1.7%)	21 (2.1%)
Paternal grandfather	4 (2.5%)	15 (12.0%)	5 (9.3%)	0 (-)	7 (1.5%)	31 (3.1%)
Population (%)	156(15.6%)	125(12.5%)	54(5.4%)	196 (19.6%)	469(46.9%)	1000(100%)

*Number (%) of probands with affected grandparent p<0.001 * Significance observed between maternal grand mothers vs paternal grandmothers*

Table 2.3 Diabetes status of the siblings and children of probands grouped by parental diabetes status

Siblings & children	Parental status of diabetes					
	Maternal	Paternal	Both	Unknown	Neither	All
Affected siblings	84 (53.8%)*	53 (42.4%)*	33 (61.1%)	32 (16.3%)	128(27.3%)	333 (33%)
Living affected siblings	77 (49.4%)	50 (40%)	30 (55.6%)	23 (11.7%)	110 (23.5%)	290 (29%)
Affected children	7 (4.5%)	2 (1.6%)	1 (1.9%)	10 (5.1%)	25 (5.3%)	45(4.5%)
Living affected children	7 (4.5%)	2 (1.6%)	1 (1.9%)	9 (4.6%)	24 (5.1%)	43(4.3%)
Population (%)	156 (15.6%)	125 (12.5%)	54 (5.4%)	196 (19.6%)	469 (46.9%)	1000(100%)

*Number (%) probands having an affected sibling or child p<0.001 * Significance observed between affected siblings with diabetic mothers vs diabetic fathers*

Table 2.4 Age of diabetes and age of onset of diabetes of probands grouped by parental diabetes status

	Maternal	Paternal	Both	Neither	Unknown
Age of onset	43.9 (11.0)	42.5 (11.2)	41.2 (10.7)*	47.7 (11.9)	49.8 (12.5)
Duration	8.4 (5.7)	10.3 (8.2)	11.3 (6.3)*	8.74 (6.1)	9.9 (7.5)
Age	52.3 (11.5)	52.1 (14.3)	51.6 (11.5)	47.5 (14.0)	49.6 (14.3)

Mean (sd).* $p<0.05$ compared with maternal, neither and paternal

2.5 Discussion

One of the aims of this study was to estimate the degree of familial aggregation of type 2 diabetes in a Sri Lankan population and to investigate the transmission patterns of this disorder and their relationship with parents' status of diabetes. Moreover the effect of stronger family history on age of onset of diabetes in the offspring was investigated. A family history database which spans several generations of probands family history of diabetes was created with information regarding the living status of the relatives in each generation.

The results of this present study agree with the concept of familial aggregation of type 2 diabetes in that 59.4% of those with diabetes had at least one affected first degree relative in the family. A familial aggregation for type 2 diabetes has previously been reported by researchers in France (66%), England (43%), South India (54%), Japan (43-49%) and Tunisia (70%) (Thomas et al. 1994, Alcado & Alcado 1991, Viswanathan et al. 1996, Royko et al. 1994, Arfa et al. 2007). Family history studies conducted in the USA have observed that people with a family history of diabetes are 2-6 times more likely to develop diabetes than those without (Harrison et al. 2003) and in Japan the observed familial aggregation in non diabetic population is 15% (Royko et al. 1994). The present study did not observe the family history of non diabetic population which is one of the major limitations of the study.

The results of the present study show a significant difference in parental transmission pattern of type 2 diabetes. The prevalence of diabetes in mothers was 15.6% when compared to 12.5% in fathers. Analysis of a parental history of diabetes based on questionnaires by Alcolado and Alcolado in the UK found 36% of mothers and 15% of fathers were affected with diabetes. Similarly the CODIAB study (33% mothers to 17.1% fathers) and a study conducted in Tunisia found (21% of mothers and 3% of fathers) in their studies that there was an excess maternal influence for the transmission of the disease. Our results are in agreement with these studies. However, they contradict similar studies in south India, Korea and Mexican-Americans who observed that there was no excess maternal influence (Viswanathan et al. 1996, Kim et al. 2004, Mitchell et al. 1995). Viswanathan and co-workers suggested that there may be ethnic variation to this phenomenon. The results of the present study however, suggest clearly that there is an

excess maternal influence on type 2 diabetes in Sri Lanka (15.6% vs. 12.5%). This contradicts the suggestion of Viswanathan and co-workers that there is ethnic variation in maternal influence (Viswanathan et al. 1996). This excess extends to the grandparents (42.9% in maternal to 38.4% in paternal), siblings (49.5% maternal to 40% paternal) and children (4.5% maternal to 1.6% paternal) of probands. The study by Viswanathan and co workers was clinic-based. Although the current study was not community-based, patients were selected from general practices in a defined area, and this should not have incurred significant bias.

An excess of maternal transmission was found by Alcolado and Alcolado but their sample had a female preponderance (Alcado & Alcado 1991). A study by Mitchell and co-workers reported that excess maternal influence might be attributed to reporting bias, as women in some countries use health-care facilities more often than men, and individuals may be more likely to know about their mother's health status than that of their father's (Mitchell et al. 1993). A UK study showed that 14% of South Asians and 27% of Europeans were unaware of their parental history (Fischbacher et al. 2001). In the present study, 19.6% of subjects were unaware of any parental history of diabetes. Of these, fathers were not alive in 90.1% and mothers not alive in 83.3% of cases. It is likely that the lack of awareness of diabetes status of parents is mainly because they were dead.

Previous studies may have been influenced by an unequal male/female ratio. The South Indian study had excess male (668/308) preponderance (Viswanathan et al. 1996). According to the Sri Lankan labor force survey conducted by the Department of Census and Statistics, the percentage of total labor force in work is 90.3% and 78.3% for men and women, respectively (Sri Lanka Labor Force Survey, Quarterly Report 1999). In the present study the clinic from which the patients were recruited was held outside normal working hours, and was attended equally by men and women. The present sample consisted of 502 males and 498 females. Hence, any effect due to female preponderance and women's use of excess health-care facilities can be excluded in this study. Unlike in developed nations, routine screening for gestational diabetes does not take place in Sri Lanka. Therefore, mothers are unlikely to be detected as having diabetes more often than fathers because of pregnancy screening (Siribaddana et al. 1998).

According to the current data, more than half of subjects with both parents affected developed diabetes before the age of 40 years, and accordingly, mean age at onset was less and mean duration of the condition longer, compared with other groups suggesting double gene dose effect. Individual risk of developing diabetes increases with the extent of diabetes in the previous generation. It has been reported in India that offspring of diabetic parents develop diabetes at a younger age (Ramachandran et al. 1988, Vishwanathan et al. 1985). The early onset of type 2 diabetes is characterized by a high prevalence of diabetes in both parents and siblings and onset of the disease between 25 and 40 years age (Fajans 1989). Indeed, early onset of type 2 diabetes mellitus is common among Sri Lankans.

The most serious limitation in this questionnaire-based study is its reliance on the information from respondents about their family histories. Diabetes is an asymptomatic disease. Undiagnosed diabetes is found in 36% of subjects during community surveys (Katulanda et al. 2008). Ideally, therefore, when using questionnaire-based databases to perform family studies, glucose tolerance tests should be done to assess the glycaemic status of relatives reported to have no diabetes or where diabetic status is not known. However, on the basis of the reported data from those with knowledge of maternal and paternal status of diabetes, it may be concluded that there is an excess maternal influence for the disease in the Sri Lankan community.

Neel (1962) described type 2 diabetes as the major ingredient of a geneticists' nightmare. According to him the major obstacles for the genetic studies of this disorder is the late and variable age of onset, that members are middle-aged and have moved from their place of birth and the paucity of suitable genetic materials for the study and extensive heterogeneity (Editorial, the Lancet 1992). Accordingly non parametric methods for association studies family based studies are encouraged to prevent spurious associations due to population stratification. In Sri Lanka, as extended families maintain contact suitable genetic material for linkage and genetic association studies could be found. This study revealed 145 of the 1000 probands were trios. It is also clear that the probands with no parental history have reported the maximum number of affected siblings and total of 290 living affected siblings thus, indicating feasibility of probands and affected siblings for sibpair analyses. Thus it is evident from the present study that it is possible to extract suitable pedigrees for genetic

studies for type 2 diabetes (case control studies, sib pair analysis or transmission disequilibrium test analysis).

In conclusion, this study observed that 59.4% of the probands had first degree relatives with diabetes, indicating a familial aggregation for type 2 diabetes. An excess maternal transmission pattern has also been observed, with 15.6% of diabetics having a positive maternal family history compared to 12.5% with positive paternal family history. Interestingly, the onset of the disease was early among the probands who had both parents with diabetes indicating a double gene dose effect. This part of the study provides useful family history information which can be expanded to further study the feasibility of using stratified family history data as a method of diagnosing people at high risk of developing diabetes.

Chapter 3

Type 2 diabetes, periodontal status and association with metabolic parameters

3.1 Metabolic abnormalities in type 2 diabetes mellitus

The normal glucose level in the blood is coordinately regulated by tissue uptake by skeletal muscle, liver, adipose tissue, kidney and brain and glucose stimulated insulin secretion by pancreatic beta cells (Lowell & Shulman 2005). In type 2 diabetes mellitus a failure in this coordination causes insulin resistance or insufficient insulin secretion by the pancreas, ultimately leading to high blood glucose levels (Alberti & Zimmet 1998). Deviations from normal levels of fats in the blood (dyslipidemia) are consistently associated with type 2 diabetes mellitus. In both type 1 and type 2 diabetes, hyperglycaemia is often accompanied by hyperlipidemia (Kim et al. 1993, Abdel-Aal et al. 2008, Okafor et al. 2008). These serum lipid abnormalities are caused by disruption in fatty acid metabolism. The condition is characterised by hypertriglyceridemia, with increased very low density lipoprotein (VLDL), increased levels of small dense low density lipoprotein particles (LDL), low high density lipoprotein (HDL) and increased levels of omega -6 polyunsaturated fatty acids (Taskinen 2003, De Mann et al. 1996, Tilvis & Meittinen 1985). Several lines of evidence indicate that high triglycerides play a role in insulin resistance and type 2 diabetes (Mingrone et al. 1999, Yang et al. 2003a). Recent evidence suggests that a fundamental defect is an overproduction of large very low-density lipoprotein (VLDL) particles, which initiates a sequence of lipoprotein changes, resulting in higher levels of remnant particles, smaller LDL, and lower levels of high-density lipoprotein (HDL) cholesterol. These atherogenic lipid abnormalities precede the diagnosis of type 2 diabetes by several years (Adiels et al. 2008).

Underlying mechanisms could be activation of hormone sensitive lipase in adipocytes which is suppressed under normal conditions by insulin. In insulin resistance states, this enzyme is activated and free fatty acids are released into the plasma. As a result there is huge influx of free fatty acids to the liver which stimulate synthesis and secretion of VLDL (Rivellese 2004). Lipoprotein lipase, the enzyme which catalyses the release of triglycerides from lipoproteins is under the influence of insulin (Howard 1999). Thus in the insulin resistant state, the prolonged presence of triglyceride rich VLDL particles in the circulation promotes the enrichment of HDL with triglycerides. This alteration results in faster catabolic rates of the triglyceride rich HDL in comparison with the normal HDL under the action of hepatic lipase resulting in low levels of HDL in circulation (Krentz 2003).

The elevated levels of lipid also cause lipotoxicity. This lipotoxic effect can also act synergistically with glucose to produce even greater deleterious effects known as glucolipotoxicity (Such as destruction of pancreatic beta cells) in patients with type 2 diabetes (Khan et al. 2006).

3.1.2 Periodontitis and diabetes: relationship with hyperlipidemia

Studies performed on patients with systemic infections have documented a relationship between infection and hyperlipidemia (Lopes-Virella 1993). Infections induce changes in the plasma concentrations of cytokines and hormones leading to a catabolic state characterised by altered lipid metabolism (Samara et al. 1996). The main features of this altered metabolism are hypertriglyceridemia and lipid oxidation. There is evidence to suggest that even low levels of chronic exposure to Gram negative microorganisms and their LPS can manifest a similar response (Lopes-Virela 1993). Consistent with this theory, a study conducted to assess the degree of periodontal infection and lipid profiles in diabetic subjects has found that elevated levels of LDL cholesterol were significantly associated with antibody titers to *Porphyromanas gingivalis* (Nishimura et al. 2006). Inflammation due to infection could induce the prediabetic status characterised by insulin resistance and dyslipidemia. Infection induced proinflamatory cytokines (TNF-α and IL-1β) are known to exert effects on lipid metabolism (Van der Poll & Saurwein 1993, Moldwer 1994), resulting in elevated levels of free fatty acids FFA, LDL/TRG (Feingold et al. 1992, Fried & Zechner 1989). People with diabetes are at a higher risk of infection-induced hyperglycaemia and hypertriglyceridemia. According to a study by Rufail and coworkers prevalence of atherogenic lipoprotein parameters, very low density lipoprotein (VLDL) was 8.3%, 33.3% and 66.6% in healthy controls, localised aggressive periodontitis and generalized aggressive periodontitis patients respectively. This is an indication that periodontitis leads to dyslipidemia (Rufail et al. 2007).

3.1.3 Chapter aims and objectives

To determine the impact of type 2 diabetes on lipid parameters

To determine the impact of periodontal status on lipid parameters

To determine the effect of diabetes parameters on periodontal health

3.2 Method

3.2.1 Patient identification

To study the effect of diabetes on periodontal status a cross sectional study was designed. A total of 400 patients were identified from the family history database. Letters were sent to their last recorded address three weeks prior to the clinics inviting them to participate in a special diabetic clinic, followed by telephone calls three days in advance, reminding them to attend the clinics. The clinics were held over several days at each of five occasions at Endocrinology and Metabolic Disease Trust in Colombo, Sri Lanka between 2000 and 2006. Periodontal health status was examined by a team of dental professionals from The University of Newcastle Upon Tyne (Professor Peter Heasman and Dr. Philip Preshaw) and general health status was recorded by an endocrinology team from the University of Sri Jayawardanapura (Professor Devaka Fernando, Dr. Namal Weerasuriya, Dr. Noel Somasundaram, Dr. Nimantha De Alwis, and Dr. Chandima Idampitiya).

3.2.2 Clinical diagnosis of diabetes and periodontitis

The diabetes status of patients was determined according to WHO diagnostic criteria (Table 1.1). The fasting plasma glucose level, lipid profile and glycated heamoglobin levels were assessed in the clinical laboratory of Durdan's Hospital in Sri Lanka. Clinical diagnosis of periodontitis was assigned according to methods described in sections 4.2.5. and 4.2.6.

3.2.3 Study population

A total of 285 type 2 diabetes patients, 62 biological parents and 73 controls responded to the invitation. The present analysis included 285 patients with type 2 diabetes and 72 (excluding one edentulous control) age matched controls who underwent periodontal examination.

3.2.4 Coding data for analysis

Patients were examined for periodontal variables and other relevant data were collected according to the method described in chapter 4.2. Statistical analysis was performed using SPSS version 16. Categorical variables considered in this study were gender, smoking status and genotype status. Continuous variables included age, body mass index (BMI) and blood pressure data. Diabetes-related continuous variables included glycated haemoglobin (HbA1c), fasting blood glucose (FBG), diabetes duration and lipid parameters including

cholesterol (TOC), triglycerides (TGL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Mean probing depth (Mean PD), Mean Recession (Mean Rec), Mean Loss of Attachment (Mean LOA), Maximum Probing Depth (Maximum PD), Maximum loss of attachment (Maximum LOA), % bleeding on probing (% BOP) , % sites with probing depth (PD)(> 4mm, >5mm, >6mm), % sites with LOA (> 2mm, 3mm, 4mm, 5mm and 6mm) and number of missing teeth were the recorded periodontal variables.

3.2.5 Control classification

Controls were screened for possible diabetes taking into account both the HbA1c value and fasting blood glucose value (FBG). They were considered as having impaired glucose metabolism if HbA1c was >6.1% and FBG was > 100 mg/dl. (Saudek et al. 2008). Secondly, they were screened for impaired fasting glycaemia or diabetes. WHO criteria were used for diagnosis and if the control subject had FBG values greater than 126mg/dl they were considered to have possible (though not confirmed) diabetes. If FBG values were within the range 110mg/dl -125mg/dl they were catogerised as impaired fasting glycaemia (IFG) and if the value was < 110mg/dl they were considered as normoglycaemic. In this study, 72 controls who were recruited under the normoglycaemic category 12 exceeded the normal threshold values of diabetes diagnosis according to the WHO diagnostic criteria. These 12 individuals did not have a confirmed diagnosis of diabetes, but also could not be definitively assigned to the non-diabetic control group as they had evidence of IFG. Therefore, they were either excluded from some of the analyses, or, where appropriate, they were considered as a separate group (IFG, n=12). Therefore, three different groups; patients with type 2 diabetes (diabetes), confirmed non-diabetic controls, and a further group with IFG (i.e. possible undiagnosed diabetes) were considered.

3.2.6 Statistical Analysis

Statistical analyses were conducted using the statistical software SPSS 16. Frequency distributions were determined and descriptive statistics were calculated including means, standard deviations, and ranges. All variables were assessed for normality using the Kolmogorov-Smirnov test, supplemented with histograms. Where there was no evidence to reject normality, means and standard deviations of the parametric variables were calculated. Where the assumption of normality was rejected, means and interquartile ranges of these non-parametric variables were calculated. Differences in serum mediator levels and subject

characteristics between the type 2 diabetic subjects and controls, and periodontal status and metabolic parameters were assessed using chi- squared tests for discrete variables. Differences between groups in continuous measures were evaluated by ANOVA for parametric variables. Box plots were constructed and Kruskal Wallis tests were conducted for non-parametric variables. Correlations between continuous variables were assessed using Pearsons correlations coefficients for parametric data i.e. if both variables were normally distributed, or Spearmans correlation coefficients if variables were not normally distributed. Scatter diagrams were constructed to illustrate these associations. Statistical significance of all tests was assessed at the 5% level.

3.3 Results

3.3.1 Normality distribution of data

Normality distribution of the variables was tested using Kolmogorov-Smirnov test. In general a p value >0.05 for this test indicates that the data have a normal distribution (i.e. the distribution is not significantly different from normal). Table 3.1 presents Kolmogorov-Smirnov p values for all the variables. As can be seen, only low density lipoprotein (LDL) and total cholesterol (TOC) levels showed a normal distribution (Figure 3.1). When compared to the mean distribution of LDL and TOC between diabetic cases and controls (Table 3.2) there were no significant differences between the groups for TOC (p=0.482) but LDL values were significantly lower in cases than controls and the IFG group (P<0.001).

Table 3.2. presents mean (standard deviation) and median (interquartile ranges) values and respective p values for all categorical, general and diabetes related variables for the three groups. When comparing between patients with type 2 diabetes and controls, significant differences were observed for systolic blood pressure (Systolic BP) (p=0.005), diastolic blood pressure (Diastolic BP) (p=0.006), glycated heamoglobin (HbA1c) (p=0.001), fasting blood glucose (FBG) (p=0.001) and triglycerides (TG) (p=0.001). Both systolic BP and diastolic BP values were significantly higher in control patients (p=0.007) and IFG group (p=0.028) than in patients with type 2 diabetes. The highest mean systolic BP values were recorded in the IFG group 142.6 (126.8-151.8 mm/Hg) when compared to diabetic cases 129.6 (120-140 mm/Hg) and controls 136.9 (125.3 - 145.8mm/Hg). A similar trend was observed for diastolic BP, with mean values of 81 mm/Hg (73.8-90.0), 85.6 mm/Hg (76.3 - 93.5) and 88.8 mm/Hg (82.0-95.5) respectively in type 2 diabetes patients, controls and patients with IFG. TG and FBG values were significantly higher among patients with diabetes than non-diabetic controls (p=0.001). BMI distribution did not differ between the three groups. However, age of the IFG group was significantly different from patients with type 2 diabetes (p=0.013) and non diabetic controls (p=0.004). There was no significant difference in HDL between the diabetic cases and the non-diabetic controls.

Table 3.1 **Normality distribution data**

Kolmogorov-Smirnov[a]	P	Kolmogorov-Smirnov[a]	P
HbA1c (%)	0.000	Mean Probing depth (mm)	0.000
Total Cholesterol (mg/dl)	**0.200**	Mean Recession (mm)	0.000
Triglycerides (mg/dl)	0.000	Mean Bleeding on Probing (%)	0.000
Low Density Lipoproteins (mg/dl)	**0.200**	Percentage bleeding on probing (%)	0.000
High Density Lipoperoteins (mg/dl)	0.000	Maximum probing depth (mm)	0.000
Fasting blood glucose (mg/dl)	0.000	Mean Loss of Attachment(mm)	0.000
		Maximum loss of attachment (mm)	0.000
Diabetes duration (years)	0.000	% sites with LOA ≥ 1mm	0.000
BMI(kg/m^2)	0.003	% sites with LOA ≥ 2mm	0.000
Age (years)	0.000	% sites with LOA ≥ 3mm	0.000
Systolic Blood pressure (mm/Hg)	0.000	% sites with LOA ≥ 4mm	0.000
Diastolic blood pressure (mm/Hg)	0.000	% sites with LOA ≥ 5mm	0.000
		% sites with LOA ≥ 6mm	0.000

a= Lilliefors Significance Correction

Kolmogorov-Smirnov test statistics shows that total cholesterol and low-density lipoprotein levels are normally distributed whereas all other variables are not. (Table 3.1, Figure 3.1)

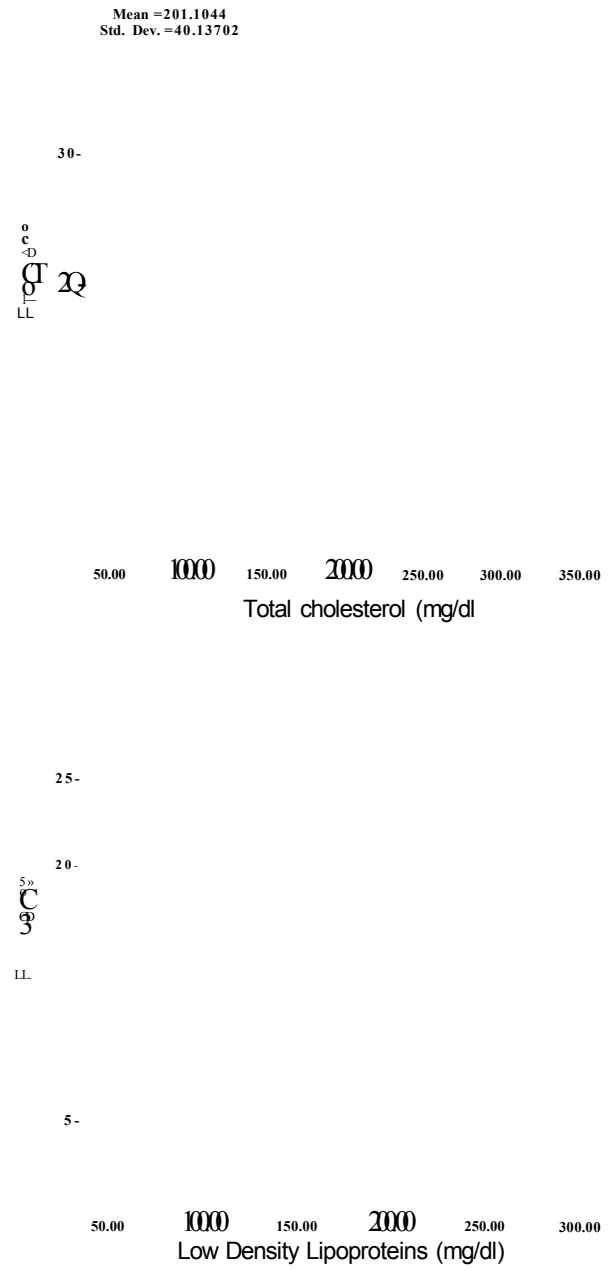


Figure 3.1 Histograms demonstrating normal distribution of total cholesterol (TOC) and low density lipoprotein (LDL)

According to Kolmogorov-Smirnov test statistics only TOC and LDL data were normally distributed. Figure 3.1 shows the histograms for cholesterol and LDL

Table 3.2 **Comparison of continuous variables between patients with type 2 diabetes and controls.**

	Type 2 diabetes (n=285)	Controls (n=60)	IFG group (n=12)	p
Age (years)	45.70 (37.-54.5)	45.05 (38-51.8)	53.17(48.3-5.5)	0.964*
Systolic Blood pressure (mm/Hg)	129.63 (120 - 140)	136.9 (125.3-145.8)	142.6(126.8-151.8)	**0.003****
Diastolic blood pressure (mm/Hg)	81 (73.8 – 90)	85.6(76.3 – 93.5)	88.8(82-95.5)	**0.006****
HbA1c (%)	7.37 (6.00 – 8.00)	5.82 (5.5–6.1)	7.3(6.8-7.4)	**0.001****
Fasting blood glucose (mg/dl)	160.81(110-189)	88.4(81.3-93)	121(107.5-125.8)	**0.001****
Total Cholesterol (TOC) (mg/dl)	199 (40.50)	207.01 (37.1)	211(17.3)	0.482
Triglycerides (mg/dl)	152.06 (89- 174.5)	108 (70 – 116.3)	109.92(90.5-120.5)	**0.001*****
Low Density Lipoproteins (mg/dl)	123.45 (37.35)	140.54 (32.59)	140.85(18.76)	**0.006****
High Density Lipoproteins (mg/dl)	47.54 (44.4-51.8)	48.4 (46-50)	48.17(46.5-50)	0.986
BMI (kg/m^2)	24.65 (22.4 – 26.7)	24.86 (22.6– 27.4)	26.79(23.6-31.9)	0.741

Mean and standard deviation are presented for total cholesterol and low-density lipoproteins. Medians and interquartile ranges are presented for all other variables. *p* values were determined using ANOVA for TOC and LDL and Kruskall Wallis test for all other variables between diabetes and control group

* *IFG group is significantly different with Type 2 diabetes and controls*
** *IFG group and type 2 diabetes significantly different from controls*
*** *Type 2 diabetes significantly different from IFG group and controls*
**** *Type 2 diabetes significantly different from controls*

Table 3.3 Correlations between diabetes related variables.

	Systolic BP (mm/Hg)	Diastolic BP (mm/Hg)	Age (years)	FBG (mg/dl)	TOC (mg/dl)	HDL (mg/dl)	HbA1C (%)	Diabetes duration (years)
Age (years)	0.388**(**0.001**)	0.290**(**0.001**)	1.000	-0.146 (0.073)	0.093 (0.124)	-0.007 (0.924)	0.001(0.983)	0.506**(**0.001**)
TOC (mg/dl)	0.179**(**0.010**)	0.172**(**0.013**)	0.093(0.124)	0.104(0.272)	1.000	0.312**(**0.001**)	0.004 (0.943)	0.025(0.722)
LDL (mg/dl)	0.212**(**0.003**)	0.205**(**0.005**)	0.190**(**0.008**)	-0.111(0.507)	0.889**(**0.000**)	0.173*(**0.015**)	-0.094 (0.203)	0.002(0.987)
BMI (kg/m^2)	0.159**(**0.011**)	0.183**(**0.004**)	-0.087(0.105)	0.070(0.390)	-0.023(0.700)	0.062(0.389)	0.092 (0125)	-0.179**(**0.003**)
TGL(mg/dl)	-0.040(0.585)	-0.006 (0.936)	-0.114 (0.113)	0.345*(**0.039**)	0.347**(**0.001**)	-0.007(0.924)	0.309* (**0.000**)	-0.231(0.011)
HbA1c (%)	0.031(0.659)	-.002(0.975)	0.001(0.983)	0.602**(**0.001**)	0.004 (0.943)	0.000 (0.999)	1.000	0.053(0.458)
Systolic BP (mm/Hg)	1.000	0.651**(**0.001**)	0.388**(**0.001**)	-0.04(0.735)	0.179**(**0.010**)	0.122 (0.094)	0.031(0.659)	0.175*(0.020)

** correlation is significant ($p<0.01$)
* correlation is significant ($p<0.05\%$)
Pearson's correlation coefficient value is presented for TOC and LDL
Spearman's rho values are presented for the remaining correlations

3.3.2 Associations between metabolic parameters

Statistically significant correlations were observed between TOC and LDL (p=0.000), TG (p= 0.000), systolic BP (p=0.010), diastolic BP (p=0.013) and HDL (p= 0.000) (Figure 3.2). In addition age was positively correlated with systolic BP (p=0.000), diastolic BP (p=0.000), LDL (p=0.008) and diabetes duration (p=0.000) (Figure 3.3). Apart from positive correlations with TOC, TGs correlated significantly with FBG (p=0.039), and FBG with HbA1c (Figure 3.4). LDL levels were correlated with HDL cholesterol, systolic BP (p=0.003), diastolic BP (p=0.005) and HDL (p=0.015) (Figure 3.5). BMI was positively correlated with systolic BP (p=0.011), diastolic BP (p=0.004) and negatively correlated with diabetes duration (p=0.003). Positive correlations were also observed between HbA1c and FBG (p=0.000), and between diabetes duration and systolic BP (p=0.020) (Table 3.3).

3.3.3 Correlation between periodontal parameters and biochemical parameters

When assessing correlations between periodontal parameters and biochemical parameters, significant positive correlations were observed between TG and maximum PD (p=0.02). Significant negative correlations were observed between TG and the % number of sites with PD>4mm (p=0.034) (Table 3.4, Figure 3.6). HDL cholesterol had a negative significant correlation with mean Rec (p=0.048) and maximum LOA (p=0.004) (figure 3.7). In addition FBG was positively correlated with all the periodontal parameters except with % number of sites with PD>5mm and PD> 6mm. {(maximum PD (p=0.001), mean PD (p=0.047), mean Rec (p=0.002), mean % BOP (p=0.000), mean loss of LOA (p=0.01), maximum LOA (p=0.003), % BOP (p=0.000) and % sites with PD ≥4mm (p=0.001) (Figure 3.8). Diabetes duration was significantly correlated with mean Rec (p=0.002), mean LOA (p=0.023) and maximum LOA (p=0.005) (Figure 3.9). No significant correlations were identified between HbA1C, TOC, LDL and BMI and periodontal parameters.

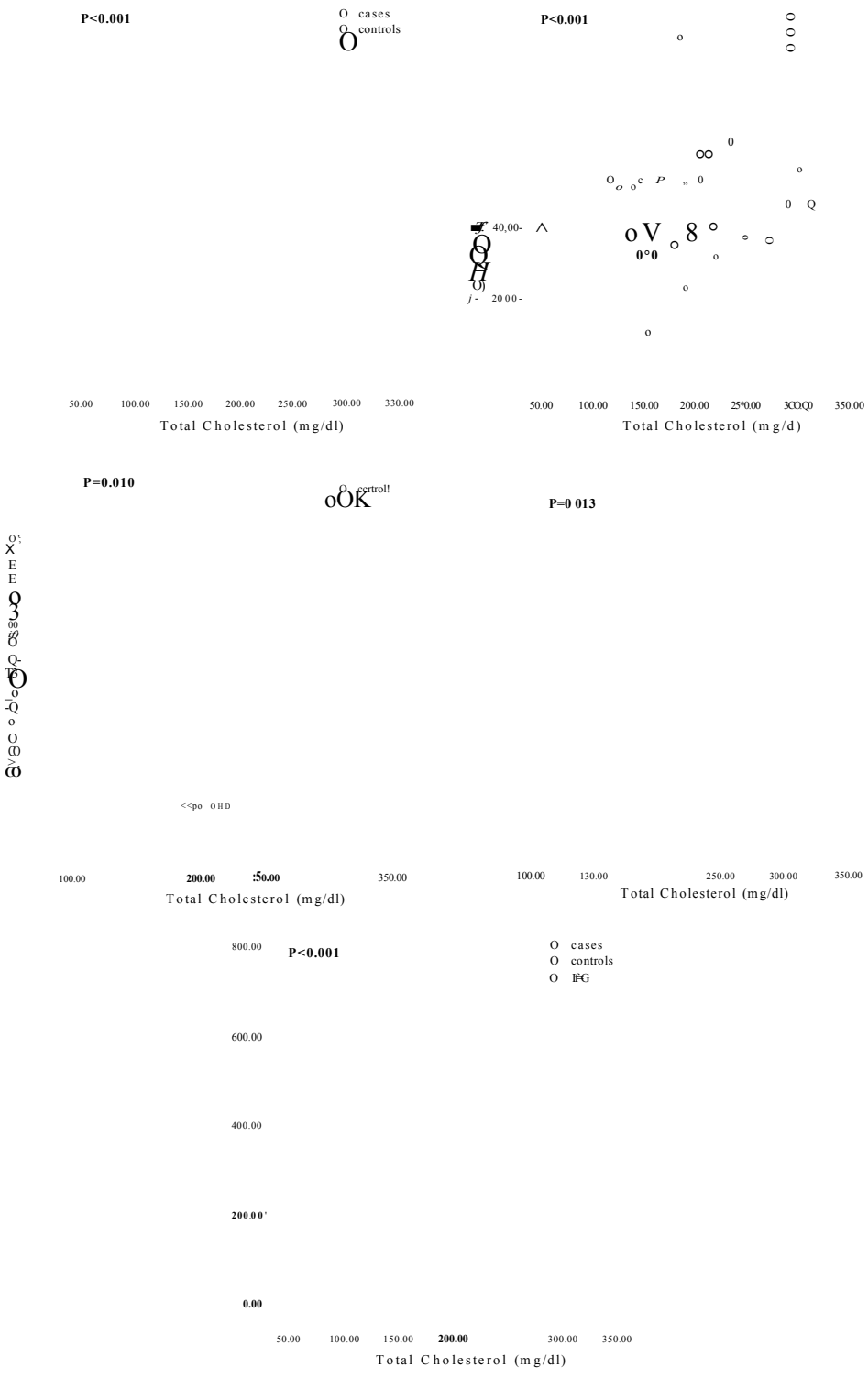


Figure 3.2. Significant correlations of TOC

TOC showed significant correlations with HDL, LDL, systolic BP, diastolic BP and TG.

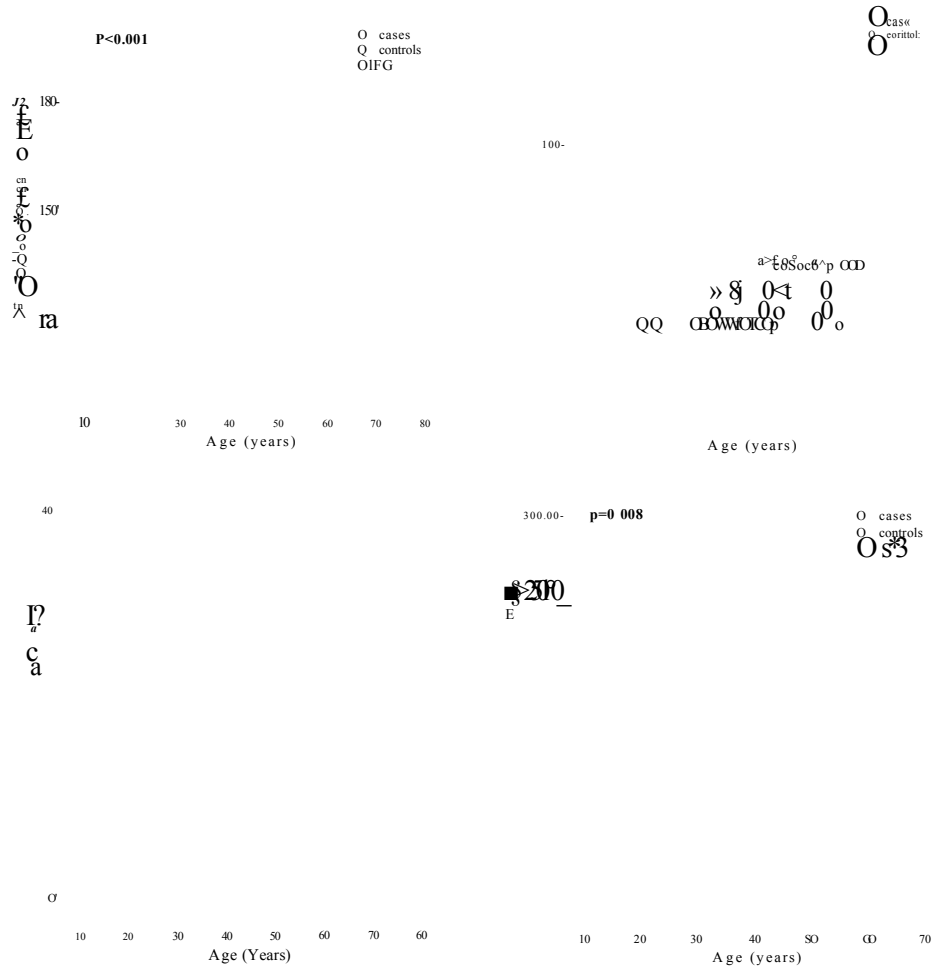


Figure 3.3 Significant correlations of age

Age significantly correlated with systolic bloodpressure diastolic bloodpressure, diabetes duration and low density lipoproteins

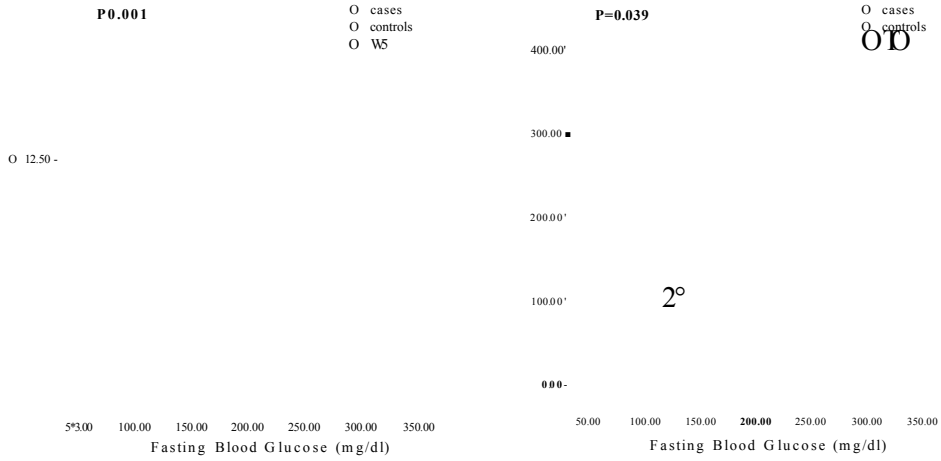


Figure 3.4 significant correlations of fasting blood Glucose (FBG)

FBG significantly with HbAlc and triglycerides

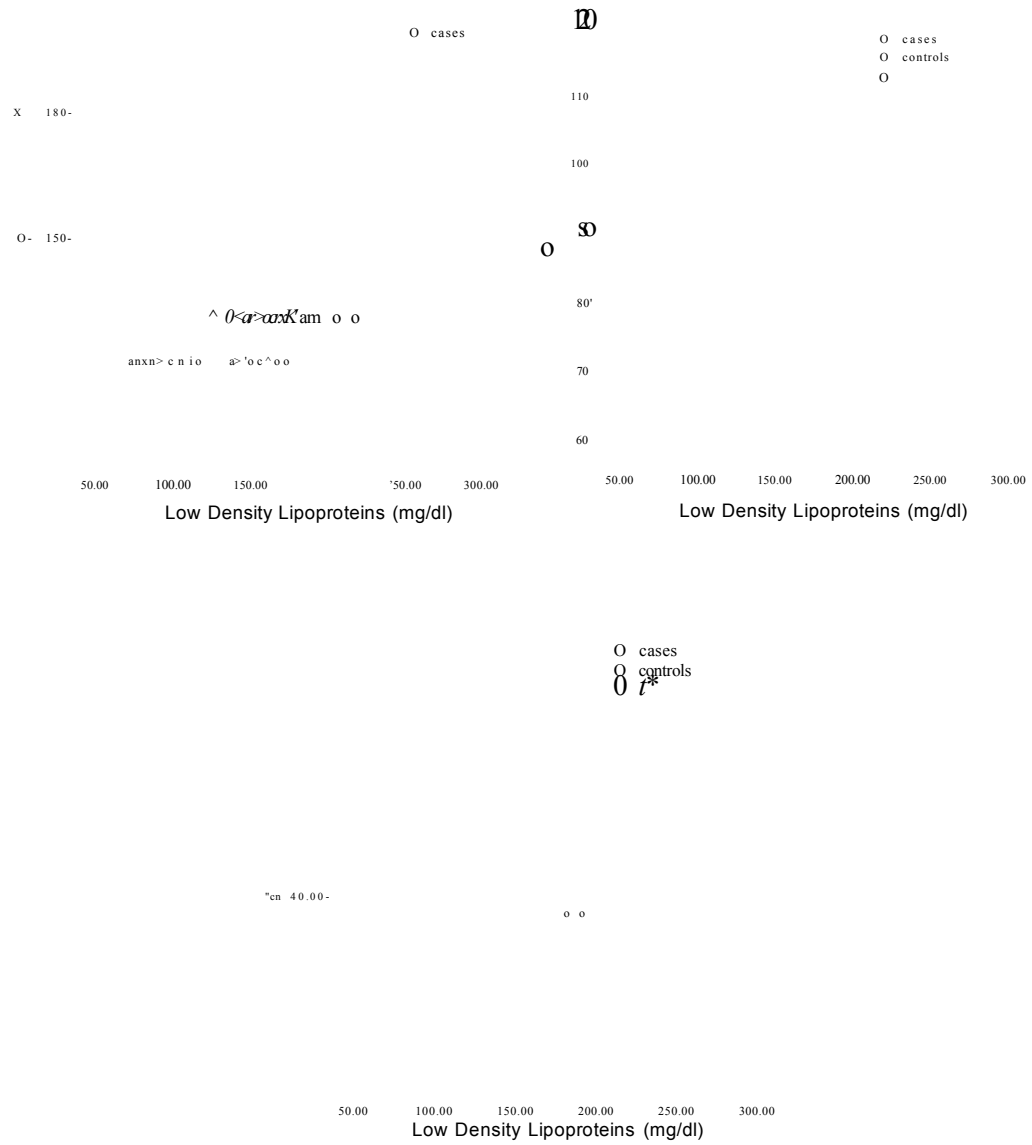


Figure 3.5 Significant correlations of low density lipoproteins (LDL)

LDL was significantly correlated with systolic bloodpressure, diastolic bloodpressure, high density lipoproteins.

Table 3.4. Correlations of biochemical parameters, BMI and diabetes duration with periodontal parameters

	Mean PD (mm)	Mean Rec (mm)	Mean BOP	Mean LOA (mm)	Maximum PD (mm)	Maximum LOA (mm)	%BOP	% of sites with PD ≥4mm	% of sites with PD ≥5mm	% of sites with PD ≥6mm
BMI (kg/m^2)	-0.022	-0.036	-0.030	0.028	-0.042	-0.037	-0.028	-0.053	-0.028	-0.011
	0.688	0.508	0.573	0.608	0.440	0.493	0.608	0.327	0.606	0.834
Diabetes duration (years)	0.093	**0.185***	-0.019	**0.138***	0.058	**0.170***	-0.023	0.080	0.116	0.066
	0.127	**0.002**	0.755	**0.023**	0.344	**0.005**	0.702	0.190	0.057	0.284
High Density Lipoproteins	-0.035	**-0.142***	-0.048	-0.083	-0.079	**-0.203***	-0.049	-0.028	-0.095	-0.104
(mg/dl)	0.626	**0.048**	0.503	0.251	0.271	**0.004**	0.500	0.699	0.187	0.150
Low Density Lipoproteins (mg/dl)	0.023	-0.012	0.069	0.052	0.031	0.042	0.068	0.018	-0.018	0.014
	0.751	0.872	0.342	0.472	0.665	0.559	0.559	0.801	0.808	0.842
Triglycerides(mg/dl)	0.026	0.098	0.104	0.044	**0.167***	0.108	0.105	**-0.153***	0.076	0.026
	0.717	0.176	0.150	0.543	**0.020**	0.137	0.149	**0.034**	0.292	0.718
Fasting Blood Glucose (mg/dl)	**0.134***	**0.206***	**0.268***	**0.172***	**0.225***	**0.198***	**0.269***	**0.223***	0.121	0.071
	0.047	**0.002**	**0.000**	**0.010**	**0.001**	**0.003**	**0.000**	**0.001**	0.074	0.299

*Spearman's rho values and its significant value for each correlation is given * indicates correlation is significant at 0.01 level.*
Mean PD – Mean Probing Depth , Mean Rec- Mean Recession, Mean BOP- Mean Bleeding On Probing, Mean LOA- Mean Loss of Attachment , Maximum PD- Maximum Probing Depth, Maximum LOA - Maximum Loss of Attachment BOP- Bleeding on Probing

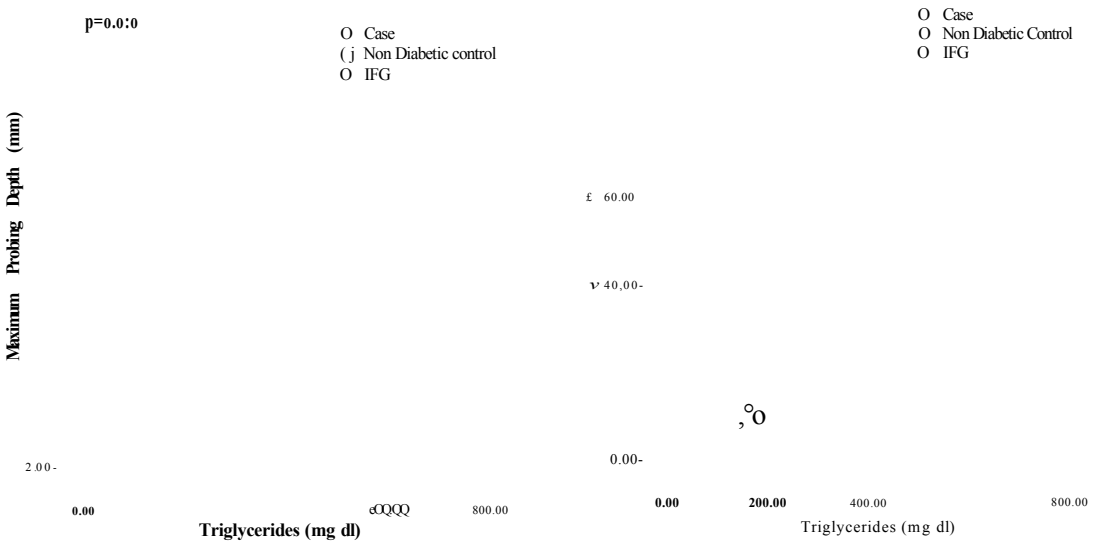


Figure 3.6 Significant correlations of triglycerides with periodontal parameters

Triglycerides were significantly correlated with mean probing depth and percentage sites with probing depth > 4mm

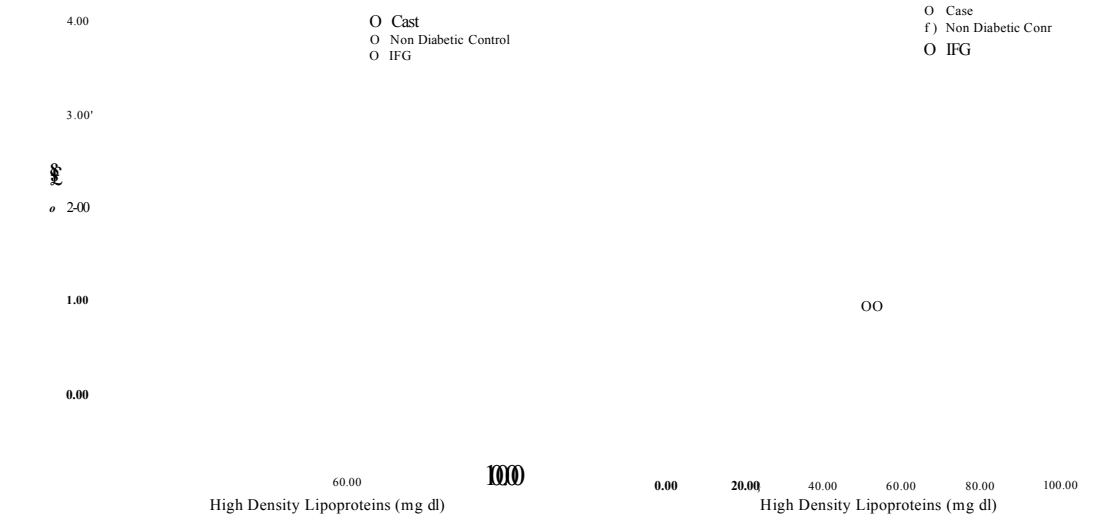


Figure 3.7 Significant correlations of HDL with periodontal parameters

HDL significantly correlated with mean recession and mean loss of attachment

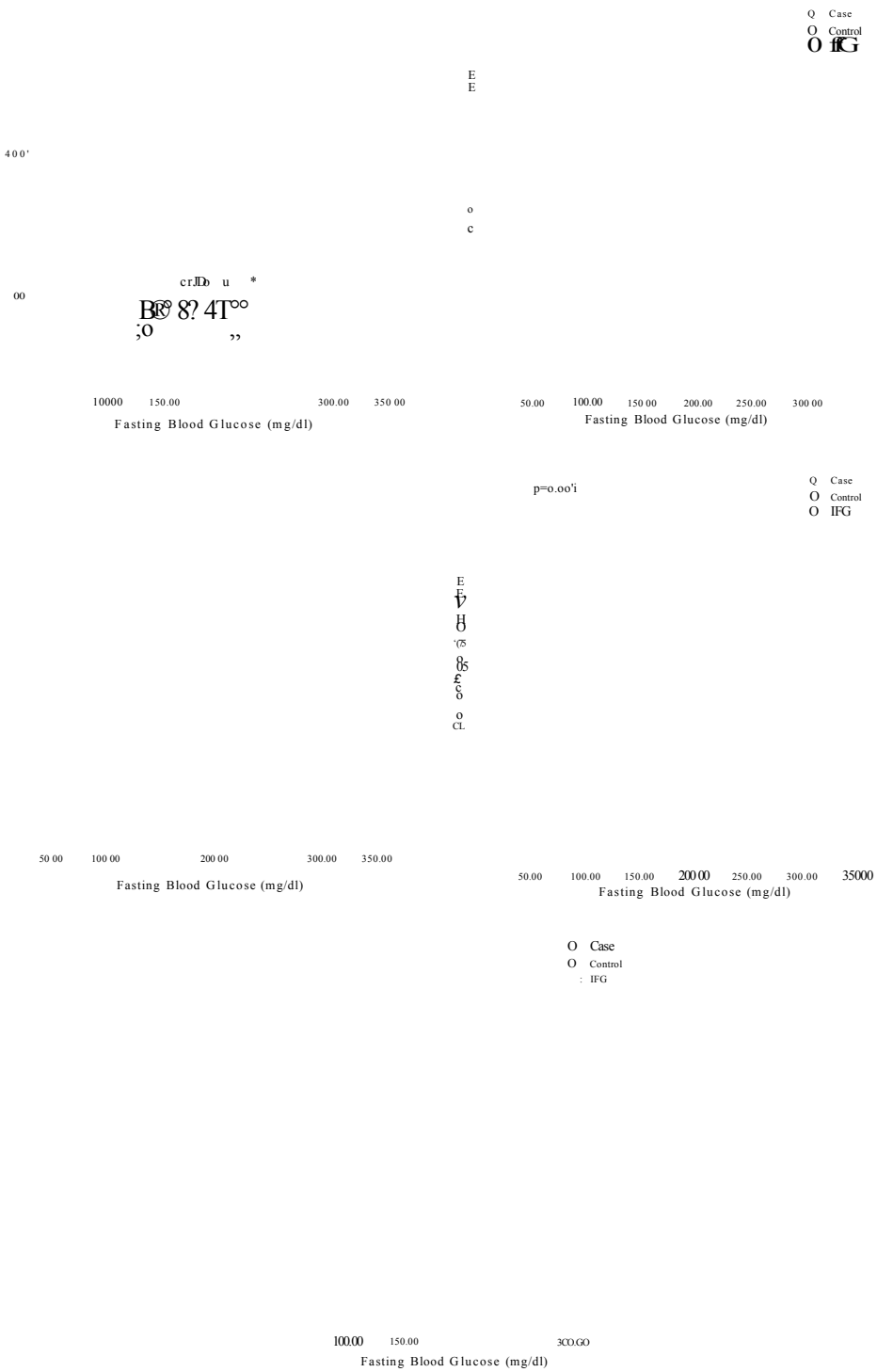


Figure 3.8. Significant correlations of Fasting Blood Glucose (FBG) with periodontal parameters

FBG significantly correlated with mean bleeding on probing, mean recession, mean probing depth, % sites > 4mm, mean and maximum loss of attachment

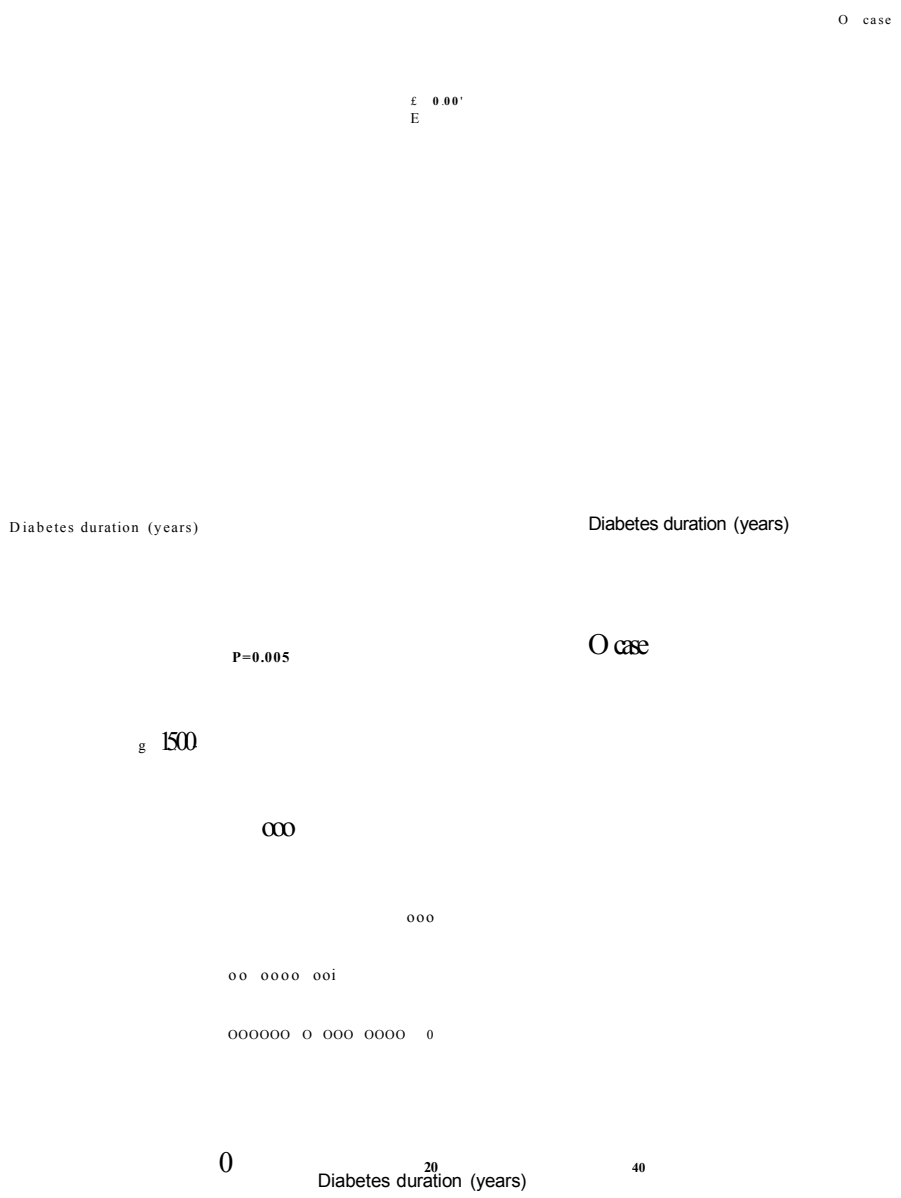


Figure 3.9. Significant correlations of diabetes duration with periodontal parameters,

Diabetes duration significantly correlated with mean recession, mean loss of attachment and maximum loss of attachment.

3.3.4 Impact of periodontal status on the diabetes parameters

The impact of periodontal status (i.e. a diagnosis of health, gingivitis or chronic periodontitis is presented in Table 3.5. Other than for fasting blood glucose (p=0.033) (Figure 3.10) there were no significant differences in biochemical parameters of diabetes care between the three periodontal diagnosis groups. Mann Whitney tests for group comparisons revealed that FBG is higher in the periodontal group than healthy group (p=0.012). Flowever, the difference was not significant between gingivitis and periodontitis (p= 0.847) or between health and gingivitis (p=0.039) (> 0.0167 which would be expected for Mann Whitney comparisons).

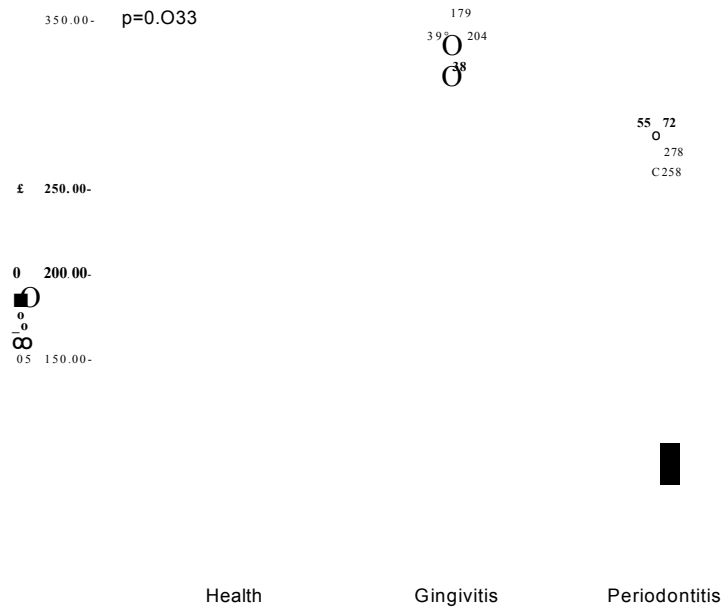


Figure 3.10 Box plots demonstrating fasting blood glucose (FBG) according to periodontal status

Significance difference was between health and periodontal group (p=0.033)

Table 3.5 Diabetes parameters according to periodontal status

	HbA1c (%)	TOC (mg/dl)	TGL (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	FBG (mg/dl)	Diabetes duration (years)
Health (n=137)	6.97 (5.95 - 8.00)	201.21 (40.14)	132.20 (171.00 -225.00)	126.82 (36.92)	48.11 (46.00 -52.00)	123.34 (87.00 -157.20)	7.30 (2.00 -12.00)
Gingivitis (n=104)	7.11 (5.70-7.80)	206.69 (39.84)	140.09 (83.00-177.0)	132.51 (36.28)	47.77 (46.00-51.00)	150.27 (92.00-179.5)	6.38 (2.00-9.00)
Periodontitis (n=116)	7.04 (6.00-7.80)	196 (40.19)	137.62 (87.00-158.15)	131.67 (36.49)	47.41 (45.00-50.00)	140.73 (100.23-164.25)	8.67 (3.5 - 12.50)
P	0.959	0.237	0.627	0.595	0.192	**0.033***	0.105

Mean and Standard deviation for TOC and LDL Median and interquatile ranges were present for other variables

* *Significant difference was observed between health and periodontal groups.*

3.4 Discussion

Patients with diabetes have an altered carbohydrate and lipid metabolism. As a result they typically have elevated levels of serum glucose, triglycerides, LDL, total cholesterol and normal serum level of HDL decreases. It is evident from the literature that infections have a negative impact on glycaemic control and other parameters of diabetes status, and it is presumed that this is also true for periodontitis (Lopes-Virella 1993). The present analysis aimed to investigate alterations in carbohydrate and lipid metabolism in patients with type 2 diabetes and non-diabetic controls according to their periodontal status

Similar to previous findings (Kim et al. 1993) HbA1c (7.37% vs. 5.82%), FBG 160.81 vs. 88.4 mg/dl) and TG (152.06 vs. 108.00 mg/dl) were significantly higher in patients with diabetes than non-diabetic controls. Furthermore and perhaps not initially anticipated, significantly lower systolic BP (129.63 vs. 136.9 mm/Hg), diastolic BP (81.00 vs. 85.60 mm/Hg) and LDL levels (123 vs. 140 mg/dl) were observed in patients with diabetes compared to controls. It has previously been reported that hypertension is present in 23%, hypercholesterolemia in 11%, hypertriglyceridaemia in 14% and high density lipoprotein cholesterol in 12% in the diabetic population from where the present sample were drawn (Weerasuriya et al. 1998). Patients with diabetes remain at high risk for cardiovascular events despite appropriate blood pressure management, LDL cholesterol and blood glucose control (Huxley et al. 2006). Therefore, the lower blood pressure and LDL levels reported by people with diabetes in this study may reflect an increase in the use of lipid and hypertension lowering medication and people with diabetes may be under appropriate medical care, whereas the controls are not.

According to National Cholesterol Education Program – Adult treatment Panel III (ATP III) of USA the treatment target is < 130mg/dl for serum cholesterol in the absence of diabetes or cardiovascular diseases. The target LDL level is reduced to <100mg/dl in patients with diabetes or coronary heart disease (CHD) (NCEP Adult Treatment Panel III, 2001). Based on 2005 American Diabetes Association guidelines, LDL cholesterol goal status (<100mg/dl), HDL cholesterol goal status (>45mg/dl), TG (<150mg/dl) and TOC (<240mg/dl). (American Diabetes Association 2006). The results of the present study in patients with diabetes demonstrate mean values which were higher than the expected goal

standards status for LDL and triglycerides except for total cholesterol which is 199mg/dl. When considering both non diabetic control group and IFG groups, they showed TOC 207.0 (non diabetic group) & 211.0 (IFG group) and LDL levels 140.5 (non diabetic) & 140.85 (IFG group) which were higher than the expected goal standards for a non diabetic population. Moreover the IFG group had elevated HbA1c and FBG levels. These data shows that the control population of the study is not metabolically healthy despite their FBG values.

This study recruited age and sex matched controls randomly from the same population that the type 2 diabetes patients were recruited from. These control patients had no previous history of diabetes. However, 16.67% of this group (12 patients) showed abnormal FBG and HbA1c levels. This is similar to the findings of a recently conducted study in Sri Lanka that identified that the prevalence of IFG was 14.2% in a group of (non-diabetic) people aged 30-65 years (Wijewardene et al. 2005) and 13.6% in urban populations of Sri Lankans aged ≥ 20 years (Katulanda et al. 2008). The individuals with possible IFG in the present study had a mean age of 53.17(48.3-5.5) years. Katulanda and co workers report in their study the prevalence of pre-diabetes (IFG and IGT) is 15.5% for the Sri Lankan population for the age group of 50-59 which is very similar to the percentage observed in the present study (Katulanda et al. 2008).

According to one recently published report, systemically healthy patients with moderate periodontitis demonstrated a significantly higher blood glucose levels than patients without periodontitis (Losche et al.2000). Although the numbers of subjects are low, 67% of the IFG group (i.e. 8 of 12) in the present study were diagnosed with chronic periodontitis. When considering serum lipid levels in this cohort of 12 IFG group, levels of LDL, TOC and TG , HbA1c, and FBG were higher than those identified in the normoglycaemic controls. It is possible that the low number of subjects in the IFG group contributed to the lack of statistical significance but studying a large cohort would demonstrate significant results. These carbohydrate and lipid abnormalities may be due to infection-induced (i.e. periodontitis-induced) abnormalities in metabolism. The IFG group showed the symptoms of hypertension and were significantly older (53.17(48.3-5.5) than the type 2 diabetes (45.70 (37.-54.5) and control group (45.05 (38-51.8). In addition the mean BMI value of the IFG group was 26.79(23.6-31.9) which indicate that they are at moderate risk of

morbidity and belong to the category of obese 1 (BMI within the range 25-29.9) for adult Asians (WHO technical report series 894 (2000). These results indicate that there is a possibility of older obese people to be at an increased risk of developing diabetes and associated complications like periodontitis.

The present study observed that people with periodontitis and gingivitis have FBG values which are significantly higher compared to people with periodontal health (140.73 mg/dl with periodontitis vs. 123.34 mg/dl in periodontal health, 150.27 mg/dl with gingivitis vs. 123.34 mg/dl in periodontal health). In addition, higher levels of HbA1c were observed in periodontitis (7.04%) and gingivitis (7.11%) than people with periodontal health (6.97%). Moreover, this study has observed longer diabetes duration, low HDL values and increased levels of triglycerides in gingivitis and periodontitis groups compared to healthy group but failed to reach statistical significance. Furthermore, FBG significantly correlated with all periodontal parameters except % sites with PD $\geq$ 4mm and % sites with PD $\geq$ 5mm. Moreover, positive significant correlation of triglyceride levels was observed with maximum probing depth indicating possible evidence of an impact of periodontitis on lipid metabolism. Other studies have reported similar findings. A study by Hisayama in Japan revealed a significant difference in deep pockets with impaired glucose tolerance in a subgroup of subjects with normal glucose tolerance ten years previously. This study also identified that each additional millimeter in mean pocket depth corresponded to 0.13% increase in HbA1c (Satio et al. 2004). Other studies have observed elevated serum total cholesterol levels was associated with periodontal damage assessed by the mean depth of periodontal pockets (Furukawa et al. 2007). These results suggest the possibility of an infection-induced (i.e. periodontitis induced) metabolic imbalance resulting in hyperlipidemia and hyperglycaemia.

The present study also identified significant associations between diabetes duration and mean recession, mean loss of attachment and maximum loss of attachment. This may result from the long term presence of periodontal disease in patients with diabetes, leading to the observed association between these periodontal variables and the duration of diabetes.

In summary it can be concluded that the subjects with diabetes in this population have hyperglycaemia and hyperlipidemia. This is to be expected, and is consistent with

previously reported research. Patients with periodontitis and gingivitis had significantly higher FBG values than healthy controls suggesting the possibility of periodontitis- induced hyperglycaemia. The severity of periodontitis as measured by Mean PD, Maximum PD, Maximum LOA, Mean Rec, mean BOP, %BOP and % sites with PD ≥4mm were significantly correlated with serum FBG values. Moreover, Mean Rec, Mean LOA and Maximum LOA correlated with diabetes duration and serum triglyceride levels were significantly correlated with Maximum PD and % sites with PD≥ 4mm, suggesting an underlying metabolic imbalance associated with periodontitis. Furthermore, 16.7% of the non diabetic population who come under the obese category according to Asian standards were at mean age of 53 showing impaired glycaemic control as measured by HbA1c and FBG values and 66.7% of this group were diagnosed with periodontitis. Clearly, the individuals with IFG may have undiagnosed diabetes and are at a high risk of developing diabetes related complications.

Chapter 4

Type 2 diabetes: Periodontal association

4.1 Periodontitits

Periodontal diseases encompass a range of conditions with variable clinical presentation ranging from gingivitis to chronic periodontitis and aggressive periodontitis. Gingivitis is highly prevalent, probably affecting (to some degree) the great majority of the population. The prevalence of advanced periodontitis is generally estimated to be 10-15% in most populations studied. The extent and severity of periodontitis can be assessed by the number of sites affected and degree of clinical attachment loss. Thresholds for defining severity of disease are not well defined, but severe periodontitis would certainly be characterised by loss of attachment ≥ 6mm (Preshaw et al. 2007). There is emerging consensus that although periodontal diseases are localised to the oral cavity, they may also have systemic impact too. In the context of diabetes, periodontal inflammation is associated with an elevated systemic inflammatory state which appears to alter glycaemic control. Furthermore, intervention trials suggest that periodontal therapy, which decreases the intra-oral bacterial bio-burden and reduces periodontal inflammation, can have a significant impact on systemic inflammatory status and is associated with improved glycaemic control in many patients with both diabetes and periodontal diseases (Mealey 2008).

4.1.1 Epidemiological data

Chronic periodontitis is considered as a disease of middle age with a majority of patients presenting in their 40s & 50s (Preshaw et al. 2007). In epidemiological terms, mild to moderate chronic periodontitis is common, affecting 40-60% of adults in Western populations whereas the prevalence of severe periodontitis is approximately 8-15% (Burt 1996, Fox et al. 1994, Kelly et al. 2000, Oliver et al. 1998). The UK Adult Dental Health Survey identified that in adults, dental plaque was visible in 72% and calculus was visible in 73% of patients, signifying a poor level of oral hygiene. However, moderate periodontal pocketing was observed in only 54% of people. In this population, approximately 43% of adults have moderately advanced chronic periodontitis (with attachment loss ≥ 4mm), and it was reported that 8% have severe periodontitis (attachment loss >6mm) (Office for National statistics 2000, Kelly et al. 2000). These data are, of course, prevalence data, and identifying incidence of periodontal disease is extremely difficult. However, according to recent statistics in the UK the annual incidence of patients presenting with symptoms of periodontal disease in general medical practice is reported as 6 per 10,000 people per year

(Birmingham Research Unit 2005). The prevalence of gingivitis in the U.S. population was estimated as 50% (Albandar et al. 1999). Furthermore, according to the National Health and Nutrition Examination survey (NHANES III) data, it has been identified that approximate values of moderate to severe periodontitis is 13% and 5.6% respectively for the US population aged 20 years older. Analysis of the data of this survey further revealed that 22.5% (23.8 million persons) have gingival recession (one or more tooth surfaces with ≥ 3mm). In addition, the presence of calculus and subgingival calculus were reported in 91.8% and 55.1% of the population, respectively (Albandar et al. 1999).

4.1.2 Periodontal disease severity and type 2 diabetes

In addition to the evidence observed for an increased prevalence of periodontitis in patients with type 2 diabetes (See section 1.10 and Table 1.2 for further details) increased severity of periodontal disease in patients with diabetes has been reported. A study conducted in Native Americans indicated an association of probing depth (>5mm) to the level of glycated haemoglobin (Nelson et al. 1996) thus suggesting more generalised infection may have a negative control on blood glucose level (Rees 2000). A recently conducted study in Jordan identified a higher prevalence of periodontitis in patients with diabetes than in normoglycaemic people based on clinical measures of PD, LOA and tooth mobility (Khader et al. 2008). In a similar study in Hispanic Americans, periodontal status was evaluated by measuring PD, LOA, plaque, BOP, visual gingival inflammation and calculus. This study reported that type 2 diabetes is associated with significantly more calculus formation, tooth loss and increased extent and severity of periodontal disease. In this population, subjects with diabetes demonstrated nearly three times greater LOA and PD >6mm, and approximately twice the frequency of moderate to advanced LOA (>3mm) than the non-diabetic population. In addition to the increased extent and severity of periodontal destruction, the Hispanic Americans with type 2 diabetes had more supra- and subgingival calculus, and an increased frequency of tooth loss due to periodontitis. This study also found synergistic effects of diabetes and smoking resulting in significantly higher frequency of sites with attachment loss ≥ 3mm (Novak et al. 2008).

Another case control study which was designed to study the prevalence of periodontitis among patients with diabetes had measured clinical and microbiological periodontal

parameters of periodontal disease among adult Sardinians. Patients with type 2 diabetes had significantly fewer remaining teeth, and more PD ≥4mm, BOP and plaque levels (Campus et al. 2005). Al-Otaibi and co-workers also identified that probing depths and clinical attachment levels were significantly higher in patients with diabetes than those without diabetes in Saudi Arabia (Al-Otaibi et al. 2008). These studies collectively provide evidence that measures of periodontal disease such as clinical attachment loss, probing depth and bleeding on probing are more advanced in type 2 diabetes.

4.1.3 Periodontitis and level of glycaemic control

Glycaemic control is clearly important when considering the role of diabetes as a risk factor for periodontal disease. It is clear from the literature that the severity of periodontal disease is greater in diabetic patients with poor metabolic control (Mangan et al. 2000). An increased level of glucose is observed in the gingival crevicular fluid of poorly controlled patients with diabetes mellitus (Kjellman 1970) which may alter plaque microflora with a resultant influence on the development of dental caries and possibly periodontal disease (Mealy 1998). It has been shown that the loss of periodontal attachment is most rapid during the elevation of blood glucose and the severity of periodontal disease has been found to decrease as control of diabetes improves (Ainamo et al. 1990). The monitoring of the effectiveness of diabetes control is done by measuring the levels of glycated serum proteins especially HbA1c which because of its incorporation into red blood cells gives an indication of the serum glucose levels over the proceeding 2 to 3 months (The Diabetes Control and Complication Trial Research Group 1993).

Taylor and colleagues have observed that individuals with poorly controlled type 2 diabetes compared to those without type 2 diabetes, or with better-controlled type 2 diabetes have a significantly greater risk for alveolar bone loss progression, with more severe bone loss than in subjects without type 2 diabetes (Taylor et al. 1998). Based on NHANES III data, individuals with poorly controlled type 2 diabetes had a significantly higher prevalence of severe periodontitis than those without diabetes after controlling for age, education, smoking and calculus (Tsai et al. 2002). In another study, the prevalence of severe attachment loss increased with decreasing control of diabetes as determined using HbA1c (Guzman et al. 2003). It has also been reported that people with impaired glucose tolerance

also have a higher prevalence of periodontitis in Pima Indians (Shlossman et al. 1987). In non diabetic patients, Loesche and co workers found fasting plasma glucose (FPG) levels to be on average 15% higher in periodontitis patients compared to controls. (Losche et al. 2000). In contrast, Noack and co-workers did not demonstrate an association between increased blood glucose levels and periodontal disease (Noack et al. 2000) and Marugame and co-workers found no association between alveolar bone loss and impaired glucose tolerance and periodontitis (Marugame et al. 2003).

4.1.4 Periodontal treatment and metabolic control of diabetes

A systemic effect of periodontal disease has been suggested in periodontal treatment studies which incorporate systemic antibiotics. Conventional mechanical periodontal therapy (subgingival scaling, periodontal surgery and dental extractions) had shown no effect on levels of HbA1c levels in subjects with poorly controlled type 1 diabetes (da Cruz et al. 2008, Seppala & Ainamo 1997, Aldrige et al. 1995, and Smith et al. 1996). Scaling and root planing with adjunctive subgingival irrigation with 0.2% chlorohexidine had no effect on levels of HbA1c, concentration of C-peptide, creatinine, C-reactive protein and fibrinogen in well controlled type 1 and type 2 diabetic patients (Christgau et al. 1998). However, a significant reduction of probing depth and clinical attachment level were observed three months after mechanical therapy (ultrasonic bactericidal curettage) (Grossi et al. 1997). In contrast, studies incorporating systemic antibiotics with conventional mechanical periodontal treatment consistently reported improvement in metabolic control following the periodontal therapy. Grossi et al (1997) studied the effect of periodontal therapy on glycaemic control in patients with type 2 diabetes. 85 patients were divided into four treatment groups and they were followed for a period of one year post treatment period. All patients in the 4 groups received scaling and root planing, and then received topical irrigation with water, 0.12% chlorohexidine, or 0.05% iodine during therapy. Three of the four groups also received 14 days of systemic doxycycline therapy. Significant reductions in HbA1c values were observed three months after the treatment in the three groups who received doxycycline therapy irrespective of the irrigant used. However, this beneficial effect was lost at the subsequent 6-9-and 12 months visit. This study supports the hypothesis that anti-inflammatory therapy used for the treatment of periodontal disease can potentially improve metabolic control of diabetes (Grossi et al. 1997) In a later study, Kiran and co-workers found that periodontal treatment in a cohort of patients with type 2

diabetes resulted in improved glycaemic control three months after treatment. In this study, HbA1c scores reduced from 7.3% to 6.5% compared to controls, who demonstrated a (non significant) increase in HbA1c from 7% to 7.3% over the same timescale (Kiran et al. 2005).

A study conducted in China also evaluated the effect of non-surgical periodontal therapy on glycaemic control in patients with type 2 diabetes. In this study, one group of patients was treated with scaling and root planing, and both study groups received systemic doxycycline. This study revealed significant reductions in plaque index and bleeding on probing scores in the group that underwent mechanical periodontal therapy, and both study groups demonstrated reductions in HbA1c values, which was postulated as being possibly related to the systemic antibiotic (Yun et al. 2007). A similar study was recently conducted in the USA, in which HbA1c scores were evaluated following minimal or enhanced non-surgical, non antibiotic treatment in patients with type 2 diabetes. Patients with mildly elevated (>7% and <9%) and severely elevated (> 9%) HbA1c scores were randomized to one of two non-surgical periodontal therapy protocols. One group received minimal therapy (scaling, root planing and oral hygiene instruction) once in six months whereas the other group received frequent episodes of scaling, root planing and oral hygiene instructions on two occasions six months apart, together with additional 0.12% chlorhexidine rinse twice a day. This study observed that clinical loss of attachment (LOA) unchanged in both groups but other measures such as probing depth (PD) and gingival index (GI) were improved in both groups. Moreover an overall modest improvement in HbA1c was observed in both groups with a trend towards the frequent periodontal therapy having a greater impact (3.7% reduction) than the minimal therapy with 1.6% reduction (Madden et al. 2008).

A meta-analysis of 10 intervention studies to estimate the effect of periodontal treatment on HbA1c identified a weighted mean absolute reduction in HbA1c values of 0.66% in patients with type 2 diabetes following periodontal treatment, though this did not achieve statistical significance (Janket et al. 2005). A similar reduction level of HbA1c scores (0.6%) was observed in the study by Madden and co-workers referred to above (Madden et al. 2008). A study that evaluated the impact of mechanical periodontal therapy combined with subgingival application of minocycline gel also identified a similar reduction in HbA1c levels (0.8% reduction) in 13 Japanese patients with type 2 diabetes (Iwamoto et al.

2001). Collectively, these results suggest that periodontal treatment has the potential to improve glycaemic control in patients with type 2 diabetes.

4.1.5 Glycaemic control and complications

The control of diabetes focuses on controlling blood glucose levels, and there is clear evidence that diabetes complications can be prevented by tight glycaemic control. The Diabetes Control and Complication Trial (DCCT) conducted in 1993 concluded that, after 6.5 years of follow-up, that strict glycaemic control can delay the onset of complications and reductions in the progression of existing complications (DCCT) (DCCT group 1993, The diabetes control and complication trial/ Epidemiology of Diabetes Interventions and Complications Research Group 2000). Similar results were obtained from the UK Prospective Diabetes Study (UKPDS) (UKPDS 1998) in which, after following patients for an average of 10 years, it was concluded that tight control of blood sugar is associated with reduction in microvascular complications of disease. This study identified a gradational effect with a 1% reduction in HbA1c scores accounting for a 35% reduction in diabetes complications and a 25% reduction in diabetes related deaths. In both studies outcome measures were the onset and progression of diabetic retinopathy, nephropathy and neuropathy. However, improved glycaemic control (as measured by reduction of HbA1c) achieved with intensive insulin treatment in patients with type 1 diabetes did not result in any detectable improvements in periodontal parameters (PD, LOA or BOP) (Sastrowijoto et al. 1990).

4.1.6 Periodontitis and diabetes duration

Several studies have suggested that the age at onset of diabetes, and the duration of diabetes may be a more critical factor than the level of glycaemic control when considering oral and systemic complications (Bacic et al. 1998, Taylor et al. 1998, Cerda et al. 1994). Al-shamari and co-workers have investigated the association of periodontal disease severity with diabetes duration and diabetic complications in patients with type 1 diabetes in Kuwait and identified that patients who had diabetes for more than 5 years had more severe attachment loss and a higher number of missing teeth than those patients who had diabetes for less than 5 years (Alshammari et al. 2006). In another study, the severity of periodontal disease as demonstrated by periodontal pocket depth was significantly higher in patients with duration of diabetes of more than 5 years (Khader et al. 2008). This parallels findings

from other studies of complications of diabetes, in that the greater the diabetes duration, then the greater the prevalence and severity of the complication (Nathan 1993).

4.1.7 Obesity and periodontitis

Obesity has also been linked to increased periodontal disease (Alabdulkarim et al. 2005). Saito and coworkers found that relative risk of periodontitis after adjustment for confounders (age, gender, oral-hygiene, smoking) was 3.4 in persons with BMI of 25-29.9 kg/m2 and 8.6 in those with BMI >30kg/m2 (Saito et al. 2001). Genco and coworkers based on data from the third NHANES III survey demonstrated that BMI was positively correlated with the severity of periodontal attachment loss (Genco et al. 2005).

4.1.8 Chapter aims and objectives

There is clear, and firmly established evidence to support that there is an association between type 2 diabetes and periodontitis in different populations. In Sri Lanka, the prevalence of type 2 diabetes rate is moderately high (14.2%) in adults of 30-65 years (Wijewardena et al. 2005) and 10.3% for Sri Lankans ≥ 20 years of age (Katulanda et al. 2008). The diagnosis in many cases is incidental and it has been reported that 36% of the people with diabetes were previously undiagnosed in this population. The prevalence of pre-diabetes was 11.5% (Katulanda et al. 2008). Usually by this time of diagnosis of diabetes complications have developed and progressed. The prevalence of diabetes complications at the time of diagnosis has been reported previously in this population (Weerasuriya et al. 1998). Records of periodontal status in Sri Lankans date back to late 1970 where the periodontal examinations were conducted in rural tea workers who had no access to treatment and periodontal care (Loe et al. 1978, Anerud et al. 1979). However, the association of periodontitis with type 2 diabetes has not been evaluated.

Therefore, the aims of this chapter were to

1. evaluate the prevalence and severity of periodontal disease in type 2 diabetes and a matched control population of urban Sri Lankans
2. study the relationship between glycaemic control and periodontal disease severity in this population
3. investigate the impact of age and duration of diabetes on periodontal status.

4.2 Method

4.2.1 Ethical Clearance and Informed consent

Ethical clearance for the study was obtained from the Ethics Committee of University of Sri Jayewardenepura, the Ethics committee of the Sri Lanka Medical Association and the Newcastle and North Tyne side Health Authority and Joint Ethics Committee. The informed consent was obtained from patients and controls after the nature of the procedure, purpose of the study, and possible risks and benefits had been fully explained verbally in addition to the Patient Information Leaflet provided. They were also notified that they were free to discontinue their participation in the study at anytime without any negative effect on their usual medical care. Those who agreed to participate, gave written consent by signing a consent form.

4.2.2 Inclusion criteria and exclusion criteria

Patients with type 2 diabetes and normoglycaemic controls who underwent periodontal examination were included in the study. Patients who were edentulous were excluded from the primary dental analysis (n=2). In addition people with other known systemic conditions such as rheumatoid arthritis or HIV infections were excluded from the study.

4.2.3 Study population

Age and sex matched subjects in the non diabetic control group was based on a power calculation to detect a difference between groups in attachment level of 0.6mm with 90% power assuming a significance of 5% and 271 subjects with type 2 diabetes and 50 controls were required. Statisticians help was sought in the power calculation and subjects of Sri Lankan origin were invited to participate in this study. There were 285 patients with type 2 diabetes and 72 controls responded. The control group was selected from the same population as the patients were recruited from and had no previous records of diabetes. Based on control classification (Sec 3.2.5) controls who exceeded normoglycaemia (n=12) were excluded from the present analysis. Therefore, the analysis of impact on diabetes status, periodontal status and periodontal variables were based on 345 subjects with confirmed type 2 diabetes (n=285) and confirmed non diabetic controls (n=60).

4.2.4 General health status

All the patients were examined by a medical professional for their systolic blood pressure, and diastolic blood pressure. Height and weight measurements of the subjects were recorded. Weight in Kilograms (Kg) divided by height in square meters (M^2) were used to calculate body mass index (BMI) of each subject (Kg/M^2). In addition data regarding age, date of birth, smoking status and alcohol consumption were recorded. In patients with diabetes, age of onset of diabetes, diabetes duration and hypo treatment were recorded.

4.2.5 Measuring probing depth (PD) and loss of attachment (LOA)

The prevalence and severity of destructive periodontal disease was determined by measuring the PD and clinical LOA. The manual TPS periodontal probe (Vivadent, Schaan, Lichtenstein) was used to record probing depths at 6 sites per tooth on all teeth present (excluding 3rd molars). The distance between the free gingival margin (FGM) and the apical depth of the probe tip penetration was measured as the PD at each site. The distance between the CEJ and the gingival margin was recorded. Gingival recession (GR) was recorded by measuring the distance between CEJ and FGM. All measurements taken were rounded up to the nearest millimetre and clinical LOA was computed using GR and PD values. Measured values were used to calculate periodontal parameters PD (% sites with PD > 4mm, % sites with PD > 5mm and % sites with PD > 6mm), Mean PD, Maximum PD, loss of attachment (LOA) (% sites with LOA >1mm, % sites with LOA > 2mm, % sites with LOA > 3mm, % sites with LOA > 4mm, % sites with LOA > 5mm), Mean LOA, Maximum LOA, and mean recession which were the continuous variables considered in this study. Number of missing teeth from each subject were counted and recorded.

4.2.6 Diagnosis

Subjects were clinically diagnosed using the diagnostic criteria for periodontal diseases that were proposed by the European Workshop of periodontology 2005 and the centre for disease control and American academy of periodontology(CDC-AAP) collaboration 2007 (Tonetti 2005, Page 2007).

Periodontal health : If there is no probing depth (PD) of the true pocket is ≥ 3mm and minimal bleeding on probing is ≤15%.

Gingivitis : In addition to clinical features if the PD ≤ 4mm and demonstrate ≥ 15% BOP the subjects were diagnosed for gingivitis

Chronic periodontitis : diagnosis was made when there were ≥ 6 sites with PD of ≥ 5mm

4.2.7 Grouping cases according to HbA1c values

Patients were categorized into good, moderate and poor control of diabetes based on their HbA1c value. HbA1c value is considered as a good measure of glycaemic control as they reflect the blood glucose levels over the previous three months (Waugh et al. 2007). They were considered to be in good control if the HbA1c values were less than or equal 7% (HbA1c ≤ 7%). If the values were between >7% -8.5% they were considered as moderate control and above 8.5% were catogorised as poor diabetes control (Qaseem et al. 2007).

4.2.8 Statistical analysis

Categorical grouping variables in this study included periodontal status (health, gingivitis, periodontitis) and diabetes status (diabetic with good control, diabetic with moderate control, diabetic with poor control, non-diabetic). A cohort of subjects (n=12) who, according to medical history were initially believed non-diabetic, but who transpired to have blood results suggestive of impaired glucose tolerance or possible diabetes, were excluded from analyses. Impact of periodontal status on diabetes status (and vice versa) was determined using chi-squared statistics. The same statistics were applied to find the difference in distribution of periodontal diagnosis according to gender smoking and alcoholism. To compare the distribution of continuous variables, independent sample t-test statistics were employed for parametric data. Comparisons were made between the groups using analysis of variance with post-hoc Bonferroni corrections. When the data were not normally distributed comparisons between groups were made using Kruskal-Wallis test

with post hoc Mann Whitney tests. When employed Mann Whitney test to compare groups the critical value p-0.05 was adjusted according to the number of post-hocs performed to avoid inflating type 1 error. For some analyses the diabetic patients were collapsed into one group, and compared against the non-diabetic patients. In these analyses, comparisons between the groups were made using unpaired t tests (parametric data) or Mann Whitney tests (non-parametric data). Box plots were plotted for all variables, to indicate median values, interquartile ranges, outliers (1.5-3.0 interquartile ranges from the box) and extreme values (>3.0 interquartile ranges from the box). Correlations between the variables were assessed by Spearman's correlation coefficient for non parametric data. Scatter diagrams were constructed to illustrate these associations. Multiple linear regression analyses were employed to identify the ability of key predictor variables to predict outcomes. Significance of all the tests was assessed at 5% level.

4.3 Results

4.3.1 Subject characteristics

A total of 357 subjects recruited represent 285 patients with type 2 diabetes and 72 controls. According to HbA1c criteria, 12 (16.6%) of the control subjects were identified as having impaired metabolic conditions hence the final periodontal analysis included 285 patients and 60 normoglycaemic controls. Females in the study group were 51.5% (184/357). Mean (SD) of age was 45.84(±11.99). There were 7.8% current smokers and 12.3% ex-smokers. 26.1% of the study population were alcohol consumers.

4.3.2 Periodontal status

Table 4.1. shows the proportions of individuals within the diabetes and control groups according to periodontal diagnoses (Group 1= health, gingivitis and chronic periodontitis), (Group 2 = Health/Gingivitis and Chronic periodontitis). Among patients with diabetes 38.2% were periodontally healthy compared to 41.7% of the controls. 28.4% of diabetes group and 36.7% of the controls had gingivitis. Chronic periodontitis was present in 33.3% of the patients with type 2 diabetes and 21.7% of controls. However the difference was not statistically significant (p=0.18) A strong trend towards a greater proportion of the people with diabetes also having chronic periodontitis compared to non-diabetic controls was observed when health and gingivitis patients were considered as a single group (p=0.077)

Table 4.1 Periodontal diagnosis in patients with type 2 diabetes and controls

Periodontal Health	**Diabetes /control**		**Total**	
Group 1	**Diabetes (n=285)**	**Control (n=60)**	(n=345)	
Health	109 (38.2%)	25 (41.7%)	134 (38.8%)	
Gingivitis	81 (28.4%)	22 (36.7%)	103 (29.9%)	p= 0.180
Chronic Periodontitis	95 (33.3%)	13 (21.7%)	108 (32.5%)	
Total	285 (100%)	60 (100%)	345 (100%)	
Group 2				
Health/Gingivitis	190(66.7%)	47(78.3%)	237 (68.7%)	
Periodontitis	95(33.3%)	13 (21.7%)	108 (31.3%)	p=0.077
Total	285(100%)	60(100%)	345(100%)	

P > 0.05 in group 1 and group 2 periodontal diagnosis categories

4.3.3 Impact of diabetic status on periodontal parameters

Table 4.2 shows the distribution of periodontal parameters between patients with type 2 diabetes and controls. Subjects with type 2 diabetes generally showed increased disease severity compared to controls although all periodontal parameters failed to achieve statistical significant difference. Measures for mean recession (mean Rec) (p=0.005) mean bleeding on probing BOP (p=0.001), maximum PD (p=0.001), maximum LOA (p= 0.001), and % BOP (p=0.015), % sites with PD ≥ 4mm, (p=0.001), ≥ 5mm (p=0.007) and ≥ 6mm (p=0.020) were significantly higher in the group with type 2 diabetes than controls (Figure 4.1).

4.3.4 Periodontal status according to level of glycaemic control

Patients with type 2 diabetes were catagorised into good, moderate and poor control diabetes according to HbA1c values. 37.5% of the good control, 38.3% of the moderate control and 26.8% of the poorly controlled group were affected with periodontitis (table 4.3). Table 4.4 presents data of periodontal parameter distribution according to level of glycaemic control. When the impact of glycaemic control on periodontal parameters was examined, statistically significant effects of diabetes status were identified for maximum PD (p=0.025) % BOP (p=0.002), Maximum LOA (p=0.015) % sites with probing depth (PD) ≥ 4mm and % sites with PD ≥ 5mm (p=0.05). For % BOP the differences were more likely to be between good (18.97%), (p=0.001) and poor (18.78%), ((p=0.002) vs non diabetic controls (8.29%). In addition significant differences were observed for maximum PD (4.47mm vs. 3,8mm) (p=0.003) and maximum LOA (6.24mm vs. 5.14mm) (p=0.002) in subjects with diabetes who have good glycaemic control and non diabetic controls. Moreover subjects with moderate glycaemic control displayed significant difference in LOA (6.27mm) compared to non diabetic controls (5.14mm) (p=0.011). Considering the % sites with PD ≥ 4mm, a significant difference exists between diabetes with good glycaemic controls (12.93%) and non diabetic controls (5.08%) (p=0.002). Similarly, % sites with PD ≥ 4mm showed a significant difference between poor glycaemic control (6.74%) and non diabetic controls (5.07%) (p=0.021). When considering the % sites with PD ≥ 5mm the difference was again significant between good glycaemic control (5.45%) and non diabetic controls (2.77%) (p=0.007). Collectively these data provide evidence that in people with

diabetes, the good glycaemic control group have greater disease severity than non diabetic controls (Figure 4.2).

Table 4.2. Periodontal parameter according to type 2 diabetes and control

	Type 2 diabetes (n=285)	Controls (n=60)	p value
Mean probing depth (mm)	2.13 (1.61-2.39)	2.0(1.51-2.32)	0.552
Mean recession (mm)	0.48 (0.04-0.49)	0.34 (0.02-0.27)	**0.005**
Mean bleeding on probing	0.17 (0.18-0.23)	0.08 (0.01-0.11)	**0.001**
Mean loss of attachment (mm)	2.61(2.14-2.87)	2.34(1.71-2.44)	0.214
Maximum probing depth (mm)	4.47 (3.0-5.0)	3.8 (3.0-5.0)	**0.001**
Maximum loss of attachment (mm)	6.37 (4.0-8.0)	5.14 (3.0-6.0)	**0.001**
%BOP	16.75 (1.80-22.9)	8.3 (0.6-10.7)	**0.015**
% of sites with PD ≥4mm	10.47(0.00-9.9)	5.08 (0.0-2.4)	**0.001**
% of sites with PD ≥5mm	4.52 (0.00-2.08)	2.77 (0.00-0.60)	**0.007**
% of sites with PD ≥6mm	0.91(0.00-0.00)	1.04 (2.0-2.0)	**0.020**
% sites with LOA ≥ 1mm	99.60(100-100)	99.73 (100-100)	0.873
% sites with LOA ≥ 2mm	72.71 (58.83-91.85)	72.57(60.50-95.60)	0.520
% sites with LOA ≥ 3mm	37.54(13.70-58.00)	29.66 (7.90-44.20)	0.066
% sites with LOA ≥ 4mm	21.53 (2.4-32.50)	15.88 (1.60-20.88)	0.109
% sites with LOA ≥ 5mm	18.05 (1.73-24.47)	13.15 (1.20-11.55)	0.211
% sites with LOA ≥ 6mm	15.46 (1.30-20.57)	15.06 (0.78– 24.75)	0.429
Missing teeth	4.83 (1.00-7.00)	5.33 (1.00-7.00)	0.437

Median and inter-quartile ranges are present for all the variables. Overall significance between two groups was assessed at 0.05 level (Kruskal-Wallis test for non parametric data)

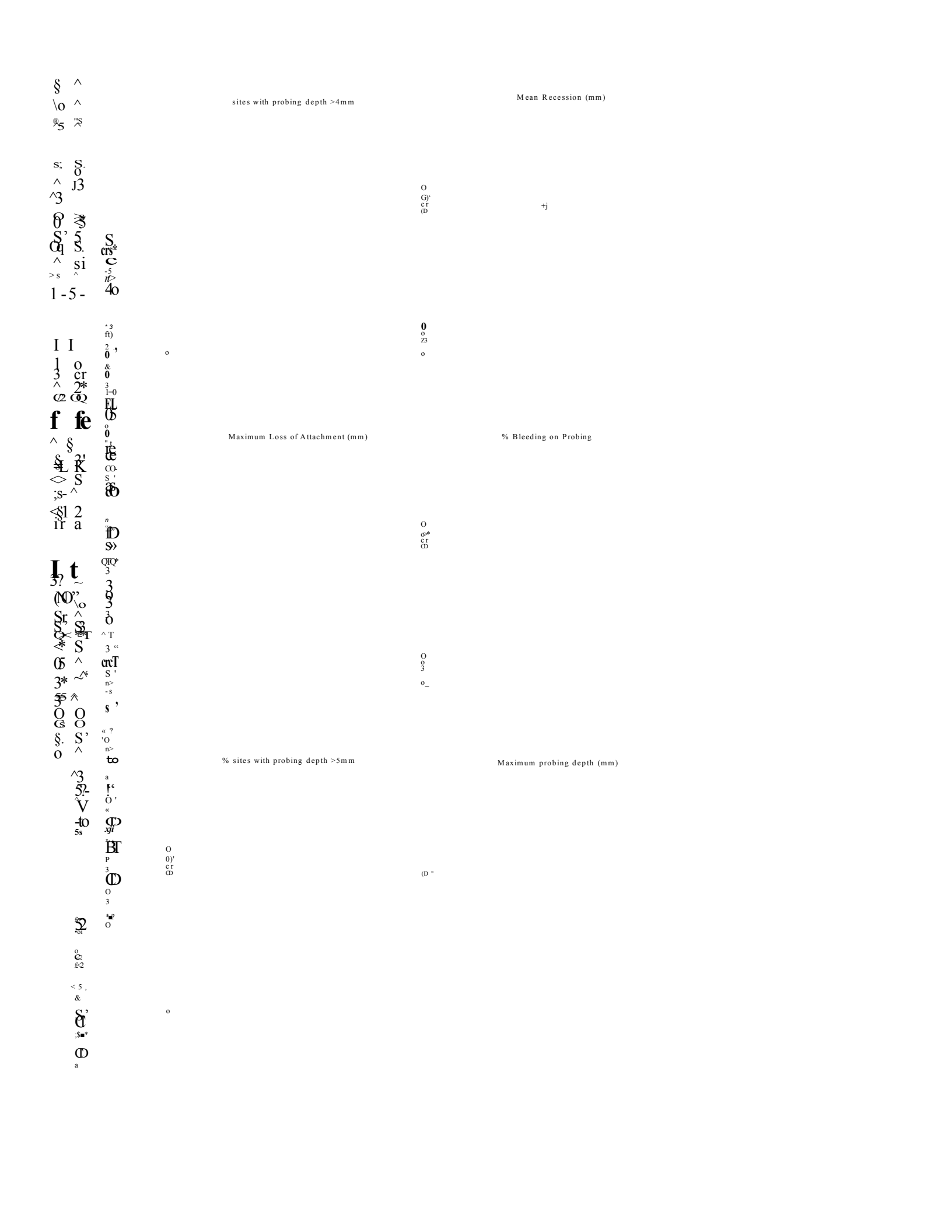

sites with probing depth >4mm
Mean Recession (mm)
Maximum Loss of Attachment (mm)
% Bleeding on Probing
% sites with probing depth >5mm
Maximum probing depth (mm)

Table 4.3. Periodontal health according to level of glycaemic control

Periodontal diagnosis	Cases			Controls
	good glycaemic control (n=112)	Moderate glycaemic control (n=53)	Poor glycaemic control (n=41)	(n=60)
Health (n=109)	41(36.6%)	26 (49.1)	14(34.1%)	25(41.7%)
Gingivitis (n=81)	29(25.9%)	12(22.6%)	16(39%)	22(36.7%)
Chronic periodontitis (n=95)	42(37.5%)	15(28.3%)	11(26.8%)	13(21.7%)
Total	112 (100%)	53(100%)	41(100%)	60(100%)

$\chi^2 = 0.172$
n= number and percentages in brackets

4.3.5 Correlations between periodontal parameters

Table 4.5 presents data for spearman's correlation coefficient for periodontal parameters. All the periodontal parameters (MeanPD, MeanRec, MeanBOP, MeanLOA, Maxumum LOA, % BOP, % sites with PD >4mm, >5mm and >6 mm) significantly correlated with each other at $p<0.01$ level. (Figure 4.3 represent some of these correlations).

Table 4.4. Periodontal scores according to level of glycaemic control

Periodontal parameter	Cases			Controls	p
	good glycaemic control (n=112)	Moderate glycaemic control (n=53)	Poor glycaemic control (n=41)		
Mean probing depth (mm)	2.21 (1.58-2.73)	1.98 (1.59-2.24)	2.05 (1.63-2.25)	1.99 (1.51-2.32)	0.756
Mean recession (mm)	0.46 (0.05-0.42)	0.45 (0.05-0.62)	0.49 (0.44-0.43)	0.34 (0.22-0.27)	0.074
Mean loss of attachment (mm)	2.66 (1.74-3.33)	2.44 (1.82-2.59)	2.53 (1.81-2.73)	2.34 (1.71-2.44)	0.691
Maximum probing depth (mm)	**4.47 (3.00-5.00)**	4.25 (3.00-5.00)	4.37 (3.00-5.00)	**3.80 (3.00-5.00)**	**0.025**
Maximum loss of attachment	**6.24 (4.00-8.00)**	**6.27 (4.00-8.00)**	6.39 (4.00-7.50)	**5.14 (3.00-6.00)**	**0.015**
%BOP	**18.97 (1.88-30.7)**	12.65 (0.60-19.85)	**18.78 (3.10-24.75)**	**8.29 (0.60-10.70)**	**0.002**
% of sites with PD ≥ 4mm	**12.93 (0.00-18.53)**	6.28 (0.00-4.68)	6.74 (0.00-5.85)	**5.07 (0.00-2.40)**	**0.013**
% of sites with PD ≥ 5mm	5.45 (0.00-4.68)	2.45 (0.00-1.35)	3.35 (0.00-2.30)	2.77 (0.00-0.60)	**0.050**
% of sites with PD ≥ 6mm	0.87 (0.00-0.00)	0.29 (0.00-0.00)	1.15 (0.00-0.00)	1.04 (0.00-0.00)	0.076
Missing teeth	5.38 (1.00-9.00)	4.45 (1.00-6.00)	3.90 (0.25-5.00)	5.33 (1.00-7.00)	0.480
% sites with LOA ≥ 1mm	99.81 (100-100)	99.25 (100-100)	99.68 (100-100)	99.73 (100-100)	0.533
% sites with LOA ≥ 2mm	72.35 (52.1-96.2)	72.63 (59.60-87.48)	72.21 (64.15-86.55)	72.43 (60.5-95.6)	0.662
% sites with LOA ≥ 3mm	41.03 (13.45-71.30)	32.98 (12.30-46.40)	35.15 (13.35-49.05)	29.66 (7.90-44.20)	0.241
% sites with LOA ≥ 4mm	24.50 (2.25-43.20)	17.39 (3.35-20.50)	18.69 (1.83-23.05)	15.88 (1.60-20.88)	0.328
% sites with LOA ≥ 5mm	20.01 (1.75-29.1)	33.99 (1.30-13.38)	17.80 (1.98-25.00)	13.15 (1.20-11.55)	0.374
% sites with LOA ≥ 6mm	16.70 (2.10-21.18)	11.43 (0.70-9.33)	14.69 (0.70-26.6)	15.06 (0.78-2475)	0.246

Significance were accessed at 0.05% level

Maximum probing depth and % sites with probing depth >4mm and > 5mm was significantly different between DGC and NDC/ maximum loss of attachment was significantly different between DGC, DMC and NDC/ % BOP was significantly different between DGC, DFC and NDC.

>Bleeding On Probing (%BOP)

sites wilh probing depth :-4mm

Maximunn Probing depth (mm)

% sites witli probing depth >5mm

Maximum Loss of Attachment (mm)

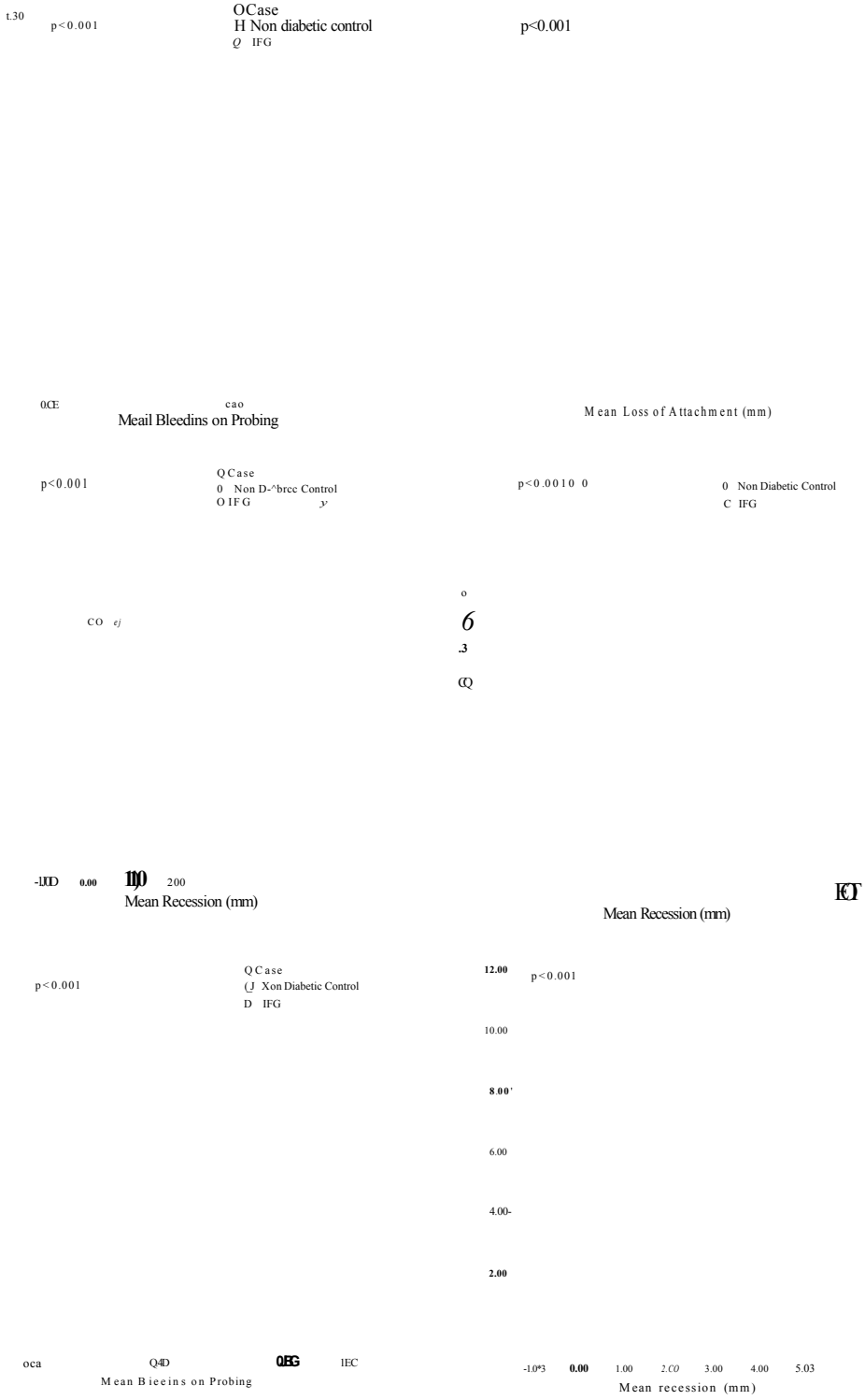


Figure 4.3. Significant correlations between periodontal parameters

Significant correlations were observed between all the periodontal parameters at 5% level.

Table 4.5. Correlation coefficient of periodontal parameters

	MeanPD (mm)	MeanRec (mm)	Mean BOP (%)	Mean LOA (mm)	Maximum PD (mm)	Maximum LOA (mm)	%BOP	% of sites with PD ≥4mm	% of sites with PD ≥5mm
MeanRrec (mm)	0.344*	-	-	-	-	-	-	-	-
MeanBOP	0.515*	0.251*	-	-	-	-	-	-	-
MeanLOA (mm)	0.899*	0.646*	0.470*	-	-	-	-	-	-
Maximum PD (mm)	0633*	0.455*	0.502*	0.669*	-	-	-	-	-
Maximum LOA(mm)	0.561*	0.796*	0.369*	0.757*	0.714*	-	-	-	-
%BOP	0.518*	0.252*	0.999*	0.473*	0.504*	0.371*	-	-	-
% of sites with PD ≥4mm	0.736*	0.488*	0.575*	0.751*	0.904*	0.714*	0.577*	-	-
% of sites with PD ≥5mm	0.639*	0.479*	0.484*	0.664*	0.876*	0.703*	0.485*	0.835*	-
% of sites with PD ≥6mm	0.498	0.383*	0.413*	0.510*	0.649*	0.516*	0.414*	0.557*	0.649*

Spearman's rho correlation coefficients * indicates values were significant at 0.01 level

MeanPD – Mean Probing Depth MeanRec-Mean Recession MeanBOP- Mean Bleeding On Probing, MeanLOA- Mean Loss of Attachment Maximum PD- Maximum Probing Depth Maximum LOA - Maximum Loss of Attachment BOP- Bleeding on Probing

4.3.6 Impact of gender on periodontal status

Table 4.6 presents the data on periodontal status according to gender. 33.7% of the females and 31.2% of the males were affected with chronic periodontitis, and there were no statistically significant differences between males and females according to periodontal diagnosis.

Table 4.6. Periodontal health according to gender

Periodontal Health	Gender		Total
	Female	Male	
Health	67(36.4%)	70(40.5%)	137(38.4%)
Gingivitis	55(29.9%)	49(28.3%)	104(29.1%)
Chronic periodontitis	62(33.7%)	54(31.2%)	16(32.5%)
Total	184(100%)	173(100%)	357(100%)

Group 1 χ^2=0.579

4.3.7 Periodontal status according to age, blood pressure and BMI

The mean (inter-quartile range) age was 42.0 (36.0-47.0) years for people with periodontal health, 42.5 (36.0-49.75) for gingivitis and 53.4 (43.3-63.0) for chronic periodontitis. Age differed significantly between the three groups ($p<0.01$) (figure 4.4). Comparison between groups revealed that people with periodontitis were significantly older than those with periodontal health or gingivitis ($p=0.001$). Systolic blood pressure showed a trend towards being higher in the periodontal group (135.26 mm/Hg), but failed to achieve statistical significance however ($p=0.079$). Diastolic blood pressure and BMI had no effect on periodontal status (Table 4.7).

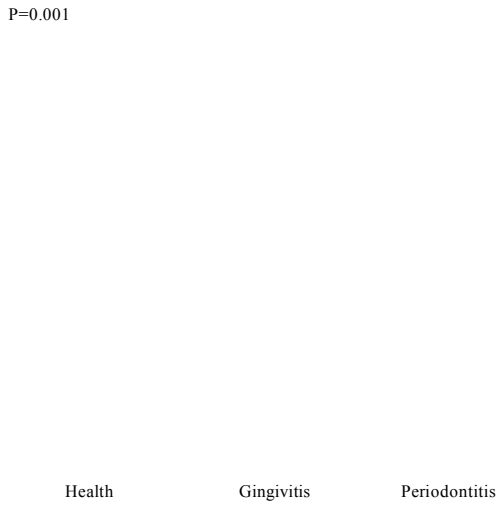


Figure. 4.4 Box plots that illustrate age distribution according to periodontal status

Age distribution was significantly different between health and periodontitis and gingivitis and periodontitis (p<0.001)

4.3.8 Age, BMI and blood pressure distribution according to levels of glycaemic control

Statistically significance difference was observed between level of glycaemic control and systolic BP and diastolic BP. When compared the three glycaemic control groups and non diabetic controls, systolic and diastolic blood pressure was higher in non diabetic controls compared to diabetes with good control. (p=<0.01) No statistical significance difference was observed for age and BMI according to glycaemic levels (Table 4.8, Figure 4.5).

4.3.9 Periodontal health and Alcohol consumption

A total of 26.1% of the subjects were current alcohol consumers whereas 72.8% were not. It was observed that far fewer females in this population consumed alcohol (0.8%) compared to males (25.5%). Among those who consume alcohol, 44.1% were periodontally healthy, 24.7% were affected with gingivitis and 31.2% had chronic periodontitis. Of those who never consumed alcohol, 36.5% were periodontally healthy, 30.8% had gingivitis, and 32.7% had chronic periodontitis. However, there were no significant differences between these groups with regard to periodontal status (p=0.798).

Table 4.7 Age, blood pressure and BMI distribution according to periodontal health

Periodontal Health	Age (years)	Systolic BP (mm/Hg)	Diastolic BP (mm/Hg)	BMI (kg/m^2)
Health (n=137)	42.01 (36.00-47.00)	130.98 (120.00-140.00)	81.54 (76.00-87.00)	24.83 (22.46-26.96)
Gingivitis(n=104)	42.45 (36.00-49.75)	130.83 (120.00-135.25)	82.29 (74.24-90.00)	24.70 (22.43-26.81)
Chronic periodontitis (n=116)	53.41 (43.25-63.00)	135.26 (125.00-145.00)	84.89 (80.00-90.00)	24.74 (22.46-26.67
Total (n=357)	45.84 (37.00-54.00)	131.96 (120.00-140)	82.59 (76.00-90.00)	24.76 (22.46-26.78)
Significance	0.001	0.079	0.172	0.770

Median and interquatile ranges were present for other variables All variables were tested at 0.05 significant levels

SP- Systolic blood pressure DP-Diastolic Blood Pressure

Table 4.8. Impact of diabetes control on age, BMI, and blood pressure

	Diabetes			Non diabetic controls
	Good glycaemic control	Moderate glycaemic control	Poor glycaemic control	
Age (years)	47.35 (36.00-47.00)	45.49 (39.00-49.00)	43.98 (35.50-54.50)	45.05 (38.0-51.75)
BMI (kg/m^2)	24.49 (21.94-26.36)	24.93 (23.14-26.74)	24.50 (23.00-27.26)	24.86 (22.55-27.43)
Systolic BP (mm/Hg)	127.5 (120.0-130.0)*	133.06 (120.0-140.0)	133.25 (120.0-147.5)	136.88 (125.3-145.8)*
Diastolic BP(mm/Hg)	80.28 (78.75-84.25)*	81.94 (70.00-88.75)	83.14 (75.75-90.00)	85.55 (76.25-93.50)*

*p=<0.05

True significance exists between good glycaemic controls with non diabetic controls.

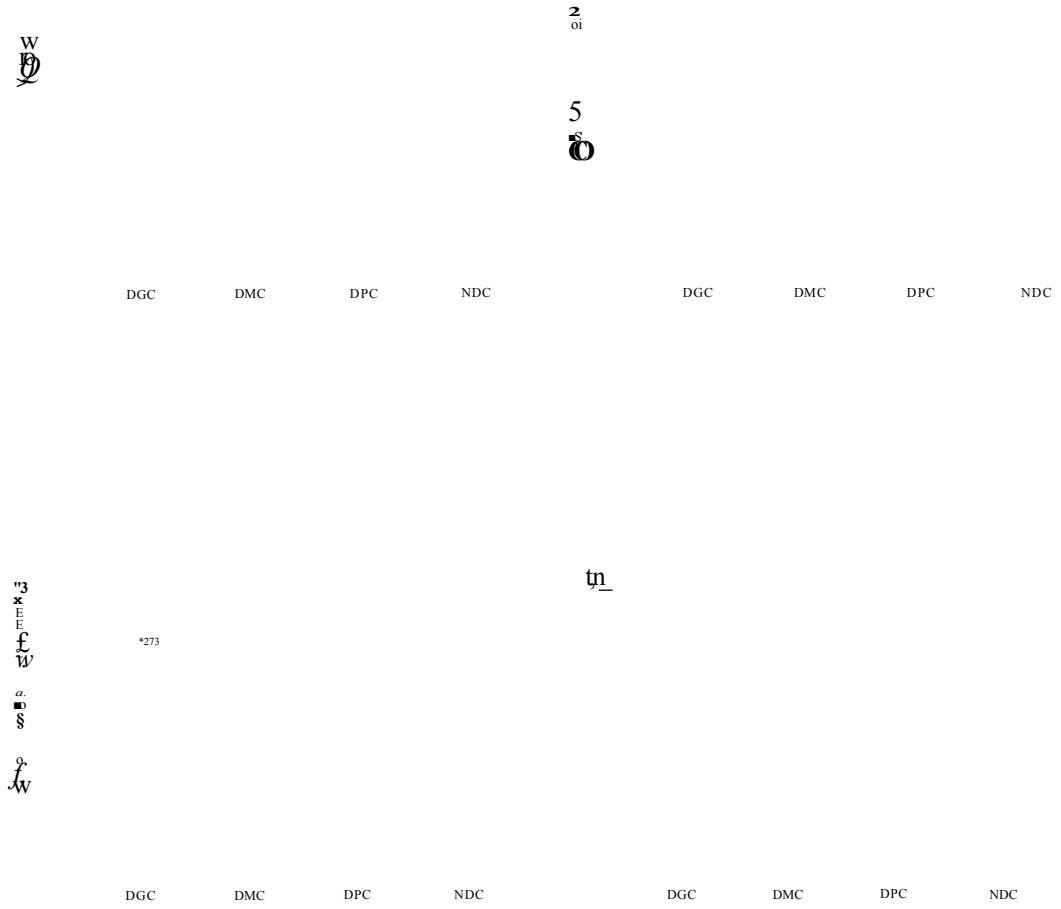


Figure 4.5 Box plots illustrating age, BMI and blood pressure according to glycaemic control in type 2 diabetes compared to controls

Legend :

DGC - Diabetes with good glycaemic control *DMC - Diabetes with moderate glycaemic control*

DPC - Diabetes with poor glycaemic control *NDC - Non diabetic control group*

Systolic blood pressure and diastolic blood pressure were significantly lower in people with DGC than NDC (p< 0.05)

4.3.10 Effect of biochemical parameters on periodontal parameters after adjusting for confounding factors age, sex, smoking and BMI

Results of multiple linear regression analysis revealed that glycaemic control (HbA1c value) is a significant predictor of mean recession (p=0.009) and maximum loss of attachment (0.014). HbA1c was not a significant predictor for the remaining periodontal parameters ($p > 0.05$). When considering the TOC, TG, LDL, HDL and their effect on periodontal parameters after adjusting for age, sex, BMI and smoking, only FBG was a significant predictive indicator of mean BOP (p=0.034), % BOP (p=0.034) and maximum LOA ($p< 0.024$) (Table 4.11). Significant correlations were observed for diabetes durations with mean Rec, mean LOA, and maximum LOA (Figure 3.9)

Table 4.9. **Effect of biochemical parameters on periodontal health after adjusting for the confounding factors (age, sex, smoking and BMI)**

	Mean PD (mm)	MeanRec (mm)	MeanBOP (%)	MeanLOA (mm)	Maximum LOA (mm)	%BOP	% of sites with PD ≥4mm	% of sites with PD ≥5mm	% of sites with PD ≥6mm
HbA1c	0.909	**0.009***	**0.019**	0.156	**0.014***	0.373	**0.012***	0.175	0.834
Total Cholesterol (mg/dl)	0.681	0.633	0.473	1.000	0.177	0.473	0.515	0.639	0.580
High Density Lipoproteins (mg/dl)	0.694	0.296	0.795	0.396	0.404	0.794	0.555	0.570	0.588
Low Density Lipoproteins (mg/dl)	0.631	0.602	0.800	0.943	0.948	0.803	0.710	0.696	0.760
Triglycerides(mg/dl)	0.997	0.996	0.797	0.974	0.383	0.799	0.658	0.361	0.340
Fasting Blood Glucose (mg/dl)	0.547	0.149	**0.034***	0.250	**0.024***	**0.034***	0.829	0.483	0.517

* $p<0.05$ significant level

MeanPD – Mean Probing Depth MeanRec-Mean Recession MBOP- Mean Bleeding On Probing MLOA- Mean Loss of Attachment Minimum PD – Minimum Probing Depth Maximum PD- Maximum Probing Depth Maximum LOA - Maximum Loss of Attachment BOP- Bleeding on Probing

4.4 Discussion

This cross sectional study was designed with the primary aim to determine the prevalence of periodontitis in patients with diabetes compared to non diabetic controls. This study further aimed to investigate the effect of glycaemic control on periodontal health. A number of studies reported high incidence of periodontal disease in people with diabetes compared to non diabetic controls (Emrich et al. 1991, Taylor et al. 1998). Most of these early studies were confined to Pima Indians, a population which has the highest recorded incidence and prevalence of diabetes in the world. However, literature published during recent years coming from different populations all over the world was consistently in agreement with this finding (Novak et al. 2008, Al-Otaibi 2008, Peck et al. 2006, Cutler et al. 1999). The prevalence of type 2 diabetes in Sri Lanka is moderately high, between 10.3%-14.2% (Katulanda et al. 2008, Wijewardena et al. 2005). There is evidence of rapidly progressing periodontal disease among the age group of 15-45 in Sri Lankan tea workers (Anerud et al. 1979, Loe et al. 1978, Loe et al. 1986) from previous studies. However, evaluations of chronic burden of periodontal disease in diabetes is extremely limited in this population.

4.4.1 Periodontal health in diabetes and controls

In this study 285 people with type 2 diabetes and 60 aged matched controls were examined for periodontal health and severity of the disease was determined by level of attachment loss and probing depth. Results of the present study indicate that the prevalence rate of chronic periodontitis is 33% among people with diabetes and 22% in the control population. When compared the prevalence rates observed in the present study with the results of National Health and Nutrition Examination Survey (NHANES) III data in the USA, prevalence of periodontitis in people with diabetes is extremely high (33% in Sri Lankan patients with diabetes to 17.3% in people with diabetes in the USA). The figures are also high for the non-diabetic population, 22% vs. 9% in the Sri Lanka and USA respectively.

In 1994 Perus and coworkers have analysed 536 sites of teeth of 256 male Sri Lankan tea workers. Their study found out of the 536 sites, 87 (16.2%) had pathologically deepened pockets ≥6mm (Preus et al. 1995). This group of people were recruited from tea plantations that represent a minor percentage of the Sri Lankan community who have no access to proper dental care and treatment. The prevalence of chronic periodontitis observed in the

present study (22%) from an urban non diabetic population shows comparable results with the prevalence of 16% observed by Perus in 1995. These figures are comparatively high compared to 9% in the USA (Soskolne & Klinger 2001) and 8-15% in the UK general population (Kelly et al. 2000). There is a general agreement that Asians are particularly susceptible to periodontitis (Corbert 2006) and the present and previous studies in Sri Lanka confirm this statement.

If the relationship between periodontitis and diabetes is a two way relationship then it would be expected that the prevalence of diabetes should be higher among people who have periodontitis than those who do not. The USA NHANES III survey shows that the prevalence of diabetes in patients with periodontitis is 12.5% which is double when compared to people with no periodontitis (6.3%) (Soskolne & Klinger 2001). The present study lacks data about the incidence of diabetes among patients with periodontitis. However, 67% (n=8) of the excluded 12 subjects from the control group due to impaired metabolic status as determined by higher HbA1c and fasting blood glucose values were diagnosed with chronic periodontitis. Similar incidences were reported in other studies. For example in a longitudinal study, type 2 diabetes subjects with severe periodontitis at baseline has demonstrated significantly worse glycaemic control than diabetic subjects with minimal periodontal destruction (Taylor et al. 1996). Loesche and co workers have observed that glucose levels are on average 15% higher in periodontitis patients compared to controls in a non diabetic population (Loesche et al. 2000). This may be an indication of periodontitis induced metabolic abnormalities. This group of 12 subjects in the present study may represent either undiognosed diabetes progressing to complications or periodontal status may have led metabolic imbalance as observed with raised FBG or HbA1c values. However, the number of subjects in this group is limited (n=12) and cannot come to a definite conclusion from these results but this area remains to be further explored.

The observed high prevalence of periodontitis in type 2 diabetes compared with and non-diabetes controls did not reach a statistical difference in this population. However, considering health and gingivitis together in a single group showed a trend ($p= 0.077$) towards people with diabetes to have higher prevalence of chonic periodontitis than non-diabetic controls. Based on the results of the present study it is evident that there is 1.5 fold increase to develop chronic periodontitis in the diabetic group.

4.4.2 Periodontal disease severity

When considering the severity of chronic periodontitis the present study adds to the evidence that the people with diabetes have more severe destructive periodontal disease than non diabetic controls. Severity of periodontal disease is measured by LOA and PD (Glavind & Loe 1967 & Goodson et al. 1984). The results of this study indicate most of the periodontal parameters: MeanRec (0.48mm vs 0.34mm), MeanBOP (0.17 vs 0.08), maximum PD (4.47 vs 3.8), maximum LOA (6.37mm vs. 5.14mm), % BOP (16.75% vs 8.3%) , % of sites with PD ≥4mm (10.47 vs 5.08), % of sites with PD≥ 5mm (4.52% vs 2.77) and % sites with PD≥ 6mm (0.91 vs 1.04) were significantly higher in patients with type 2 diabetes than non diabetic controls. The measured values for Mean PD and Mean LOA were higher in people with diabetes than those without but failed to reach any statistical significance. Shlossman et al. observed in Pima Indians, that median attachment loss and alveolar bone loss is higher in diabetic subjects for all age groups and for both sexes. (Shlossman et al. 1990). Similarly higher attachment loss and probing depth were observed in patients with diabetes in some other studies (Novak et al. 2008, Cutler et al. 1999, Morton et al. 1995, Bridges et al. 1996, Oliver and Trevonen 1993, Emrich et al. 1991, Lu and Yang 2004 and Mansour et al. 2005). Results observed in the present study are therefore consistent with the existing literature indicating increased severity of the disease among patients with diabetes compared to non diabetic controls in the present study population.

4.4.3 Diabetes control and periodontal health

Of the total number of people with chronic periodontitis there were 37.5% with good glycaemic control, 28.3% with moderate control and 26.8% with poor glycaemic control. 8.2% of the people with chronic periodontitis did not have HbA1c values. There is a general agreement that poorly controlled diabetes is a risk factor for the development of periodontitis. A greater prevalence of severe periodontitis is observed in both type 1 and type 2 long-term patients with diabetes with poor metabolic control (Mangan et al. 2000). Taylor and co-workers also reported that patients with poorer glycaemic control are at increased risk of alveolar bone loss and more severe progression of periodontitis compared with those without type 2 diabetes (Taylor et al. 1998). The results observed in this study follow the opposite trend, more people with good glycaemic control were diagnosed with

chronic periodontitis than those with moderate or poor glycaemic control. The reasons for the observed differences may be due to an imbalance in the number of patients in good {(n=112 (54%)}, moderate {(n=53 (25.7%)} and poor {n=41(19.9%)} glycaemic control groups. Higher number of patients falling into good glycaemic control may have had an impact on the negative trend observed with respect to glycaemic control in this study. Some other authors have stated previously that if there were a large number of poorly controlled diabetics in a study group, then they were more likely to suffer periodontitis than the control group. Conversely, if the study group contained mainly people with diabetes with good glycaemic control the difference in periodontal status between the two groups is likely to minimal (Oliver and Trevonen 1993). Since the present study group largely represents patients with controlled diabetes the prevalence rates observed may not represent true data for the diabetic population. This also may be a reason for the results of the present study not agreeing with previously reported data in the literature to support the notion of poorly controlled people with diabetes being more likely to be affected with chronic periodontitis.

Patients with type 2 diabetes for the present study were selected from a clinic based database created for patients in a defined area of Colombo suburbs in Sri Lanka (Weerasuriya et al. 1998). The database was created to assess the complications of newly diagnosed patients with type 2 diabetes. At the time of diagnosis neuropathy was present in 25.1% of subjects, nephropathy in 29%, retinopathy in 15%, coronary vascular disease in 21%, stroke in 5.6%, and peripheral vascular disease in 4.8%. The present study group was managed for at least two years and was under medication. In fact, they were advised to attend the diabetic clinic every three months, were on diet control or on medication (personal communications) and checked for their FBG, HbA1c and lipid profile regularly and received advice from a general practitioner and received newsletters in their native language explaining the importance of controlling their blood glucose values since the diagnosis. This may have improved their periodontal health in general as their glycaemic control improved. Also when patients were randomly selected and invited to participate in a research study, there is a likelihood that patients who are concerned about their health will attend the clinic more often than those who are not. This is evident from the present study that 54% of the study group was under good glycaemic control. It is thus evident that the

present study group is not a representative sample of good, moderate and poor glycaemic control. It under represents people with moderate and poor glycaemic control and results obtained in this study cannot be generalized to the status of diabetes in the general population.

When periodontal parameters were evaluated according to level of glycaemic control, significant differences were observed for maximum probing depth, % BOP Maximum loss of attachment, % sites with probing depth (PD) ≥ 4mm and % sites with PD ≥ 5mm between diabetes with good control and non-diabetic control. Generally people with good glycaemic control express a similar incidence of periodontitis. The results of the present study were largely affected by the higher number of subjects representing the good glycaemic control group. Poorly controlled diabetes showed significant difference with % BOP and % sites with probing depth ≥ 4mm.

Bacic and co-workers have observed that the association between periodontal disease and the duration of diabetes is consistent with trends seen in other complications of diabetes and that the duration of diabetes may be a more critical factor in developing periodontitis (Bacic et al. 1988). The duration of diabetes in the present study population was mean (sd) 7.5 (±6.28) years. Significant correlations were observed between diabetes duration with mean recession, mean loss of attachment and maximum loss of attachment in this group. Thus this may be the critical factor that determines the severity of periodontitis.

4.4.4 Age in relation to periodontal health in type 2 diabetes

One of the aims of the present study was to evaluate the impact of age on periodontal status. Subjects with chronic periodontitis were significantly older 53.41(±11.17) than subjects with health 42.01 (± 9.58) (p=0.001) and gingivitis 42.45 (±11.23) (p=0.001). Regression analysis also revealed that age is a significant predictor of all the periodontal parameters when adjusted for gender, smoking and BMI. Ainamo and co workers too demonstrated that age, severity of diabetes and oral hygiene of patients contributes to the severity and disease progression (Ainamo et al. 1990). The principal finding of a study conducted by Emrich and co workers (1991) found that diabetes and age are strong

predictors of the development of periodontal disease. Another study regarded age as a strong predictor of periodontal disease despite the presence of diabetes (Nelson et al.1990). Thus results in this analysis were in agreement with previous studies implicating age may be a significant indicator of chronic periodontitis. When assessed for the effect of age according to glycaemic control patients with good glycaemic control were slightly older than {47.35 (36.00-47.00)} those with moderate {45.49 (39.00-49.00)} and poor glycaemic control {43.98 (35.50 – (54.50)} patients. However, age had no impact on level of glycaemic control. Older people with diabetes in this study population shows good glycaemic control compared to moderate and poor glycaemic control. The reasons for the observed higher prevalence of chronic periodontitis in good glycaemic control group may be due to age or diabetes duration.

This study also observed that HbA1c is a significant predictor of mean recession and maximum loss of attachment. FBG was identified as a significant predictive indicator of mean BOP, % BOP and maximum LOA. Similar results were reported from a study in the USA revealing a significant association with baseline level of HbA1c with probing depth ≥ 3mm (Nelson et al. 1990). Similarly Native Americans with type 2 diabetes and periodontal disease demonstrated that the percent of sites with probing depth ≥ 5mm was significantly associated with the level of HbA1c (Papapanou 1996). These results may be an indication of peridontitis induced metabolic imbalance that causes hyperglyceamia and subsequent elevated levels of HbA1c levels.

In summary the prevalence of periodontitis in patients with diabetes in the Sri Lankan population is 33% compared to 22% aged matched controls and these rates are comparatively high compared to other populations. The present study adds to the evidence that severity of periodontal disease is significantly higher in patients with type 2 diabetes compared with controls. Glycaemic control had no impact on periodontal disease in the present analysis however age can be considered as a significant predictor of periodontal health in this population.

Chapter 5

Cytokine gene polymorphisms: association with periodontitis and type 2 diabetes

5.1 Introduction

Periodontal disease is a multifactorial complex disease. The occurrence and severity of the disease shows differences between individuals which cannot be explained by their level of plaque control. Twin studies suggest that this individual variation could be due to genetic predisposition and it has been estimated that genetic factors account for up to 50% of the increased susceptibility to periodontitis (Michalowicz et al. 1991). The disease includes a broad spectrum of inflammatory and destructive responses to oral microbe infections mediated by cytokines that include TNF-α and IL-1. Recent studies provide evidence that polymorphisms in cytokine genes may contribute to these genetic factors. Therefore, studies have now focused on the polymorphisms in genes encoding proinflammatory cytokines as the genetic markers for periodontitis (Kornman & di Giovine 1998).

5.1.1 Periodontal Associated Genotype (PAG) and smoking; Impact on periodontal pathogenesis

Polymorphisms in the IL-1 gene (*IL-1A* and *IL1B* SNPs) have been extensively studied in association with periodontal disease. It has been suggested that these polymorphisms influence the transcription of gene products and thereby the pathophysiology of periodontitis. Kornman and coworkers reported that a composite genotype of *IL1B (+3954)* allele 2 and *IL1A (-889)* allele 2 (designated as PAG or Periodontal Associated Genotype) could be a predictor of periodontal disease in non smoking Caucasian patients (Kornman et al. 1997). Additional supporting evidence was provided by some other studies (Papapanou et al. 2001, McDevitt et al. 2000). They observed that PAG is correlated with severity of periodontal disease as measured by mean probing depth (Cullinan et al. 2001) and attachment loss in non smokers (Papapanou et al. 2001, McDevitt et al. 2000). Inconsistent with these results other studies failed to establish the relationship (Gore et al. 1998, Meisel et al. 2002). For example Meisel and colleagues observed the PAG to be associated with periodontitis in smokers. In subsequent studies *IL1A (-889)* was replaced by *IL1A (+4845)* and it was suggested that both positions generate the same genetic information (Kornman & di Giovine 1998). Analysis of the PAG with the latter *IL1A* polymorphism by Agrawel and coworkers found that PAG is a significant risk indicator of periodontal pathogenesis in an Asian population (Agrawel et al 2006). Contradictory results were observed by Laine and colleagues. Their findings state PAG was not significantly associated in either smokers

with chronic periodontitis or non smokers with chronic peiodontitis (Laine et al. 2001). However, a meta analysis performed considering data from 13 studies concluded that the minor allele of *IL1B (+3954)* (TT) and minor allele of *IL1A (-889)* (TT) positive genotype has significant association with chronic periodontitis. This analysis has revealed that positive genotype is more frequent among cases with periodontitis when compared to controls, even though the estimates were marginally insignificant (Nikolopoulos et al. 2008).

5.5.2 Population differences in PAG status

While the true association between PAG and periodontal status is yet to be elucidated due to conflicting results obtained from different studies the percentage distribution of this PAG shows variation in different ethnic groups. A frequency percentage of 40.6% has been reported from Australia (Cullinan et al. 2000) and a study conducted in people of Northern European origin (Sweden) observed 42.9% (Papaponou et al. 2001). A high prevalence rate was also observed for the positive PAG in 38.2% of the controls and 44.4% of the patients in a Greek population (Sakellari et al. 2003). However, this study failed to demonstrate that PAG can act as a differential indicator between chronic periodontitis and controls. A recent case control study in European Caucasians reported a high percentage (46.4%) of PAG between periodontally healthy individuals and generalized early onset periodontitis patients regardless of smoking status (Hodge et al. 2001). A study on periodontally healthy individuals of Hispanic origin has reported a lower rate of 23% for the PAG genotype compared to European study (Caffesse et al. 1998). Another study conducted in the Chinese population revealed an overall frequency of PAG genotype of 2.3% which is the lowest recorded percentages thus far observed (Armitage et al. 2000). Most of these data of different populations suggest, these polymorphisms are associated with the severity of adult periodontitis and there is a possibility of the difference being race related.

5.1.3 Diabetes, periodontitis and IL-1 genotype

Apart from smoking, diabetes is one of the major risk factors for periodontitis. Diabetes patients with periodontitis have significantly higher levels of proinflammatory mediators (IL-1β, TNF-α, and PGE_2) in gingival crevicular fluid compared to non diabetic controls. It has been hypothesized that IL-1 participates in the pathogenesis of both periodontitis and

disturbed glycaemic control as seen in type 1 and 2 diabetes (Kirkovsky et al. 2002) with an association of IL-1β polymorphism with type 1 diabetes reported (Krikovsky et al. 2002). A population based study conducted in Germany evaluated the association of *IL1A* and *IL1B* haplotype in relation to periodontitis in patients with diabetes. It has been observed that diabetic subjects with increased levels of HbA1c had more widespread and severe periodontal disease than normoglycaemic subjects and diabetic subjects bearing a variant *IL1B* genotype C/T or T/T had an enhanced risk for periodontal disease in comparison with their IL-1 wild type counterparts. This study also reports the periodontal parameters; bleeding on probing, attachment loss and number of teeth were associated significantly with diabetes and the IL-1 genotype (Struch et al. 2008).

5.1.4 TNF- α and Periodontitis

A significant association between *TNFA* allele 2 and chronic periodontitis and severity of the disease was observed in some studies (Kornman et al. 1997, Kornman and di Giovine 1998, Galbraith et al. 1999, Lin et al. 2003). However, the *TNFA (-308)* SNP was not found to be associated with disease severity in some other studies (Galbraith et al. 1998, Craandijik et al. 2002, Kinane et al. 1999, Endo et al. 2001, Folwaczny et al. 2004, Fassmann 2003). A recently conducted meta analysis of cytokine SNPs in association with periodontitis analysed 15 studies that have examined the *TNFA (-308)* polymorphism. The majority were carried out in Caucasian populations and reported no association with chronic periodontitis. (Nikolopoulos et al. 2008).

5.1.6 *TNFA* in association with diabetes and biochemical parameters

Perezl et al. studied the association of aggressive periodontitis and the *TNFA (-308)* polymorphism in Chile. Their study group involved two groups, type 1 diabetes patients with aggressive periodontitis and patients with type 1diabetes only. They concluded that allele 2 of (-308) has no significant association with the disease, circulating TNF-α levels or production of TNF-α in whole blood in an *ex vivo* culture system. However, the study group with aggressive periodontitis and type 1 diabetes showed higher frequency of *TNFA* allele 2 compared to the other group with type 1 diabetes only suggesting that in people with both periodontitis and type 1 diabetes, *TNFA* allele 2 may have an increased risk of developing the disease (Perezl et al. 2004).

Fernandes-Real and coworkers reported that *TNFA* genetic polymorphism was associated with insulin resistance. Indeed, TNF-α is believed to suppress insulin signaling via interaction with insulin receptor substrate (IRS) (Fernaders Real et al. 1997). Shiau et al studied the *TNFA (-308)* polymorphism and type 2 diabetes in Taiwanese population. This study failed to find a possible association with respect to *TNFA (-308)* polymorphism. However, they observed the *TNFA (-308)* allele 2 had significant association with fasting blood glucose levels. The biological explanation to this phenomenon could be that TNF-α induced insulin resistance would increase plasma glucose concentration in affected individuals. A significant association was also observed with *TNFA (-238)* polymorphism with HDL cholesterol. Their study concluded that people who have these two promoter polymorphisms together are more susceptible to diabetic complications than others (Shiau et al. 2003). Another study conducted in the Netherlands also reported an association of *TNFA (-308)* polymorphism with risk of diabetes (Heijmans et al. 2002).

5.1.7 *TNFA (-308)* polymorphism in ethnically different populations

The studies of *TNFA (-308)* promoter polymorphism have failed to achieve consistent significant associations with aggressive periodontitis, chronic periodonitis or diabetes. Ethnic differences likely play roles in these conflicting results, because the distribution of *TNFA* promoter polymorphism is different among study subjects with different racial origins. The *(-308)* and *(-238)* polymorphisms were found in comparatively high prevalence in Caucasians (Endo et al. 2001). A study conducted in Germany revealed that The GA and AA genotypes were present in 33.3% and 2.5% of patients with periodontitis whereas in the control group frequencies of the same were 27.5% and 0%. There was no significant difference observed between patients and controls with respect to the frequency of *TNFA (-308)* A allele (Folwaczny et al. 2004). It has been observed that the allele 2 distribution ranges from 9% in Hong Kong Chinese (Lee et al. 2000), 16% in French and Scandinavian populations (Hoffstedt et al. 2000, Herrmann 1998), 24% in Australians (Dalziel et al. 2002) and 1.78% in Taiwanese population (Shiau et al. 2003). The genotype distribution of *TNFA (-308)* G/A polymorphism was 65.4% (G/G), 32.2% (G/A) and 2.4% (A/A) in Netherlands (Heijmans et al. 2002).

5.1.8 Chapter aims and objectives

1. To estimate the prevalence of the polymorphism of *IL1A (+4845)*, *IL1B (+3954)* and *TNFA (-308)* genes in the Sri Lankan population
2. To investigate whether or not an association exists between *IL1A (+4845), IL1B (+3954)* and *TNFA (-308)* cytokine gene polymorphisms and periodontitis in patients with type 2 diabetes.
3. To investigate whether the PAG *IL1A (+4845)* and IL1B *(+3954)* has any association with periodontal status in never smokers.
4. To investigate whether or not *IL1A* and *IL1B* polymorphisms separately or PAG has any association with periodontal or biochemical parameters.
5. To investigate whether *TNFA (-308)* polymorphism has any association with diabetes or diabetes related biochemical parameters or periodontal parameters.

5.2 Materials and Method

5.2.1 Blood sample collection

Blood samples were collected in parallel to clinical data collection (see sec.3.2.1 for further details) from a total of 124 parents, 272 patients and 73 controls. Samples were collected either by finger prick blood drop collected in a DNAase free absorbent filter paper or by peripheral blood in sodium EDTA containers. Filter papers had been air dried and stored in a sealed container at 4°C and blood samples were separated into two aliquots and stored at -20°C until further analysis.

5.2.2 Laboratory methods

All techniques relating to *IL1B, IL1A* and *TNFA* genotyping were carried out at the Oral Biology Department of the School of Dental Sciences, University of Newcastle upon Tyne, UK and Biomedical Research Centre Sheffield Hallam University UK. DNA extraction was done at the Faculty of Science, University of Colombo, Sri Lanka.

DNA extraction from whole blood

Two mL of whole blood was used in the extraction process. DNA extraction was carried out using QIAamp DNA blood midi column purification kits following manufacturer's instructions (QIAGEN Ltd). Extracted DNA was separated into two aliquots and stored at -20°C. One aliquot was transferred to the Biomedical Research Centre, Sheffield Hallam University, UK for genotype analysis. The second aliquot was stored separately to establish a DNA bank to conduct further genetic studies in relation to type 2 diabetes.

DNA extraction from dried blood spots

One quarter of the dried blood spots was cut into small pieces and boiled in 100µl of 50mM NaOH for 10 minutes at 100°C. 20µl of 1M Tris HCl was added to neutralize the medium and the tubes were briefly microfuged at 4000g. The supernatant was transferred into a fresh tube and stored at –4°C.

Gene frequency analysis in Sri Lankan population

There were 272 patients from whom blood samples were collected. 62 parents from 62 patients were genotyped for the three SNPs analysed. Thus gene frequency analysis included genotyped data from 210 patients, 124 parents and 73 controls (n =407). (There were 124 parents. The children of 124 parents (n=62) were excluded from the gene frequency analysis of the population since they are dependant variables of their parents and are therefore bound to share their alleles). All the subjects were genotyped for *IL1A (+4845)*, *IL1B (+3954)* and *TNF A (-308)* polymorphisms according to the protocols defined in methods section 5.2.4.

5.2.3 Periodontal analysis

After excluding edentulous patients [patients who had no teeth and those who were not examined for periodontal health (n=10)] final genotype analysis included 262 patients with type 2 diabetes. There were 72 controls after excluding one edentulous subject, thus a total of 334 subjects were included in cytokine gene polymorphism analysis in relation to periodontitis.

5.2.4 Protocol for genotyping

IL1B (+3954)

This single nucleotide polymorphism (SNP) was described as Taq I restriction fragment length polymorphism (RFLP) of *IL1B*. (Pociot et al. 1992). Sequencing of the region has discovered a C/T single base transition at +3954 in exon V that fully explains the RFLP (di Giovine et al.2000).

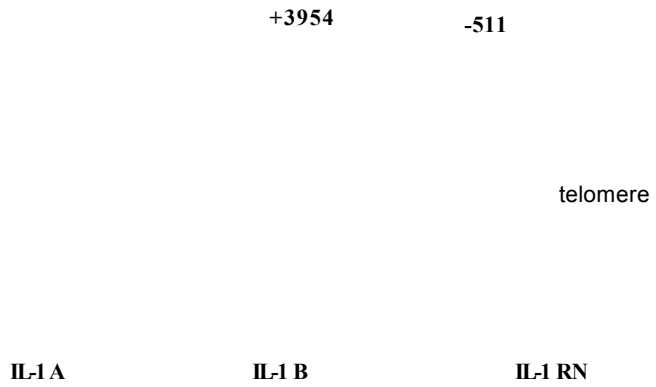


Figure 5.1 Position of *IL1B* mapped oil 430 Kb region of chromosome 2q14.

Primers

The specific primers were designed to amplify the region containing the SNP that has a control Taq site. Therefore the PCR product will contain constant non polymorphic and one polymorphic restriction site for Taq I (di Giovine et al. 2000). The Taq 1 polymorphic site, allows identification of two alleles of the single nucleotide polymorphism corresponding to the presence or absence of the restriction site. Taq 1 Non polymorphic site will indicate the status of digestion. The *IL1B* allele 1 was the digested fragment. Therefore, three possible genotypes can be expected from a given population (Figure 5.4). DNA samples were PCR amplified using oligonucleotide primers (Fig.5.2) which has been described previously in the literature (di Giovine et al. 2000).

Primer 1 : 5' CTC AGG TGT CCT CGA AGA AAT CAA A 3'

Primer 2 : 5' GCT TTT TTG CTG TGA GTC CCG 3'

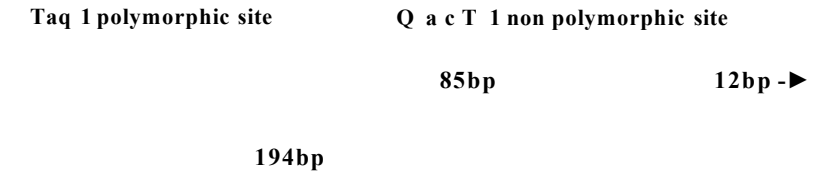
Figure 5.2 Primers that amplify the fragment of *IL1B* containing the *(+3954)* SNP

Polymerase Chain Reaction

The sequence of the primers designed to flank the region of exon 5 of human *IL1B (+3954)* and primers were obtained from Helena Biosciences (Sunderland, UK). PCR reaction volume was 50pl. Two pL of template DNA obtained from each patient and control were amplified in a reaction mixture containing 25pl of 2 X Biomix1MRed (BIOTAQIM DNA polymerase, 125mM Tris-HCl (pH 8.8 at 25°C), 32mM (NH4)2S04, 3mM MgCF, and 2mM of each dATP, dCTP, dGTP and dTTP) 4pl of reverse and forward primer mix to the final concentration of 0.8pM each and 19pl of distilled water. Amplifying conditions consisted of an initial denaturation at 94°C for 6 minutes, followed by 35 cycles at 94°C for 1 minute, annealing at 59°C for 1minute, elongation at 72°C for 1minute and a final extension at 72°C for 5 minutes and cooling to 4°C.

PCR/RFLP genotyping

The PCR product was 194 base pairs in size. Following restriction digestion the resulting products of 12bp + 85bp + 97bp (allele 1- presence of restriction site is more common allele) and 12bp+182bp (allele 2 absence of a restriction site are diagnostic (Figure 5.3). A non-polymorphic Taq 1 site gives rise to a 12 base pair fragment, which could not be seen on the agarose gel. However, the presence of the non polymorphic site enables the detection of the complete digestion when the digested products were run in parallel to undigested products.



Allele 1 - presence of a restriction site, common form (C)

Allele 2 - absence of a restriction site (T)

Figure 5.3 Graphical representation of the 194bp segment *oilLIB* that shows the polymorphic and non polymorphic sites

Restriction digestion of the PCR products with Taq 1 endonuclease

25µl of the PCR products were digested with Taq 1 endonuclease enzyme from *Thermus aquaticus* (fermetars). Digest mix, which contained 4µl of 10x buffer and 0.25µl of 1.0mg/ml BSA and 0.5µl (5U) of Taq 1 and 5.25µL of deionised water. Reagents were mixed and incubate at 65°C for three hours.

IL1A (+4845) genotyping

IL1A gene encodes for interleukin 1 alpha (IL-1α) which like IL-1β, is a pro-inflammatory cytokine. Sequencing of this gene had revealed a single base variation (G/T) in exon 5 at position *(+4845)*, which explains the restriction fragment length polymorphism (Gubler et al. 1989).

Primers

The primers were designed to create an Fnu4HI restriction site (di Giovine et al.2000) (Figure 5.6). These two alleles are in 100% linkage disequilibrium with alleles of *IL1A (-889)*. The enzyme Fnu4H1 cuts a constant band of 76bp at the non polymorphic site, absence of this band indicates an incomplete digestion. In allele one Fnu4HI cuts a constant band of 76bp and two further bands of 29 and 124 bp and in the absence of polymorphic site, it gives a single band of 153bp for allele 2 (Figure 5.7).

Polymerase chain reaction

The primers designed to flank the region of exon 5 of human *IL1A (+4845)* containing the Fnu4HI restriction site were obtained from Helena Biosciences (Sunderland, UK). One µL of template DNA obtained from each patient and control were amplified in a reaction mixture containing 12.5µL of 2 X Biomix™ Red (BIOTAQ™ DNA polymerase, 125mM Tris-HCl (pH 8.8 at 25°C), 32mM $(NH_4)_2SO_4$, 3mM $MgCl_2$, and 2mM of each dATP, dCTP, dGTP and dTTP) 2µL of reverse and forward primer mix to the final concentration of 0.8pM each and 9.5µL of distilled water. Amplifying conditions consisted with an initial denaturation at 95°C for 1 minute, followed by 35 cycles at 94°C for 1minute, annealing at

56°C for 1minute, elongation at 72°C for 2 minutes and a final extension at 72°C for 5 minutes and cooling to 4°C.

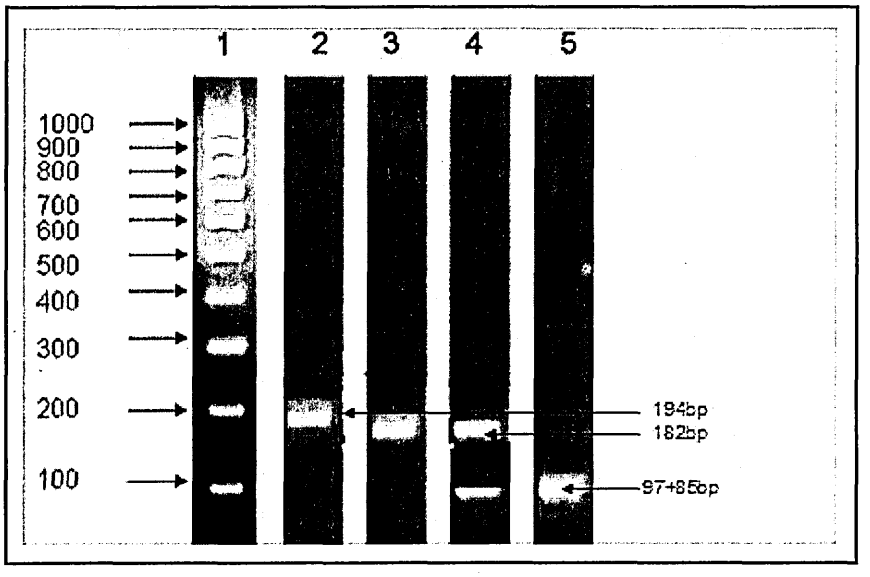


Figure 5.4 Three possible genotypes of *IL1B (+3954)*

Lane 1 – Hyper ladder V

Lane 2 – Positive control (194bp PCR product)

Lane 3 – Homozygous (TT) – Allele 2 (Absence of polymorphic sites on both chromosomes)

Lane 4 - Heterozygous

Lane 5 – Homozygous (CC) -Allele 1(Presence of polymorphic site in both chromosomes)

Figure 5.4 is a computer generated image of three possible genotypes expected for *IL1B (+3954)* SNP after digesting the PCR products with Taq 1. Three possible genotypes are a homozygote for the presence of the restriction site (CC) indicated by two bands of 97 and 85 base pairs. A homozygote for the absence of restriction site (TT) has one band of 182 base pairs. The heterozygotes will have all three bands (12bp fragment cannot be visualised in the gel).

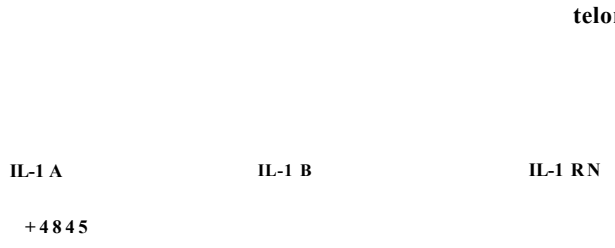


Figure 5.5 Position of *ILIA* mapped on 430 Kb region of chromosome 2ql2.1

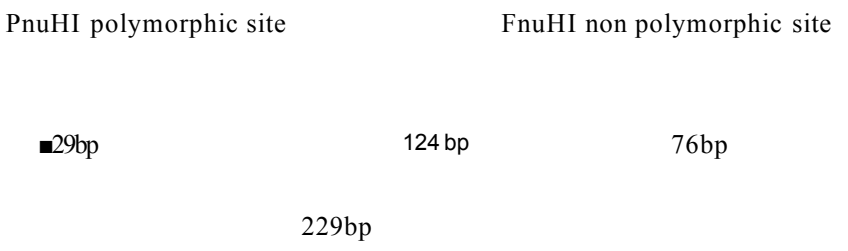
Primer 1-5'-ATG.GTT.TTA.GAA.ATC.ATC.AAG.CCT.AGG.GCA-3' (+4814/+4843)

Primer 2- 5'- AAT.GAA.AGG.AGG.GGA.GGA.TGA.CAG.AAA.TGT.-3' (+5015/+5044)

Figure 5.6 Primers that amplify a fragment of *ILIA* containing the *(+4845)* SNP

PCR/RFLP genotyping

The *ILIA* PCR product was 229 base pairs in size. Following restriction digestion the resulting products of a polymorphic site were 76bp + 29bp + 124bp fragments for allele 1 and 76bp+153bp for non polymorphic site which is allele 2. Three possible genotypes are a homozygote for the presence of the restriction site indicated by three bands of 76, 29 and 124 base pairs. A homozygote for the absence of restriction site has two bands of 153 and 76 base pairs. The heterozygotes have all three bands (Figure 5.8). 29 base pair band would not be visualised in an agarose gel.



Allele 1 - presence of a restriction site, common form (G)

Allele 2 - absence of a restriction site (T)

Figure 5.7 Graphical representation of 229bp segment of *ILIA* with polymorphic and non polymorphic sites

Restriction digestion of the PCR products with Fnu4H1 endonuclease

lOpl of the PCR products were mixed with digest mix, which contains 3pl of lOx buffer and 2pl (20U) of Fnu4H1 endonuclease enzyme and 15pl of deionised water. Reagents were gently mixed and incubated at 37°C overnight.

Figure 5.8 Three possible genotypes of *ILIA (+4845)*

Lane 1 - Hyper ladder V

Lane 2 - Positive control

Lane 3 - Homozygous - Absence of polymorphic site on both chromosomes

Lane 4 - Heterozygous

Lane 5 - Homozygous - Presence of polymorphic site on both chromosomes

Figure 5.8 is a computer generated image of the three possible genotypes expected for *ILIA (+4845)* SNP after digesting the PCR products with FnuH41. Three possible genotypes are a homozygote for the presence of the restriction site (GG) indicated by a single band of 124 base pairs. A homozygote for the absence of restriction site (TT) has one band of 153 base pairs. The heterozygotes will have 153 and 124 base pair bands. The PCR product is 229 base pairs will cut a constant band of 76 base pairs which can be seen in all three genotypes.

TNFA (-308) genotyping

TNFA gene encodes for tumour necrosis factor 1 alpha (TNF-a) (chromosome 6p21.3). Sequencing of this gene had revealed that a single base variation (G/A) in the *TNFA* promoter at position *(-308),* which explains the restriction fragment length polymorphism. (Wilson et al, 1992).

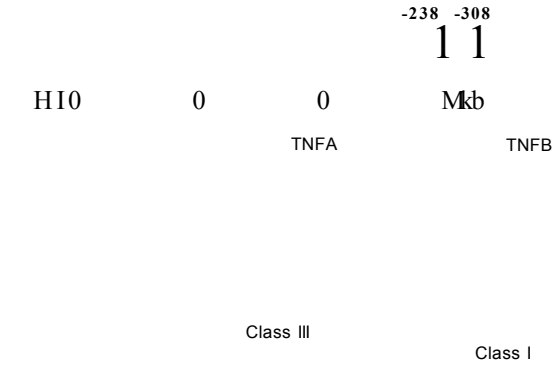


Figure 5.9 Structure of Human MHC complex- chromosome number 6q21, 4 - 5Mb region where TNFA gene is located

Primers

Primers were used which create an Ncol restriction site when amplifying allele 1. Amplified PCR product will be of 107bp nucleotides in length, (di Giovine et al.2000) (Figure 5.10)

Primer 1 - 5'- AGG.CAA.TAG.GTT.TTG.AGG.GCC.AT-3' (-331/309)

Primer 2 - 5'- TCC.TCC.CTG.CTC.CGA.TTC.CG.-3, (-244/-226)

Figure 5.10 Primers that amplify a fragment containing the *TNFA (-308)* SNP

Polymerase chain reaction

The primers designed to flank the promoter region of human *TNFA (-308)* were obtained from the Helena Biosciences (Sunderland, UK). One pL of template DNA obtained from each patient and control were amplified in a reaction mixture containing 12.5pL of 2 X Biomix™ Red (BIOTAQ™ DNA polymerase, 125mM Tris-HCl (pH 8.8 at 25°C), 32mM (NH4)2S04, 3mM MgCl2, and 2mM of each dATP, dCTP, dGTP and dTTP) 2pL of reverse and forward primer mix to the final concentration of 0.8pM each and 9.5pL of distilled water. Amplifying conditions consisted an initial denaturation at 95°C for 1 minute, followed by 35 cycles at 94°C for 1 minute, annealing at 60°C for 1 minute, elongation at 72°C for 2 minutes and a final extension at 72°C for 5 minutes and cooling to 4°C.

PCR/RFLP genotyping

PCR products were 107 base pairs in size. Following restriction digestion the resulting products of a polymorphic site were 87bp + 20bp fragments for allele 1 and 107bp for non polymorphic site which is allele 2. Three possible genotypes are a homozygote for the presence of the restriction site indicated by two bands of 87 and 20 base pairs. A homozygote for the absence of restriction site has one band of 107 base pairs. The heterozygotes have all three bands (Figure 5.12). 20 base pair band would not be visualised in an agarose gel.

Ncol site

87bp

107 bp

Allele 1 - presence of a restriction site, common fonn (G)

Allele 2 - absence of a restriction site (A)

Figure 5.11 Graphical representation of the 107bp segment of *TNFA (-308)* SNP

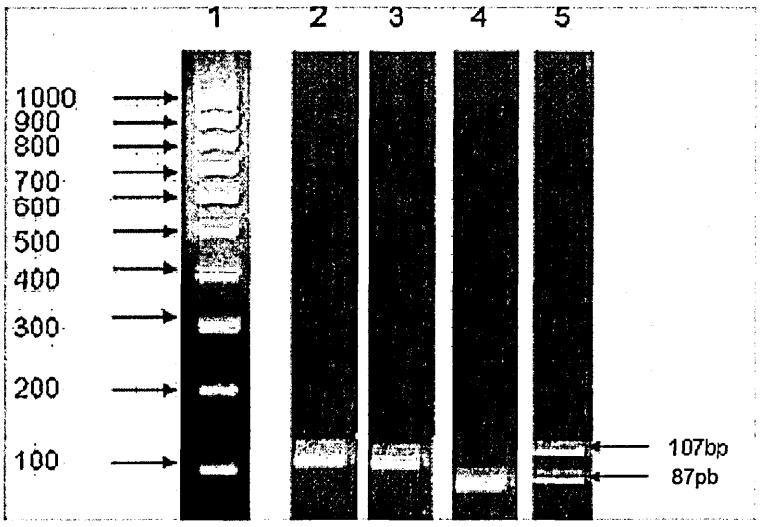


Figure 5.12 Three possible genotypes of *TNFA (-308)*

Lane 1- Hyper ladder maker IV

Lane 2 - PCR amplified product

Lane 3 - Homozygous (Allele 2)

Lane 4 - Homozygous (Allele 1)

Lane 5 - Heterozygous

Figure 5.12. represents a computer generated image for the expected three possible genotypes for *TNFA (-308)* SNP after digesting the PCR products with NcoI. Homozygote for the presence of the restriction site (GG) will be indicated by a two bands of 87 base pairs and 20 base pairs. A homozygote for the absence of restriction site (AA) has one band of 107 base pairs. The heterozygotes will have 107 and 87 base pair bands. 20 base pair band would not be visualised under UV light.

Restriction digestion of the PCR products with NcoI endonuclease

15µl of the PCR products were mixed with digest mix, which contains 2µl of 10x buffer Tango and 1µl (5U) of NcoI endonuclease enzyme (Fermantas, Life Science) and 18µl of distilled water. The total volume of each component was calculated by multiplying the number of samples. A control mixture free of enzyme containing 2µl buffer and 18µl of distilled water was prepared in each RFLP set up to serve as a control. Reagents were mixed gently and spun down and incubated over night at 37°C in the GeneE Thermocycler (Techne, Cambridge Ltd, 1994). Digested products were checked on a 4% agarose gel electrophoresis at 40mA after one hour.

5.2.5 Control procedures

DNA Extraction control procedure

All the extractions were carried under aseptic conditions. When DNA were extracted from dried blood spots, samples were grouped into batches and with every batch of extractions a negative control was run in parallel and assayed for amplification. In cases of positive results in a negative control, the samples with that batch were discarded and DNA re-extracted.

PCR Control procedures

A100 base pair DNA ladder (Bioline UK) was run simultaneously in agarose gels along with the PCR products to determined the sizes of the DNA fragments of each polymorphism. Every PCR reaction included negative controls to which no template DNA was added to ensure that no contamination of the sample had occurred. In the case of contamination PCR was repeated. Every PCR reaction included a positive control with known human DNA templates. When negative results (absence of bands) for the positive control were obtained, PCR was repeated.

5.2.6 DNA visualization and gel electrophoresis

All PCR products were resolved in 3% agarose gel and digested products were resolved in 4% agarose gel in TBE buffer. Separated fragments were visualised by UV excitation of the Ethidium bromide bound to DNA within the gel. 5µl of 0.5mg/ml of ethidium bromide was added to the agarose gel and mixed before setting. Five µl of PCR

products and total volume of digested products were loaded into the gel and subjected to electrophoresis at 70 volts for one hour. The image of the gel was taken.

5.2.7 Statistical Analysis

The number of three different genotypes for each of the polymorphism was calculated by direct counting as observed in the gel pictures. Allele frequencies were determined from the observed number of genotypes. Data were coded into PAG positive if both allele 2 of *IL1A* and *IL1B* were present. The significance of difference between both groups (PAG positive and PAG negative) according to periodontal health was determined by cross tabulating to determine chi square test statistics. Similar test statistics were applied to determine the association *TNFA (-308)* polymorphisms and periodontal diagnosis. Periodontal parameters and biochemical parameters were tested within different groups of each genotype using Kruskal Wallis tests for multiple comparisons.

5.3 Results

5.3.1 SNP/ RFLP pattern generated for *IL1B (+3954)*

Figure 5.13. represents the genotypes observed for *IL1B (+3954)* SNP. Bands were observed (85bp and 97bp) for homozygotes for allele 1 in lanes 2 to 7. Heterozygotes for presence of allele 1 (85bp + 97bp) and allele 2 (182bp) were visualized in lane 1.

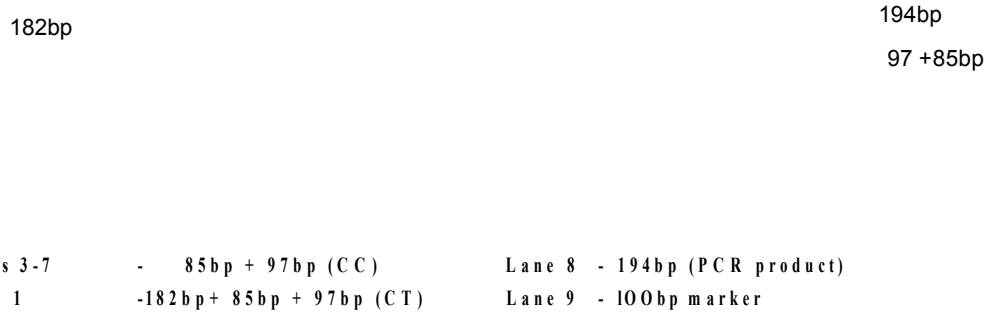


Figure 5.13 Gel images generated for *IL1B (+3954)* RFLP that shows two of the three possible genotypes

5.3.2 SNP/RFLP pattern for *ILIA (+4845)* SNP

Figure 5.14 shows all three genotypes expected *fox ILIA (+4845)* SNP/RFLP. Lane 8 shows the 229bp PCR product. The digested product generated a 153bp fragment for a homozygote for allele 2 as seen in lane 7. Heterozygotes for allele 1 (124bp and allele 2 (153bp) can be seen in lanes 2, 5 & 6. Homozygote for allele 1 (124bp) can be seen in lane 1. All lanes from 1-7 shows the constant band of 76bp which is cut from 229bp PCR product.

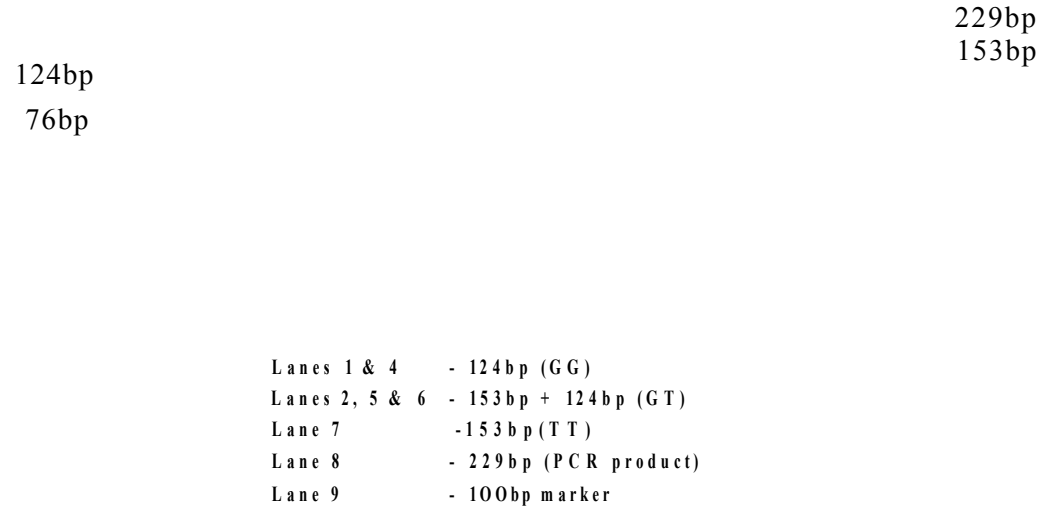


Figure 5.14 Gel images for *ILIA (+4845)* RFLP demonstrating three possible genotypes

5.3.3 SNP/RFLP pattern *TNFA (-308)* SNP

Figure 5.15 shows the RFLP pattern for *TNFA (-308).* Homozygotes for allele 1 digested the 107bp PCR product into two fragments of 87bp and 20bp which can be seen in lanes 2, 3 and 7. Heterozygotes generated 107bp, 87bp and 20bp fragments as observed in lanes 1, 5, and 6. Homozygous allele 2 was not digested and gives a 107bp fragment which is seen in lane 4.

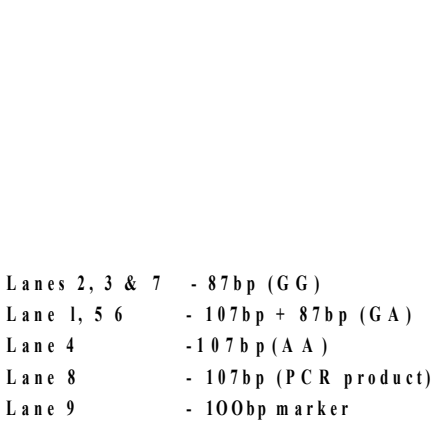


Figure 5.15 Gel images generated for *TNFA (-308)* RFLP with three possible genotypes

5.3.4 Population distribution of gene frequencies *oilLIB (+3954), ILIA (+4845)* and *TNFA (-308)*

A total of 407 subjects' genotyped data were included in the present analysis to estimate population frequencies. Genotype percentages for *IL1B (+3954)* CC, CT and TT were 80.3%, 18.9% and 0.7% respectively. ¥or *ILIA (+4845)* the distribution of GG, GT and TT alleles were 55.8%, 36.9% and 7.4% respectively. 82.8% of the group possessed GG genotype of *TNFA (-308)* SNP whereas GA and AA were 15.2% and 2.0% only (Figure 5.16).

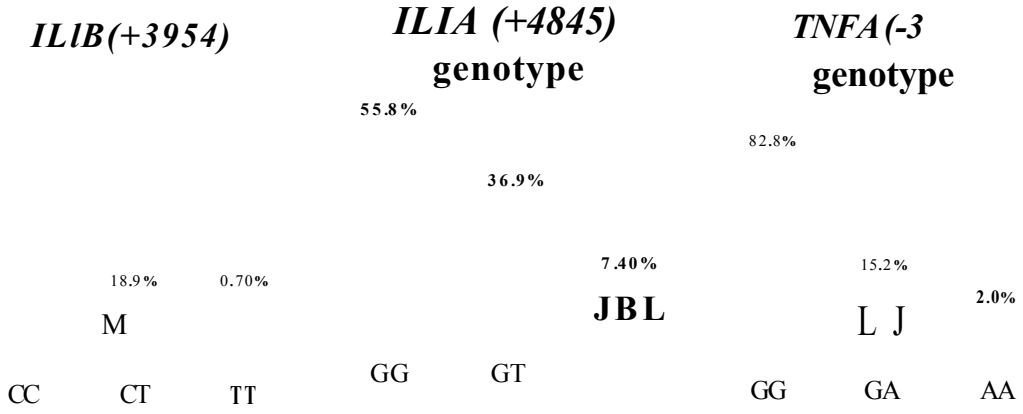


Figure 5.16 Allele distribution of *IL1J3 (+3954), ILIA (+4845),* and *TNFA (-308)*

5.3.5 Test of Hardy Weinberg Equilibrium for genotype distribution in the population

Allele (1) and allele (II) frequencies for all the SNPs were calculated manually (Table 5.1) and checked for whether they were in agreement with Hardy Weinberg Equilibrium. The distribution of *ILIB* and *TNFA* genotypes was in agreement with Hardy Weinberg Equilibrium distribution for this population whereas *ILIA* genotype distribution slightly deviates from the expected frequency of 1.

Table 5.1 Observed gene frequency for the Sri Lankan population

	P	*Q*	$P^2 + 2pq + q^2$	*Observed frequency*
IL1B (+3954)	*0.90*	***0.10***	*0.81 + 0.18 +0.01*	*1*
IL1A (+4845)	*0.74*	*0.26*	*0.55 + 0.10 +0.07*	*0.71*
TNFA (-308)	*0.91*	*0.09*	*0.83 +0.164 + 0.0081*	*1.0*

p -frequency observed for allele 1
q- Frequency observed for allele 2

5.3.6 *IL1B (+3954)* genotype and periodontal health

IL1B genotype distribution showed no significant difference according to periodontal health (p=0.849). CC (~80%) and CT (~20%) genotype distributions were approximately equal in all three groups (health, gingivitis and chronic periodontitis) (Table 5.2).

Table 5.2 *IL1B* genotype according to periodontal health

IL1B Genotype	CC	CT	TT	Total
Health	105 (80.8%)	24 (18.5%)	1 (0.8%)	130(100%)
Gingivitis	73 (78.5%)	20(21.5%)	0 (0%)	93 (100%)
Chronic periodontitis	91 (81.5%)	20(18.5%)	0(0%)	111(100%)

Pearson χ^2=0.84

5.3.7 *IL1A (+4845)* genotype and periodontal health

Distribution of GG genotype among health, gingivitis and chronic periodontitis were 58%, 50.5% and 55.9% respectively. Similarly GT distribution was 37.7%, 44.1% and 36.9% respectively. TT genotype distribution was 7.2% in the chronic periodontal group when compared to 3.9% in health and 5.4% in gingivitis group. However there was no significant difference of genotype distribution among the three groups (p=0.529) (Table 5.3).

Table 5.3 *IL1A* genotype according to periodontal health

IL1A Genotype	GG	GT	TT	Total
Health	76 (58.5%)	49 (37.7%)	5 (3.9%)	130 (100%)
Gingivitis	47 (50.5%)	41(44.1%)	5 (5.4%)	93 (100%)
Chronic periodontitis	62 (55.9%)	41(36.9%)	8(7.2%)	111(100%)

Pearson χ^2*=0.529*

5.3.8 PAG status in patients with diabetes and controls

Composite genotype of allele 2 of both *IL1B* (T) and *IL1A* (T) is considered as periodontal association genotype (PAG) (Kornman et al.1997). It has been observed that 12.6% of patients with diabetes and 12.5% of controls were PAG positive in this population and there was no significant difference in PAG status between the two groups (p=0.983) (Table 5.4).

Table 5.4 PAG status between cases and controls

	Patients		Controls		Total	
	n	%	n	%	n	%
PAG positive	33	12.6	09	12.5	42	12.6
PAG negative	229	87.4	63	87.5	292	87.4
Total	262	100	72	100	334	100

Pearson's Chi squared test for difference in PAG status between cases and controls
$\chi 2 = 0.983$

5.3.9 PAG status and periodontal status

Regardless of the diabetes status the PAG status was analysed against periodontal health among the total subject population. 38.9% (130/334) of the study group were found to be periodontally healthy and among them 13.1% (17/130) were PAG positive. 27.8% (93/334) and 32.3% (108/334) had gingivitis and Chronic periodontitis respectively. Among gingivitis 16.1% and 9.3% in chronic periodontal group were PAG positive. This analysis

of PAG status in relation to periodontal status showed no significant association.(Table 5.5).

Table 5.5. Cross tabulation of PAG status and periodontal diagnosis

Pearson's Chi squared test for PAG status and periodontal diagnosis
$\chi 2 = 0.456$

	PAG Positive n=32	PAG negative n=292	Total n=334
Health	17(13.1%)	113(86.9%)	130(100%)
Gingivitis	15(16.1%)	78(83.9%)	93(100%)
Chronic Periodontitis	10(9.0%)	101(91.0%)	111(100%)

5.3.10 PAG status in association with never smokers

There were a total of 265 subjects with no history of smoking in the study group, 212 from the cases and 53 from the controls. Periodontal status in never smoking group and PAG status showed no significant association among cases (p= 0.517), controls (p=0.988) or in the entire never smoking group (p = 0.582) (Table 5.6).

Table 5.6. PAG in association with never smoking

	Cases			Controls		
	PAG +)ve n (%)	PAG(-)ve n (%)	**Total** n (%)	PAG (+)ve n (%)	PAG (-) ve n (%)	**Total** n (%)
Health	11(13.8)	69 (86.3)	80 (100)	2 (9.5)	19(90.5)	21(100)
Gingivitis	9 (15.8)	48 (84.2)	57(100)	2 (10)	18(90)	20(100)
Chronic Periodontitis	6 (8.3)	66 (91.7)	72 (100)	1(8.3)	11(91.7)	12(100
Total	26(12.3)	186(87.7)	212 (100)	5(9.4)	48(90.6)	53(100)

Pearson $\chi 2 = 0.582$)

5.3.11 PAG status in association with periodontal parameters

When comparing the periodontal parameters with PAG status a significant difference was observed between PAG status and mean probing depth (p=0.032). However, the mean probing depth was higher in PAG negative group (2.15 (±0.80) than the PAG positive 1.87 (±0.57) (Table 5.7).

Table 5.7. PAG status in relation to periodontal parameters.

Periodontal parameter	PAG (+ve)	PAG (-ve)	p value
Mean Probing depth (mm)	1.87 (1.47-2.15)	2.15 (1.64-2.39)	0.039
Mean Recession (mm)	0.40 (0.05 - 0.37	0.48 (0.04-0.51)	0.714
Mean Bleeding on probing	0.12 (0.01-0.16)	0.15 (0.01-0.21)	0.434
Mean Loss of Attachment (mm)	2.27 (0.58-2.52)	2.63 (1.79-2.82)	0.052
Maximum probing depth (mm)	4.22 (3.00-5.00)	4.37 (3.00-5.00)	0.754
Maximum loss of attachment (mm)	5.90 (4.00-7.00)	6.22 (4.00-8.00)	0.826
% BOP	12.24 (1.05-16.00)	15.14 (1.13 - 20.78)	0.448
% sites with PD ≥ 4mm	4.66 (0.00 - 4.75)	10.19 (0.00 – 9.25)	0.513
% sites with PD ≥ 5mm	1.94 (0.00-1.36)	4.52 (0.00 - 1.95)	0.448
% sites with PD ≥ 6mm	0.29 (0.00-0.00)	1.00 (0.00-0.00)	0.549

Mean and interquartile ranges are given in brackets

5.3.12 *TNFA (-308)* genotype in association with type 2 diabetes

Table 5.8 shows the *TNFA (–308)* allele distribution between patients with diabetes and controls. Distribution of Allele 1 (GG) was 83.6% among the cases and 79.2% in controls. Heterozygotes (G/A) carriers were 14.1% and 19.4% in cases and controls respectively. Homozygotes for allele 1(AA) was rare among the study population and observed percentages were 2.3 and 1.4 in cases and controls respectively. However, there was no significant difference in the distribution of these alleles between the cases and controls (p = 0.497).

Table 5.8. ***TNFA (-308)*** **genotype distribution among cases and controls**

TNFA genotype	Case or control		Total
	Case n (%)	Control n (%)	n (%)
GG	219(83.6)	57(79.2)	276(82.6)
GA	37(14.1)	14(19.4)	51(15.3)
AA	6 (2.3)	1(1.4)	7(2.1)
Total	262(100)	72(100)	334(100)

Number (%) of Probands with genotype
$\chi^2 = 0.497$

5.3.13 *TNFA (-308)* genotype in association with periodontitis

Table 5.9 represents the data for *TNFA* genotype distribution according to periodontal health. Among those who were diagnosed as health, 80% possess the GG genotype. There were 18.5% with GA and 1.5% with AA genotype. Distribution of the GG genotype in the gingivitis group was 81.7% and 86.1% in chronic periodontal group. 15.1% and 3.2% of the subjects in gingivitis group had GA and AA genotypes respectively. In chronic periodontal group 12% and 2% demonstrated GA and AA genotypes. These genotypes in differently diagnosed groups showed no significant difference in distribution (p=0.770).

Table 5.9. ***TNFA*** **genotype distribution and periodontal health**

TNFA Genotype	GG	GA	AA	Total
Health	104 (80.0%)	24 (18.5%)	2 (1.5%)	130(100%)
Gingivitis	76 (81.7%)	14 (15.1%)	3 (3.2%)	93 (100%)
Chronic periodontitis	96 (86.5%)	13(11.7%)	2(1.80%)	111(100%)

Pearson's Chi-Square =0.770
Numbers and percentages given in parenthesis

5.3.14 *TNFA* allele distribution and periodontal parameters

Significant correlations were observed between *TNFA (-308)* genotype with mean recession mean loss of attachment and maximum loss of attachment. This genotype had no impact on other periodontal parameters (Table 5.10).

Table 5.10 Significant correlations of *TNFA* genotypes with periodontal parameters

	Correlation	*P*
Mean recession (mm) (Mean Rec)	-0.119*	0.031
Mean loss of attachment (mm) (Mean LOA)	-0.113*	0.040
Maximum loss of attachment (mm) MAximum LOA)	-0.117*	0.033

Correlations were significant at 0.05 level. Correlations were significant for mean Rec, Mean LOA and Maximum LOA

5.3.15 *TNFA (–308)* and blood chemical parameters

Table 5.11 presents the relationship between *TNFA* (-308) genotype and biochemical parameters. When tested the *TNFA (-308)* genotype in association with blood chemical parameters the only statistical significant difference was found with HDL levels. The values, median (SD) for GG, GA and AA were 48.46mg/dl (6.56), 46.67 mg/dl (5.81) and 30.40mg/dl (19.45) respectively. Post hoc Bonferroni test has identified that the difference was with AA genotype (p = 001). Usually level below 40mg/dl is considered as a risk of developing coronary artery diseasaes and <35mg/dl belongs to a high risk group (National Cholesterol Education Program 2007). Multiple linear regression analysis for association of *TNFA (–308)* genotype and blood chemistry parameters after adjusting for age, sex, diabetes status, and BMI resulted in no significant association with HbA1c (p=0.496), total cholesterol (TOC) (p= 0.177), triglycerides (TGL) (p= 0.980), low density lipoproteins (LDL) (p=0.340), and fasting blood glucose (FBG) (p=0.649). However, there is an effect of *TNFA* genotype status on HDL (p=0.001) after adjusting for age, sex, diabetes status and BMI again proving a statistically significant association between the two. Spearman's rho Correlation coefficient (r) indicates a correlation was a negative correlation, (Figure 5.17).

Table 5.11 Effect of *TNFA* (-308) genotypes in biochemical parameters and BMI

Biochemical parameter	*p value*	*Biochemical parameter*	*p value*
Systolic Blood pressure	0.599	Low Density Lipoproteins	0.726
Diastolic blood pressure	0.793	High Density Lipoperoteins	0.001
HbAlc	0.841	Fasting blood glucose	0.597
Total Cholesterol (TOC)	0.333	Diabetes duration	0.486
Triglycerides	0.880	BMI	0.511

p is significant at 0.05 level

Figure 5.17. Significant correlations of *TNFA (-308)* genotype with HDL

TNFA (-308) genotype AA shows significantly different low level of High Density Lipoproteins (HDL) (p<0.001)

5.4 Discussion

A genetic polymorphism in the interleukin 1 gene has been implicated as a factor in determining the severity of adult periodontitis. The proinflammatory cytokines such as *IL-1* (IL-1α and IL-1β) and TNF-α have a key role in the initiation and perpetuation of the innate response in the periodontium. These cytokines levels appear to be higher in sites of infection and contribute to tissue destruction and alveolar bone resorption in a susceptible individual and the presence of polymorphisms make them ideal candidate genes for association studies. At the outset it should be emphasized however, that genetic predisposition represents only one of several dimensions of patient based risk factors for disease progression.

The primary aims of this part of the study was to analyze the role of cytokine gene polymorphisms (*IL1A (+4845)*, *IL1B (+3954)* and *TNFA (-308)* in association with chronic periodontitis in type 2 diabetes. In addition further investigations were carried out to determine the association of composite PAG, i.e. the occurrence of allele 2 of both *IL1A (+4845) & IL1B (+3954)* SNPs with periodontitis. Moreover, *TNFA (-308)* polymorphism in association with diabetes or diabetes related biochemical parameters or periodontitis and related parameters was investigated.

5.4.1 Gene frequency analysis

Population frequencies analysis of *IL1B (+3954)* for Sri Lankan population revealed gene frequencies for allele 1 and allele 2 were 0.9 and 0.1 respectively. Similarly the frequencies for *IL1A (+4845)* were 0.74 and 0.26 respectively. Frequencies previously reported for *IL1B (+3954)* in North British Caucasian populations are 0.82 and 0.18 for allele 1 and allele 2 (di Giovine et al. 2000) and for *IL1A (+4845)* are 0.71 and 0.29 for allele 1 and allele 2 respectively (Gubler et al. 1989). Thus, the present study observed gene frequencies that are similar to North British Caucasian populations for *IL1A* and *IL1B* polymorphism. When the gene frequencies of *TNFA (-308)* polymorphisms were analysed , frequencies of 0.91 and 0.09 were observed for allele 1 and allele 2 respectively. Frequencies for the same alleles reported in North British Caucasians were 0.77 and 0.23 respectively (Wilson et al.1992). The population studied in the present analysis of Sri Lankan population shows considerable difference in allele distribution for *TNFA (-308)* polymorphism. Some other

studies have reported similar frequencies to the present study. They were from two other Asian populations, Japan and Taiwan. In a Japanese population observed frequencies were 0.99 for allele 1 and 0.01 for allele 2 (Endo et al. 2001). In Taiwanese patients a frequency of 0.94 for allele 1 and 0.05 for allele 2 was observed (Shiau et al. 2003). Thus it is evident that Japanese, Taiwanese and Sri Lankan populations have similar gene frequencies for *TNFA (-308)* promoter polymorphism compared to that observed in North British Caucasians. This is an indication that some cytokine gene frequencies are distributed differently in certain populations and may be contributing differently to the pathogenesis of a disease.

5.4.2 *IL1B (+3954), IL1A (+4845)* and PAG: Impact on periodontal disease

This study next investigated the effect of composite genotype PAG and its distribution between patients with type 2 diabetes and controls. The results of this case control study does not support an association between *IL1B (+3954), IL1A (+4845)* SNPs in association with chronic periodontitis in the Sri Lankan population. Also the PAG was equally distributed among patients with type 2 diabetes (12.6%) and controls (12.5%). PAG status showed no significant association with chronic periodontitis.

Kornman and colleagues were the first to report an association between IL1 gene cluster polymorphism as a risk indicator for chronic periodontitis. Since then contradictory results have been published. A significant association between allele 2 of *IL1B (+3954)* and advanced periodontitis has been reported (Galbraith et al. 1999). Parkhill and colleagues observed an association with allele I of *IL1B (+3954)* genotype with aggressive periodontitis (Parkhill et al. 2000). When considering allele 2 of *IL1A (-889)* and *IL1B (+3954)* polymorphism as PAG, associations were observed in some studies (Gore et al. 1998, Laine et al. 2001) however, not in others (Hodge et al. 2001, Tai et al. 2002). Since *IL1A (-889)* and *IL1A (+4845)* are believed to generate similar results in genotyping and analysis of *IL1A (+4845)* is comparatively easy, in subsequent studies the position of *IL1A (-889)* was replaced with *IL1A (+4845).* Kornman et al. studied the variants *(-889)* and *(+3954)* and discovered that the PAG status was correlated with severity of periodontitis, patients had an approximately seven times greater chance of having severe periodontitis than those who were PAG negative in non-smokers (Kornman et al. 1997) and Agrawel and

co-workers found significant associations with PAG (*IL1A (+4845)* and *IL1B (+3954))* positions in association with periodontitis in India (Agrawel et al. 2006). In contrast, some studies have reported contradictory results (Gore et al. 1998, Cutler et al. 2000, De Sanctis & Zucchelli 2000). However, considering all the publication data analysed together there is evidence that PAG in *IL1A (-889)* and *IL1B (+3954)* combination is a risk factor for periodontitis (Nikolopoulos et al. 2008). Up to date there were seven studies conducted in Asian populations in relation to the IL-1 gene cluster. (Four studies were conducted in China (Duan et al. 2002, Huang & Zhang 2004, Zhong et al. 2002, Tian et al. 2006), one in Thailand (Anusaksathein et al. 2003), one in Japan (Soga et al. 2003) and one in India (Agrawel 2006)). The results of these seven studies were pooled together and analysed to find out whether there is a significant detectable relationship with the IL-1 gene cluster with periodontitis (Nikolopoulos et al. 2008). Based on this analysis it was concluded allele 2 of *IL1B (+3954)* is a significant predictor of chronic periodontitis with double the hazard in populations of Asian origin. The sample sizes of these studies varied from 30 -182. The present study conducted with a sample size of 334 subjects however contradicts the meta analysis findings failing to observe a significant association between chronic periodontitis and PAG. The present sample however consists of 272 patients with diabetes, 12 IFG subjects and 60 controls. Assessed cytokine genotype is not the only contributory factor that determines the severity of periodontitis in such a group. Diabetes is also a risk factor for periodontitis and we have not accessed the microbiological factors (Plaque microorganisms) which are primary determinants of initiation of periodontitis. Previous studies that have reported significant difference in genotype distribution in patients with chronic periodontitis and controls. The failure of the present study to demonstrate such an association may be due to the true effects of genotype (expression is not induced mainly by the genotype but other factors) being overshadowed by the effects of diabetes and microbial associated factors that influence the severity of periodontitis.

PAG status may be an indicator for periodontal disease severity as measured by probing depth measurement. According to results observed in some studies PAG was correlated with severity of periodontitis as measured by attachment loss (McDevitt et al. 2000, Papapanou et al. 2001). A, significant difference was observed between PAG status and mean probing depth (mean PD) in the present study. However, the PAG negative group shows higher mean PD {2.15 (1.64-2.39)} than PAG positive {1.87 (1.47-2.15)}.

Smoking is another major environmental risk factor associated with increased incidence and severity of periodontitis (Page and Beck 1997).This study was not designed to assess the impact on smoking but recorded the smoking status of the participants in order to exclude them and study the effect of PAG on non smokers. Observations suggest a negative impact on periodontal health of tobacco smoking. This study group represents only 7.8% smokers and 12.3% of ex-smokers. Among 79.8% of never smokers only 11.7% were demonstrated PAG positive status and only 2.5% of them had chronic periodonititis. Kornman and co-workers have found the association of PAG and aggressive periodontitis in non smokers. Those non smokers who were genotype positive had the same risk of disease as smokers who were either genotype positive or genotype negative. In addition PAG positive never smokers showed a significantly elevated chance of an increase in % bleeding on probing (BOP) (Lang et al. 2000). McGuire and coworkers found that PAG was significantly associated with extent of tooth loss after 14 years in 42 patients with chronic periodontitis and had an additive effect with smoking (McGuire et al. 1999). This provides evidence for interaction between smoking and genotype in conferring susceptibility to aggressive periodontitis. A study conducted by Parkhill and co-workers demonstrated that allele 1 carriers of *IL1B (+3954)* are significantly elevated in aggressive periodontitis in smokers. But no significant differences were found between *IL1B (+3954)* genotype or allele frequency in aggressive periodontitis non smokers and non smoking controls (Parkhill et al. 2000). In contrast to the original Kornman study, in a cross sectional study of 154 chronic periodontitis patients PAG was associated with increased severity of disease in smokers but not in non smokers (Meisel et al. 2002). The present study observed difference in smoking status between males and females. This study group includes 51.5% females. Stratification according to smoking would be applicable only to males in the study group since all the females were non smokers except for one ex smoker. The negative impact on smoking status is apparently due to low number of smokers in the present analysis.

5.4.3 Population difference of PAG status and carriage rates

Although contradictory results were obtained for PAG in association with pathogenesis of periodontal disease a noteworthy finding of this study is the low percentage of PAG positive genotype in Sri Lanka. This study revealed it was only 12.6%. Kornman et

al.demonstrated 29.1% occurrence of PAG status in the North European subjects and 36.3% prevalence in non smoking periodontal patients (Kornman et al, 1997). A 40.6% rate has been found in Australians of essentially European heritage (Cullinan et al. 2000). In Sweden the 42.9% occurrence of the positive composite genotype is comparable to Australians but found to be significantly higher than North European subjects (Papapanou et al.2001). Another study reported a higher (41%) occurrence rate of the positive composite genotype of (*IL1A (+4845)* & *IL1B (+3954)* in cases and only 28% in controls, although the difference was not statistically significant (McDevitt et al. 2000). The composite genotype was found to occur at rates 35% in a sample of periodontally healthy subjects comprising both Caucasians and African Americans (Goodson et al. 2000). 35.3% of IL-1 genotype positive was observed in Switzerland (Caucasians of central European ancestry) (Lang et al. 2000). In Hispanics the percentage declines to 26% (Caffesse et al. 2002). The prevalence of the composite genotype in Chinese heritage was 2.3% (Armitage et al. 2000). This shows variation of PAG distribution in different populations. The present study observed only 12.6% of PAG prevalence in Sri Lankans (Figure 5.18). This clearly shows that genotypes are distributed differently according to the population studied. Disease prevalence rates also vary from population to population. For example the prevalence rate of diabetes in Sri Lanka is 14.2% and the prevalence of diabetes in the UK is 5.1%. Indeed, the exposure to environment factors also vary from population to populations thus, variations observed in genetic and environment factors which are the main contributors of a common complex disease would indicate that the pathogenesis mediates differently. Therefore population studies are a timely requirement for targeting genes relevant to different population, ethnic groups and to individuals.

When considering the carriage rate for *IL1B (+3954),* allele 1 was 80.5% whereas for allele 2 it was 19.5% for present population. Carrier percentages of 55.4% and 44.6% were found for allele 1 and allele 2 of *IL1A (+4845).* When considering the carriage rates of the alleles in different populations for *IL1B (+3954)* allele 2, previous studies showed 44.1% (Kornman 1997) 40.6% (Gore et al. 1998) and 33.7% (Galbraith et al. 1999) in the USA, in Sweden 49% (Papapanou et al. 2001) With regard to *IL1A* reported rates in the literature on the carriage of allele 2 of *IL1A (-889)* locus were 50.5% in the USA (Kornman et al. 1997) and 56.4% for *IL1A (+4845)* locus in Sweden (Papapanou et al. 2001). A recent study of

subjects of Chinese heritage (Armitage et al.2000) revealed allele 2 carriage rates of 3.3% *(IL1B +3954)* and 17% *(ILIA +4845).* It becomes increasingly clear however, that such rates should be expected to vary greatly between ethnically and/or racially distinct populations. Thus the results of this study indicates the observed carrier rates for *IL1B* allele 2 is comparatively low when compared some studies but relatively higher compared to Chinese population.

Population differences of PAG status

Figure 5.18 Variations seen in PAG status in different populations.

5.4.4 *TNFA (-308)* gene variant and periodontal disease.

This study observed no impact of this gene variation on periodontal status. However, negative co-relations were observed for periodontal parameters mean recession, mean loss of attachment and maximum loss of attachment. A relatively large number of studies have published the association between periodontal disease and polymorphism at position *(-308)* in the promoter region. However the meta-analysis of all these data has revealed, overall there is lack of association between the allele 2 polymorphism and the susceptibility to chronic periodontitis (Nikolopoulos et al.2008). However, Fassmann and co-workers found a significant difference between the combined genotype *(TNFA (-308) &* Lymphotoxin Alpha *(LTA) (+252)* between the control and the patient groups (Fassmann et al. 2003). No association was found between the allele 2 polymorphism of *TNFA (-308)* and *ex vivo* TNF-a levels in Chilean patients with aggressive periodontitis and aggressive periodontitis in patients with type 1 diabetes (Perezl et al. 2004) but their study demonstrated that

aggressive periodontitis combined with diabetes express higher LPS induced TNF-α concentration than healthy individuals. The number of studies conducted in periodontitis with type 2 diabetes in relation to *TNFA (-308)* polymorphism is limited. The present study is in accordance with the previous studies conducted in literature has observed no association of *TNFA* polymorphism with periodontal status.

5.4.5 *TNFA* and diabetes

The analysis of *TNF* gene polymorphism in relation to diabetes in the present study observed that *TNFA–(308)* genotype distribution is not significantly different in type 2 diabetes patients compared to controls. When comparing three genotypes for blood chemical parameters, diabetes duration and BMI, the results implicated a significant difference in HDL cholesterol and the difference was associated with the AA genotype. Although the numbers are low for AA genotype these findings are similar to results found in a study conducted in a Taiwanese population (Shaiu et al. 2003). Shaiu and coworkers were also not able to observe a statistically significant variation in the distribution of three different genotypes between type 2 diabetes patients and controls however, high-density lipoprotein cholesterol (HDL) distribution was significantly different with TNF-α (238) genotypes (Shiau et al. 2003). These findings suggest that individuals with heterozygous (G/A) or homozygous (A/A) genotypes tended to have lower HDL and higher TOC/HDL levels. Additionally individuals with homologous *TNFA (-308)* (AA) genotype tended to have higher fasting blood glucose levels. These results imply that these two polymorphisms might be associated with HDL and glucose metabolism. TNFA (-308) genotype AA shows longer duration of diabetes (9.5 years) compared to GA (8.5 years) and GG (7.41years). TNF-α is known to play a key role in mediating metabolic imbalance through insulin resistance. Insulin resistance eventually increases lipolysis and thereby lipid metabolism. Therefore results of this study confirm the conclusion reached by Taiwanese study, by concluding that *TNFA (-308)* cytokine polymorphisms may be associated with glucose metabolism. The observed allele 2 frequency (A) is very low in these two populations (1.5% in Taiwanese and 0.8% In Sri Lankans).

Discrepancies exists concerning the role of *TNFA (-308)* in type 2 diabetes pathogenesis. An association has been reported in some (Fernandes-Real et al. 1997) but not in others

(Walston et al. 1999, Koch et al. 2000, Rasmussen et al. 2000, Endo et al. 2001). A different scenario is reported by a cohort of elderly patients with diabetes in the Netherlands, the *–308G/A* polymorphism in the promoter of the gene encoding TNF-α strongly contributed to the risk of diabetes in subjects aged 85 years and over (Heijmans et al. 2002). Homozygosity of A allele conferred a more than four fold increased risk of diabetes. This is in agreement with the extensive evidence for a direct role of TNF-α in the etiology of insulin resistance and type 2 diabetes (Uysal et al. 1997, Hotamisligil et al. 1994).

The distribution of these two alleles of *TNFA* is different among study subjects with different racial origins. The frequencies of *TNFA (-308)* allele 2 ranges from 9% in Hong Kong Chinese (Lee et al. 2000), 16% in French Scandinavian populations (Hoffstedt et al. 2000 & Herrmann et al. 1998), 11.9 % - 18% in German (Koch et al 2000, Brand et al. 2001), to 24% in Australians (Dalziel et al. 2002), 16% in Chileans (Perezl et al. 2004) and ranges from 1.25% in Taiwanese (Shue et al. 2001), 3.5% in UK Caucasian (Grove et al. 1997), 5.65% in US Caucasian (Walston et al. 1999) and 39.5% in the Spanish (Fernandes-Real et al. 1997). Therefore ethnic variation for allele distribution are suggested. In this study we have observed an allele frequency of 0.8%. When compared with available allele distribution data a low distribution of allele 2 was observed among Japanese, Taiwanese and Sri Lankan populations, further strengthening the fact that ethnic variation may exist and contribute differently to the pathogenesis of a disease.

Summary

In summary this study does not support an association between periodontal diagnosis and *IL1A (+4845)*, and *IL1B (+3954)* SNP polymorphisms. PAG percentage (12.6%) was relatively low when compared to PAG percentages of European populations but similar to the trend in other Asian populations. PAG positive status positively correlated with mean attachment loss. Thus PAG could be considered as a risk indicator for severity of periodontitis but may not act as a predictor. *TNFA (-308)* genotype showed no association with periodontal status and negatively correlated with some periodontal parameters. However, significant negative correlation of HDL with *TNFA (-308)* allele 2 implies this gene may have a role in diabetes pathogenesis.

6.1 Introduction

The world is experiencing a pandemic of type 2 diabetes and its complications due to hyperglycaemia that is causing significant morbidity and mortality. Sri Lanka is a developing country in which the prevalence of type 2 diabetes has been observed at the very high rate of 10.3% in people ≥ 20 years of age (Katulanda et al. 2008) and 14% in people above 35 years of age (Wijewardena et al. 2005). The projected diabetes prevalence in Sri Lanka for the year 2030 is 13.9%, anticipating large numbers of people developing the disease in the near future (Katulanda et al. 2008). Insulin resistance is also very common. Impaired glucose tolerance (IGT) and impaired fasting glycaemia (IFG) also constitute a major public health problem. This pre-diabetic state is now recognized as a stage in the transition from normality to diabetes. Thus individuals with IGT are at high risk of progressing to diabetes, with 70% of affected individuals expected to develop the disease (Diabetes atlas 2003). In Sri Lanka, 36% of the people with diabetes were previously undiagnosed and 11.5% had pre-diabetes in the form of IFG or IGT (Katulanda et al. 2008). The challenge for the medical community lies in testing the right people in a timely fashion in order to identify high risk individuals because early treatment of insulin resistance before the condition progresses to diabetes may have beneficial metabolic effects thus reducing the development of complications.

Previous studies have identified the burden of diabetes complications in the diabetic population at the time of diagnosis in Sri Lanka (Weerasuriya et al. 1998). In this study, periodontal disease was not recognized as a complication and data regarding the prevalence of the periodontal disease in patients with diabetes were lacking in this community. With the anticipated increasing number of patients with diabetes, the resulting burden of complications, morbidity and premature mortality is a major growing public health problem with huge economic impact in Sri Lanka.

Type 2 diabetes and periodontitis are both considered to be common complex diseases in which genetic and environmental factors play a role in the pathogenesis. These two diseases are systemically related as evidenced by observational and longitudinal studies. Therefore, people with diabetes are more prone to develop periodontal disease than those without and once periodontal disease is established in people with diabetes, it is probable that their

underlying metabolic control is adversely impacted when compared to people with diabetes but not periodontitis. Chronic periodontal disease is an inflammatory disease initiated by bacterial plaque accumulation in the periodontium and inter-individual variation in cytokine secretion and tissue destruction has been observed in different individuals after controlling for the confounding factors of plaque, age, smoking and diabetes.

Within this context the principal aims of the present study were to determine the genetic contribution to type 2 diabetes based on family history in order to identify people who are at high risk of developing diabetes and to investigate the prevalence of periodontal disease in patients with type 2 diabetes. It has been observed that poorly controlled diabetes leads to increased prevalence of periodontitis in patients with diabetes; therefore the study investigated the effect of level of glycaemic control on periodontal status. Identifying genetic risk factors for disease is also likely to become as important as an early diagnostic tool to identify people at risk for periodontitis. According to the periodontal literature, higher levels of cytokines have been observed in the periodontium of patients with destructive periodontitis. Furthermore, the genetic variants of cytokine genes IL-1 (*IL1B (+3954)* and *IL1A (+4845)* have been implicated as possible genetic markers for periodontitis. However, variable and contradictory results from different studies limit the conclusions that may be drawn with regard to this potential risk factor. Therefore, this study aimed to investigate the genetic variants of cytokine genes IL-1 (*IL1B (+3954)* and *IL1A (+4845)*) in association with periodontal disease. TNF-α which is mainly secreted by adipocytes is found in high levels in the plasma of patients with diabetes. This adipokine is known to inhibit insulin action by interacting with insulin receptor substrate 1 (IRS-1) thus leading to insulin resistance. Serum TNF -α levels have also been reported to be higher in patients with periodontitis. This may have a potential role in contributing to poorer metabolic control in patients with periodontitis. Therefore, this study also aimed to investigate any relationship between *TNFA (-308)* polymorphism and periodontitis.

6.2 Implications of family history study and future directions

The relevance of genetic studies in diabetes is to achieve early diagnosis of people at risk of developing disease, and to minimise suffering and disability due to complications (Diabetes atlas 2003). In fact, family history data provide useful insights to identify people with a

probable risk of developing disease compared to those people with no history of diabetes. The present analysis of family histories in one thousand patients indicates that the disease has a familial aggregation in 59.4% of the diabetic population studied in Sri Lanka. A stronger family history of diabetes associated with an earlier age of diagnosis of diabetes has also been observed in India (Vishwanathan et al. 1995). The results of the present study are therefore in agreement with those results observed in India, that the age of onset of diabetes is earlier if both parents of the patient have diabetes.

A recently conducted study in the USA concluded that family history of diabetes has a graded association with prevalence of diabetes. They also identified that this association is detectable within strata of several well established risk factors for diabetes such as age and BMI. The basis of categorisation of family history was high, moderate and average in the US study. When a person has at least two first degree relatives or one first degree and at least two second degree relatives with diabetes from the same lineage, they are considered to have a higher risk of developing diabetes. If a person has just one first degree and one second degree relative with diabetes or only one first degree relative with diabetes or at least two second degree relatives with diabetes from the same lineage, this is considered to be moderate risk. If there is no family history of diabetes or at most, one second degree relative with diabetes, then these individuals are considered to have just an average risk of developing diabetes. Reliable estimates of the prevalence of familial risk of diabetes in the US population suggest that about 1 in every 3 adults has a moderate risk and about 1 in every 13 adults has a high risk of developing diabetes (Valdez et al. 2007).

This US study provides evidence for the feasibility of incorporating family history data as a risk factor for development of diabetes. It has also indicated that compared to people without a family history of diabetes, those that do have a family history of diabetes are two to six times more likely to have type 2 diabetes (Harrison et al. 2003). Family history evaluation in another study in the USA also suggested that it may provide a useful screening tool for the detection and prevention of diabetes (Hariri et al. 2006). The present study lacks family history data for people who do not have diabetes in Sri Lankan population. Therefore, future studies should be directed at obtaining family histories in diabetes from a group of people who do not themselves have diabetes in order to establish

and confirm the strength of using family history data as a predictor in relation to identify risk of developing diabetes.

Several genetic variants related to the risk for diabetes have been reported, but their use to estimate diabetes risk in populations is limited and has not been considered to be economical. A family history is relatively easy to obtain and conveniently conveys information on genes and environment shared by close relatives (Valdez et al. 2007). The results of the US study show that stratified familial risk combined with other readily available indicators of diabetes risk could be a great tool to identify segments of the populations at high risk for the disease (Valdez, et al. 2007). A universally agreed upon definition of what constitutes a positive family history of diabetes that is also simple to collect would be necessary to establish and compare familial risk across Sri Lanka. Thus, incorporating family histories into screening programmes would be a cost effective method of identifying people at high risk of diabetes that could be used in a developing country like Sri Lanka. This area remains to be explored in the future.

The present study also established a DNA bank of samples from patients with type 2 diabetes together with controls. Presently, DNA from 100 trios and 285 patients and 60 controls are available. It is expected to expand this DNA bank in order to conduct genome wide association studies to identify genetic determinants of diabetes in relation to this population.

6.3 Implications of periodontal study and future directions

The major risk factors for periodontal disease are smoking (Tomar & Asma 2000) and diabetes (Nelson et al. 1990, Jansson et al. 2006). Over recent decades, in countries such as the USA, the proportion of individuals who smoke has reduced and it has been suggested that this has led to a reduction in the incidence of periodontitis of about 31% between 1955 and 2000 (Hujoel et al. 2003). However, the projected prevalence of diabetes is increasing and we know that people with diabetes are more susceptible to develop periodontitis. Therefore, although decreases in the incidence of periodontitis may be attributable to reduced smoking until now, it is possible that the future will see an increase in the numbers

of patients with periodontitis as a result of the increasing proportion of the population with diabetes.

Regarding an impact of periodontal treatment on glycaemic control, when mechanical periodontal therapy has been combined with systemic antibiotics, improvements in glycaemic control have been reported (Grossi et al. 1997, Kiran et al. 2005, Yun et al. 2007). Data from experimental and observational studies suggest that the chronic inflammatory burden conferred by advanced periodontitis may contribute to a heightened inflammatory state, potentially contributing to poorer glycaemic control and the potential for increased development of complications via altered metabolic pathways. An association between inflammatory periodontal disease and diabetic nephropathy as well as kidney dysfunction has been reported (Naugle et al. 1998). Moreover, periodontal disease increases insulin resistance. Hyperinsulinemia, hyperglycaemia and dyslipidemia are all independent risk factors for arthrosclerosis (Bierman 1992). Undiagnosed, untreated diabetic patients with periodontitits are therefore potentially at increased risk of development of significant complications, and management of this high risk group should be a health care priority when treating people with diabetes. In other words, periodontal assessment and treatment would be of likely benefit as a routine management strategy in patients with diabetes.

A comparatively high prevalence rate of periodontitis was observed in both type 2 diabetes and control populations (33% and 21% respectively) in this study in Sri Lanka compared to prevalence data reported in the UK and USA (Kelly et al. 2000, Albandar et al. 1999). In addition, periodontal parameters such as mean recession, percent bleeding on probing, maximum probing depth, maximum loss of attachment, number of sites with probing depth $\geq$4mm and $\geq$ 5mm were all significantly higher in people with diabetes when compared to controls. HbA1c values recorded in this study indicate that 54% of the patients had good glycaemic control. Moreover blood pressure scores and LDL values were significantly lower in the people with diabetes than the control group. This is likely because of effective medical management of the known diabetes patients, who were receiving medication for hypertension, together with cholesterol lowering drugs and their blood glucose level is controlled. Therefore this group of patients with managed diabetes may not represent a normal diabetic population in Sri Lanka. Therefore, contradictory results observed in this

study compared to previous studies which reported significant associations between periodontitis and diabetes could have arisen because of the effective medical management of the diabetes patients in this study. However, when evaluating periodontal disease severity as measured by periodontal scores, it is clear that periodontitis is a serious health problem among the diabetic population in Sri Lanka. This statement extends to the control population as well. According to the results, roughly 1 in every 3 patients with type 2 diabetes are affected with periodontitis and 1 in every 5 of the people in the general population are affected with the condition. When considering the group of people with IFG, 66.7% of these were diagnosed with periodontitis.

It appears from the data that patients with diabetes may be more prone to develop periodontitis due to altered immune-inflammatory responses and susceptibility to infection. Once periodontal disease is established, the chronic nature of this infection may aggravate glycaemic control and contribute to worsening of diabetic status leading to increased risk of microvascular and macrovascular complications. Evidence observed for this relationship was reported in a longitudinal case control study which compared the development of diabetes complications over a period of 11 years in people with diabetes who also had periodontitis to a health / gingivitis control population. They reported significantly greater prevalence of proteinuria and cardiovascular complications in those subjects who had periodontitis at baseline (Thorstensson et al. 1996). Similarly, controlled clinical trials in which mechanical periodontal therapy was combined with systemic administration of antibiotics have reported improved glycaemic control (Positional paper 2000). These studies support the concept that periodontal disease and diabetes share common pathogenic pathways, and that successful treatment of either condition may have a positive impact on the other.

Development of diabetes complications is a long term process that occurs as a consequence of elevated hyperglycaemia. Periodontal destruction occurs as a result of inflammatory response of the immune system to the subgingival bacterial challenge. Type 2 diabetes is likely to modify the inflammatory response to the subgingival biofilm, and the net result of this is an increased severity of periodontitis that will require specific strategies for early intervention and treatment in this high risk population (Novak et al. 2008). Therefore,

routine dental examinations and adequate oral hygiene should be emphasized as part of the standard care of people with diabetes.

One of the limitations of the present study is that there are unequal numbers of patients in each category of glycaemic control, with relatively fewer patients with moderate and poor control, compared to good control. This may also contribute to the variance between the results identified in this study, and those reported in previously published literature. Specifically, poor glycaemic control has usually been reported as a risk factor for periodontitis in populations with diabetes (Guzman et al. 2003) whereas such a finding was not identified in the present study. The lack of statistically significant findings may be related to the relatively smaller number of patients with poor glycaemic control in this study. Moreover, periodontal disease in diabetes is likely to be a long term complication as a consequence of progression of hyperglycaemia. This cross sectional study thus limits the interpretability regarding precise relations between development of periodontitis and glycaemic control. Although adjustments were made for confounding factors in the analyses, in observational studies such as this, the observed associations may be caused by unidentified underlying factors. It has been suggested that to circumvent these problems, prospective cohort studies should be designed (Pischon et al. 2006). Cohort studies would ideally be conducted by enrolling people without periodontitis or diabetes to assess disease progression over many years while monitoring individuals for a wide range of quantitative traits and lifestyle and exposure data which enable an evaluation of the joint effect of genes and environment. Clearly, in the context of a slowly progressing disease such as periodontal disease, the costs of such an approach would be prohibitive, however.

In their review Lamster and Lalla suggested that periodontal disease can be considered a microvascular complication of diabetes. They also suggested that conducting studies to determine the early development of gingival inflammation and periodontal disease in people with diabetes represents a risk for development of other complications (Lamster & Lalla 2001). Examining a larger cohort with IFG for periodontitis may provide useful information regarding the systemic links between the two diseases and increased risk of progression to diabetes in patients with both IFG and periodontitis.

6.4. Implications of the genetic study and future directions

Research into human molecular genetics is now focused on identifying the genetic factors involved in complex diseases. If successful, identification of genetic risk factors could lead to early identification of individuals with an increased risk for a particular disease. Early diagnosis is always preferable, and is likely to result in much better treatment outcomes than late diagnosis. Furthermore, establishing the underlying genetic risk factors removes one variable from the study of the environmental/genetic interaction. Identification of environmental risk factors thus becomes easier and more precise. If the actual genetic cause of the disease can be elucidated by genetic testing, then treatment can be better fitted to the actual cause rather than the phenotype. Finally, cloning the genes and characterisation of the proteins they code for could provide opportunities for better understanding of disease pathogenesis and development of therapeutics (Sudbery 2002).

The *IL1B* , *IL1A* genes were considered as ideal candidate genes for genetic studies in relation to periodontitis and *TNFA* in diabetes since the protein levels of these genes are elevated in periodontal tissues and diabetic plasma. Moreover, these genes contain SNPs and therefore act as molecular markers and are easy to identify by PCR based genotyping.

In this study *IL1B (+3954), IL1A (+4845)* and *TNFA (-308)* polymorphisms were studied in relation to periodontitis and diabetes. Population frequency analysis of *IL1B (+3954), IL1A (+4845)* and *TNFA (-308)* revealed that allele 1 and allele 2 frequencies were 0.9 & 0.1, 0.74 & 0.26 and 0.91 & 0.09 respectively. The *TNFA (-308)* polymorphism deviated from the frequencies observed in northern British Caucasians (Wilson et al. 1992) but was similar to that observed in Taiwan (Shiau et al. 2003) and Japan (Endo et al. 2001). This is an indication that certain gene frequencies are distributed differently in different populations and may have a direct interaction with the environment in that particular population and susceptibility to disease.

The composite genotype (PAG) demonstrated no significant associations with periodontal status, either in diabetes patients, controls or non smokers ($p>0.05$) in the present population. A noteworthy finding is that PAG shows great variation in people with European ancestry. Greenstein and Hart questioned the rationale for testing for PAG as a

predictor of susceptibility to periodontitis (Greenstein & Hart 2002). In the original Kornman study, PAG status was determined by *IL1A (-889)* and *IL1B (+3954)* (Kornman et al. 1997). In subsequent studies *IL1A (+4845)* and *IL1B (+3954)* was considered because *IL1A (+4845)* is in strong linkage disequilibrium with *IL1A (-889)*. In their meta-analysis, Nikolopoulos and colleagues stated that when considering *IL1A (+4854),* only two studies yielded marginally insignificant results whereas for *IL1A (-889)* six studies were included the results reached significance (Nikolopoulos et al. 2008). Functional differences in the two SNP positions may be the result of observed differences.

IL-1α is produced as a 31KDa precursor protein and processed into a 17 KDa mature protein for export from the cell. Cleavage of precursor IL-1α involves the intracellular enzyme calpain. The polymorphism in the IL1A promoter at position -889 (C>T) is reported to affect the generation of IL-1α protein but not mRNA in short term cultures, with individuals carrying the rare TT genotype having higher levels than those with the CC genotype. The *IL1A (+4845)* polymorphism involve a substitution of 114th amino acid of pre IL-1α. Pre IL-1α is processed into mature form by the enzymatic cut between 112 and 113 amino acids (Dinarello 1996).

Several reports suggested the influence of -889 SNP on the transcriptional activity of *IL1A* gene (Dominici et al.2002). However, recent results on human skin fibroblasts indicate that the -889 SNP was not involved in transcriptional activity, possibly because the transcriptional regulation of the *IL1A* gene might be different among different human cell types. Thus, further investigations of transcription of the *IL1A* gene with -889 *SNP* in human cells would be needed (Kawaguchi et al. 2007). His results indicate that IL1A *(-889)* has no functional significance in the human fibroblast. However polymorphism analysis of *IL1A (-889)* variant with IL1-α levels in gingival crevicular fluid of patients with periodontitis demonstrated that in the diseased population there was at least a four fold increase in the mean level of IL-1α protein in patients who carried one or two copies of the allele 2 of the *IL1A (-889)* gene variant when compared with those who were homozygous for allele 1 (Shirodaria et al. 2000). Thus multiple risk factors in which all are not genetic may determine concentrations of functional protein levels.

Since *IL1A (+4845)* substitutes an amino acid in the *IL1A* pre-protein this may have a direct functional consequence (Dinarello 1996). Phenotypic expression is a result of protein secretion. This could be a reason for the insignificant result observed with *IL1A (+4845)* when compared to *IL1A (-889)* (Nikolopoulos et al. 2008) similar to those reported in the present study. Thus, using PAG as a predictor for periodontitis still remains controversial. It would be interesting to see how the leucocytes or fibroblasts of the patients who are PAG positive and PAG negative would respond to LPS. Determining the IL-1α and IL-1β levels from such a group will help to elucidate the functional relationship between PAG status and clinical phenotype. It should also be remembered that common disorders with multifactorial pathogenesis such as periodontal disease are not likely to result from single, highly penetrant genetic variants, but are likely to be influenced by subtle phenotypic variations caused by common alleles of a number of polymorphic genes relevant to disease pathogenesis (Hart & Kornman 1997). Therefore, future genetic tests should focus on number of polymorphic alleles that are involved in coding for proteins that are involved in periodontal pathogenesis.

Final conclusions

In conclusion this study was able to determine the genetic component of type 2 diabetes in Sri Lanka. When considering periodontal status, it is clear that periodontal disease is highly prevalent in Sri Lanka, and the condition is exacerbated in patients with diabetes. This should be taken into consideration when treating people with diabetes, to diagnose periodontal disease early, to implement treatment early, and to potentially improve quality of life. PAG status as a predictor for periodontitis still remains ambiguous and does not seems to correlate with severity of disease in this population. Functional studies (perhaps involving in *vitro* studies of PAG positive and PAG negative cell lines) would help to overcome the ambiguity. It is likely that testing for multiple polymorphisms of the genes that encode proteins that play a role in periodontal pathogenesis will help to identify genetic determinants that contribute to disease susceptibility. The constructed DNA bank provides valuable resource to study the genetic determinants of type 2 diabetes in this population.

Abdel-Aal, N.M., Ahmad, A.T., Froelicher, E.S., Batieha, A.M., Hamza, M.M., Ajlouni, K.M. (2008). Prevalence of dylipidemia in patients with type 2 diabetes. *Saudi Med J* **29**(10), 1423-8.

Abraham, L.J., Kroeger, K.M (1999). Impact of the -308 TNF promoter polymorphism on the transcriptional regulation of the TNF gene: relevance to disease *J Leukoc Biol* **66(4)**, 562-6.

Adiels, M., Olofsson, S.O., Taskinen, M.R., Boren, J. (2008). Overproduction of very low-density lipoproteins is the hallmark of the dyslipidemia in the metabolic syndrome. *Arterioscler Throm Vasc Biol* **28(7)**, 1225-36

Agrawal, A.A., Kapley, A., Yeltiwar, R.K., Purohit, H.J. (2006). Assessment of single nucleotide polymorphism at *IL-1A+4845* and *IL-1B+3954* as genetic susceptibility test for chronic periodontitis in Maharashtrian ethnicity *J Periodontol* **77(9)**, 1515-21.

Alabdulkarim, M., Bissada, N., Al-Zahrani, M., Ficara, A., Siegel, B. (2005). Alveolar bone loss in obese subjects. *J Int Acad Periodontol* **7**, 34-38.

Alberti, K.G.M.M.& Zimmet, P.Z. (1998). Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus, provisional report of a WHO Consultation. *Diabetic Medicine* **15**, 539-553.

Albandar, J.M., Kingman, A. (1999). Gingival recession, gingival bleeding and dental calculus in adults 30 years of age and older in the United States (1988-1994). *J Periodontol* **70**, 30-43.

Albandar, J.M., Brunelle, J.A., Kingman, A. (1999). Destructive periodontal disease in adults 30 years of age and older in the United States (1988-1994). *J Periodontol* **70**, 13-29.

Alcolado, J.C. & Alcolado, R. (1991). Importance of the Maternal history on non insulin dependent diabetic patients. *British Medical Journal* **302**, 1178-80.

Aldridge, J.P., Wilson, R.F., Viberti, G., Collins, A., Watts, T.L. & Lester, V. (1995). Single-blind studies of the effect of improved periodontal health on metabolic control in type 1 diabetes mellitus. *J Clin Periodontol* **22**, 271-275.

Alexander, M.B. & Damoulis, P.D. (1994). The role of cytokines in the pathogenesis of periodontal disease. *Curr Opin Periodontol* **2**, 39-53.

Alfonso, S., Richiardi, P.M. (1994). A polymorphic variation in a putative regulation box of the TNFA promoter region *Immunogenetics* **39(2)**, 150-4.

Alheim, K., McDowell, T.L., Symons, J.A., Duff, G.W.& Bartfai, T. (1996). An AP-1 site is involved in the NGF induction of IL-1α in PC12 cells. *Neurochem Int* **29**, 487-496.

Al-Otaibi, D.H.,Babay, N.A., Habib, S.S., Almas, K (2008). Assessment of lipid profile in Saudi type 2 diabetic and non-diabetic periodontal patients *Saudi Med J* (Abstract) **29(5)**, 723-7.

Altshuler D, Hirschhorn J.N, Klannemark M., Lindgren C.M, Vohl M., Nemesh J., Lane C.R., Schaffner S.F, Bolk A., Brewer, C., Tuomi T., Gaudet D., Hudson T.J., Daly M., Groop L., & Lander E.S. (2000). The common PPARγ Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes *Nature Genet*, **26**, 76.

Al-Shammari, K.F., Al-Ansari, J.M., Al-Arouj, M., Wang, H. (2006). Association of periodontal disease severity with diabetes duration and diabetic complications in patients with Type 1 diabetes. *J Int Acad Periodontol* **8(4)**, 109-14.

American Diabetes Association (2006). Standards in medical care in diabetes 2006. (Position Statement) *Diabetes Care* **29(1)**, S4-S42.

Andersen, P.C.C., Buschard, K., Flybjerg, A, Stoltze, K., Holmstrup, P. (2006). Periodontitis deteriorates metabolic control in type 2 diabetic Goto-Kakizaki rats. J *Periodontol* **77(3)**, 350-6.

Anerud, A., Loe, H., Boysen, H., Smith, M (1979). The natural history of periodontal disease in man. Changes in gingival health and oral hygiene before 40 years of age. *J Periodontal Res* **14(6)**, 526-40.

Arfa, I., Abid, A., Malouche, D., Alaya, N.B., Azegue, R.T., Mannai, I., Zorgati M.M., Rayana, M.C.B., Ammar S.B., Blousa-Chabchoub,S., Romdhane, H.B., Zouari, B., Dellagi M.K. and ,Abdelhak, S.(2007). Familal aggregation and excess maternal transmission of type 2 diabetes in Tunisia. *Postgrad Med J* **83**, 348-351

Armitage, G.C., Wu,Y., Wang, H.Y., Sorrell, J., di Giovine, F.S., Duff, G.W. (2000). Low prevalence of a periodontitis-associated interleukin-1 composite genotype in individuals of Chinese heritage. *J Periodontol* **71(2)**, 164-71.

Australian Bureau of Statistics (2006). *National Aboriginal and Torres Strait Islander Health Survey: Australia, 2004-05* Canberra: Australian Bureau of Statistics

Bacic, M., Plancak, D. & Granic, M. (1988). CPITN assessment of periodontal disease in diabetic patients. *J Periodontol* **59**, 816-22.

Bailly, S., di Giovine, F.S., Blakemore, A.I.F., Duff, G.W. (1993). Genetic polymorphism of -human Interleukin 1 alpha due to a variable number of intronic repeats. *Eur J Immunol* **23**, 1240-1245.

Bailly, S., Fay, M., Israel, N.& Gougerot-Pocidalo, M.A. (1996). The transcription factor AP-1 binds to the human interleukin 1-α promoter. *Eur Cytokine Netw* 7, 125-128.
Baker, P.J. & Roopenian, D.C. (2002). Genetic susceptibility to chronic periodontitis disease. *Microbes Infect* **4**, 1157-1167.

Barker, D.J.P., Eriksson, J.G, Forsen, T., Osmond, C. (2002). Fetal origins of adults diseases: Strength of effects of biological basis. *International Journal of Epidemiology* **31**, 1235-1239.

Banyer, J.L. Hamilton, N.H. Ramshaw, I.A. & Ramsay, A.J.(2000). Cytokines in innate and adaptive immunity. *Rev Immunogenet* **2**, 359-373.

Bartolucci, E.G. & Parks, R.B. (1981). Accelerated periodontal breakdown in uncontrolled diabetes: Pathogenesis and treatment. *Oral Surg Oral Med Oral Pathol Oral radiol Endod* **52**, 387-390.

Bergman, R.N. (2007). Orchestration of Glucose Homeostasis: From a Small Acorn to the California Oak. *Diabetes* **56**(6), 1489-1501.

Bernstein, B.E., Meissner, A., Lander, E.S. (2007). The Mammalian epigenome. *Cell* **128**, 669-681.

Bernzweig, E., Payne, J.B., Reinhardt, R.A., Dyer, J.K. & Patil, K.D. (1998). Nicotine and smokeless tobacco effects on gingival and peripheral blood mononuclear cells. *Journal of Clinical Periodontology* **25**, 246-252.

Bierman, E.L. (1992). George Lyman Duff Memorial Lecture Atherogenesis in diabetes *Arterioscler Thromb* **12**, 647-656.

Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes Dev* **16**, 6-21.

Birkedal-Hansen, H. (1993). Role of cytokines and inflammatory mediators in tissue destruction. *J Periodontol Res* **28**, 500-510.

Birmingham Research Unit (2005). Weekly returns service: annual prevalence report. *Royal College of General Practitioners*. www.rcgp.org.uk

Bisanda, N.F., Manouchehr-Pour, M., Haddow, M.& Spagnuolo, P.J. (1982). Neutrophil function activity in juvenile and adult onset diabetic patients with mild and severe periodontitis. *J Periodontol Res* **17**, 500-502.

Blackmore, A., Tarlow, J., Cork, M., Gordon, C., Emery, P., Duff, G. (1994). Interleukin -1 receptor antagonist gene polymorphism as a disease severity factor in systemic lupus erythematosis *Arthritis Rheum* **37**, 1380-1385

Boden, G., Chen, X., Ruiz, J., White, J.V., Rossetti, L. (1994). Mechanisms of fatty acid induced inhibition of glucose uptake *J Clin Invest* **93**, 2438-2446.

Bouma, G., Crusius, J. B. A., Pool, M.O., Kolkman, J.J., Von Blomberg, B.M.E., Kostense, P.J., Giphart, M.J., Schreuder, G.M.T., Muewissen, S.G.M. & Pena, A.S. (1996). Secretion of tumor necrosis factor α and lymphotoxin factor α in relation to polymorphisms in the TNF genes and HLA-DR alleles. Relevance for inflammatory bowel disease. *Scandinavian Journal of immunology* **43**, 456-463.

Brand, E., Schorr, U., Kunz, I., Kertmen, E., Ringel, J., Distler, A., Sharma, A.M. (2001). Tumor necrosis factor alpha –308 G/A polymorphism in obese Caucasians *Int J Obes Relat Metab Disord* **25(4)**,581-5.

Bridges, R.B., Anderson, J.W., Saxe, S.R., Gregory, K., Bridges, S.R. (1996). Perodontal status of diabetic and non diabetic men: effects of smoking, Glycaemic control and socioeconomic factors. *J Periodontol* **67(11)**, 1185-92.

Brownee, M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature* **414**, 813-820.

Brownlee, M. (2005). Banting Lecture 2004: The pathology of Diabetic Complications A unifying mechanism *Diabetes* **54**, 1615-1621.

Braun, N., Michel, U., Ernst, B.P., Metzner, R., Bitsch, A., Weber, F. & Rieckmann, P (1996). Gene polymorphism at position -308 of the tumor necrosis factor –alpha (TNF-alpha) in multiple sclerosis and its influence on regulation of TNF-alpha production. *Neuroscience letters* **215**, 75-78.

Burt, B.A. (1996). Position paper: epidemiology of periodontal disease. American Academy of Periodontology. *J Periodontol* **67**, 935-945.

Caffesse, R.G. De LaRosa, M.R., DLaosa M.G. (1998). PST genotypes in a periodontally healthy population treated for mucogingival surgery *Journal of Dental Research* **77**, (Abstract)

Caffesse, R.G., De LaRosa, M.R., De LaRosa, M.G., Mota, L.F. (2002). Prevalence of interleukin 1 periodontal genotype in a Hispanic dental population. *Quintessence Int.* **33(3),** 190-4.

Campus, G., Salem, A., Uzzau, S., Baldoni, E., Tonolo, G. (2005). Diabetes and periodontal disease: a case-control study. *J Periodontol* **76(3)**, 418-25.

Cardon, L.R.& Bell, J.I. (2001). Association study designs for complex diseases. *Nat Rev Genet* **2**, 91-9.

Cattabriga, M., Rotundo, R., Muzzi, L., Nieri, M., Verrocchi, G., Cairo, F., Pini, P.G. (2001). Retrospective evaluation of the influence of the interleukin-1 genotype on radiographic bone levels in treated periodontal patients over 10 years. *J Periodontol* **72**, 767-773.

Centers for disease control and prevention. National Center for Health Statistics (content online) www.cdc.gov/nchs/nhis/htm.

Chakravarti, A. (1999). Population genetics - making sense out of sequence. *Nat Genet* **21,** 56-60.

Christgau, M., Palitzsch, K.D., Schmaiz, G., Kreiner, U., Frenzel, S. (1998). Healing response to non surgical periodontal therapy in patients with diabetes mellitus; clinical microbiological and immunological results. *J Clin Periodontol* **25**, 112-124.

Clarke, N.G. & Hirsch, R.S (1995). Personal risk factors for generalized periodontitis. *J Clin Periodontol* **22(2)**, 136-45.
Clarke, N.G., Shephard, B.C., Hirsch, R.S. (1981). The effects of intra-arterial epinephrine and nicotine on gingival circulation. *Oral Surg Oral Med Oral Pathol* **52**, 577-582.

Cerda, J.G, Torre, C.V.D., Malacara, J.M. & Nava, L.E (1994). Periodontal Disease in Non-Insulin Dependant Diabetes Mellitus (NIDDM). The effect of age and time since diagnosis. *J Periodontol* **65(11)**, 991-995.

Chinnery, P.F., Mowbary, C.,Patel, S.K., Elson, J.L., Sampson, M., Hitman, G.A., McCarthy, M.I., Hattersley, A.T., Walker, M (2007). Mitochondrial DNA haplogroups and type 2 diabetes : a study of 897 cases and 1010 controls. *J Med Genet* **44**,80

Ciaraldi, T.P., Carter, L., Mudaliar, S., Kern, P.A., Henry R.R. (1998). Effects of tumor necrosis factors alpha on glucose metabolism in cultured human muscle cells from nondiabetic and type 2 diabetic subjects *Endocrinology* **139**, 4793-4800.

Corbet, E.F. (2006). Periodontal diseases in Asians *J Int Acad Periodontol* **8(4)**, 136-44.

Collins-Schramm, H.E., Phillips, C.M., Operario, D.J., Lee, J.S., Weber, J.L. & Hanson, R.L. (2002). Ethnic-difference markers for use in mapping by admixture linkage disequilibrium. *Am J Hum Genet* **70**, 737-50.

Collota, F., Re, F., Muzio, M., Bertini, R., Polentarutti, N., Sironi, M., Giri, J.G., Dower, S.K., Sims, J.E. & Mantovani, A. (1993). Interleukin -1 type II receptor : a decoy target for IL-1 that is regulated by IL-4. *Science* **261**, 472-475.

Conti, F. (2001). Claude Bernard: primer of the second biomedical revolution *Nature Reviews Molecular Cell Biology* **2**, 703-708.

Cox, A., Camp, N.J., Nicklin, M.J.H., Di Giovine, F.S. & Duff, G.W. (1998). An analysis of linkage disequilibrium in the interleukin-1 gene cluster, using novel grouping method for multiallelic markers. *American Journal of Human Genetics* **62**, 1180-1188.

Cropley, J.E., Suter, C.M., Beckman, K.B., Martin, D.I. (2006). Germ-line epigenetic modifications of the murine A vy allele by nutritional supplementation. *Proc Natl Acad Sci USA* **103**, 17308-17312.

Craandijik, J., van Krugten, M.V., Verweij, C.L. van d V. &, Loos, B.G. (2002). Tumor necrosis factor-α gene polymorphism in relation to Periodontitis. *J Clin Periodontol* **29**, 28-34.

Cullinan, M.P., Westerman, B., Hamlet, S.M., Palmer, J.E., Faddy, M.J., Lang, N.P., Seymour, G.J.(2001). A longitudinal study of interleukin-1 gene polymorphisms and periodontal disease in a general adult population *J Clin Periodontol* **28(12)**, 1137-44.

Cutler, C.W., Machen, R.L., Jotwani, R., Iacopino A.M. (1999). Heightened gingival inflammation and attachment loss in type 2 diabetics with hyperlipidemia. *J Periodontol* **70(11)**, 1313-1321.

Cutler, C.W., Stanford, T.W., Abraham , C., Cederberg, R.A., Boardman, T.J. & Ross, C. (2000). Clinical benefits of oral irrigation for periodontitis are related to reduction of pro-inflammatory cytokine levels and plaque. *J Clin Periodontol* **27**, 134-143.

da Cruz, G.A., de Toledo, S., Sallum, E.A., Sallum, A.W., Ambrosano, G.M., de Cassia, O.S.J, da Cruz S.E., Goncalves, R.B. (2008). Clinical and laboratory evaluations of non-surgical periodontal treatment in subjects with diabetes mellitus. *J Periodontol* **79(7)**, 1150-7.

D'Aiuto, F., Parkar, M., Andreou, G., Suvan, J., Brett, P.M., Ready, D., Tonetti, M.S. (2004). Periodontitis and systemic inflammation: control of the local infection is associated with a reduction in serum inflammatory markers. *J Dent Res* **83**, 156-160.

D'Aiuto, F., Nibali, L., Parkar, M., Suvan, J., Tonetti, M.S. (2005). Short term effects of intensive periodontal therapy on serum inflammatory markers and cholesterol *J Dent Res* **84**, 269-273.

Dahlman, I., Eaves, I.A., Kosoy, R., Morrison, V.A., Heward, J.& Gough, S.C. (2002). Parameters for reliable results in genetic association studies in common disease. *Nat Genet* **30,** 149-50.

Delima, A.J. Oates, T., Assuma, R., Schwartz, Z., Cochran, D., Amar, S., Graves, D.T (2001). Soluble antagonists to interleukin-1 (IL-1) and tumor necrosis factor (TNF) inhibits loss of tissue attachment in experimental periodontitis *J Clin Periodontol* **28(3)**, 233-40.

Dalziel, B., Gosby, A.K., Richman,R.M., Bryson, J.M., Caterson, I.D. (2002). Association of the TNF-α -308 G/A promoter polymorphism with insulin resistance and in obesity. *Obes Res* **10**, 401-7

Danis, V.A., Millington, M., Hyland, V.J. & Grennan, D. (1995). Cytokine production by normal human monocytes : inter subject variation and relationship to an IL-1 receptor antagonist (IL-1Ra) genepolymorphism. *Clin Exp Immunol* **99**, 303-310.

Darveau, R.P., Tanner, A. & Page, R.C. (2000). The microbial challenge in periodontitis. *J Periodontol* **14,** 12-32.

De Mann, F.H., Cabezas, M.C., Van Barlingen, H.H., Erkelens, D.W., de Bruin, T.W. (1996). Triglycerides rich lipoproteins in non insulin dependant diabetes mellitus; Post-prandial metabolism and relation to premature arthrosclerosis. *Eur J Clin Invest* **26**, 89-108.

De Sanctis, M. & Zucchelli, G. (2000). Interleukin-1 gene polymorphisms and long term stability following guided tissue regeneration therapy. *J Periodontol* **71**, 606-613.

Diabetes Atlas (2003). The global burden of diabetes. International Diabetes Federation, Second Ed 7-13.

Diabetes in Canada (2008). NDSS data files contributed by provinces and territories Public Health Agency of Canada http://www.phac-aspc.gc.ca/cd-mc/diabetes-diabete/face-eng.php

Dinarello, C.A. (1996). Biologic basis for interleukin-1 in disease. *Blood* **87**,2095–2147.

Dinarello, C.A., Ikejima, T., Warner, S.J., Orencole, S.F., Lonnemann, G., Cannon, J.G. & Libby, P. (1987). Interleukin 1 induces interleukin1.I.Induction of circulating interleukin 1 in rabbits in vivo and in human mononuclear cells in vitro. *J Immunol* **139**, 1902-1910.

Dominici, R., Cattaneo, M., Malferrari, G. et al. (2002). Cloning and functional analysis of the allelic polymorphism in the transcription regulatory region of interleukin-1 alpha *Immunogenetics* **54**, 82-6.

Donahue, R.P. & Tiejian, W.U. (2001). Insiulin resistance and periodontal disease: An epidemiologic overview of research needs and future directions. *Ann Periodontol* **6(1)**, 119-124.

Duff G.W. (2006). Evidence for genetic variation as a factor in maintaining health. *Am J Clin Nutr* **83**, 431S-45S.

Dullaart, R.P., de Vries, R., Sluiter, W.J., Voorbij, H.A. (2008). High plasma reactive C protein is related to low paraoxonase-1 activity independently of high leptin and low adiponectin in type 2 diabetes. *Clin Endocrinol (*Oxf) **70(2)**, 221-6.

Dunn, E., Sims, J.E., Nicklin, M.J. & O'Neill, L.A. (2001). Annotating genes with potential roles in the immune system: six new members of the IL-1 family. *Trends Immunol* **22,** 533-6.

Duan, H., Zhang, J., Zhang, Y. (2002).The association between IL-1 gene polymorphisms and susceptibility to severe periodontitis. *Hua Xi Kou QiangYi Xue Za Zhi* **20(1)**, 48-51. (Abstract)

Eason, R.J., Pada, J., Wallace, R., Henry, A. & Thornton, R. (1987). Changing patterns of hypertension, diabetes, obesity and diet among Melanesians and Micronesians in the Soloman Islands. *Med J Aust* **146,** 465-473.

Editorial. (1992). Genetic Basis of NIDDM. *The Lancet* **340**, 455-456.

Egeler, R.M., Zntinga, A.R., Coppes, M.J. (1994). Paul Langerhans Jr. (1847-1888): A short life, yet two eponymic legacies. *Med Pediatr Oncol* **22,**129-132.

Ehmke, B., Kress, W., Karch, H., Grimm, T., Klaiber, B., Flemming, T.F. (1999). Interleukin -1 haplotype and periodontal disease progression following therapy. *J Clin Periodontol* **26**, 810-813.

Emrich, L.J., Shlossman, M. & Genco, R.J. (1991). Periodontal disease in non-insulin dependant diabetes mellitus. *J Periodontol* **62,** 123-130.

Enders, S., Ghorbani, R., Lonnemann, G., van der Meer, J.W. & Dinarello, C.A. (1988). Measurement of immunoreactive interleukin-1β from human mononuclear cells: optimization of recovery, intrasubject consistency and comparison with interleukin 1α and tumor necrosis factor. *Clin Immunol Immunopathol* **49**, 424-438.

Enders, S., Cannon, J.G., Ghorbani, R., Dempsey, R.A., Sisson, S.D., Lonnemann, G., Vandermeer, J.W.M., Wolff, S.M. & Dinarello, C.A. (1989). In vitro production of IL-1 beta, IL-1 alpha, TNF and IL-2 in healthy subjects – distribution, effect of cyclooxygenase inhibition and evidence of independent gene regulation. *European Journal of immunology* **19**, 2327 -2333.

Endo, M., Tai, H., Taβ, K., Kobayashi, T., Yamazaki, K.& Yoshie, H. (2001). Analysis of single nucleotide polymorphisms in the 5'- flanking region of tumor necrosis factor -α gene in Japaneese patients with early-onset periodontitis. *J Periodontol* **72,** 1554-1559.

Engebretson, S.P., Lamster, I.B., Herrera-Abreu, M., Celenti, R.S., Timms, J.M., Chaudhary, A.G., di Giovine, F.S. & Kornman, K.S. (1999). The influence of interleukin gene polymorphism on expression of interleukin-1beta and tumor necrosis factor-alpha in periodontal tissue and GCF. *J Periodontol* **70**, 567-573.

Engebretson, S.P., Hey-Hadavi, J., Ehrhardt, F.J., Hsu, D., Celenti, R.S., Grbic, J.T., Lamster, I.B. (2004). Gingival crevicular fluid levels of interleukin-1beta and glycaemic control in patients with chronic periodontitis and type 2 diabetes. *J Periodontol* **75(9)**, 1203-8.

Engebretson, S, Chertog, R., Nichols, A., Hey-Hadavi, J., Celenti, R., Grbic, J. (2007). Plasma levels of tumour necrosis factor-alpha in patients with chronic periodontitis and type 2 diabetes. *J Clin Periodontol* **34(1)**, 18-24.

Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) (2001). *JAMA* **285**, 2493.

Fajans, S.S. (1989). Maturity-onset diabetes of the young (MODY) *Diabetes Metabol Rev* **5**, 579-606.

Fallin, D., Cohen, A., Essioux, L., Chumakov, I., Blumenfeld, M. & Cohen, D. (2001). Genetic analysis of case/control data using estimated haplotype frequencies: application to APOE locus variation and Alzheimer's disease. *Genome Res* **11,** 143-51.

Fassmann, A., Holla, L.I., Buckova, D., Vasku, A., Znojil, V., Vanek, J. (2003). Polymorphisms in the +252 (A/G) lymphotoxin alpha and the -308 (A/G) tumor nectrosis factor alpha genes and susceptibility to chronic periodontitis in Czech population *J. Perriodontol Res* **38,** 394.

Feingold, K.R., Staprans, I., Memon, R.A. (1992). Endoroxin rapidly induces changes in lipid metabolism that produce hypertriglyceridemia: Low doses stimulate hepatic triglyceride production while high doses inhibit clearance. *J Lipid Res* **33**, 1765-1776.

Fenton, M.J., Vermeulen, M.W., Clark, B.D, Webb, A.C.& Auron, P.E. (1988). Human Pro-IL-1β gene expression in monocytic cells is regulated by two distinct pathways. *J Immunol* **140**, 2267-2273.

Fernando, D.J.S. (1992). The prevalence of Complications of Diabetes in Patients Attending Diabetic Clinics in Sri Lanka and UK. Thesis (M.Sc.). University of Manchester.

Fermin, A. C. (2002). Carranza's clinical periodontology, 9th edition. 44.

Fernando, D.J.S. & Dewapura, G.H.K.N. (2000). Guidelines on management .of diabetes mellitus in Sri Lanka. Sri Lanka Medical Association.

Fernandes-Real, J.M., Gutierrez, C. & Richar W. (1997). The TNF alpha gene NcoI polymorphism influences the relationship among insulin resistance, percent body fat and increased serum leptin levels. *Diabetes* **46**, 1468-72.

Ficara, A.J., Levin, M.P., Grower, M.F. & Kramer, G.D.J. (1975). A comparison of the glucose and protein content of gingival fluid from diabetics and non-diabetics *Periodontal Res* **10**, 171-175.

Firali, E. (1997). The relationship between clinical periodontal status and insulin dependent diabetes mellitus. Results after five years. *J Periodontol* **68**, 136-140.

Fischbacher, M., Bhopal, R., Unwin, N., Walker, M.,White, M., Alberti, K.G.M.M. (2001). Maternal transmission of Type 2 diabetes varies by ethnic group. *Diabetes Care* **24** , 1685-1686.

Folwaczny, M., Glas, J., Torok, H.P., Mende, M. & Folwaczny, C. (2004). Lack of association between the TNFα G – 308 A promoter polymorphism and periodontal disease *J Clin Periodontol* **31**, 449-453.

Fox, C.H., Jette, A.M., McGuire, S.M., Feldman, H.A., Douglass, C.W. (1994). Periodontal disease among New England elders. *J Periodontol* **65**, 676-684.

Francis, S., Camp,N., Dewberry, R., et al.(1999). Interleukin-1 gene polymorphism and coronary artery disease *Circulation* **99**, 861-866.

Fried, S.K., Zechner, R. (1989). Cachectin/tumor necrosis factor decreases human adipose tissue lipoprotein lipase mRNA levels synthesis and activity. *J Lipid Res* **30**, 1917-1923.

Froguel P, Velho G (1999). Molecular Genetics of Maturity-onset of the young. *Trends Endocrinol Metab*. **10(4)**, 142-146.

Furutani, Y. (1994). Molecular studies on interleukin -1α. *Eur Cytokine Netw* **5**, 533-538.

Furutani, Y., Notake, M., Fukui, T., Ohue, M., Nomura, H., Yamada, M. & Nakamura, S. (1986). Complete nucleotide sequence of the gene for human interleukin 1 alpha. *Nucleic Acids Res*. **14 (8)**, 3167-3179.

Gabay, C., Kushner, I. (1999). Acute-phase proteins and other systemic responses to inflammation *N Engl J Med* **340**, 448-454.

Galbraith, G.M.P., Hendley, T.M., Sanders, J.J., Palesch, Y., Pandey, J.P. (1999). Polymorphic cytokine genotypes as markers of disease severity in adult periodontitis. *J Clin Periodontol* **26**, 705.

Galbraith, G.M, Steed, R.B., Sanders, J.J. Pandey, J.P (1998). Tumor necrosis factor alpha production by oral leukocytes: influence of tumor necrosis factor genotype. *J Periodontol* **69(4)**, 428-33.

Girard, J. (1997). Is leptin the link between obesity and insulin resistance? *Diabetes Metab* **23(3)**, 16-24.

Gemmell, E., Marshall, R.I.& Seymour G.J. (2000). Cytokines and prostaglandins in immune homeostasis and tissue destruction in periodontal disease. *Periodontol 2000* **14**, 112-143.

Genco, R.J., Grossi, S.G., Nishimura, F., Murayama, Y. (2005). A proposed model linking inflammation to obesity, diabetes and periodontal infections *J periodontol* **76(11)**, 2075-84.

Giardino, I., Edelstein, D., Brownlee, M. (1994). Non enzymatic glycation in vitro and in bovine endothelial cells alters basic fibroblast groeth factor activity: a model for intracellular glycosylation in diabetes. *J Clin Invest* **94**, 110-117.

Gillett, I.R., Johnson, N.W., Curtis, M.A., Griffiths, G.S., Sterne, J.A., Carman, R.J., Bampton, J.L., Wilton, J.M. (1990). The role of histopathology in the diagnosis and prognosis of periodontal diseases *J Clin Periodontol* **17(10)**, 673-84.

Glavind, L. & Loe, H. (1967). Errors in clinical assessment of periodontal destruction. *J Periodontol Res* **2**, 180-84.

Gonzalez-Ortiz, M., Martinez-Abundis, E., Cardona-Munoz, E.G., Lifshitz, A. & Quinones Galvan, A. (1997). Metabolic profile and insulin sensitivity in healthy young Mexicans with a strong family history of non insulin dependent diabetes mellitus in the paternal Branch. *Archives of Medical Research* **28(3)**, 421-424.

Goodson, J.M., Haffajee, A.D., Socransky, S.S. (1984). The relationship between attachment loss and alveolar bone loss. *J Clin Periodontol* **11**, 348-59.

Gorden, P. (1997). Non Insulin Dependant Diabetes–The Past, Present and Future. *Annals Academy of Medicine* **26 (3)**, 326-330.

Gore, E.A., Sanders, J.J., Pandey, J.P., Palesh, Y. & Galbraith, G.M.(1998). Interleukin 1β+3953 allele 2: Association with disease status in adult Periodontitis. *J Clin Periodontol* **25**, 781-85.

Goteiner, D., Vogel, R., Deasy, M. & Goteiner, C. (1986). Periodontal and caries experience in children with insulin dependant diabetes mellitus. *J Am Dent Assoc* **113**, 277-279.

Gouni, I., Oka, K., Etienne, J., Chan, L. (1993). Endotoxin-induced hypertriglyceridemia is mediated by suppression of lipoprotein lipase at a post transcriptional level *J Lipid Res* **34**, 139-146.

di Giovine, F., Camp, N.J., Cox, A., Chaudhary, A.G., Sorrell, J., Crane, A. & Duff, G.W. (2000). "Detection and population analysis of IL-1 and TNF polymorphisms" In: Balkwill, F. ed. *Cytokine Molecular biology*. Oxford: Oxford University press. 21- 46.

Granner, D.K & O'Brien, R.M. (1992). Molecular physiology and genetics of NIDDM. *Diabetes Care* **15 (3)**, 369-395.

Grant, S.F., Thorleifsson, G., Reynisdottir, I., Benediktsson, R., Manolescu, A., Sainz, J., Helgaon, A., Stefansson, H., Emilsson, V., Helgadottir, A (2006). Variant of transcriptional factor 7 like 2 (TCF7L2) gene confers the risk of type 2 diabetes. *Nat Genet* **38**, 320-232.

Greenstein, G., Hart, T.C. (2002). A critical assessment of interleukin-1 genotyping when used in a genetic susceptibility test for severe chronic periodontitis *J Periodontol* **73**, 231-247.

Grossi, S.G. & Genco, R.J. (1998). Periodontal disease and Diabetes Mellitus: A two way relationship. *Annals of Periodontology* **3(1)**, 51-61.

Grossi, S.G., Skrepcinski, F.B., DeCaro, T., et al. (1997). Treatment of periodontal disease in diabetes reduces glycated haemoglobin. *J Periodontol* **68**, 713-719.

Grove, J., Daly, A.K., Bassendine, M.F., Day, C.P. (1997). Association of a tumor necrosis factor promoter polymorphism with susceptibility to alcoholic stearohepatitis. *Hepatology* **26**, 143-6.

Grrop, L.C., Forsblum, C., Lehitovirta, M., Tumoi, T., Karanko, S., Nissen, M., Ehrnstrom, O., Forsen, B., Isomaa, B., Snickars, B., & Taskinen, M.R. (1996). Metabolic consequences of a family history of NIDDM (the BOTNIA study). *Diabetes* **45**, 1585-1593.

Gubler, U., Chua, A.O., & Lugg, D.K. (1989). In: Bomford, R., Henderson, B. (eds). *Interleukin-1, inflammation and disease*. Oxford: Elsevier. 31-45.

Guerrero-Romero, F., Rodriguez-Moran, M. & Sandoval-Herrera, F. (1997). Low prevalence of non insulin dependant diabetes mellitus in indigenous communities of Durango Mexico. *Archives of Medical Research* **28(1)**, 137-140.

Guzman, S., Karima, M., Wang, H. & Van Dyke, T.E. (2003). Association between interleukin-1 genotype and periodontal disease in a diabetic population. *J Periodontol* **74(8)**, 1183-90.

Haffajee, A.D. & Socransky, S.S. (1994). Microbial etiological agents of destructive periodontal disease. *Periodontol* **5,** 78-111.

Hagura, R., Matsuda, A., Kuzuya, T. (1994). Family history of diabetic patients in Japan. *Diabetes Res Clin Pract* **24,** 69–73.

Hales, C.N., Barker, D.J.P., Clark, P.M.S., Cox, L.J., Fall, C., Osmond, C. & Winter, P.D. (1991). Fetal and infant growth and impaired glucose tolerance at age 64. *British Medical Journal* **303**, 1019-1022.

Hao, J.M., Meng, H.X., Ji, L.N. (2007). The investigation of the periodontal status of type 2 diabetes mellitus families *Zhonghua Kou Qiang Yi Xue Za Zhi* **42(7),**408-11. (Abstract)

Hariri, S., Yoon, P.W., Qureshi, N., Valdez, R., Scheuner, M.T., Khoury, M.J. (2006). Family history of type 2 diabetes: a population based screening tool for prevention *Genet Med* **8(2),** 102-108

Harrison, T.A., Hindorff, L.A., Kim, H., Wines, R.C., Bowen, D.J., McGrath, B.B., Edwards, K.L., (2003). Family history as a potential public health tool. *Am J Prev Med* **24**, 152-159.

Hart, T.C. & Kornman, K.S. (1997). Genetic factors in the pathogenesis of periodontitis. *Periodontology* **14**, 202-215.

Harrison, R. & Bowen, W.H. (1987). Periodontal health, dental caries and metabolic control in insulin dependant diabetic children and adolescents. *Pediatr Dent* **9**, 283-286.

Havemose-Poulsen, A., Sorensen, L.K., Bendtzen, K., Holmstrup, P. (2007). Polymorphism within the IL-1 gene cluster: effects on cytokine profiles in peripheral blood and whole blood cell cultures of patients with aggressive periodontitis, juvenile idiopathic arthritis and rheumatoid aethritis *J Periodontol* **78(3)**, 475-92.

Hawkes, C.H. (1997). Twin Studies in Diabetes Mellitus. *Diabetic Medicine* **14**, 347-352.

Health Survey for England 2006 (2008). Cardiovascular disease and risk factors in adults National Statistics, Copyright © 2008 The Information Centre, 63-72.

Heesom, M.A., Hibberd, M.L., Millward, A. & Demaine, A.G. (1997). Polymorphism in the 5' end of the aldolase reductase gene is strongly associated with the development of diabetic nephropathy in type 1 diabetes. *Diabetes* **46**, 287-291.

Hegele R.A., Cao H, Harris S.B., Hanley A.J., Zinman B. (1999). The Hepatic Nuclear Factor-1a G319S Variant Is Associated with Early-Onset Type 2 Diabetes in Canadian Oji-Cree. *Diabetes Care* **22**, 524.

Heijmans, B.T., Westendorp R.G.J., Droog, S., Kluft, C., Knook D.L., and Slagboom, P.E.(2002). Association of the tumor necrosis factor α -308 G/A polymorphism with the risk of diabetes in an elderly population based cohort *Genes and Immunity* **3**, 225-228.

Heresbach, D., Ababou, A., Bourienne, A., Alizadeh, M., Quelvennec, E., Pagenault, M., Dabadie, A., Berre, N.H., Campion, J.P., Launois, B., Gosselin, M., Genetet, B., Bretagne,J.F., Semana, G. (1997). Significance of interleukin -1beta and interleukin-1 receptor antagonist genetic polymorphism in inflammatory bowel diseases *Am J Gastroenterol* **92**, 1164-1169.

Herrmann, S.M., Ricard, S., Nicaud, V., Mallet, C., Arveiler, D., Evans, A., Ruidavets, J.B., Luc, G., Bara, L., Parra, H.J., Poirier, O., Cambien, F. (1998). Polymorphisms of the tumour necrosis factor-alpha gene, coronary heart disease and obesity. *Eur J Clin Invest* **28(1)**, 59-66.

Hodge, P. & Michalowicz, B. (2001). Genetic predisposition to periodontitis in children and young adults. *Periodontol 2000* **26**, 113-134.

Hoffstedt, J. Eriksson, P., Hellstrom, L. Rossner, S., Ryden, M., Arner, P. (2000). Excessive fat accumulation is associated with the TNF alpha-308 G/A polymorphism in women but not men. *Diabetologia* **43**, 117-20.

Hotamisligil, G.S., Murray, D.L., Choy, L.N., Spiegelman, B.M. (1994). Tumor necrosis factor alpha inhibits signaling from the insulin receptor. *Proc Natl Acad Sci USA* **91**, 4854-4858.

Hotamisligil, G.S., Arner, P., Caro, J.F., Atkinson R.L., Spiegelman, B.M.(1995) Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* **95**, 2409-2415.

Howard, B.V. (1999). Insulin resistance and lipid metabolism. *Am J Cardiol* **84**(1A), 28J-32J.

Hujoel, P.P., del Aguila, M.A., DeRouen, T.A., Bergstrom, J. (2003). A hidden periodontitis epidemic during the 20th century? *Community Dent Oral Epidemiol* **31(1)**, 1-6.

Huxley, R., Barzi, F., Woodward, M. (2006). Excess risk of fatal coronary heart disease associated with diabetes in men and women. Meta-analysis of 37 prospective cohort studies. *BMJ* **332**, 73-78.

Huxtable SJ, Saker PJ, Haddad, L., Walker M, Frayling TM, Levy JC, Hitman GA, O'Rahilly S, Hattersley AT, McCarthy MI (2000). Analysis of parent-offspring trios provides evidence for linkage and association between the insulin gene and type 2 diabetes

mediated exclusively through paternally transmitted class III variable number tandem repeat alleles. *Diabetes* **49**, 126.

Illangasekera, U., Nugegoda, D.B. & Perera, L.S. (1994). Prevalence of Diabetes and impaired glucose tolerance in a rural Sri Lankan Community. *Postgrad Med J* **70**, 347-9.

Illangasekera U. (2004). Temporal trends in the prevalence of diabetes mellitus in a rural community in Sri Lanka *The Journal of the Royal Society for the Promotion of Health* **124 (2)** 92-94.

Iwamoto, Y., Nishimura, F., Nakagawa, M., Sugimoto, H., Shikata, K., Makino, H., Fukuda, T., Tsuji, T., Iwamoto, M., Murayama, Y (2001). The effect of antimicrobial periodontal treatment on circulating tumor necrosis factor alpha and glycated hemoglobin level in patients with type 2 diabetes *J Periodontol* **72**, 774-778.

Iwamoto, Y., Nishimura, F., Soga, Y., Takeuchi, K., Kurihara, M., Takashiba, S., Murayama, Y (2003). Antimicrobial periodontal treatment decreases serum C-reactive protein tumor necrosis factors alpha, but not adiponectin levels in patients with chronic periodontitis, *J Periodontol* **74**, 1231-1236.

Jablonka, E. & Lamb, M.L. (2005). Evolution in four dimensions: Genetic, Epigenetic, Behavioral and Symbolic variation in the history of life. Cambridge, Massachusetts: MIT Press. 462p.

Jacob, C.o., Fronek, Z., Lewis, G.D., Koo, M., Hansen, J.A. & McDevitt, H.O. (1990). Heritable major histocompatibility complex class II associated differences in production of tumor necrosis factor α : relevance to genetic predisposition to systemic lupus erythromatosus. *Proc Natl Acad Sci USA* **87**, 1233-1237.

Janket, S.J., Wightman, A., Baird, A.E., Van Dyke, T.E., Jones, J.A. (2005). Does periodontal treatment improve glycaemic control in diabetic patients? A meta analysis of invention studies *J Dent Res* **84**, 1154-1159

Jansson, H., Lindholm, E., Lindh, C., Groop, L., Bratthall, G. (2006). Type 2 diabetes and risk for periodontal disease: a role for dental health awareness. *J Clin Periodontol* **33**, 408–414.

Jarrett, R.J. (1991). "The epidemiology of diabetes Mellitus" In: Pickup, J.C. & Williams, G. (eds). *Text book of Diabetes Mellitus Vol.1*. Oxford: Blackwell Scientific Publications. 49-53.

Jarrous, N. & Kaempfer, R. (1994). Induction of human interleukin -1 gene expression by retinoic acid and its regulation at processing of precursor transcripts. *J Biol Chem* **269**, 23141-23149.

Johnson, J.D., Houchens, D.P., Kluwe, W.M., Craig, D.K., Fisher, G.L. (1990). Effects of mainstream and environment tobacco smoke on the immune system in animals and humans: A review. *Crit Rev Toxicol* **20**,369-395.

Kaati, G., Bygren, L.O. & Edvinsson, S. (2002). Cardiovascular and diabetes mortality determined by nutrition during parents' and grandparents' slow growth period. *European Journal of Human Genetics* **10**, 682-688.

Kaprio, J., Tuomilehto, J., Koskenvuo, M., Romanov, K., Reunanen, A., Eriksson, J., Stengard, J. & Kesaniemi, Y.A. (1992). Concordance for type 1 (insulin dependant) and type 2 (non insulin dependant) diabetes mellitus in a population based cohort of twins in Finland. *Diabetologia* **35,** 1060-1067.

Katz, J., Flugelman, M.Y., Goldberg, A., Heft, M. (2002). Association between periodontal pockets and elevated cholesterol and low density lipoprotein cholesterol levels *J Periodontol* **73**, 494-500.

Katulanda,P., Constantine,G.R., Mahesh,J.G., Sheriff, R., Seneviratne, R.D.A.,Wijeratne, S., Wijesuriya, M., McCarthy, M.I., Adler, A.I., Matthews D.R. (2008). Prevalence and projections of diabetes and pre-diabetes in adults in Sri Lanka--Sri Lanka Diabetes, Cardiovascular Study (SLDCS). *Diabetic Medicine.* **25(9)**, 1062-9.

Katulanda, P., Groves, C.J., Barrett, A., Sheriff, R., Matthews, D.R., MaCarthy, M.I. & Gloyn, A.L. (2007). Prevalence and clinical characteristics of maternally inherited diabetes and deafness caused by the mt3243A>A mutation in young adult diabetic subjects in Sri Lanka. *Diabet Med* **25**, 315-374.

Kawaguchi, Y., Tochimoto, A., Hara, M., Kawamoto, M., Sugiura T., Saito, S., Kamatani, N. (2007). Contribution of single nucleotide polymorphism of the *IL1A* gene to the cleavage of precursor IL-1α and its transcription activity. *Immunogenetics* **59**, 441-448.

Kelly, M., Steele, J., Nuttall, N., Bradnock, G., Morris, J., Nunn, J., Pine, C., Pitts, N., Treasure, E. & White, D. (2000). "The condition of supporting structures" In: Walker, A.& Cooper, I. (eds.) *Adult dental health survey. Oral health in the United Kingdom* London: The Stationery Office. 123-146.

Kjellman, O. (1970). The presence of glucose in gingival exudate and resting saliva of subjects with insulin treated diabetes mellitus. *Swed Dent J* **63**, 11-19.

Khan, S.E., Hull, R.L., Utzschneider K.M. (2006). Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* **444,** 840-847.

Khan, S.R., Ayub, N., Nawab, S., Shamsi, T.S. (2008). Triglyceride profile in dyslipidaemia of type 2 diabetes mellitus. *J Coll Physicians Surg Pak* **18(5)**, 270-3.

Khader, Y.S., Albashaireh, Z.S., Hammad, M.M. (2008). Periodontal status of type 2 diabetics compared with nondiabetics in north Jordan. *East Mediterr Health J* **14(3)**, 654-61. (Abstract)

Khoury, M.J., Beary, T.H. & Cohen, B.H. (1993). "Fundamentals of genetic epidemiology" In: Kelsey, J., Marnot, M., Stolley, P. & Vessey, M. (eds). *Monographs in epidemiology and biostatistics Vol 22*. New York: Oxford University Press. 373-383.

Khovidhunkit, W., Memon, R.A., Feingold, K.R., Grunfeld, C. (2000). Infection and inflammation – induced proatherogenic changes in lipoproteins *J infect Dis* **181**, S462-S472.

Klover, P.J., Zimmers, T.A., Koniaris, L.G., Mooney, R.A. (2003). Chronic exposure to IL-6 causes hepatic insulin resistance in mice. *Diabetes* **52**, 2784-2789.

Krentz, A.J. (2003). Lipoprotein abnormalities and their consequences for patients with type 2 diabetes. *Diabetes Obes Metab* **5(SI)**, 19-27.

Krikovsky, D., Vasarhelyi, B., Treszl, A., et al. (2002). Genetic polymorphism of Interleukin -1β is associated with risk of type 1 diabetes mellitus in children. *Eur J Peadiatr* **161,** 507-508.

Kim, D.J., Cho, N.H., Noh, J.H., Lee, M.K., Kim, K.W. (2004). Lack of excess maternal transmission of type 2 diabetes in a Korean population. *Diabetes Res Clin Pract* **65**, 117-24.

Kim, D.K., Escalante, D.A., Garber, A.J. (1993). Prevention of atherosclerosis in diabetes: Emphasis on treatment for the abnormal lipoprotein metabolism of diabetes. *Clin Ther* **15,** 766-778.

Kinane, D.F. & Chestnutt, I.G. (2000). Smoking and periodontal disease. *Crit Rev Oral Biol Med* **11**, 356-365.

Kinane, D.F., Hodge, P., Ellis, R. & Gallagher, G. (1999). Analysis of genetic polymorphisms at the interleukin -10 and tumor necrosis factor loci in early onset periodontitis. *J Periodontol Res* **34**, 379-386.

King, H. & Rewers, M.(1993). Global estimates for prevalence of diabetes mellitus and impaired glucose tolerance in adults. *Diabetes care* **16**, 157-77.

Kiran, M., Arpak, N., Unsal, E., Erdogan, M.F. (2005). The effect of improved periodontal health on metabolic control in type 2 diabetes mellitus. *J Clin Periodontol* **32,** 266–72.

Kjellman, O. (1970). The presence of glucose in gingival exudates and resting saliva of subjects with insulin treated diabetes mellitus. *Swed Dent J* **63**, 11-19.

Kjellman, O., Henriksson, C.O., Bergnagen, N. & Anderson, B. (1970). Oral conditions in 105 subjects with insulin treated diabetes mellitus. *Swed Dent J* **63**, 99-110.

Knowler, W.C., Bennett, P.H., Hamman, R.F. & Miller, M. (1978). Diabetes incidence and prevalence in Pima Indians: a 19 fold greater incidence than in Rochester, Minnesota. *Am J Epidemiol* **108**, 497-505.

Koch, M., Rett, K & Volk, A. (2000). The tumour necrosis factor alpha -238G - A and -308G - A promoter polymorphism do not associate with insulin sensitivity and insulin secretion in young healthy relatives of type 2 diabetic patients. *Diabetologia* **43**, 181-184.

Kominato, Y., Galson, D., Waterman, W.R., Webb, A.C. & Auron, P.E. (1995). Monocyte expression of the human prointerleukin 1β gene (IL1β) is dependent on promoter sequences which bind the hematopoietic transcription factor Spi-1/PU.1. *Mol Cell Biol* **15**, 59-68.

Kornman, K.S., Page, R.C. & Tonetti, M.S. (2000). The host response to the microbial challenge in periodontitis: assembling the players. *Periodontol* **14**, 33-53.

Kornman, K.S., Crane, A., Wang, H.Y., di Giovine, F.S., Newman, M.G., Pirk, F.W., Wilson, T.G., Higinbottom, F.L. & Duff, G.W. (1997). The interleukin-1 genotype as a severity factor in adult periodontal disease. *J Clin Periodontol* **24**, 72-77.

Kornman, K.S.and Di Giovine, F.S. (1998). Genetic variations in cytokine expression: a risk factor for severity for of adult periodontitis. *Ann Periodontol* **3,** 327-338.

Koya, D. & King, G.L. (1998). Protein Kinase C activation and he development of diabetic complications. *Diabetes* **47**, 859-866.

Kroeger, K.M., Steer, J.H., Joyce, D.A., Abraham, I.J. (2000). Effects of stimulus and cell type on the expression of the –308 tumor necrosis factor promoter polymorphism. *Cytokine* **12**, 109- 110.

Lagathu, C., Bastard, J.P., Auclair, M., Maachi, M., Capeau, J., Caron, M. (2003). Chronic interleuin-6 (IL-6) treatment increased IL-6 secretion and induced insulin resistance in adipocyte: prevention by rosiglitazone. *Biochem Biophys Res Comun* **311**, 372-379.

Laine, M.I., Farre, M.A., Gonzalez, G., van Dijk, L.J., Ham, A.J., Winkel E.G., Crusius, J.B., Vandenbroucke, J.P., van Winkelhoff, A.J., Pena, A.S. (2001). Polymorphisms of the interleukin-1 gene family oral microbiological pathogens and smoking in adult periodontitis *J Dent Res* **80**, 1695-1699.

Lamster I.B & Lalla E (2001). Periodontal disease and Diabetes mellitus: Discussion Conclusions and Recommendations *Ann Periodontol* **6(1)**, 146-149.

Landi, L. Amar, S., Polins, A.S. & Van Dyke, T.E. (1997). Host mechanisms in the pathogenesis of periodontal disease. *Curr opin Periodontol* **4**, 3 -10.

Laine, M.L., Farre, M.A., Gonzalez, G., van Dijik, L.J., Ham, A.J, Winkel, E.G., Crusius, J.B., Vandenbroucke, J.P., van Winkelhoff, A.J. & Pena, A.S. (2001) Polymorphism of the interlukin-1 gene family, oral microbial pathogens, and smoking in adult periodontitis. *J Dent Res* **80**, 1695-1699.

Lang, N.P., Adler, R., Joss, A., Nyman, S. (1990). Absence of bleeding on probing. An indicator of periodontal stability. *J Clin Periodontol.* **17(10)**, 714-21.

Lang, N.P., Tonetti, M.S., Suter, J., Sorell, J., Duff, G.W., Kornman, K.S. (2000). Effect of interleukin-1 gene polymorphisms on gingival inflammation assessed by bleeding on probing in a periodontal maintenance population. *J Periodontal Res* **35(2)**, 102-7.

Lanza-Jacoby, S., Wong, S.H., Tabares, A., Baer, D., Schneider, D. (1992). Disturbance in the composition of plasma lipoproteins during gram-negative sepsis in the rat. *Biochim Biophys Acta* **1124**, 233-240.

Lalla, E., Lamster, I.B., Stern, D.M. & Schmidt, A.M. (2001). Receptor for advanced glycation end products, Inflammation and accelerated periodontal disease in Diabetes: Mechanisms and insights into therapeutic modalities. *Ann Periodontol* **6(1)**, 113-118.

Lee, A.Y.& Chung S.S.(1999). Contributions of polyol pathay in oxidative stress in diabeteic cataract *FASEB J* **13**, 23-30.

Lee, S., Temple, S., Roberts, S., Price, P. (2008). Complex effects of IL1A polymorphism and calpain inhibitors on interleukin 1α (IL-1α) mRNA levels and secretion of IL-1α protein. *Tissue Antigens* **72**, 67-71

Lee, S.C., Pu, Y.B., Thomas, G.N., Lee, Z.S., Tomilinson, B., Cockram, C.S., Critchley, J.A., Chan, J.C. (2000). Tumor necrosis factor alpha gene G-308A polymorphism in the metabolic syndrome. *Metabolism* **49(8)**, 1021-4.

Lehner, T. (2003). The mouth and salivary glands. In Warrell, D.A., Cox, T.M., Firth, J.D. and Benz, E.J.Jr (Eds) Oxford textbook of medicine. 4th edn. Oxford: Oxford University Press. Section 14.5

Lernmark, A. (1996). Glutamic acid decoarboxylase- gene to antigen to disease. *Journal of Internal Medicine* **240**, 259-277.

Lien, Y.H., Stern, R., Fu, J.C.C. & Siegel, R.C. (1984). Inhibition of Collagen fibril formation in vitro and subsequent cross linking by glucose. *Science* **225**, 1489-1491.

Lin, L., Pan, Y.P., Yin, L.Y. (2003). Study on the correlation of cytokine gene polymorphism with chronic periodontitis. *Shanghai Kou Qiang Yi Xue* **12**,456. (Abstract)

Little, J.W., Falace, D.A., Miller, C.S. & Rhodus, N.L. (1997). Dental management of the medically compromised patient. 5th ed. St Louis: Mosby, 387-409.

Loe, H., Anerud, A., Boysen, H., Smith, M (1978). The natural history of periodontal diseases in man. The rate of periodontal destruction before 40 years of age. *J Periodontol* **49(12)**, 607-20.

Loe, H., Anerud, A., Boysen, H.,Morrison, E. (1986). Natural history of periodontal disease in man. Rapid, moderate and no loss of attachment in Sri Lankan laborers 14 to 46 years of age. *J Clin Periodontol* 13(5), 431-45.

Loe, H (1993). Periodontal disease. The sixth complication of diabetes mellitus *Diabetes Care* **16(1)**, 329-34.

Loos, B.G., John, R.P. & Laine, M.L. (2005). Identification of genetic risk factors for periodontitis and possible mechanism of action. *J Clin Periodontol* **32(6)**,159-179.

Loos, B.G., Craandijk, J., Hoek, F.J., Wertheim-van Dillen, P.M., van der Velden U (2000). Elevation of systemic markers related to cardiovascular diseases in the peripheral blood of periodontitis patients *J Periodontol* **71**, 1528-1534.

Lopes-Virella, M.F. (1993). Interactions between bacterial lipopolysaccharides and srum lipoproteins and their possible role in coronary heart disease. *Eur Heart J* **14**(Suppl. K), 118-124.

Loesche, W., Karapetow, F., Pohl, C., Kocher, T. (2000). Plasma lipid and blood glucose levels in patients with destructive periodontal disease. *J Clin Periodontol* **27(8),** 537-41.

Lowell, B.B., Shulman, G.I. (2005). Mitochondrial dysfunction and type 2 diabetes. *Science* **307**, 384-7.

Lu, H.K., Yang, P.C. (2004). Cross-sectional analysis of different variables of patients with non-insulin dependent diabetes and their periodontal status. *Int J Periodontics Restorative Dent* **24**, 71–79.

Madden, T.E., Heriges, B., Boyd, L.D., Laughlin, G., Chiodo, G., Rosenstein, D. (2008). Alterations in HbA1c following minimal or enhanced non-surgical, non-antibiotic treatment of gingivitis or mild periodontitis in type 2 diabetic patients: a pilot trial *J Contemp Dent Pract* **9(5)**, 9-16.

Malo, D. & Skanmene, E. (1994). Genetic control of host resistance to infection. *Trends Genet* **10,** 365-371.

Mangan, D., Selwitz, R.H. & Genco, R. (2000). Infection associated with diabetes mellitus. *N Engl J Med* **342**, 896.

Manouchehr-Pour, M., Spagnuolo, P.J., Rodman, H.M. & Bisanda, N.F. (1981). Comparison of neutrophil chemotactic responses in diabetic patients with mild and severe periodontal disease. *J Periodontol* **52**, 410-14.

Manouchehr-Pour, M., Spangnuolo, P.J. & Bissada, N.F. (1981). Impaired neutrophil chemotaxis in diabetic patients with severe periodontitis. *J Dent Res* **60**, 729-730.

Mansour,A.A., Abd-Al-sada, N. (2005). Periodontal Disease among Diabetics in Iraq *Medscape General Medicine* 7**(3),** 2

Mattout C, Bourgeois D, Bouchard P (2006). Type 2 diabetes and periodontal indicators: epidemiology in France 2002- 2003. *J Periodontal Res* **41**, 253–258.

Marugame, T, Hayasaki, H., Lee, K., Eguchi, H., Matsumoto, S. (2003). Alveolar bone loss associated with glucose tolerance in Japanese men. *Diabetic Medicine* **20**, 746-751.

Martin, A.O., Simpson, J.L., Ober, C. & Freinkel, N. (1985). Frequency of diabetes mellitus in mothers of probands with gestational diabetes: possible maternal influence on the predisposition to gestational diabetes. *American Journal of Obstetrics and Gynecology* **151(4),** 471-475.

McDevitt, M.J., Wang, H.Y., Knobelman, C., Newman, M.G., di Govinine, F.S., Timms, J., Duff, G.W. & Kornman, K.S. (2000). Interleukin-1 genetic association with periodontitis in clinical practice. *J Periodontol* **71**, 156-63.

McGuire, M.K. & Nunn, M.E. (1999). Prognosis versus actual outcome IV. The effectiveness of clinical parameters and IL-1 genotype in accurately predicting prognoses and tooth survival. *J Periodontol* **70**, 49-56.

McLellan, A.C., Thornalley, P.J., Benn, J., Sonksen, P.H. (1994). Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. *Clin Sci (Lond)* **87**, 21-29.

McMullen, J.A., van Dyke, T.E., Horoszewicz, H.V. & Genco, R. (1981). Neutrophil chemotaxis in individuals with advanced periodontal disease and a genetic predisposition to diabetes mellitus. *J Periodontol* **52**, 167-73.

Mealey, B. L., Rose, L.F. (2008). Diabetes mellitus and inflammatory periodontal diseases; Diabetes and the endocrine pancreas. *Current Opinion in Endocrinology, Diabetes & Obesity* **15**(2), 135-141.

Mealy, B.L., Oates, T.W., American Academy of Periodontology (2006). Diabetes mellitus and periodontal diseases. *J Periodontol* **77(8)**, 1289-303.

Mealey, B.L., Moritz, A.J. (2003). Hormonal influences: effects of diabetes mellitus and endogenous female sex steroid hormones in the periodontium. *Periodontol 2000* **32**, 59-81.

Mealey, B.L. (1998). Impact of advances in diabetes care on dental treatment of the diabetic patient. Compendium. *Contin Educ Dent* **19**, 41-58.

Meisel, P., Siegemund, A., Dombrowa, S., Sawaf, H., Fanghaenel, J. & Kocher, T. (2002). Smoking and polymorphism of the interleukin-1 gene cluster (IL-1α, IL-1β and IL-1RN) in patients with periodontal disease. *J Periodontol* **73**, 27-32.

Melander, A. (1996). Oral antidiabetic drugs: an overview. *Diabet Med* **13(S6)**, S143-S147.

Michalowicz, B.S., Aeppli, D., Virag, J.G., Klump, D.G., Hinrichs,J.E., Segal, N.L., Bouchard, T.J. & Pihlstrom, B.L. (1991). Periodontal findings in adult twins. *J Periodontol* **62**, 293-299.

Michalowicz, B.S., Diehl SR, Gunsolley JC, Sparks BS, Brooks CN, Koertge TE, Califano JV, Burmeister JA, Schenkein HA.(2000). Evidence of a substantial genetic basis for risk of adult periodontitis, *J Periodontol* **71,** 1699–1707.

Mingrone, G., Henriksn, F.L., Greco, A.V., Krogh, L.N., Capristo, E., Gastaldelli, A., Castagneto, M., Ferrannini, E., Gasbarrini, G., Beck-Nielsen, H. (1999). Triglyceride induced diabetes associated with familial lipoprotein lipase deficiency. *Diabetes* **48**, 1258-63.

Mitchell, B.D., Valdez, R., Hazuda, H.P., Haffner, S.M., Monterrosa, A. & Stern, M.P. (1993). Differences in the prevalence of diabetes and impaired Glucose Tolerance According to Maternal or Paternal History of Diabetes. *Diabetes Care* **16(9)**, 1262-1267.

Mitchell, B.D., Kammerer, C.M., Reinhart, L.J., Stern, M.P. & Maccluer, J.W. (1995). Is there an excess maternal transmission of NIDDM. *Diabetologia* **38**, 314-317.

Moldwer, L.L. (1994). Biology of Poinflammatory cytokines and their antagonists. *Crit Care Med* **22 (Sl)**, S3-S7.

Molvig, J., Beak, L., Christensen, P., Manogue, K.R., Vlassara, H., Platz, P.,Nielsen, L.S., Svejgaard, A. & Nerup, J.(1988). Endotoxin-stimulated human monocyte secretion of interleukin – 1, tumor necrosis factor-alpha and prostaglandin – E2 shows stable interindividual differences. *Scandinavian Journal of Immunology* **27**, 705-716.

Morohoshi, M., Fujisawa K., Uchimura, I., Numano, F. (1995). The effect of glucose and advanced glycosylation end products on IL-6 production by human monocytes. *Ann N Y Acad Sci* **748**, 562-570.

Morohoshi, M., Fujisawa K., Uchimura, I., Numano, F. (1996). Glucose-dependant interleukin 6 and tumour necrosis factor production by human peripheral blood monocytes in in vitro. *Diabetes* **45**, 954-959.

Mooradian AD. (1996). Drug therapy of non-insulin-dependent diabetes mellitus in the elderly. *Drugs* **51**: 931-41.

Moore T.R (2005). Diabetes Mellitus and Pregnancy. e*Medicine* Jan 27.

Mori, N. & Prager, D. (1996). Transactivation of the interleukin -1α promoter by human T cell leukemia virus type I and type II Tax proteins. *Blood* **87**, 3410-3417.

Morton, A.A., Williams, R.W., Watts, T.L.(1995). Initial study of periodontal status in non-insulin dependant diabetics in Mauritius. *J Dent* **23(6)**, 343-5.

Nair, S.P., Meghji, S., Wilson, M., Reddi, K., White, P. & Henderson, B. (1996). Bacterially induced bone destruction: mechanisms and misconceptions. *Infection and immunity* **64**, 2371-2380.

Nathan D.M. (1993). Long term complications of diabetes mellitus. *N Engl J Med* **328**, 1676-1685.

Clark, N.G., Fox, K.M., Grandy, S. (2007). Symptoms of diabetes and their association with the risk and presence of diabetes *Diabetes Care* **30(11),** 2868-2873.

Neale, M.C. & Cardon, L.R. (1990). "Methodology for Genetic Studies of Twins and Families" In: *NATO ASI Series D,* Behavioural *and Social Sciences Vol. 67.* Dordrecht, Netherlands: Kluwer Academic Publishers.

Neel, J.V. (1962). Diabetes Mellitus : a thrifty genotype rendered detrimental by progress. *Am J Hum Gent* **14**, 353-62.

Nelson, R.G., Shlossman, M., Budding, L.M., Pettitt, D.J., Saad, M.F., Genco, R.J. & Knowler, W.C. (1990). Periodontal disease and NIDDM in Pima Indians. *Diabetes Care* **13(8)**, 836-840.

Naugle, K., Darby, M.I., Bauman, D.B., Lineberger, L.T., Powers, R. (1998). The oral health status of individuals on renal dialysis. *Ann Periodontol* **3**, 197-205.

Nawman, B., Selby, J.V., King, M.C., Slemenda, C., Fabsitz, R., & Friedman, G.D. (1987). Concordance for type 2 diabetes mellitus in male twins. *Diabetologia* **30**, 763-768.

National Diabetes Statistics (2007). National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. U.S. Department of Health and Human Services

Nicklin, M.J., Weith, A. & Duff, G.W.(1994). A physical map of the region encompassing the human interleukin-1 alpha, interleukin-1 beta, and interleukin-1 receptor antagonist genes. *Genomics* **19**, 382-4.

Nikolopoulos, G.K., Dimou, N.I., Hamodrakas, S.J., Bagos, P.G. (2008). Cytokine gene polymorphisms in periodontal disease: a meta analysis of 53 studies including 4178 cases and 4590 controls. *J Clin Periodontol* **35**, 754-767.

Nishimura, F., Taniguchi, A., Yamaguchi-Morimoto, M., Soga, Y., Iwamoto, Y., Kokeguchi, S., Kuroe, A., Fukushima, M., Nakai, Y., Seino, Y. (2006). Periodontal infection and dyslipidemia in type 2 diabetics: association with increased HMG-CoA reductase expression. *Horm Metab Res* **38(8)**, 530-5.

Nishimura, F., Murayama, Y. (2001). Periodontal inflammation and insulin resistance – lessons from obesity *J Dent Res* **80**, 1690-1694.

Noack, B., Jachmann, I., Roscher, S., Sieber, L., Kopprasch, S., Luck, C. et al.(2000). Metabolic disease and their possible link to risk indicators of periodontitis. *J Periodontol* **71(6)**, 898-903.

Novaes, A.B. Jr, Gutierrez, F.G., Novaes, A.B. (1996). Periodontal disease progression in type II non-insulin dependant diabetes mellitus patients (NIDDM). Part-I probing pocket depth and clinical attachment. *Braz Dent J* **7(2),** 65-73. (Abstract)

Novak, M.J., Potter, R.M., Blodgett, J., Ebersole, J.L. (2008). Periodontal disease in Hispanic Americans with type 2 diabetes. *J Periodontol* **79(4)**, 629-636.

Offenbacher, S. (1996). Periodontal Diseases Pathogenesis. *Ann Periodontolo* **1**, 821-878.

Offenbacher, S., Heasman, P.A. & Collins, J.G. (1993) Modulation of host PGE_2 secretion as a determinant of periodontal disease expression. *Journal of Periodontology* **64**, 432-444.

Office for National Statistics (2000). Adult dental health survey: oral health in the United Kingdom 1998. www.statistics.gov.uk

Okada, H. & Murakami, S. (1998). Cytokine expression in periodontal health and disease. *Critical Reviews in Oral Biology and Medicine* **9**, 248-266.

Okafor, C.I., Fasanmade, O.A., Oke, D.A. (2008). Pattern of dyslipidaemia among Nigerians with type 2 diabetes mellitus. *Niger J Clin Pract* **11(1)**, 25-31.

Oliver, R.C. & Tervonen, T. (1993). Periodontitis and tooth loss: comparing diabetes with the general population. *J Am Dent Assoc* **124**, 71-76.

Oliver, R.C. & Tervonen, T. (1994). Diabetes – A risk factor for Periodontitis in adults?. J Periodontol **107**, 530-538.

Oliver, R.C., Brown, L.J., Loe, H. (1998). Periodontal disease in the United States population. *J Periodontol* **69**, 269-278.

Oral Health U.S. (2002). Third National Health and Nutrition Examination Survey (NHANES III) 1988-1994, National Centre for Health Statistics, Centres for Disease Control and Prevention. Section 3 periodontal diseases, 21-26.

Osei, K., Daryl, A.C., Christopher, A.A., Ernest, C.E., Abayomi, O.A. & Thomas, M.O. (1993). Serum Insulin and glucose concentrations in people at risk for type 2 diabetes, a comparative study of African Americans and Nigerians. *Diabetes Care* **16(10)**, 1367-1375.

Osei, K., Gaillard, T. & Schuster, D.P. (1999). Significance of the parental history of type 2 diabetes on Insulin Sensitivity and Glucose effectiveness in obese Nondiabetic offspring of African American Patients. A letter. *Diabetes Care* **22(9)**, 1594-1596.

Page, R.C. and Beck, J.D. (`1997). Risk assessment in periodontal disease. *International Dental Journal* **47**, 61-87.

Page, R. C. & Eke, P. I. (2007) Case definitions for use in population-based surveillance of periodontitis. *Journal of Periodontology* **78**, 1387–1399.

Papapanou, P.N., Neiderud, A.M., Sandros, J. & Dahlen, G. (2001). Interleukin-1 gene polymorphism and periodontal status. A case control study. *J Clin Periodontol* **28**, 389-396.

Parkhill, J.M., Hennig, B.J.W., Chapple, I.L.C., Heasman, P.A. & Taylor, J.J. (2000). Association of interleukin- 1 gene polymorphism with early onset Periodontitis. *J Clin Periodontol* **27**, 682-689.

Peck, T., Price, C., English, P., Gill, G. (2006). Oral health in rural South African type 2 diabetic patients *Trop Doct* **36,**111-112.

Pérez1, C., González1, F.E., Pavez, V., Araya, A.V., Aguirre, A., Cruzat, A., Contreras-Levicoy, J., Dotte, A., Aravena, O., Salazar, L., (2004). The –308 polymorphism in the promoter regionof the tumor necrosis factor-alpha (TNF-a) gene and *ex vivo*lipopolysaccharide-induced TNF-a expression in patients with aggressive periodontitis and/or type 1 diabetes mellitus. *Eur Cytokine Netw* **15 (4)**, 364-70.

Permutt, M.A. (1990). Genetics of NIDDM. *Diabetes Care* **13,** 11-14.

Philips D.I.W. (2004). Commentary: Twins, low birthweight and type 2 diabetes. *Int J Epidemiol* **33**, 1-2.

Physicians' Desk Reference (1998). Oradell, N.J. Medical Economic Company, 52nd ed. 2173-2175, 2182-2186, 2280-2282.

Pickup, J.C., & Crook, M.A. (1998). Is type 2 diabetes mellitus a disease of the innate system? *Diabetologia* 41(10), 1241-1248.

Pihistrom, B.L., Michalowicz, B.S., Johnson, N.W. (2005). Periodontal diseases *www.thelancet.com* **366,** 1809- 1820.

Position Paper (1996). Diabetes and Periodontal Disease. *J Periodontol* **67(2)**, 166 175.

Pozzilli P., Di Mario U (2001). Autoimmune diabetes not requiring insulin at diagnosis (latent autoimmune diabetes of the adult): definition, characterization, and potential prevention. *Diabetes Care* **24(8)**, 1460-7.

Position paper (1996). Epidemiology of periodontal disease. American Academy of Periodontology. *J Periodontol* **67**, 935-945.

Position paper (2000). Diabetes and periodontology. Academy reports *J Periodontol* 71(4),664-678.

Poulton J, Luan J, Macaulay V, Hennings S, Mitchell S.J, Wareham N.J. (2002). Type 2 diabetes is associated with a common mitochondrial variant: evidence from a population-based case–control study. *Hum Mol Genet* **11**, 1581.

Pociot, F., Molving, J., Wogensen, L., Worsaae, H., & Nerup, J. (1992). A Taq 1 polymorphism in the human interleukin-1β (IL-1β) gene correlates with IL-1β secretion in vitro. *Eur J Clin Invest* **22**, 396-402.

Pratley, R.E. (1998). Gene Environment interactions in the pathogenesis of type 2 diabetes Mellitus: lessons learned from the Pima Indians. Plenary Lecture: *Proceedings of the Nutritional Society* **57,** 175-181.

Preshaw, P.M., Seymour, R.A., Heasman P.A. (2004). Current concepts in periodontal pathogenesis. *Dental update* **31,** 570-578.

Preshaw, P.M., Foster, N., Taylor, J.J. (2007). Cross susceptibility between periodontal disease and type 2 diabetes mellitus: an immunobiological perspective. *Periodontol 2000* **45,** 138-57.

Preshaw, P.M., Schifferle, R.E., Walters, J.D. (1999). Porphyromonas gingivalis lipopolysaccharide delays human polymorphonuclear leukocyte apoptosis in vitro. *J Periodontal Res* **34(4)**, 197-202.

Preus, H.R., Anerud, A., Boysen, H., Dunford, R.G., Zambon, J.J., Loe, H. (1995). The natural history of periodontal disease. The correlation of selected microbiological parameters with disease severity in Sri Lankan tea workers. *J Clin Periodontol* **22(9)**, 674-8.

Qaseem, A., Vijan, S., Snow, V., Cross, J.T., Weiss, K.B., Owens, D.K. (2007). Glycaemic control and type 2 diabetes mellitus: the optimal hemoglobin A1c targets. A guidance statement from the American College of Physicians. *Ann Intern Med* **147(6)**, 417-22.

Qureshi, S.T. Skamene, E. & Malo, D. (1999). Comparative genomics and host resistance against infectious diseases. *Emerg Infect Dis* **5,** 36-47.

Ramachandran, A., Mohan, V., Snehalatha, C. & Viswanathan, M. (1988). Prevalence of non-insulin dependent diabetes mellitus in Asian Indian families with a single diabetic parent. *Diabetes Research and Clinical Practice* **4**, 241-245.

Rasmussen, S.K., Urhammer, S.A, Jensen, J.N., Hansen, T., Borch-Johnsen, K & Pedersen, O. (2000). The -238 and -308 G to A polymorphism of the tumour necrosis factor alpha gene promoter are not associated with features of insulin resistance syndrome or altered birth weight in Danish Caucasians. *J. Clin Endocrinol Metab* **85**, 1731-1734.

Report of a WHO/IDF Consultation (2006). Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia: Printed by the WHO Document Production Services, Geneva, Switzerland.

Report of a World Health Organization consultation (1999). Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications. Department of Noncommunicable Disease Surveillance.

WHO technical report series 894 (2000). Obesity preventing and managing the global epidemic. Geneva

Rees, T.D. (2000). Periodontal management of the patients with diabetes mellitus. *Periodontology* **23**, 63-72.

Risch, N. & Merikangas, K. (1996). The future of genetic studies of complex human diseases. *Science* **273**, 1516-7.

Risch, N. (2000). Searching for genes in complex diseases: lessons from systemic lupus erythematosus *The Journal of Clinical Investigation* **105 (11)**, 1503-1506.

Rivellese, A.A., De Natale, C., Di Marino, L. (2004). Exogenous and endogenous postprandial lipid abnormalities in type 2 diabetic patients with optimal blood glucose control and optimal fasting triglyceride levels. *J Clin Endocrinol Metab* **89,** 2153-9.

Rufail, M.L., Schenkein, H.A., Koertge, T.E., Best, A.M., Barbour, S.E., Tew, J.G., van Antwerpen, R. (2007). Atherogenic lipoprotein parameters in patients with aggressive periodontitis .*J Periodont Res* **42**, 495–502.

Romero, R., Kuivaniemi, H., Tromp, G. & Olson, J.M. (2002). The design, execution, and interpretation of genetic association studies to decipher complex diseases. *Am J Obstet Gynecol* **187**, 1299-3.

Rotter, V., Nagaev, I., Smith, U. (2003). Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin resistance subjects. *J Biol Chem* **278**, 45777-45784.

Ryoko, H., Ayako, M., Takeshi, K., Hideyo, Y. & Kinori, K. (1994). Family history diabetic patients in Japan. Diabetes *Res.Cli.Prac* **24**, 569-573.

Sakellari, D., Koukoudetsos, S., Arsenakis, M., Konstantinidis, A (2003). Prevalence of IL-1A and IL-1B polymorphisms in a Greek population. *J Clin Periodontol* **30(1)**, 35-41.

Salvi, G.E., Yalda, B., Collins, J.G., Jones, B.H., Smith, F.W., Arnold, R.R. & Offenbacher, S.(1997a). Inflammatory mediator response as a risk marker for periodontal disease in insulin dependant diabetes mellitus. *Journal of Periodontology* **68**, 127-135.

Salvi, G.E., Collins, J.G., Yalda, B., Arnold, R.R., Lang, N.P. & Offenbacher, S.(1997b). Monocytic TNF α secretion patterns in IDDM patients with periodontal disease. *Journal of Periodontology* **24**, 8-16.

Salzberg, T.N., Overstreet, B.T., Rogers, J.D., Califano, J.V., Best, A.M., Schenkein, H.A. (2006). C – reactive protein levels in patients with aggressive periodontitis. *J Periodontol* **77(6)**, 933-9.

Saito, T., Shimazaki, Y., Koga, T., Tsuzuki, M., Ohshima, A. (2001). Relationship between upper body obesity and periodontitis. *J Dent Res* **80**, 1631-1636.

Samara, J.S., summers, L.K.M., Frayn, K.N. (1996). Sepsis and fat metabolism *Br J Surg* **83**, 1186-1196.

Sandberg G.E., Sundberg, H.E., Fjellatrom, C.A., Wikblad, K.F. (2000). Type 2 diabetes and oral health: a comparison between diabetic and non-diabetic subjects. *Diabetes Res Clin Pract* **50(1)**, 27-34.

Sastrowijoto, S.H., van der Velden, U., van Steenberen, T.J.M., Hillemans, P., Hart, A.A.M., de Graaff. J. & Abraham-Inpijin, I. (1990). Improved metabolic control, clinical periodontal status and subgingival microbiology in insulin dependant diabetes mellitus. A prospective study. *J. Clin Periodontol* **17**, 233- 242.

Saudek, C.D., Herman, W.H., Sacks, D.B., Bergenstal R.M. Edelman, D. & Davidson, M.B. (2008). A new look at screening and dignosing diabtes mellitus. *The Journal of Endocrinology & Metabolism* **93(7)**, 2447-2453.

Sauty, A., Mauel, J., Philippeaux, M.M. & Leuenberger, P. (1994). Cytostatic activity of alveolar macrophages from smokers and non smokers: role of interleukin-1 β, interleukin-6, and tumor necrosis factor-α. *American Journal of Respiratory Cell and Molecular Biology* **11**, 631-637.

Schenkein, H.A. (2002). Finding genetic risk factors for periodontal diseases: is the climb worth the view?. *Periodontol 2000* **30**, 79-90.

Schindler, R., Ghezzi, P.& Dinarello, C.A. (1990). IL-1 induces IL-1.IV.IFN-γ suppresses IL-1 but not lipopolysaccharides-induced transcription of IL-1. *J Immunol* **144**, 2216-2222.

Schmidt, A.M., Hori, O., Brett, J., Yan, S.D., Wautier, J.L. & Stern, D. (1994). Cellular receptors for advanced glycation end products: implications for induction of oxidant stress and cellular dysfunction in the pathogenesis of vascular lesions. *Arterioscler Thromb* **14**, 1521-1528.

Scott, W.B., John, M.S., Ellis, V.H., Ian, T., Meraida, A.P., Deborah, A.K.& Douglas, C.W. (1992). Maternally transmitted diabetes and deafness associated with a 10.4kb mitochondrial DNA deletion. *Nature Genetics* **1**, 11-15.

Scully, C. (2004). The oral cavity and lips. In. Burns, T., Breathnach, S., Cox, N. and Griffiths, C. (Eds). Rook's text book of dermatology: Volome 4. 7th edn. Oxford: Blackwell Science.

Seppala, B., Sorsa, T. & Ainamo, J. (1997). Morphometric analysis of cellular and vascular changes in gingival connective tissue in long term insulin dependant diabetes. *J Periodontol* **68**, 1237-1245.

Senn, J.J., Klover, P.J., Nowak, I.A., Mooney, R.A. (2002). Interleukin -6 induces cellular insulin in hepatocytes. *Diabetes* **51**, 3391-3399.

Seymour, G.J. and Taylor, J.J. (2004). Shouts and Whispers: An introduction to immunoregulation in periodontal disease. *Periodontal 2000* **35**, 9-13.

Seymour, G.J. & Gemmell, E. (2001). Cytokines in periodontal disease: where to from here? *Acta Odontol Scand* **59**, 167-173.

Shapira, L., Stabholz, A., Rieckmann, P. & Kruse, N. (2001). Genetic polymorphism of the Tumor Necrosis Factor (TNF)-alpha promoter region in families with localised early onset periodontitis. *Journal of Periodontal Research* **36**, 183-186.

Shapiro A.M. J.,Ricordi C., Hering B.J., Auchincloss H., Lindblad R., Robertson R.P., Secchi A., Brendel M.D., Berney T., Brennan D.C., Cagliero E., Alejandro R., Shiau, M.Y., Wu, C.Y., Huang, C.N., Hu, S.W., Lin, S.J. & Chang, Y.H. (2003). TNF-α polymorphisms and type 2 diabetes mellitus in Taiwanese patients. *Tissue Antigens* **61** 393-397.

Shiau, M.Y., Wu, C.Y., Huang, C.N., Hu, S.W., Lin, S.J., Chang, Y.H. (2003). TNF-alpha polymorphisms and type 2 diabetes mellitus in Taiwanese patients. *Tissue Antigens* **61(5)**, 393-7.

Shirakawa, F., Saito, K., Bonagura, C.A., Galson, D.L., Fenton, M.J., Webb, A.C. & Auron, P.E. (1993). The human prointerleukin 1β gene requires DNA sequences both proximal and distal to the transcription start site for tissue specific induction. *Mol Cell Biol* **13**, 1332-1344.

Shirodaria, S., Smith, J., McKay, I.J., Kennett, C.N. & Hughes, F.J. (2000). Polymorphisms in the IL-1A gene are correlated with levels of interleukin -1alpha protein in gingival crevicular fluid of teeth with severe periodontal disease. Journal of Dental Research **79**, 1864-1869.

Shlossman, M., Knowler, W.C., Pettitt, D.J.,Arevalo, A. & Genco, R.J. (1987). Type 2 diabetes and periodontal disease. (Abstract) *J Dent Res* **66**, 256.

Shlossman, M., Knowler, W.C., Pettitt, D.J. & Gneco, R.J. (1990). Type 2 diabetes mellitus and periodontal disease. *J. Am Dent Assoc* **121**, 532-536.

Shue, W.H., Lee, W.J., Lin, L.Y., Chang, R.L., Chen, Y.T. (2001). Tumor necrosis factor α-238 & 308 polymorphism do not associate with insulin resistance in hypertensive subjects. *Metabolism* **50**, 1447-51.

Sims, J.E., Nicklin, M.J., Bazan, J.F., Barton, J.L., Busfield, S.J., Ford, J.E., Kastelein, R.A., Kumar, S., Lin, H., Mulero, J.J., Pan, J., Pan, Y., Smith, D.E. & Young, P.R. (2001). A new nomenclature for IL-1-family genes. *Trends Immunol* **22**, 536-7.

Siribaddana, S.H., Deshabandu, R., Rajapakse, D., Silva, K., Fernando, D.J.S. (1998). The prevalence of gestational diabetes in a Sri Lankan ante-natal clinic. *Ceylon Med J* **43** 88–91.

Smirnova, M.G., Kiselev, S.L., Gnuchev, N.V. (2003). Role of the pro-inflammatory cytokines tumor necrosis factor-alpha, interleukin-1 beta, interleukin-6 and interleukin-8 in the pathogenesis of the otitis media with effusion. *Eur. Cytokine Netw* **13** (2), 161–72.

Smith, G.T., Greenbaum, C.J., Johnson, B.D., Person, G.R. (1996). Short term responses to periodontal therapy in insulin dependant diabetic patients. *J Periodontol* **67**, 794-802.

Soliman, D.M. & Twigg, H.L. (1992). Cigarette smoking decreases bioactive interleukin-6 secretion by alveolar macrophages. *American Journal of Physiology* **263**, 471-478.
Soskolne, W.A. & Klinger, A. (2001). The relationship between periodontal diseases and diabetes: an overview. *Annals of Periodontology* **6**, 91-8.

Southerland, J.H., Taylor, G.W., Moss, K., Beck, J.D., Offenbacher, S. (2006). Commonality in chronic inflammatory diseases: periodontitis, diabetes and coronary artery disease. *Periodontal 2000* **40**,130-143.

Sri Lanka Labor Force Survey (1999). Quarterly Report

Steinberg, A.G. (1959) The Genetics of Diabetes A review *Ann.N.Y.Acad Sci* **82**,197-207.

Stashenko, P., Jandinski , J.J., Fujiyoshi, P., Rynar, J & Socransky, S.S. (1991). Tissue levels of bone resorptive cytokines in periodontal disease. *Journal of Periodontology* **62**, 504-509.

Strachan, T. & Read, A.P. (1999). "Genetic mapping of mendelian characters" In: *Human molecular genetics*. 2nd edn, Oxford: BIOS Scientific Publishers Ltd. 270-294.

Stöger, R. (2008). The thrifty epigenotype: an acquired and heritable predisposition for obesity and diabetes? *BioEssays* **30,** 156-66.

Struch, F., Dau, M., Schwahn, C., Biffar, R., Kocher, T., Meisel, P. (2008). Interleukin-1 gene polymorphism, Diabetes and periodontitis : Results from the study of health Pomerania (SHIP). *J Periodontol* **79(3)**, 501-507.

Sudbery, P. (2002). The genetic components of complex disease In: *Human molecular genetics* 2nd edn. Pearson Education Limited. 180- 224.

Surani, M.A. (1998). Imprinting and the Initiation of Gene Silencing in the Germ Line. *Cell* **93**, 309

Svejgaard, A. (1996). "MHC and disease associations" In: Herzenberg, L.A., Weir, D.M. & Blackwell, C. (eds). *Handbook of Experimental Immunology*, 5th edn, Cambridge: Blackwell Science. 1-37.

Szalkowski, D., White-Carrington, S., Berger, J., Zhang, B. (1995). Antidiabetic thiazolidinediones block the inhibitory effect of tumor necrosis factor alpha on differentiation insulin stimulated glucose uptake and gene expression in 3T3-L1 cells. *Endocrinology* **136**, 1474-1481.

Tabor, H.K., Risch, N.J. & Myers, R.M. (2002). Opinion: candidate-gene approaches for studying complex genetic traits: practical considerations. *Nat Rev Genet* **3**, 391-7.

Taskinen, M.R. (2003). Diabetic dyslipidemia: from basic research to clinical practice. *Diabetologia* **46**, 733-49.

Tsai, C., Hayes, C., Taylor, G.W. (2002). Glycaemic control of type 2 diabetes and severe periodontal disease in the US adult population. *Community Dent Oral Epidemiol* **30**,182-192.

Taylor, G.W., Burt, B.A., Becker, M.P., et al (1996). Severe periodontitis and risk for poor glycaemic control in patients with non insulin dependant diabetes mellitus. *J Periodontol* **67 (SI)**, 1085-1093.

Taylor, G.W., Burt, B.A., Becker, M.P., Genco, R.J. & Shlossmans, M. (1998). Glycaemic control and alveolar bone loss progression in type 2 diabetes. *Annals of Periodontology* **3(1)**, 30-39.

Taylor, G.W., Burt, B.A., Becker, M.P., Genco, R.J., Shlossmans, M., Kinder, W.C., & Pettitt, D.J. (1998). Non-Insulin dependent diabetes mellitus and alveolar bone loss progression over two years *J Periodontol* **69**, 76-83.

Taylor, S.L., Renshaw, B.R., Garka, K.E., Smith, D.E. & Sims, J.E. (2002). Genomic organization of the interleukin-1 locus. *Genomics* **79**, 726-33.

Taylor, J.J., Preshaw, P.M. & Donaldson, P. (2004). Cytokine gene polymorphism and immunoregulation in periodontal disease. *Periodontol 2000* **35**, 158-182.

Tervahartiala, T., Koski, H., Xu, J.W., Hayrinen-Immonen, R., Hietanen, J., Sorsa, T., & Konttinen, Y.T. (2001). Tumor necrosis factor-alpha and its receptor, p55 and p75, in gingiva of adult periodontitis *Journal of Dental Research* **80**, 1535-1539.

Tervonen, T. & Oliver, R.C. (1993). Long term control of diabetes mellitus and periodontitis. *Journal of clinical periodontology* **20,** 431-435.

The Ethics of Clinical research in Developing countries (1999). A discussion paper, Nuffield Council on Bioethics, 7.

The Diabetes Control and Complications Trail Research Group (1993). The effect of intensive treatment of diabetes on the development and progression of long term complications in insulin dependent diabetes mellitus *N Engl J Med* **329**, 977-986.

The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group (2000). Retinopathy and nephropathy in patients with type 1 diabetes four years after a trail of intensive therapy. *N ENgl J Med* **342**, 381-389.

Thomas, F., Balkau, B., Vauzelle-Kervroedan, F. & Papoz, L. (1994). Maternal effect and familial aggregation in NIDDM. The CODIAB study. CODIAB-inserm-zeneca study group. *Diabetes* **43**, 63-67.

Thorsby, E. (1997). Invited anniversary review: HLA associated diseases. *Human Immunology* **53**, 1-11.

Tian, Y.G., Pan, Y.P., Lin, L., Zhang, D.M., Zhao, J. (2006). The relationship of IL-1beta expressed in buccal cells and the polymorphisms of IL-1beta (+3953) with chronic periodontitis. *Shanghai Kou Qiang Yi Xue* **15 (5)**, 456-60. (Abstract)

Tilvis, R.S., Miettinen, T.A. (1985). Fatty acid compositions of serum lipids, erythrocytes and platelets in insulin dependant diabetic women. *J Clin Endocrinol Metab.* **61,** 741-745.

Tomar, S.L. & Asma, S. (2000). Smoking-attributable periodontitis in the United States: findings from NHANES III. National Health and Nutrition Examination Survey. *J Periodontol* **71**,743-51.

Tonetti, M. S. & Claffey, N. (2005). Advances in the progression of periodontitis and proposal of definitions of a periodontitis case and disease progression for use in risk factor research. Group C consensus report of the 5th European Workshop in Periodontology. *Journal of Clinical Periodontology* **32**, 210–213.

Tsuchiya, T., Schwarz, P.E., Bosque-Plata, L.D., Geoffrey, H.M., Dina, C., Froguel, P., Wayne, T.G, Fischer, S., Temelkova-Kurktschiev, T., Rietzsch, H. (2006). Association of the calpain-10 gene with type 2 diabetes in Europeans: results of pooled and meta analysis. *Mol Genet Metab* **89**, 174-184.

Tsigos, C., Kyrou, I., Chala, E., Tsapogas, P., Stavridis, J.C., Raptis, S.A., Katsilambros, N. (1999). Circulating tumor necrosis factor alpha concentrations are higher in abdominal versus peripheral obesity. *Metabolism* **48**, 1332-1335.

Tuskada, J., Saito, K., Waterman, W.R., Webb, A.C. & Auron, P.E. (1994). Transcription factor NF-IL6 and CREB recognize a common essential site in the human prointerleukin 1β gene. *Mol Cell Biol* **14**, 7285-7297.

Turner, R.C., Levy, J.C. & Clark, A. (1993). Complex genetics of type 2 diabetes: Thrifty genes and previously neutral polymorphisms. Mini review. *QJM* **86**, 413-417.

United Nations Resolution 61/225: World diabetes day. www.worlddiabetesday.org, 11.

Uysal, K.T., Wiesbrock, S.M., Mariono, M.W., Hotamisligil, G.S. (1997). Protection from obesity induced insulin resistance in mice lacking TNFα function *Nature* **389**, 610-614.

UK prospective Diabetes Study (UKPDS) group (1998). Effect of intensive blood glucose control with metformin on complications in overweight patients with type 2 diabetes *Lancet* **352,** 854-865.

Valdez, R., Yoon, P.W., Liu, T., Khoury, M.J. (2007). Family history and prevalence of diabetes in the US population: the six year results from the National Health and Nutrition Examination Survey (1999 – 2004). *Diabetes Care* **30(10),** 2517-2522

Valverde, A.M., Teruel, T., Navarro, P., Benito, M., Lorenzo, M. (1998). Tumor necrosis factor-α causes insulin receptor substrate -2-mediated insulin resistance and inhibits insulin induced adipogenesis in fetal brown adipocyts. *Endocinology* **139**, 1229-1238.

Valsania, P. & Micossi, P. (1994). Genetic epidemiology of non insulin dependant diabetes. *Diabetes/Metabolism Reviews* **10(4)**, 385-405.

Van der Poll, T., Saurwein, H.P. (1993). Tumor necrosis factor α: its role in the metabolic response to sepsis. *Clin Sci* 84, 247-256.

Verweij C.L., Bayley J.P., Bakker A., Kaijzel E.L. (2002). Allele specific regulation of cytokine genes: monoallelic expression of the IL-1A gene. *Adv. Exp. Med. Biol.* **495,** 129–39.

Vincelj, J., Sucic, M., Bergovec, M. et al.(1997). Serum total, LDL, HDL cholesterol and triglycerides related to age, gender and cigarette smoking in patients with first acute myocardial infarction. *Collegium Antropol* **21**, 517-524.

Viswanathan, M., Mohan, V., Snehalatha, C., Ramachandran, A. (1985). High prevalence of type 2 diabetes among the offspring of conjugal type 2 diabetes patients. *Diabetologia* **28**,907-10.

Viswanathan, M., McCarthy, M.I., Snehalatha, C., Hitman, G.A. & Ramachandra, A. (1996). Familial Aggregation of type 2 (non insulin dependent) diabetes mellitus in south India; Absence of Excess Maternal Transmission. *Diabetic Medicine* **13,** 232-237.

Vlassara, H., Palace, M.R. (2002). Diabetes and advanced glycation end products. *J Intern Med* **251**, 87-101.

Walston, J., Seibert, M., Yen, C.J., Cheskin, L.J. & Andersen, R.E. (1999). Tumor necrosis factor alpha -238 and -308 polymorphisms do not associated with traits related to obesity and insulin resistance. *Diabetes* **48**, 2096-8.

Waugh, N., Scotland, G., McNamee, P., Gillett, M., Brennan, A., Goyder, E. (2007). Screening for type 2 diabetes: literature review and economic modeling. *Health Technol Assess* **11(17),** 1-125.

Weerasuriya, N., Siribaddana, S.H., Dissanayake, A., Subasinghe, Z., Wariyapola, D. & Fernando, D.J.S. (1998). Long-term complications in newly-diagnosed Sri Lankan patients with Type 2 diabetes mellitus, *Q. J. Med.* **91**, 439-443.

Wijewardene, K., Mohideen, M.R., Mendis, S., Fernando, D.S., Kulathilaka, T., Weerasekara, D., Uluwitta, P. (2005). Prevalence of hypertension, diabetes and obesity: baseline findings of a population based survey in four provinces in Sri Lanka. *Ceylon Med J* **50(2),** 62-70.

Weiss, K.M. & Terwilliger, J.D. (2000). How many diseases does it take to map a gene with SNPs? *Nat Genet* **26**, 151-7.

Wells, J.C. (2007). The thrifty phenotype as an adaptive maternal effect. *Biol Rev Camb Philos Soc* 82(1), 143-72.

Wiebe, C.B. & Putnins, E.E. (2000). Th periodontal disease classification system of the Americal Academy of Periodontology – an update. *J Can Den Assoc,* **66** 594-597.

Wild, S., Roglic, G., Green, A., Sicree, R., King, H. (2004). Global Prevalence of Diabetes: Estimates for the year 2000 and projections for 2030. *Diabetes Care* **27,** 1047-1053.

Wilson, A.G., di Giovine, F.S., Blakemore, A.I.H. & Duff, G.W. (1992). Single base polymorphism in the human tumor necrosis factor alpha (TNF alpha) gene detected by NcoI restriction of PCR Product. *Hum. Mol Genet* **1**, 353.

Wilson, A.G., Symons, J.A., McDowell, T.L., McDevitt, H.O., & Duff, G.W. (1997). Effects of a polymorphism in the human necrosis factor alpha promoter on transcriptional activation. *Proceedings of the National Academy of Science USA* **94**, 3195-3199

Xie, Y., Chen, C., Stevenson, M.A., Auron, P.E. & Calderwood, S.K. (2002). Heat shock factor 1 repress transcription of the IL-1β gene through physical interaction with the nuclear factor of interleukin 6. *J Biol Chem* **277**, 11802-11810.

Yang, T., Pang, C.P., Tsang, M.W., Lam, C.W., Poon, P.M., Chan L.Y., Wu, X.Q. Tomlinson, B., Baum, L. (2003). Pathogenic mutatons of the lipoprotein lipase gene in Chinese patients with hypertriglyceridemic type 2 diabetes. *Hum Mutat* **21**, 453-62.

Yang, Y.Y., Tsai, H.F., Lu, S.C., Huang, Y.F., Chang, Y.C. (2002). Regulation of tisuue inhibitors of metalloproteinase -1 gene expression by cytokines in human gingival fibtroblasts *J Endod* **28(12)**, 803-5.

Yliharsila, H., Tuomilehto, J., Mackay, I.R., Zimmet, P., Tuomilehto-Wolf, E., Rowley, M.J., Nissinen, A (1999). GAD antibodies in early men in different categories of glucose tolerance *Diabetes Care* **22(6)**, 996-997.

Yun, F., Firkova, E.I., Jun-oi, L., Xun, H. (2007). Effect of non surgical periodontal therapy on patients with type 2 diabetes mellitus. *Folia Med (Plovdiv)* Abstract **49**(1-2), 32-6.

Zaldivar, F., Nugent, D.J., Imfeld, K. & Berman, M.A. (2002). Identification of a novel regulatory element I the human interleukin 1α (IL-1α) gene promoter. *Cytokine* **20**, 130-135.

Zambon, J.J., Grossi, S.G., Machtei, E.E., Ho, A.W., Dunford, R., Genco, R.J. (1996). Cigarette smoking increases the risk for subgingival infection with periodontal pathogens. *J periodontal* **67**, 1050-1054.

Zhang, Y. & Rom, W.N. (1993) Regulation of the interleukin-1β (IL-1β) gene by mycobacterial components and lipopolysaccharide is mediated by two nuclear factor –IL-6 motifs. *Mol Cell Biol* **13**, 3831-3837.

Zhong, L., Zhang, Y., Zhang, J., Yang, A., Huang, H. (2002). The association of interleukin-1 gene polymorphisms with the susceptibility to chronic periodontitis in Uighur. Zhonghua Yi Xue Yi Chuan Xue Za Zhi (Abstract) 19(5), 405-8.