The role of resistin as a mediator of cross-susceptibility between periodontal disease and type 2 diabetes mellitus

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This is to certify that the work presented in this thesis represents original research carried out by Rana Majid Shareef Al-Shahwani in fulfilment of the requirements for the degree of Doctor of Philosophy according to the regulations of Newcastle University.

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Abstract

Resistin is a cytokine involved in insulin resistance, inflammation, and immunity. Evidence suggests that resistin expression is elevated in diabetes and inflammatory diseases. Diabetes and periodontitis are associated with each other; however, the pathogenic links between these two diseases are not completely understood. Both diseases are deemed to be inflammatory conditions and, therefore, resistin may possibly play a pathogenic role in the two diseases. Therefore, the objective of this study was to investigate the possible relationship between resistin levels in saliva and serum, and periodontal disease in patients with or without type 2 diabetes mellitus (T2DM). The regulation of resistin expression and release from human monocytes and macrophages by LPS, as well as the impact of resistin on cytokine expression and secretion *in vitro* were also investigated.

The present study demonstrated that salivary resistin was significantly elevated in periodontitis subjects as compared to gingivitis and periodontally healthy subjects in both T2DM and non-diabetic groups. However, there were no significant differences in salivary resistin between T2DM and non-diabetic groups irrespective of periodontal status. These data suggest that there is an association between salivary resistin and periodontitis rather than diabetic status. This hypothesis is supported by the finding that salivary resistin was significantly associated with bleeding on probing (BOP), mean probing depth (PD), mean loss of attachment (LOA) and periodontal inflamed surface area (PISA). Furthermore, saliva samples from both T2DM and non-diabetic subjects showed significant reductions in resistin levels at 3, 6 and 12 months after non-surgical periodontal management, which suggests that salivary resistin, may reflect improvements in periodontal inflammation following periodontal treatment. Serum levels of resistin were significantly higher in T2DM subjects compared to non-

diabetic controls, confirming the association between serum resistin and diabetes. This hypothesis is supported by the finding that serum resistin positively correlated with HbA1c, BMI and hsCRP. There were no significant differences in serum resistin between subjects with healthy periodontal tissues, gingivitis and periodontitis within both the T2DM and non-diabetic groups. However, serum resistin was positively correlated with BOP, mean PD and PISA. The relationship of serum resistin to periodontal disease therefore remains unclear. *In vitro*, LPS from both *P.gingivalis* and *E.coli* significantly enhanced resistin expression and secretion in human monocytes and macrophages, suggesting that resistin is induced by inflammatory stimuli and probably involved in inflammatory responses. Resistin displays potent proinflammatory properties itself as it upregulated the expression and secretion of several proinflammatory mediators such as TNF-α, IL-1β, IL-6, MIP-1α and CXCL10 in THP-1 monocytes.

In conclusion, salivary resistin could provide a novel local biomarker for periodontal disease. The upregulation of serum resistin in T2DM could influence periodontitis through the induction of inflammatory mediators that are responsible for exacerbating inflammation in periodontal tissues, and this process could contribute to the shared susceptibility between periodontal disease and T2DM.

Dedication

This thesis is dedicated to my beloved mother, Fawziya Nuaman. Her support, encouragement, infinite sacrifice and great constant love have sustained me throughout my life.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
ACS	acute coronary syndrome
ADA	American Diabetes Association
ADSF	adipocyte-specific secreted factor
AGE	advanced glycation end products
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
APCs	antigen presenting cells
ATMs	adipose tissue macrophages
BMI	body mass index
BOP	bleeding on probing
BP	blood pressure
BSA	bovine serum albumin
Cab	capture antibody
CAL	clinical attachment loss
CCL	chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
cDNA	complimentary deoxyribonucleic acid
CNS	central nervous system
COX	cyclooxygenase
CRP	C-reactive protein
CSF	colony stimulating factor
Ct	cycle threshold
CVD	cardiovascular disease
CXCL	C-X-C motif chemokine
CYP7A1	cytochrome P450 7A1
Dab	detection antibody
DCs	dendritic cells
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

E.coli	*Escherichia coli*
EAT	epicedial adipose tissue
EDTA	ethylenediamine tetra-acetic acid
EDN1	endothelin 1
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
GCF	gingival crevicular fluid
G-CSF	granulocyte colony-stimulating factor
GLUT-4	glucose transporter type 4
GP	general practitioner
HbA1c	percentage glycated haemoglobin
HDL	high density lipoproteins
HOMA-IR	homeostasis model assessment-insulin resistance
hsCRP	high sensitive C-reactive protein
HUVECs	human umbilical vein endothelial cells
ICAM-1	intracellular adhesion molecule-1
IL	interleukin
INF-γ	interferon gamma
IP-10	interferon gamma-induced protein 10
IQR	inter-quartile range
IR_{AR}	insulin resistance index
IRF-1	interferon regulatory factor 1
LDL	low density lipoproteins
LDLR	low density lipoprotein receptor
LOA	loss of attachment
LPS	lipopolysaccharide
LRP-2	low density lipoprotein-related protein 2
LTA	lipoteichoic acid
LTA	lymphotoxin alpha
MAMP	microbe-associated molecular pattern
MAPKs	mitogen-activated protein kinases
MCP-1	monocyte chemoattractant protein 1
MDP	muramyl dipeptide
MIP-1α	macrophage inflammatory protein 1 alpha

MMP	matrix metalloproteinases
mRNA	messenger ribonucleic acid
MS	metabolic syndrome
n	number
Nampt	nicotinamide phosphoribosyltransferase
NF-κB	transcription factor nuclear kappa B
NHANES	national health and nutrition examination survey
NLRs	NOD-like receptors
non-HDL	non high density lipoproteins
NS	not significant
NSM	non-surgical periodontal management
OD	optical density
P.gingivalis	*Porphyromonas gingivalis*
PAI	plasminogen activator inhibitor
PAMP	pathogen-associated molecular pattern
PBEF	pre-B cell colony enhancing factor
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PD	probing depth
PGE2	prostaglandin E2
PISA	periodontal inflamed surface area
PI3K	phosphatidylinositol 3-kinases
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocytes
PRRs	pattern recognition receptors
PTGS-2	prostaglandin-endoperoxide synthase 2
RAGE	receptor for advanced glycation end-products
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SOCS-3	suppressor of cytokine signalling 3
SMAD7	mothers against decapentaplegic homolog 7

TBX21	T-box transcription factor
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TGF-β	transforming growth factor beta
Th	T helper cell
TIMP	tissue inhibitor of metalloproteinases
TLDAs	Tagman low-density arrays
TLR	toll-like receptor
TNF-α	tumour necrosis factor alpha
TNFSF14	tumor necrosis factor superfamily 14
TRAF-3	TNF receptor-associated factor-3
TZD	thiazolidinedione
VCAM-1	vascular cell adhesion molecule 1
VEGFR	vascular endothelial growth factor receptor
VEGF	vascular endothelial growth factor
VitD3	vitamin D3
VSMCs	vascular smooth muscle cells
WHO	World Health Organisation
WHR	waist-to-hip ratio
β-cell	beta cell

Chapter 1 Introduction

The link between periodontal disease and type 2 diabetes Mellitus (T2DM) has been suggested through a number of clinical and epidemiological studies (Preshaw *et al.*, 2007; Preshaw, 2008b). Several studies have shown that the prevalence, incidence and severity of periodontitis is increased in the presence of diabetes (Soskolne and Klinger, 2001; Novak *et al.*, 2008; Fernandes *et al.*, 2009; Matu *et al.*, 2009; Um *et al.*, 2010). Thus, diabetes is considered to be a risk factor for gingivitis and periodontitis (Mealey and Oates, 2006; Preshaw *et al.*, 2007). In fact, periodontal disease has been described as the sixth complication of diabetes (Loe, 1993). The affiliation of periodontal disease and diabetes has been examined through a wide range of research over the past 20 years (Mealey and Oates, 2006), however, the exact mechanisms by which diabetes is associated with increased risk for periodontal disease are not clearly comprehended.

There is a large volume of published studies describing the key role of cytokines for regulation of innate and adaptive immune responses. The term cytokines has come to be used to refer to a multifarious group of small protein molecules, classic examples of which are interleukins, interferons, tumour necrosis factor family, chemokines, growth factors and adipokines (Amano, 2010). A dysregulated immune response stemming from an inappropriate cytokine production may act as a possible mechanism underpinning the cross-susceptibility between periodontal disease and diabetes (Barksby *et al.*, 2007; Preshaw *et al.*, 2007; Preshaw, 2008b; Preshaw, 2009).

Although, numerous studies have attempted to explain the pathogenesis of diabetes, the exact series of events leading to diabetes is still not fully understood. It has been

suggested that the development of diabetes mellitus stems from cellular and molecular defects in both insulin action and insulin secretion (Cusi, 2010; Tripathy and Chavez, 2010) which is influenced to a large extent by inflammation and the perturbation of immune responses (Goossens, 2008). Interestingly, monocytes from diabetic patients are hyperactive, showing a profound production of a proinflammatory cytokines such as TNF-α, IL-1β and IL-6 (Giulietti *et al.*, 2007). On the other hand, an impairment of neutrophil (polymorphonuclear leukocytes PMNLs) function has been reported in patients with diabetes (Alba-Loureiro *et al.*, 2007). It has been suggested that many cytokines could possibly contribute to inflammation-mediated insulin resistance such as TNF-α, IL-6, leptin, adiponectin, visfatin, resistin and IL-1 (Wellen and Hotamisligil, 2005).

Resistin is a cytokine which may play an essential role in insulin resistance (Steppan *et al.*, 2001a) and promote inflammation (Lehrke *et al.*, 2004; Bokarewa *et al.*, 2005). Many studies have reported a resistin up-regulation in obesity, insulin resistance and diabetes (Azuma *et al.*, 2003; Lu *et al.*, 2006; Tokuyama *et al.*, 2007; Hivert *et al.*, 2008; Chen *et al.*, 2009). Furthermore, resistin is significantly elevated in serum in both rheumatoid arthritis (Migita *et al.*, 2006) and periodontal diseases (Furugen *et al.*, 2008; Saito *et al.*, 2008). Taken together, these findings suggest a role for this cytokine in linking periodontal disease and diabetes. Consequently, this study is aimed at investigating the immunological activity of resistin in periodontal disease associated with diabetes.

1.1 The pathogenesis of periodontal disease

Periodontal disease is considered to be one of the most economically significant and widely spread health problems. In the UK, 54% of the dentate adults had pocketing of 4 mm or more, while deep pocketing of over 6 mm affected 5% of the dentate population (Kelly *et al.*, 1998; Morris *et al.*, 2001). Periodontal disease represents a heterogeneous multifactorial group of infectious diseases with a destructive inflammatory pathogenesis. These diseases affect the supporting and surrounding structures of the teeth (Kornman, 2008).

Gingivitis and periodontitis are the most prevalent forms of periodontal disease found in humans (Kinane, 2001; Williams, 2008). The occurrence of both forms is reliant on the existence and persistence of microbial plaque which is assumed to be the primary etiological factor for these diseases (Page *et al.*, 1997; Kinane, 2001; Nunn, 2003; Kornman, 2008). Indeed, periodontal disease represents the inflammatory and immune responses to local microbial attack (Page and Kornman, 1997; Kinane, 2001).

The inflammatory response in the gingival tissues (gingivitis) (Nanci and Bosshardt, 2006) which is induced as a result of plaque accumulation is actually a reversible state that can be cured by efficient plaque control measures (Kinane, 2001). On the other hand, periodontitis occurs when the inflammatory processes extend to involve the hard tissue structures of the attachment apparatus e.g. periodontal ligament and alveolar bone. This irreversible condition (periodontitis) is characterised by a pathological deepening of gingival sulcus concomitant with apical migration of the epithelial attachment and destruction of underlying connective tissue and alveolar bone (Kinane, 2001). Periodontal disease is deemed to be one of the most

considerable health problems of mankind because periodontitis leads ultimately in its terminal stages to loss of teeth (Kinane, 2001; Nanci and Bosshardt, 2006).

Gingivitis was formerly deemed unavoidable following the accumulation of bacterial plaque on teeth. However, it is now acknowledged that specific patients will be more susceptible than others to gingivitis and certainly periodontitis (Kinane and Mark Bartold, 2007). In 1965, Loe et al. published a paper in which they described the role of the plaque bacteria in the development of gingivitis for the first time. Within four days following the build-up of microbial plaque, the gingivitis lesion develops. The microbial mass in the dental plaque delivers substances provoke tissue inflammation (by both direct and indirect means), which is manifested by oedema, an increase in gingival crevicular fluid, polymorphonuclear leukocytes aggregation and connective tissue loss (Page and Schroeder, 1976). The clinical symptoms of gingivitis grow into more conspicuous forms when the plaque is allowed to build up on teeth and gingiva, however, it can be resolved by resuming adequate oral hygiene measures.

Gingivitis and periodontitis can thus be viewed as a continuum of the same pathogenic process. That is, periodontitis is clinically differentiated from gingivitis by the destruction of the connective tissue attachment. However, not all patients with gingivitis will progress to periodontitis; a susceptible host is a necessary prerequisite for the disease to evolve. Suffice to say that only a fraction of population (10-15%) exhibited advanced/sever forms of periodontitis (Kinane and Mark Bartold, 2007).

Periodontitis has been deemed as a complex bacteria-induced infection, characterised by inflammatory host response to plaque microbiota and their by-products. Socransky *et al*. (1998) examined over 13,000 subgingival plaque samples from 185 adults, and identified five specific microbial groups of bacterial species; depending on their relationship to clinical parameters of inflammation and periodontal destruction. The

first group comprised of *Porphyromonas gingivalis*, *Bacteroides forsythus* (now *Tannarella Forsythia*), and *Treponema denticola*. The second group composed of closely related core group including members of *Fusobacterium nucleatum*/*periodonticum* subspecies, *Prevotella intermedia*, *Prevotella nigrescens*, and *Peptostreptococcus micros*. The species affiliated with this group included *Eubacterium nodatum*, *Campylobacter rectus*, *Campylobacter showae*, *Streptococcus constellatus* and *Campylobacter gracilis*. The third group comprised of *Streptococcus* species including *S. mitus*, *S. oralis*, *S. sanguis*, *S. gordonii* and *S. intermedius*. The fourth group consisted of three *Capnocytophaga* species, *Campylobacter concisus*, *Eikenella Corrodens*, *Actinobacillus Actinomycetmcomitans* serotype-a. The fifth group included *Veillonella parvula* and *Actinomyces odontolyticus*. Finally, *Actinomycetmcomitans* serotype-b, *Selenomonas noxia*, *Actinomyces Naeslundii* genospecies 2 (*A.viscosus*) were outliers with insignificant relationship to each other and the five major groups. It is noteworthy that the first group related remarkably to clinical measures of periodontal disease; in particular, pocket depth and bleeding on probing (Socransky et al., 1998).

Therefore, periodontitis regarded as a polymicrobial infection associated with a specific group of primarily Gram-negative, anaerobic or microaerophilic bacteria that populate the root surface in the subgingival area (Page and Kornman, 1997).

Gram-negative bacteria such as *Porphyromonas gingivalis, Aggregatibacter* (formerly termed *Actinobacillus*) *actinomycetemcomitans* (Slots and Ting, 1999; Kinane, 2001; Silva *et al.*, 2008) and *Tannerella Forsythia* are the most prevelant periodontopathic microorganisms contributing to periodontal disease pathogenesis (Page *et al.*, 1997; Kinane, 2001; Nunn, 2003; Van Dyke and Serhan, 2003; Silva *et al.*, 2008).

Although the bacterial plaque is fundamental for periodontal disease initiation and propagation, the host defensive responses represented by inflammatory and immune reactions are the essential determinants of disease occurrence (Page and Kornman, 1997; Kinane, 2001; Van Dyke and Serhan, 2003). Host based risk factors such as smoking, diabetes, systemic disease, genetics, bacterial composition of microbial plaque and socioeconomic factors, all could alter host innate susceptibility to periodontal disease (Page *et al.*, 1997; Kinane, 2001; Nunn, 2003; Kinane *et al.*, 2007; Kinane and Mark Bartold, 2007). These risk factors may exacerbate the host protective mechanisms against bacterial challenge and thereby increasing the destructive nature of these processes (Page and Kornman, 1997).

The periodontium represents those tissues supporting and investing the tooth, encompasses root cementum, periodontal ligament, alveolar bone and that part of the gingiva facing the tooth (dento-gingival unit). The dento-gingival unit comprises epithelial and connective tissue compartments, which are separated from and attached to each other by the basement lamina. Based on their architecture, the gingival epithelium is divided into oral, sulcular and junctional epithelia (Nanci and Bosshardt, 2006). The sulcular epithelium lines the gingival sulcus and extends from the keratinized oral epithelium to the junctional epithelium, which mediates the attachment of the tooth to the gingiva. Both the sulcular epithelium and the coronal

part of the junctional epithelium are in direct contact with periodontal bacteria in the gingival sulcus and seem to be pivotal site with regard to the development of periodontal disease. The location of the junctional epithelium is crucially important because it interfaces between the gingival sulcus, populated with bacteria, and the tooth attachment apparatus that needs protection from becoming exposed to bacteria and their products. In fact, it is the structural and functional characteristics of junctional epithelium which enables it to control for the constant microbial challenge. However, the antibacterial defence mechanisms of the junctional epithelium do not prohibit the development of gingival and periodontal lesion. The junctional epithelium provides a route for fluid and transmigrating leukocytes into the oral cavity. The tissue fluids exudate (GCF) conveys a wide array of molecules via the junctional epithelium to the bottom of the gingival sulcus, which together with the migrating leukocytes represent a host defence system against the bacterial challenge (Bosshardt and Lang, 2005; Andrian *et al.*, 2006). With an increasing degree of inflammation in the gingiva, both the migration of PMNs and the rate GCF traverses via the intercellular spaces of the junctional epithelium increase. An enhanced number of mononuclear leukocytes, i.e. T- and B- lymphocytes, and monocytes/macrophages, together with PMNs, are deemed as factors that are partly responsible for the focal disintegration of the junctional epithelium. Additionally, pocket formation is possibly the result of subgingival spreading of virulent bacteria under impaired defence conditions. Therefore, microbial products infiltrating the junctional epithelium at the bottom of the sulcus may directly disturb the structural and functional integrity of the junctional epithelium. The proteolytic perturbation of the epithelial integrity may not only be a crucial factor in the initiation of pocket formation, but also facilitate microbial invasion into the sub-epithelial connective

tissue in advanced stages of the lesion. When the epithelial defence mechanisms are overwhelmed by bacterial virulence factors, the gingival lesion could progress to periodontitis. Indeed, the transformation of junctional epithelium to pocket epithelium is deemed as a hallmark in the progression of gingivitis to periodontitis (Bosshardt and Lang, 2005).

When the periodontal microbes colonize the subgingival environment, they start to express various structural and metabolic substances, for instance, lipopolysaccharides (LPS) which are a surface component and a well-known virulence factor of these Gram negative subgingival bacteria (Teng, 2006). LPS plays a key role in activation and perpetuation of tissue destruction in periodontal disease. These lipopolysaccharides have the ability to trigger the host cells to release cytokines and other mediators of inflammation, in an attempt to eliminate the infectious agents and initiate a defence mechanism (Jain and Darveau, 2010). This occurs when the bacterial LPS binds with the receptors (e.g. TLR and CD14) on the surface of host cells in periodontal tissues, including resident cells and immune cells such as fibroblasts, osteoblasts/osteoclasts, macrophages, lymphocytes and dendritic cells (Bascones-Martinez *et al.*, 2009; Jain and Darveau, 2010; Hans and Hans, 2011). This binding stimulates the host cells to release inflammatory mediators and cytokines, and thereby localized periodontal inflammatory reaction ensues (Bascones-Martinez *et al.*, 2009; Jain and Darveau, 2010; Hans and Hans, 2011).

In addition to lipopolysaccharides, periodontal bacteria possess an array of other virulence factors that enhance their infectivity and provide the ability for the organisms to multiply and persist in the periodontium and to express immunogenicity and cytotoxicity. For instance, gingipains; a potent virulence factors expressed by *P. gingivalis*, consist of three cysteine proteases that are responsible for at least 85% of

the total proteolytic activity exerted by this pathogen (Amano, 2010; Guo et al., 2010). Moreover, *P. gingivalis* possesses hemagglutinin which mediates bacterial adherence to host cells as well as bacterial acquisition from erythrocytes and platelet aggregation (Amano, 2010). Furthermore, bacteria evolved a range of adhesins, ranging from fimbriae and flagella to fibrils and curli. Fimbriae, adhesins and leukotoxins are among the important virulence strategies of *A. actinomycetemcomitans* which helps the bacteria to survive harmoniously with its host, and to evade the host defence mechanisms (Henderson et al., 2010).

The host immune response in periodontal disease is initiated by the innate host recognition of LPS, which actively modulates host-microbe interactions and the inflammatory response (Jain and Darveau, 2010). The innate immune system identifies microbial attackers by recognizing molecular patterns that are common to a diversity of different microbes. These common conserved structures expressed by microbes are termed pathogen-associated molecular patterns (PAMPs). The phrase pattern recognition receptor (PRRs) has been applied to host receptors which have the ability to identify PAMPs, these include cell surface Toll-like receptor (TLRs) and intracellular NOD-like receptors (NLRs). The notion of pattern recognition suggested that host PRRs recognize PAMPs (i.e. LPS) and that lead to immediate activation of the efficient and nonspecific innate immune response. Although substantial research has been carried out to investigate the recognition and signalling of periodontal pathogens (e.g. *P. gingivalis*) via TLRs (Pathirana *et al.*, 2010; Taylor, 2010), only a limited number of studies of NLRs and oral bacteria have been reported (Bostanci *et al.*, 2009). It has been shown that *P.gingivalis* culture supernatants up-regulates NLRP3 gene expression in Mono-Mac-6 monocytic cell lines (Bostanci *et al.*, 2009).

Furthermore, the mRNA expression of both NLRP3 and NLRP2 were significantly higher in inflamed periodontal tissues compared to health (Bostanci *et al.*, 2009).

Toll-like receptors provide a first line innate defence against infection through its recognition of LPS. The interaction of LPS with TLRs in the gingival epithelium leads to the release of cytokines and chemokines which in turn result in the expression of adhesion molecules, an enhancement the gingival vascular permeability and chemotaxis of polymorphonuclear neutrophils (PMNs) via the junctional epithelium into the gingival sulcus to eliminate microbes by phagocytosis (Teng, 2006; Dumitrescu, 2010; Liu *et al.*, 2010; Hans and Hans, 2011).

In addition to lipopolysaccharides, Toll like receptors sense and respond to various other microbial structures, such as lipopeptides, lipoproteins, peptidoglycan, lipoteichoic acid, zymosan, fimbriae, and flagellin. The activation of TLR which launch by the binding of TLR to its ligand initiate a cascade of intracellular events which take place via two separate pathways: the myeloid differentiation primary response protein 88 (MyD88)-dependent pathway and the (MyD88)-independent pathway. The end products of these intracellular events will lead to activation of two distinct signalling pathways. One pathway results in activation of activator protein-1 (AP-1) through activation of mitogen-activated protein kinase (MAPK), whereas the other pathway activates transforming growth factor-β-activated kinase 1 (TAK1), which in turn enhances the activity of inhibitor of the nuclear factor-κB kinase complex. This gives rise to degradation of the inhibitor of nuclear factor-κB; and release of nuclear factor-κB (NF-κB), which translocate to the nucleus. In the nucleus, NF-κB binds to specific sequences in the promoter regions of target genes for inflammatory proteins such as cytokines, thereby control the transcription of genetic information from DNA to mRNA; the mRNA molecule is responsible for protein

synthesis in the cytoplasm (Kawai and Akira, 2006; Hans and Hans, 2011). Neutrophils forms the first line of host defence against periodontal bacteria, and, by their capability to phagocytize microbes, they can protect the host from infection. However, in addition to the pivotal role of neutrophils in combating the invading bacteria, it also plays a significant role mediating tissue destruction in the pathogenesis of inflammatory disease. In the gingival sulcus, activated neutrophils attempt bacterial elimination by phagocytosis; however, some virulent microbes are able to evade neutrophils, leading to continuous accumulation of these phagocytes in the gingival pocket (Van Dyke and Serhan, 2003; Bascones-Martinez *et al.*, 2009). Activated neutrophils release oxygen radicals and proteolytic enzymes which can directly instigate tissue damage (Entman and Smith, 1994; Hansen, 1995). Oxygen radical species can strike every biologically relevant molecule, such as proteins, lipids, carbohydrates, and nucleic acids (Badwey and Karnovsky, 1980). These molecules encompass superoxide, hydrogen peroxide, and hydroxyl radicals. Interestingly, recent studies of localized aggressive periodontitis have shown that peripheral neutrophils are hyper-responsive in respect of reactive oxygen species (ROS) generation after exposure to leukotriene B4 (LTB4) and interleukin (IL)-8 due to reduced gene and protein expression of diacylglycerol (DAG) kinase (Gronert *et al.*, 2004), inhibition of which is known to amplify the respiratory burst in normal neutrophils (Topham and Prescott, 1999). What's more, it has been shown that peripheral neutrophils from chronic periodontitis patients exhibit hyper-reactivity following stimulation with (Fcγreceptor and *F. nucleatum*) and hyperactivity in terms of excess ROS release in the absence of exogenous stimulation (Matthews *et al.*, 2007). The exacerbated neutrophilic inflammation, in terms of reactive oxygen

species (ROS) and proteolytic enzyme production, in response to periodontal pathogens is possibly a factor underpin the tissue destruction found in periodontitis. Interestingly, monocytes are a substantial component of innate immunity that plays a pivotal role in periodontal disease (Jain and Darveau, 2010). These cells migrate rapidly to the site of infection in a response to inflammatory stimuli and differentiate in the tissues into macrophages which can efficiently capture the attacking bacteria. Macrophages engulf and digest microbes resulting in cytokine release and antigen presentation which in turn drive a more efficient adaptive immune response (Teng, 2006; Liu *et al.*, 2010).

The chronic inflammation in periodontal tissues stems from inappropriate host-microbial interaction, in which chronic stimulation of host cells by bacterial PAMPs can result in excessive production of proinflammatory cytokines (e.g. IL-1β, TNF-α, IFN-γ and IL-6), leading to tissue destruction (Okada and Murakami, 1998; Preshaw, 2008a; Liu *et al.*, 2010; Hans and Hans, 2011). As a consequence of the disease development, propagation and perpetuation, the cytokines and chemokines released by innate immune reactions drive the host response toward a vigorous cell-mediated adaptive immunity. Hence, the formation of the inflammatory cell infiltrates in the connective tissues comprising predominantly of T-lymphocytes and macrophages ensue. If the T-cell response does not restrain the microbial challenge, the pathologic lesion progress to B-cell/plasma predominant one. The antibodies produced by B-cell/plasma are either protective and therefore control the infection, or non-protective resulting in connective tissue destruction and bone loss (Gemmell *et al.*, 2002; Gemmell *et al.*, 2007). The mononuclear infiltrate predominate the established periodontal lesion consist mainly of monocytes/macrophages, dendritic cells, T-cells, B-cells and plasma cells (Liu *et al.*, 2010). However, the key distinguishing feature of

chronic periodontitis is the predominance of B-cell/plasma infiltrate which is a declarative of B-cell/ plasma response (Kinane and Mark Bartold, 2007; Ohlrich *et al.*, 2009). Histologically, chronic periodontitis is characterized by the breakdown of connective tissue attachment and apical migration of junctional epithelium concomitant with plaque accumulation on root surface and subgingival calculus formation. At this phase, the cell infiltrate predominates with plasma cells, while only few macrophages are noted in connective tissue. In addition, fibroblasts undergo disfiguration and a reduction in number accompanied with encapsulation of the progression lesion by non-infiltrated fibrous band. The infiltrating cells produce high levels of IL-1 and IL-6, these cytokines trigger the production of matrix metalloproteinases (MMPs), particularly by fibroblasts which, in turn cause a degradation in extracellular matrix and lead to attachment loss (Gemmell and Seymour, 1998; Nishikawa *et al.*, 2002; Ohlrich *et al.*, 2009; Smith *et al.*, 2010). Chronic inflammatory processes in the neighbourhood of bone tissues influence the bone remodelling, and in most occasions resulting in a clinically osteolytic lesion. Of note, both bone resorption and formation are enhanced in chronic inflammatory periodontitis and it is the proportional enhancement that will decide if the osteolytic lesion or sclerotic lesion will appear clinically. The infiltrating leukocytes and resident fibroblasts release a number of stimulatory and inhibitory cytokines that influence the amount of osteoclast formation (osteoclastogenesis) in the inflammation-induced periodontal bone loss lesion (Liu *et al.*, 2010).

All in all, periodontal disease is a multi-factorial malady. Dental plaque bacteria in the oral cavity have a causal influence on periodontal disease, and a wide variety of determinants and factors also paly important roles in its manifestation and progression (Nunn, 2003). The oral microorganisms posses numerous potent virulence factors

that enhance their infectivity, provide the ability for the organism to multiply and persist in the periodontium (Van Dyke and Serhan, 2003), and also provoke a series of aberrant immune-inflammatory response in periodontal tissues (Amano, 2010). The intricate interactions of the biofilm with the host immune-inflammatory response and subsequent alterations in bone and connective tissue homeostasis represent the actual cause for the most of the tissue damage that characterises periodontal disease (Kornman, 2008). In short, bacterial plaque deposit is the primary factor initiating periodontal disease, however, the progression and perpetuation of this disease is mediated by the host-response to infection. The host response to microbial challenge is influenced by a wide array of determinants and factors which may be hereditary or environmental factors; these including subject characteristics, social and behavioural factors, genetic factors, and some systemic factors such as diabetes mellitus (Nunn, 2003; Kornman, 2008). Indeed, diabetes mellitus is deemed to be a genuine risk factor for periodontitis (Page *et al.*, 1997; Kinane, 2001; Nunn, 2003; Kornman, 2008; Preshaw, 2008b).

Therefore, a fuller understanding of the possible mechanisms for shared susceptibility between periodontal disease and diabetes could be crucial to identify novel therapeutic targets that are relevant in the management of these common conditions.

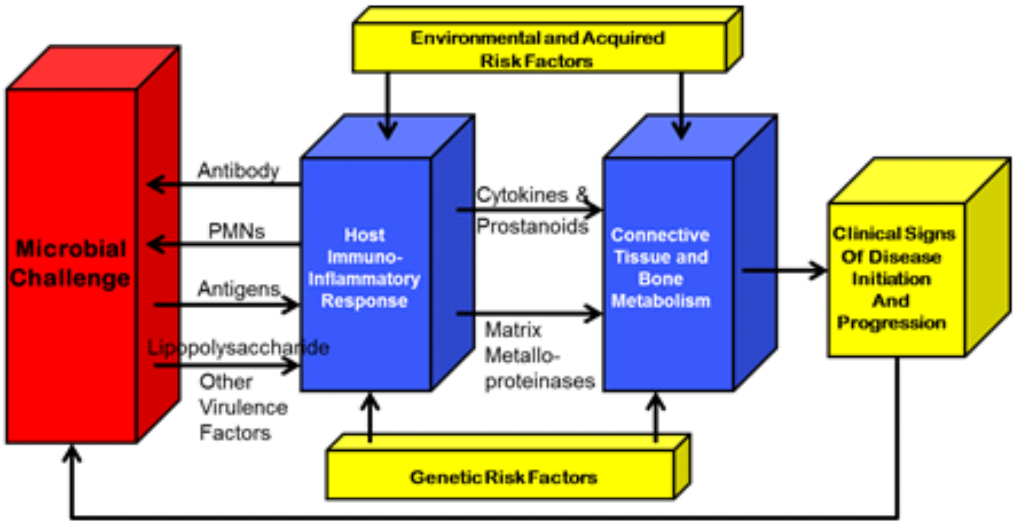


Figure 1.1 Schematic representation for the pathogenesis of human periodontitis

Invading bacteria, antigens and numerous other virulence factors constitute the microbial challenge, and the host responds with an instant inflammatory and immune response to confront the challenge. The host response drives the production of cytokines, eicosanoids and other inflammatory mediators and matrix metalloproteinases, which sustain the response and also orchestrate connective tissue and bone destruction. All of these incidents are affected by both genetic and environmental or acquired factors which act as disease modifiers. The clinical manifestation observed is a result of the aggregate of these events. Taken and modified from (Page and Kornman, 1997; Kornman, 2008).

1.2 The pathogenesis of diabetes mellitus

It is becoming increasingly difficult to ignore the importance of diabetes mellitus as one of the major threats to human health in the 21st century (Passa, 2002; King, 2008; Levitt, 2008; Preshaw, 2008). Diabetes Mellitus is a heterogeneous group of metabolic disorders, in which hyperglycemia accompanied with a disturbance in carbohydrate, fat, and protein metabolism. The chronic elevation of blood glucose levels stems from either defects in insulin secretion or insulin action or both (Mealey and Oates, 2006; Tripathy and Chavez, 2010). The most likely cause of insulin secretion deficiency is the dysfunction in the pancreatic β-cells (Mealey and Oates, 2006), whilst the hypo responsiveness of the tissues to insulin action (termed Insulin Resistance IR) may represent one of the main complications of obesity (Rasouli and Kern, 2008). Indeed, the regulation of insulin sensitivity and resistance depends on a number of factors including adipokines, inflammatory mediators, genetic factors and environmental stresses (Mealey and Oates, 2006; Preshaw et al., 2007).

Body glucose homeostasis is mainly reliant on insulin (a hormone secreted by pancreatic β-cells). Consequently, it seems that the reduction in insulin secretion and/or action can result in lowering the capability of glucose transport from blood stream into the tissue cells which give rise to hyperglycemia (Soskolne and Klinger, 2001; Preshaw *et al.*, 2007). Polydpsia, polyphagia, and polyuria are the central symptoms of diabetes mellitus. In fact, elevated blood glucose level and the resultant disturbance in osmotic homeostasis is the principal causes of these symptoms (Soskolne and Klinger, 2001). Diabetes complications have a significant detrimental influences to human health, these complications are numerous and serious, it encompass retinopathy, nephropathy, neuropathy, atherosclerosis and delayed wound

healing (Soskolne and Klinger, 2001; Preshaw *et al.*, 2007; King, 2008; Preshaw, 2008b). Indeed, periodontal disease has been considered as the sixth complication of diabetes (Loe, 1993).

Diabetes mellitus is correlated with diminished life expectancy, escalated morbidity and escalated mortality. T2DM patients in the UK have a mortality rate nearly twofold as high as those without diabetes (Mulnier *et al.*, 2006). Diabetes has a deleterious irreversible impact on the micro- and macro-vasculature, with cardiovascular disease (CVD), myocardial infarction and stroke being the fundamental cause of mortality in patients with T2DM (Dale *et al.*, 2008). What's more, diabetic nephropathy, retinopathy and neuropathy are principal causes of renal failure, blindness and sensory loss (Frank, 2004; Gilbertson et al., 2005). Type 2 diabetes probably plays a major part in escalating the risk for cardiovascular disorders which is manifested clinically as myocardial infarctions, angina, peripheral artery disease (leg claudication, gangrene), and carotid artery disease (strokes, dementia) (NICE, 2008). Both the duration of diabetes and the degree of glycaemic control are leading factors for all diabetic complications (UKPDS, 1998).

Therefore, diabetes mellitus and its affiliated complications constitute a substantial health-care burden globally. It is associated with a high morbidity and mortality worldwide (King, 2008). Diabetes has amounted to epidemic level; the international Diabetes Federation (IDF) estimates that currently diabetes affects more than 246 million of the world population and this is anticipated to increase to 380 million people by 2025 (IDF, 2006). Between 2007 and 2025, the predicted growth worldwide is 55%, with the greatest raises in the developing countries of Africa, Asia and South America (IDF, 2006). Within Europe, it is estimated that 53.2 million or 8.4% of the adult population have diabetes and this is predicted to increase by 21% to

64.1 million or 9.8% in 2025, with the highest increase being seen in the older age group (IDF, 2006). A plethora of recent research within the UK has indicated an increase in the prevalence (Newnham et al., 2002; Fleming et al., 2005; Lusignan et al., 2005; Gonzalez et al., 2009) and incidence (Ryan et al., 2005; Forouhi et al., 2007; Gonzalez et al., 2009) of diabetes cases. Furthermore, the World Health Organization (WHO) predicts that there will be around 366 million people in the United States who have diabetes by 2030 (Smyth and Heron, 2006).

The idiom 'diabetes mellitus' embraces a group of metabolic disorders, characterised by hyperglycaemia, ensuing from defects in insulin secretion, insulin action, or both. Diabetes is classified based on the underlying aetiological factor into four main categories includes; type 1, type 2, gestational and other specific types of diabetes. In effect, the two broad categories; type 1 and type 2 represent the vast majority of cases with diabetes mellitus. According to the classification by the American Diabetes Association (ADA) diabetes is generally divided on the basis of pathophysiology involved into Type 1 Diabetes Mellitus (formerly insulin dependent Diabetes Mellitus) and Type 2 Diabetes Mellitus (formerly non-insulin dependent Diabetes Mellitus) (2003). Type 1 stems from the autoimmune destruction of insulin producing islet cells which in turn stops the insulin secretion (Soskolne and Klinger, 2001; Mealey and Oates, 2006). On the other hand, a combination of insulin resistance in peripheral tissues with a defect in insulin secretion is responsible for type 2 diabetes mellitus (Mealey and Oates, 2006). Clearly, type 2 diabetes accounts for almost 90-95% of the total number of diabetic cases whilst around 5-10% of diabetic patients have type 1 (ADA, 2003). Moreover, type 1 diabetes occurs mainly in children and adolescents while type 2 usually found in adults (Mealey and Oates, 2006; King, 2008). The former term for type 2 diabetes mellitus (T2DM) was non-

insulin dependent diabetes mellitus (NIDDM). The origin of this term comes from the fact that the patient suffers from this disease can remain alive without the necessity for insulin treatment. The initial event of type 2 diabetes is a defect in insulin action at the target cells which is named insulin resistance. During the initial stages in the natural history of T2DM, the body increases insulin output which results in hyperinsulinaemia to compensate for the reduction in insulin action and maintain normal glucose tolerance. As the insulin resistance worsens, the body is not being able to control any further rise in metabolic load. There is a decline in insulin production affiliated with peripheral insulin resistance and diminished β-cell function. Ultimately, insulin secretions become diminished and inadequate to recompense for the insulin resistance, thereby driving to impaired glucose tolerance and overt type 2 diabetes. Subjects with diabetes can endure undiagnosed for several years since the hyperglycemia occur progressively and often without symptoms (Rhodes, 2005; Mealey and Ocampo, 2007; Tripathy and Chavez, 2010). There are several environmental risk factors for the pathogenesis of T2DM, among these are age, diet, lack of physical activity and obesity (Mealey and Ocampo, 2007). Indeed, obesity is considered as one of the most significantly important risk factors of this disease (Passa, 2002; Sethi and Vidal-Puig, 2005; Pischon *et al.*, 2007; Saito and Shimazaki, 2007). Alternatively, recent studies counted diabetes as one of the important metabolic complications of obesity (Rasouli and Kern, 2008). The role of obesity in the development of T2DM is now widely accepted. A body mass index (BMI) above 25 kg/m^2 is characterized as overweight, and a BMI above 30 kg/m^2 is characterized as obese. According to the World Health Organisation (WHO), there are more than one billion adults who are overweight world wide, at least 300 million of them being clinically obese. A sedentary lifestyle owing to high calorie and fat food consumption

combined with reduced physical activity represent the main reason of high obesity prevalence world wide (WHO, 2005).

A large and growing body of literature has discussed the notion of obesity as a proinflammatory condition implicated in the development of insulin resistance and type 2 diabetes (Shoelson *et al.*, 2007; Shoelson and Goldfine, 2009; Kim, 2010). Adipose tissue is an active contributor to whole body energy homeostasis; it has been granted multifarious functions involving the assimilation, preservation and synthesis of lipid, and the synthesis and secretion of a diverse range of adipokines. Apart from being the main energy depot of the body, adipose tissue is also a source of proinflammatory mediators that regulate the immune-inflammatory response and thence be partly responsible for the development of obesity associated with type 2 diabetes (Trayhurn and Wood, 2004; Lago *et al.*, 2007). Interestingly, heterogeneous types of cells constitute the adipose tissue; this encompasses adipocytes, pre-adipocytes, endothelial cells, fibroblasts, and immune-competent cells (macrophages and lymphocytes) (Juge-Aubry *et al.*, 2005; Wozniak *et al.*, 2009). Adipocytes secrete various proteins such as leptin and adiponectin which are potentially important in glucose homeostasis and lipid metabolism (Zhang *et al.*, 1994; Scherer *et al.*, 1995). The theory that obesity mediates inflammation became tangible with the discovery of the fact that an increased body mass index is positively correlated with an increase in adipose tissue mass, numbers of adipocytes and infiltrating macrophages (Weisberg *et al.*, 2003). Adipose tissue macrophages (ATMs) emerged as pivotal sources of proinflammatory mediators (Olefsky and Glass, 2010). In fact, activation of ATMs triggers the release of a diversity of chemokines, this in turn attract additional macrophages which boot to develop chronic inflammatory state. Obese individuals are well known to be associated with chronic low grade

inflammation (Zeyda and Stulnig, 2009). This can be illustrated by the elevated levels of lipids, free fatty acids, various proinflammatory mediators and acute phase proteins in the circulation of obese individual (Bergman and Ader, 2000; Das, 2001; Bullo *et al.*, 2003; Trayhurn and Wood, 2004). Exposure of various organs to free fatty acids could be partly responsible for insulin resistance by suppressing glucose uptake, glycogen synthesis, and glycolysis, and by liver glucose overproduction (Bergman and Ader, 2000). Many lines of evidence have shown that ATMs and adipocytes exhibit a pro-inflammatory activity by the production of large amounts of proinflammatory mediators such as TNF-α, IL-1β, IL-6 and resistin, also chemokines such as monocyte chemoattractant protein (MCP)-1 and IL-8 (Hotamisligil *et al.*, 1995; Mohamed-Ali *et al.*, 1997; Trayhurn and Wood, 2004; Zeyda and Stulnig, 2009). Furthermore, a significant elevation in inflammatory markers such as IL-6 and C-reactive protein (CRP) correlated with various components of insulin resistance in obese individuals (Pickup *et al.*, 1997; Festa *et al.*, 2000). It has become increasingly evident that proinflammatory mediators exaggerate the inflammatory reaction and subsidize the establishment of insulin resistance and type 2 diabetes. More specifically, it is believed that TNF-α plays a pivotal role in insulin resistance development at the receptor level. That is, TNF-α triggers serine phosphorylation of insulin receptor substrate 1 (IRS-1), blocks autophosphorylation of (IRS-1), and also, it reduces tyrosine kinase activity of the insulin receptor (Hotamisligil *et al.*, 1996; Hirosumi *et al.*, 2002). Alternatively, IL-6 provokes inhibition of the glucose stimulated insulin release. Escalated levels of IL-6 also drive to increased production of TNF-α and CRP, which in turn, may also have indirect effect on insulin resistance (Fernandez-Real and Ricart, 2003). In the last few years, inflammatory reaction was increasingly recognized as an important effector mechanism of obesity, insulin

resistance and type 2 diabetes. Given the crucial importance of inflammation in type 2 diabetes, the study of the interactions of these important pathophysiological reactions may shed light on the possible underpinning mechanism by which T2DM is linked to periodontal disease.

1.3 Periodontal disease and Diabetes Mellitus: the cross-susceptibility

In recent years, there has been an increasing amount of literature on the relationship between periodontal disease and diabetes (Soskolne and Klinger, 2001; Nishimura et al., 2003; Mealey and Oates, 2006; Preshaw et al., 2007; Preshaw, 2008). Diabetes mellitus has long been reported to act as an influential risk factor for gingivitis and periodontitis (Soskolne and Klinger, 2001; Nishimura et al., 2003; Mealey and Oates, 2006; Preshaw et al., 2007; Preshaw, 2008). The prevalence and severity of gingival inflammation have been demonstrated to be higher in type 1 diabetic children when compared with the non-diabetic control group (Mealey and Oates, 2006; Lalla et al., 2007). In addition, an increased severity (Lalla et al., 2007) and prevalence for periodontitis have been noted in children with type 1 diabetes in comparison with control non-diabetic subjects (Mealey and Oates, 2006). Likewise, type 2 diabetes has been associated with higher prevalence, incidence and severity of periodontitis when compared with non-diabetic adults. In fact, the risk for periodontal disease is considerable in diabetes (both type 1 and 2), and it increases whenever the glycaemic control is getting worse (Preshaw, 2008). Many authors have found a significant association between diabetes and gingival inflammation. The prevalence, incidence

and severity of gingivitis have been illustrated to be greater in subjects with diabetes. For instance, the prevalence of gingivitis was higher in children with type 1 diabetes when compared with non-diabetic controls with similar plaque levels (Cianciola *et al.*, 1982). Moreover, in children with similar plaque levels, the number of site with gingival inflammation was twice in diabetics comparing to non-diabetic controls (de Pommereau *et al.*, 1992). Indeed, poor glycaemic control can boost the severity of gingivitis in diabetic children (Gusberti *et al.*, 1983), whereas improvement in glycaemic control is associated with diminished gingival inflammation (Sastrowijoto *et al.*, 1990; Karjalainen and Knuuttila, 1996). In adults with type 1 diabetes, gingival bleeding was substantially greater in diabetic subjects with poor glycaemic control compared to either good controlled diabetic individuals or non-diabetics. However, when the glycaemic control was improved, the number of bleeding sites diminished (Ervasti *et al.*, 1985). In adults with type 2 diabetes, the gingival inflammation was much higher than non-diabetics, with the greatest degree of inflammation in individuals with poor glycaemic controls (Cutler *et al.*, 1999). In an experimental gingivitis study, it has been demonstrated that the development of gingival inflammation was earlier and more conspicuous in well-controlled adult type 1 diabetic subjects than non-diabetic controls, in spite of comparable levels of plaque accumulation and comparable bacterial composition of the plaque, suggesting a hyper-inflammatory gingival response in diabetes (Salvi *et al.*, 2005). These studies points out that the presence of diabetes is frequently, but not consistently, affiliated with increased gingival inflammation. Moreover, the level of glycaemic control can be influential in the gingival response to bacterial challenge in individuals with diabetes.

In a similar pattern, sufficient evidence exists that the risk for periodontitis is increased in the presence of diabetes. Several epidemiological studies have identified an increase in the extent and severity of periodontitis in diabetic adults (Bacic *et al.*, 1988; Shlossman *et al.*, 1990; Emrich *et al.*, 1991; Tervonen and Oliver, 1993). For instance, in a study of periodontal disease in Pima Indian of Arizona, a population with a remarkably high prevalence of type 2 diabetes, researchers concluded that the prevalence and severity of attachment loss and bone loss was much greater among diabetic individuals when compared to non-diabetic controls in all age groups (Shlossman *et al.*, 1990; Emrich *et al.*, 1991). Likewise, comparable results indicating a greater risk of attachment loss and bone loss in diabetic adults were reported in other cross-sectional and case-control studies (Bridges *et al.*, 1996; Collin *et al.*, 1998; Cutler *et al.*, 1999; Moore *et al.*, 1999; Tervonen *et al.*, 2000; Campus *et al.*, 2005). Furthermore, it was demonstrated that the risk of having periodontitis increased by nearly threefold in patient with type 2 diabetes compared with subjects without diabetes (Shlossman *et al.*, 1990; Emrich *et al.*, 1991). An increased risk of progressive periodontal destruction in subjects with diabetes has also been demonstrated in longitudinal studies of periodontal disease (Nelson *et al.*, 1990; Taylor *et al.*, 1998). It has been argued that the association between glycaemic control of diabetes and periodontal status is difficult to characterize definitely (Mealey and Moritz, 2003). The reason for that is the considerable heterogeneity found in diabetic community, that is to say, although many poorly controlled diabetics suffer extensive periodontal destruction (Tsai *et al.*, 2002), others do not (Barnett *et al.*, 1984). In general, therefore, it seems that glycaemic control of diabetes is a pivotal factor contributes to the onset and progression of periodontal disease. Several studies have confirmed that poor glycaemic control promotes the development and

progression of periodontitis (Tervonen *et al.*, 2000; Tsai *et al.*, 2002; Lu and Yang, 2004; Campus *et al.*, 2005; Jansson *et al.*, 2006; Peck *et al.*, 2006). In fact, data from the National Health and Nutrition examination Survey (NHANES) III were analysed to evaluate the relationship between glycaemic control of T2DM and severe periodontal disease in US adult population ages 45 years and above. From the 4343 persons in the NHANES III database, the poorly controlled diabetic subjects had a significantly greater prevalence of sever periodontitis when compared with non-diabetics (odds ratio=2.90); on the contrary, diabetics with good glycaemic control had no significant increase in the risk of periodontitis (Tsai *et al.*, 2002). Moreover, diabetics with poor glycaemic control had significantly greater risk for advanced attachment loss, progressive bone loss and deeper periodontal pockets than well controlled diabetics (Tervonen and Knuuttila, 1986; Safkan-Seppala and Ainamo, 1992; Seppala *et al.*, 1993; Tervonen and Oliver, 1993; Seppala and Ainamo, 1994; Taylor *et al.*, 1998; Guzman *et al.*, 2003).

Taken together, it can be established that people with diabetes have increased risk to develop periodontal diseases, both gingivitis and periodontitis. Also, the potential impact of glycaemic control on periodontal health is tremendous. That is to say, a poorer glycaemic control underpinning a poorer periodontal health; nonetheless, well-controlled diabetics seem to have a comparable risk of periodontal disease as the non-diabetics.

A better comprehension of the mechanisms underpinning the accelerated periodontal disease associated with diabetes is fundamental to certify the relationship between diabetes and periodontal disease. Notwithstanding, the considerable amount of studies describing the pathobiological interactions linking between diabetes and periodontal disease, the precise mechanism have yet to be fully recognized. The

potential disparities in the subgingival microflora between diabetics and non-diabetics have been investigated by previous research as a possible motive for the greater prevalence, incidence and severity of periodontal destruction in subjects with diabetes. Nevertheless, most studies demonstrated few variations in the subgingival microbiota between periodontitis patients with and without diabetes (Zambon *et al.*, 1988; Sastrowijoto *et al.*, 1989; Sbordone *et al.*, 1998). The evident deficiency of significant differences in existing periodontal pathogens between people with or without diabetes, suggests that alterations in the host immune-inflammatory response may be essentially responsible for the more aggressive periodontal destruction noted in patients with diabetes.

As described earlier in this review, it is now clearly recognized that the immune-inflammatory response perform a pivotal role in the pathogenesis of periodontal disease. Periodontal disease occurrence depends on the interaction between microbial stimulation and the host response which seems to be orchestrated by complex webs of cytokines working in synergy (Preshaw and Taylor, 2011). It has been demonstrated that chronic inflammatory process contributes to the pathology associated with both diabetes and periodontal disease. It is also suggested that diabetes modifies periodontitis principally through its effect on the normal immune and inflammatory defences (Kinane and Marshall, 2001; Southerland *et al.*, 2006). It is likely that modulations in inflammatory processes stemming from diabetes can give rise to a further dysregulation of immune-inflammatory responses in the periodontium, resulting in increased periodontal destruction (Nishimura *et al.*, 1998; Salvi *et al.*, 1998; Ryan *et al.*, 2003; Mealey and Oates, 2006; Preshaw, 2009; Santos *et al.*, 2010; Venza *et al.*, 2010). There is growing evidence that diabetes is a state of chronic inflammation, characterised by abnormal cytokine production, elevated

concentrations of acute-phase reactants in plasma, sialic acid and other stress-induced molecules (Crook *et al.*, 1993; Pickup *et al.*, 1997; Sethi and Hotamisligil, 1999). For instance, elevated levels of IL-6 and TNF-α were recorded in plasma of obese patients and those with type 2 diabetes (Dandona *et al.*, 2004). Hyperglycemia also results in elevated circulating concentrations of IL-6, IL-18, and TNF-α (Esposito *et al.*, 2002). It has been shown that a close relationship presents among periodontitis, obesity and diabetes with adipose tissue and chronic inflammation being the common denominators (Genco *et al.*, 2005; Saito and Shimazaki, 2007). Therefore, adipose tissue may be deemed as a pivotal contributor in mechanism that links diabetes and periodontal disease. Several biologically active molecules are secreted by adipose tissue; these include cytokines such as IL-6 and TNF-α, and adipokines such as resistin, leptin and adiponectin (Bastard *et al.*, 2006). The cytokines have a direct proinflammatory effects on inflammatory cells, involving those in periodontal tissues. On the other hand, adipokines are important regulators of inflammatory responses, such as leptin which act as a stimulator of neutrophil chemotaxis and cytokines releases by monocytes (Sanchez-Margalet *et al.*, 2003). Furthermore, a variety of immune-competent cells are infiltrating adipose tissue such as macrophages and lymphocytes, which are responsible for an important part of the locally produced cytokines and adipokines. It is noteworthy that the number of macrophages is much higher in adipose tissue of obese individuals than in that of lean subjects, and seems to be a pivotal source of cytokines (Weisberg *et al.*, 2003). In recent years, there has been a large and growing body of research exploring the role of adipokines in periodontal disease associated with diabetes (Johnson and Serio, 2001; Bozkurt *et al.*, 2006; Barksby *et al.*, 2007; Karthikeyan and Pradeep, 2007; Yamaguchi *et al.*, 2007; Preshaw, 2009; Shimada *et al.*, 2010).

Another mechanism may be responsible for the linkage between diabetes and periodontal disease is the significant detrimental influences of diabetes on the immune cell function including polymorphonuclear leukocytes (PMNs or neutrophils), monocytes and macrophages (Mealey, 1999; Lalla *et al.*, 2001; Mealey and Oates, 2006). Indeed, a fundamental component for establishment and maintenance of periodontal health is an intact host immune-inflammatory response. PMNs represent the first-line of nonspecific defence against the invading bacteria and are responsible for killing periodontopathic microorganisms within periodontal pocket (Van Dyke and Serhan, 2003). In diabetes, the adherence, chemotaxis and phagocytosis of neutrophil is diminished and this inhibits their essential function in bacterial killing and allowing proliferation of periodontopathic bacteria which in turn cause further periodontal destruction (Mowat and Baum, 1971; Bagdade *et al.*, 1978; Manouchehr-Pour *et al.*, 1981; Bissada *et al.*, 1982; Marhoffer *et al.*, 1992; Mealey and Oates, 2006). On the other hand, the behaviour of monocytes and macrophages in diabetes is the opposite from that of neutrophil. These cells show hyperactivity in diabetes, it produces significantly higher levels of proinflammatory cytokines and mediators in an attempt to combat the bacterial antigens (Salvi *et al.*, 1997a; Salvi *et al.*, 1997b; Naguib *et al.*, 2004; Mealey and Oates, 2006). In diabetic patients, the peripheral blood monocytes produce higher levels of tumour necrosis factor α (TNF-α) in response to *Porphyromonas gingivalis* antigen compared to monocytes from subjects without diabetes (Salvi *et al.*, 1997a; Mealey and Oates, 2006).

The formation of advanced glycation end-products (AGEs) possibly contributes to the link between the pathogenesis of diabetes and periodontal disease. As a consequence of a prolonged exposure to hyperglycaemic state owing to diabetes, circulated and immobilized proteins become glycated, resulting in irreversible formation of altered

protein molecules known as advanced glycation end-products (AGEs) (Brownlee *et al.*, 1988; Brownlee, 1994; Monnier *et al.*, 1996). Therefore, in people with diabetes and under the influence of hyperglycaemia, the levels of AGEs are elevated, and as a result of this, the expression of high affinity cell surface receptor (RAGEs) for advanced glycation end-products remarkably raise as well (Mealey and Ocampo, 2007). Intriguingly, the formation of AGEs also happens in the periodontium, and greater levels of periodontal AGEs accumulation was detected in those with diabetes than in non-diabetic controls. Furthermore, the expression of the RAGEs in gingival tissues was much higher in diabetic patients when compared with subjects without diabetes (Schmidt *et al.*, 1996b; Katz *et al.*, 2005). Receptor for AGEs (RAGEs) is present on the surface of a diversity of cells such as smooth muscle cells, endothelial cells, neurons, monocytes and macrophages (Schmidt *et al.*, 1994; Pietropaoli *et al.*, 2010). In fact, AGEs-RAGE interaction could well be involved in many cellular and connective tissue alterations occur in gingival tissues of diabetics, which contribute ultimately to increased susceptibility to infection, vascular changed and impaired healing frequently associated with diabetes (Seppala *et al.*, 1997; Soskolne and Klinger, 2001). For instance, the AGEs-RAGE binding on endothelial cells resulting in increase in vascular permeability, hyper-expression of adhesion molecules and thrombus formation (Schmidt *et al.*, 1996a; Wautier and Guillausseau, 1998; Schmidt *et al.*, 1999). Furthermore, the engagement of AGEs to RAGE on monocytes/macrophages increases cellular oxidant stress and provokes the transcription factor nuclear kappa B (NF-κB), which transforms the phenotype of monocytes/macrophages and results in hyper-production of proinflammatory cytokines such as IL-1β, TNF-α and IL-6 (Schmidt *et al.*, 1996a; Schmidt *et al.*, 1999). Therefore, the remarkable elevation in gingival crevicular fluids levels of IL-

1β, TNF-α and IL-6 found in diabetics compared to non-diabetics may be partly explained by the interaction between AGEs and RAGE in periodontal tissues (Engebretson *et al.*, 2004; Mealey and Oates, 2006). Of note, both AGEs and RAGE are elevated in diabetes. The enhanced cellular oxidant stress stemming from AGEs/RAGE interaction drive an altered cellular phenotype and cellular dysfunction which contribute to the pathogenesis of periodontitis in diabetics (Lalla *et al.*, 1998).

All in all, it can be concluded that diabetes is a complex disease characterized by multiple variables that can impact on the development of complications, including periodontitis. Although, the precise mechanism of action is not yet fully comprehended, poor glycaemic control as well as prolonged duration of hyperglycaemic state, are risk factors for periodontitis and dysregulated host function. Most likely, a combination of several factors eventually leads to the elevated prevalence and severity of periodontitis in people with diabetes. These factors may function individually or synergistically to be instrumental in the development of periodontal disease. Continued scientific investigation is fundamentally important for improving our knowledge of the pathological processes that link diabetes and periodontal disease, and thereby to determine the primary target for the treatment of periodontitis in diabetic population.

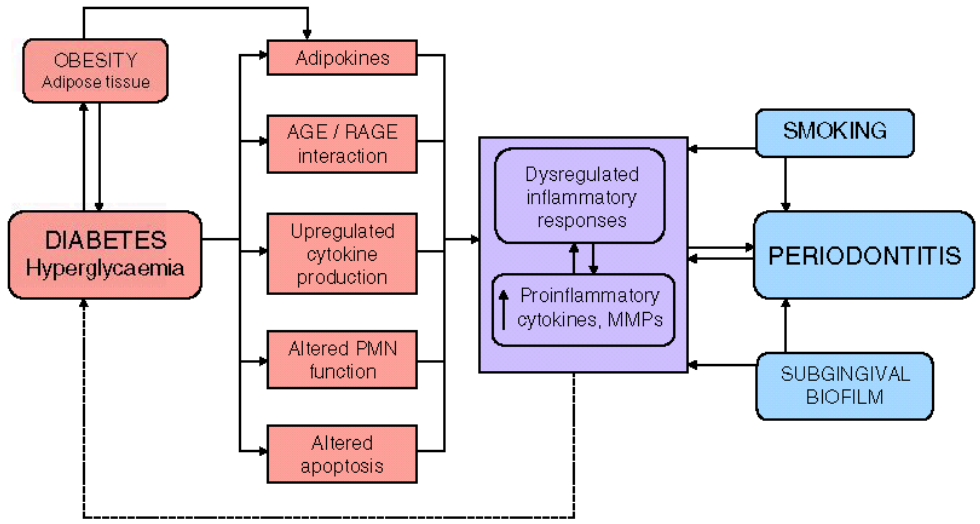


Figure 1.2 Schematic representation of the proposed bi-directional relationship between periodontal disease and diabetes

The exacerbation and dysregulation in the inflammatory responses play a central role in interrelationship between periodontal disease and diabetes. Hyperglycaemia drives numerous proinflammatory effects that influence various body tissues including the periodontium, leading to localized dysregulated immuno-inflammatory reactions. Inappropriate immune-inflammatory responses develop in the periodontium in response to perpetual challenge by subgingival bacteria, which are further aggravated by smoking. Taken from (Preshaw *et al.*, 2012).

1.4 Adipose tissue and adipokines in inflammation and immunity

In recent years there has been an increasing interest of obesity as a major global health problem (Beltowski, 2006; Guzik *et al.*, 2006; Rasouli and Kern, 2008). In fact, a remarkable increase in the prevalence (Rasouli and Kern, 2008) and incidence (Fantuzzi, 2005; Tilg and Moschen, 2006) of obesity has been reported worldwide. Obesity is a chronic metabolic disorder associated with a substantially increasing risk for numerous diseases. These diseases are termed metabolic syndrome which including insulin resistance, diabetes, dyslipidemia, hypertension and cardiovascular disease (Rasouli and Kern, 2008; Catalan *et al.*, 2009). In fact, it is becoming increasingly difficult to ignore the importance of obesity as a crucial risk factor of insulin resistance and type 2 diabetes mellitus (Bloomgarden, 2002). Indeed, there is a large volume of published studies describing the tremendous global increase in adult and children obesity and associated pathologies, specifically cardiovascular disease and type 2 diabetes mellitus (Trayhurn and Wood, 2004; Tilg and Moschen, 2006; Zavalza-Gomez *et al.*, 2008). Obesity is characterized by a pathological accumulation of body fat in an expanded adipose tissue mass. It stems from an imbalance between energy (food) intake and total energy expenditure; in other words, it occurs when the energy intake is in surplus of total energy consumption (Trayhurn, 2007).

There are various methods for appraising overweight, obesity and fat distribution such as measurements of weight, height, waist, hip, and calculations of waist-to-hip ratio (WHR), waist to height ratio (WHtR), and body mass index (BMI) (Al-Odat, 2012; Al-Odat *et al.*, 2012). For several years, the waist-hip ratio (WHR) was used for evaluation of the body fat distribution. But in some studies, it was reported that waist

circumference is more closely associated with the central fat distribution than WHR (Pouliot *et al.*, 1994; Fredriks *et al.*, 2005). Recently, another anthropometric index, waist-to-height ratio (WHtR), was shown to be better correlated with metabolic risk factors (Hsieh and Yoshinaga, 1995; Ashwell and Gibson, 2009). Nevertheless, BMI represent the most widely used measure in definition of adult obesity (Al-Odat, 2012; Al-Odat *et al.*, 2012).

Due to the extraordinary rise in the occurrence of obesity and its metabolic consequences during recent decades, adipose tissue is now the focus of much research effort. Indeed, a large body of evidence has described adipose tissue as an active contributor to whole body homeostasis, through its participation in regulating a variety of physiological and pathological processes in the body (Berg and Scherer, 2005; Matarese *et al.*, 2005; Fonseca-Alaniz *et al.*, 2007; Kralisch *et al.*, 2007; Lago *et al.*, 2007; Wannamethee *et al.*, 2007; Ahima and Osei, 2008; Antuna-Puente *et al.*, 2008; Fernandez-Riejos *et al.*, 2008; Wozniak *et al.*, 2009; Fernandez-Riejos *et al.*, 2010). Over the past few years, the belief that adipose tissue is a passive depot of fat and a layer of insulation has been altered dramatically. Recently, adipose tissue has been recognised as representing an essential and effective endocrine organ, posting and receiving signals that orchestrate appetite, energy consumption, insulin sensitivity, endocrine and reproductive systems, bone metabolism, in addition to inflammatory and immune responses (Fantuzzi, 2005; Tilg and Moschen, 2006; Catalan *et al.*, 2009). The mechanism by which adipose tissue regulates these processes is by expressing and releasing a bioactive messenger molecules such as ‘adipokines’, including leptin, adiponectin, visfatin and resistin, as well as cytokines and chemokines such as TNF-α, IL-6, MCP-1 and PAI. These molecules are secreted either by adipocytes or non-adipocyte fraction of adipose tissue such as the infiltrated

macrophages (Fain *et al.*, 2004; Juge-Aubry *et al.*, 2005; Guzik *et al.*, 2006; Gesta *et al.*, 2007). In fact, adipokines have a considerable physiological effects on various body organs including CNS, liver, bone, reproductive organs, adipose tissues, skeletal muscles, immune cells and vascular system (Hill *et al.*, 2009). Ultimately, the alterations in the production of adipokines stemming from excessive fat accumulation in obesity can lead to a dramatic adverse effect on the health by creating a state of low grade inflammation which could contribute to the development of insulin resistance and type 2 diabetes, may act as a possible linkage between diabetes and periodontal disease (Pischon *et al.*, 2007; Saito and Shimazaki, 2007; Preshaw, 2009; Shimada *et al.*, 2010). This section of the review will concentrate chiefly on discussing the role of adipose tissue and related adipokines in orchestrating inflammation and immunity.

1.4.1 The cellular subtypes of adipose tissue

There are two main forms of adipose tissue, white adipose tissue and brown adipose tissue. The white adipose tissue represents most of the adipose tissue in mammals and is deemed to be an energy depot for the body. On the other hand, brown adipose tissue occurs basically in human neonates and is responsible for body temperature modulation. It is well known that adipocytes represent the vast majority of cells in white adipose tissue, though; white adipose tissue also contains other cell types including pre-adipocytes, endothelial cells, fibroblasts, leukocytes and most significantly macrophages which play a central role in obesity. Mounting evidence highlights the considerable role of resident macrophages in adipose tissue in immune system modulations (Weisberg *et al.*, 2003; Xu *et al.*, 2003; Curat *et al.*, 2004; Fantuzzi, 2005; Tilg and Moschen, 2006; Fonseca-Alaniz *et al.*, 2007; Olefsky and Glass, 2010; Wentworth *et al.*, 2010). For instance, it has been shown that 11% of

cells in stromal-vascular fraction of human adipose tissues are composed of resident macrophages (Curat *et al.*, 2004). Indeed, an elevated numbers of adipose tissue macrophages have been observed in obesity, and is directly proportional to both body mass and adipocyte size (Weisberg *et al.*, 2003). The adipose tissue of obese human and rodents embraces elevated numbers of macrophages, and once activated, macrophages release a host cytokines such as IL1, IL-6 and TNF-α (Wellen and Hotamisligil, 2003). In subjects with obesity and insulin resistance, a high expression of macrophages marker was observed, and was also associated with the expression of IL-6 and TNF-α (Weisberg *et al.*, 2003; Di Gregorio *et al.*, 2005). It is now broadly accepted that the expansion in adipose tissue mass in obesity is correlated with an increased infiltration of a classically activated macrophages phenotype from the circulation (Coenen *et al.*, 2007). These macrophages are commonly recruited to sites of tissue injury and have been shown to be transformed into a pro-inflammatory state with elevated expression of TNF-α (Lumeng *et al.*, 2007). Taken together, increased adipose tissue mass in obese individuals is associated with increased number of activated macrophages in adipose tissue, which seems to be responsible for the low grade chronic inflammatory response observed in obesity.

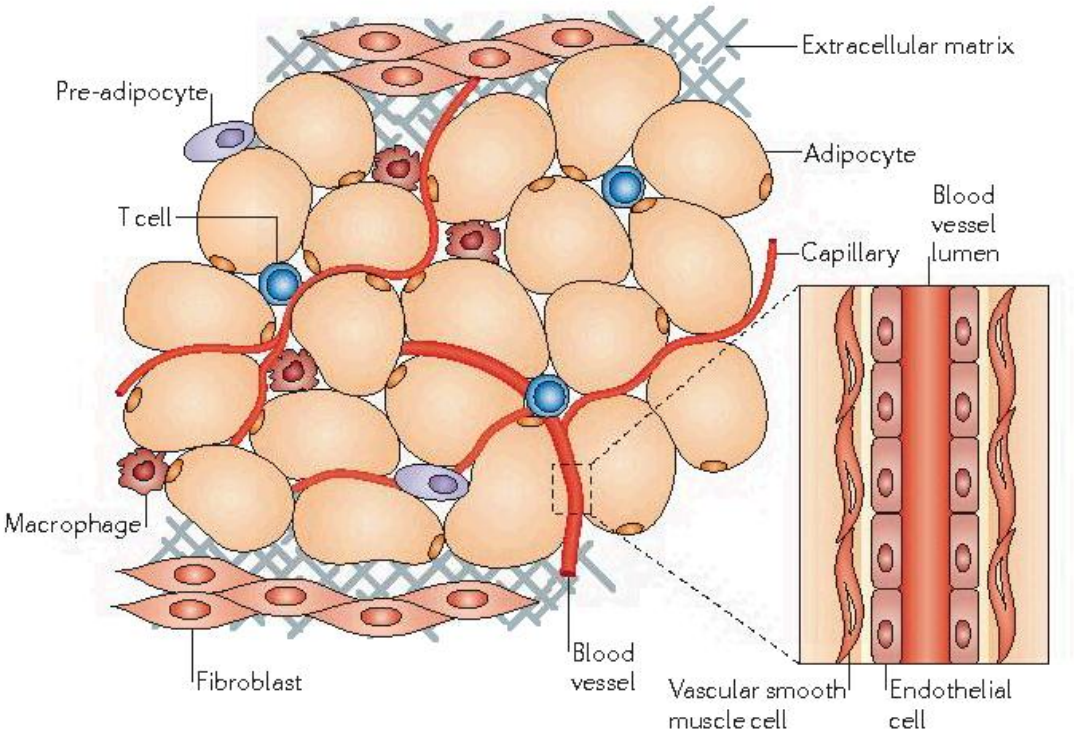


Figure 1.3 Cellular components of adipose tissue

Adipose tissue composed mainly of adipocytes, precursor cells (pre-adipocytes), fibroblasts, vascular cells (such as endothelial cells and smooth muscle cells) and immune cells. It also contains blood vessels and extracellular matrix. Macrophages and T cells represent an active components because of it pivotal role in determining the immune status of the adipose tissue. Taken from (Ouchi *et al.*, 2011).

1.4.2 Visfatin/PBEF/Nampt

Visfatin is a recently discovered adipokine, synthesized and secreted primarily by visceral fat (Fukuhara *et al.*, 2005). It is 52-kDa protein encoding a polypeptide of 491 amino acids (Moschen *et al.*, 2007; Adeghate, 2008; Sommer *et al.*, 2008). Visfatin was originally identified as a cytokine-like secreted protein that synergizes the effect of IL-7 and stem cell factor in promoting the growth and differentiation of B-cell lineage precursors. It was originally called Pre-B cell colony enhancing factor (PBEF) (Samal *et al.*, 1994). This protein has enzymatic activity and acts as a nicotinamide phosphoribosyltransferase (Nampt) (Revollo *et al.*, 2007). In fact, it was demonstrated that visfatin has insulin mimetic properties (Fukuhara *et al.*, 2005), It functions by binding to a distinct site on insulin receptors which differs from the insulin binding sites (Adeghate, 2008). Although Fukuhara et al (2005) study has been retracted, the authors continued to stand by their conclusions (Fukuhara *et al.*, 2007) and other studies suggest an insulin mimetic effect of visfatin on cultured osteoblasts (Xie *et al.*, 2007) and a correlation between plasma visfatin levels with obesity and diabetes (Chen *et al.*, 2006; Sandeep *et al.*, 2007). Despite the fact that visfatin is a visceral adipokine, it has been found in skeletal muscle, liver, bone marrow and lymphocytes, in addition it occurs as a secreted protein in the circulation.

In brief, visfatin/PBEF/Nampt may be considered as a multifunctional protein (Luk *et al.*, 2008) acting as a hormone (Fukuhara *et al.*, 2005), cytokine (Samal *et al.*, 1994) and/or enzyme (Revollo *et al.*, 2007). In other words, visfatin, PBEF, and Nampt are different terms representing an identical protein with multiple biological functions.

One of the fundamental functions of visfatin/PBEF/Nampt is the modulation of immune and inflammatory processes. Moschen et al (2007) has revealed that human

leukocytes can be activated by visfatin/PBEF/Nampt to produce several pro and anti-inflammatory cytokines. On the one hand, visfatin/PBEF/Nampt stimulates CD14+ monocytes to produce IL-1β, TNF-α and IL-6. On the other hand, anti-inflammatory cytokines such as IL-1Ra and IL-10 might be produced by monocytes as a result of visfatin stimulation. Moreover, this adipokine enhanced the surface expression of the co-stimulatory molecules CD54, CD40 and CD80 in CD14+ monocytes (Moschen *et al.*, 2007). Furthermore, it has been noted that visfatin/PBEF/Nampt was able to activate antigen presenting cells (APCs) and enhance phagocytosis in monocytes (Moschen *et al.*, 2007). In addition, trafficking CD14+ monocytes and CD19+ B-cells into sites of inflammation is another important function of visfatin which is deemed to be a strong chemotactic factor for these cells (Tilg and Moschen, 2008b).

Moreover, it has been reported that visfatin activated nuclear factor-kappaB (NF-κB) which have a crucial role in regulating immune responses (Moschen *et al.*, 2007; Adya *et al.*, 2008).

Indeed, it has been illustrated that serum visfatin levels were positively correlated with IL-6 and CRP levels in human serum, which in turn corroborate the significance of visfatin as inflammatory cytokine (Oki *et al.*, 2007). Up-regulation in the visfatin/PBEF/Nampt has been also identified in a variety of pathophysiological conditions of the immune system including rheumatoid arthritis (Nowell *et al.*, 2006; Otero *et al.*, 2006), psoriasis (Koczan *et al.*, 2005), clinical sepsis (Jia *et al.*, 2004), and acute lung injury (Garcia and Moreno Vinasco, 2006). Moschen et al (2007) have been indicated that APCs like dendritic cells and macrophages as well as epithelial cells might be potential cellular sources of visfatin besides the adipose cells. In conclusion, it seems plausible that visfatin/PBEF/Nampt could be deemed as a potentially important immunomodulating regulator.

In regard to visfatin action on the development of insulin resistance and type 2 diabetes mellitus, visceral adiposity is deemed to be more pernicious than subcutaneous obesity (Wajchenberg, 2000). It has been clearly stated that visfatin/PBEF/Nampt was predominantly found in visceral (abdominal) adipose tissue (from which the name visfatin was derived) (Fukuhara *et al.*, 2005). Interestingly, a positive correlation between circulating visfatin/PBEF/Nampt levels and the amount of visceral fat have been reported by Fukuhara et al (2005) in 101 female and male subjects; on the other hand, there was merely a weak correlation between visfatin/PBEF/Nampt concentrations and the amount of subcutaneous fat.

It has been shown that plasma visfatin/PBEF/Nampt levels correlated positively with body mass index (BMI) and percentage of body fat, as well as visceral adipose tissue visfatin gene expression. In short, it has been concluded that serum concentration of visfatin is increased in obesity (Berndt *et al.*, 2005).

Dogru et al (2007) have investigated the plasma visfatin levels in 22 subjects with newly diagnosed and untreated type 2 diabetes mellitus (T2DM), 18 subjects with impaired glucose tolerance (IGT) and 40 controls with normal glucose tolerance (NGT). Interestingly, there was no significant difference in visfatin levels between diabetic and IGT group and also between IGT group and healthy controls, even though, visfatin levels were higher in T2DM group than the controls (Dogru *et al.*, 2007). Furthermore, a significant increase in circulating visfatin/PBEF/Nampt levels in type 2 diabetic patients compared with controls has been shown by Chen et al (2006) in a study carried out on 61 type-2 diabetic patients and 59 gender and age matched controls (Chen *et al.*, 2006). Taken together, in the vast majority of studies, visfatin/PBEF/Nampt values were elevated in diabetic and obese subjects.

Recently, there has been mounting body of studies investigating the association of visfatin with periodontal disease. A pilot study exploring the gene expression signature in pathological gingival tissues has revealed Visfatin/PBEF/Nampt as one of the top 20 genes that have been distinguished in periodontitis lesions (Papapanou *et al.*, 2004). Moreover, stimulating monocytic cell line (THP-1) with *P.gingivalis* and *E.coli* LPS has yielded a differential up-regulation in Visfatin/PBEF/Nampt gene expression by *E.coli* LPS compared with *P.gingivalis* LPS (Barksby *et al.*, 2009). Interestingly, visfatin levels were higher in GCF compared to serum and levels of visfatin in both GCF and serum were elevated in patients with periodontitis, and were positively associated with all periodontal parameters (Pradeep *et al.*, 2011a; Pradeep *et al.*, 2011b). Therefore, visfatin in both GCF and serum may act as a potential marker of inflammation in periodontal disease.

1.4.3 Leptin

Leptin, a 16 kDa protein, is produced and secreted from adipocytes in response to changes in body fat mass. It is encoded by the obese gene named *ob* (also known as *Lep*) (Zhang *et al.*, 1994). Leptin controls feeding behaviour through the central nervous system (CNS). It exerts its biological actions through the activation of leptin receptor (ObR), which is primarily located in the CNS, and in other tissues including adipocytes and endothelial cells. The binding of leptin to its receptor in the hypothalamus lead to the suppression of lipogenesis in adipose tissue through activation of phosphoinositide-3 kinase pathway, sympathetic nervous system and the engagement of adipose tissue endocannabinoid system (Ahima *et al.*, 2000; Buettner

et al., 2006; Buettner *et al.*, 2008). In the CNS, leptin functions to diminish food intake and to increase energy expenditure. Leptin reduces intracellular lipid levels and prevents fat deposition in non-adipose tissue such as skeletal muscle, liver, and pancreatic β-cells, and thereby improving insulin sensitivity and prohibiting lipotoxicity of pancreatic β-cells (Hamilton *et al.*, 1995; Wang *et al.*, 1999; Minokoshi *et al.*, 2002). The lack of leptin or a mutation in leptin receptor genes drives an enormous hyperphagia (abnormally increased appetite) and obesity in animal models (Friedman and Halaas, 1998). Furthermore, it has been reported that ObR mutation and the congenital deficiency in the production of leptin contribute to hyperphagia and severe early onset obesity in human (Montague *et al.*, 1997; Farooqi *et al.*, 2007), nevertheless, the occurrence of these mutations in obese human is scarce. Leptin levels are markedly elevated in obesity (Myers *et al.*, 2008), and are correlated the fat mass and declines with weight reduction (Considine *et al.*, 1996). It has become increasingly evident that leptin plays an important role in modulating the immune response and inflammation. In effect, leptin is deemed to be a proinflammatory cytokine; it increases the production of TNF-α and IL-6 by monocytes and induces the production of CC-chemokine ligands (namely CCL3, CCL4 and CCL5) by macrophages (Santos-Alvarez *et al.*, 1999; Kiguchi *et al.*, 2009). In monocytes, leptin also promotes cell proliferation and migratory responses (Santos-Alvarez *et al.*, 1999; Zarkesh-Esfahani *et al.*, 2004). In addition, leptin levels are elevated in serum and adipose tissue in response to a proinflammatory stimuli, such as TNF-α and LPS (Grunfeld *et al.*, 1996). Moreover, leptin play an important role in regulating the reproductive system and the onset of puberty, and is correlated with hypogonadism (Rasouli and Kern, 2008). In summary, leptin serves as a fundamental

mediator in a constellation of pivotal processes including growth, metabolic control, immune regulation, insulin sensitivity regulation and reproduction.

1.4.4 Adiponectin

Adiponectin is a 30-kDa protein synthesized exclusively by adipocytes (Scherer *et al.*, 1995). It exists at relatively high levels (3-30 µg/ml) in the blood (Ouchi *et al.*, 2003). Serum adiponectin levels markedly diminished in obesity and escalate after weight loss in human and rats, and an increase in adiponectin levels are correlated with a lower incidence of diabetes (Milan *et al.*, 2002; Lin *et al.*, 2007). Also, adiponectin levels are associated inversely with insulin resistance (Arita *et al.*, 1999). In fact, adiponectin has been deemed as an insulin sensitizer and also has anti-diabetic characteristics (Berg *et al.*, 2001; Maeda *et al.*, 2002). Interestingly, it has been suggested that subjects with high adiponectin levels are less likely to develop type 2-diabetes than those with low levels (Lindsay *et al.*, 2002; Spranger *et al.*, 2003). Adiponectin is present in serum in several different isoforms including trimer, hexamer and low molecular weight isoform (Ouchi *et al.*, 2003). Different adiponectin isoforms possess discrete biological functions. Nearly all insulin-sensitizing effects of adiponectin has been contributed to high molecular weight isoform, while the central effect of adiponectin have been linked to hexamer and trimer isoforms (Wang *et al.*, 2008). Weight loss, caloric limitation, and thiazolidinedione (TZD) could elevate circulating adiponectin levels and gene expression in white adipose tissue (Bruun *et al.*, 2003). Furthermore, it has been recently reported that adiponectin has a central effect in the regulation of energy homeostasis (Kadowaki *et al.*, 2008); it enhances energy expenditure and food intake

when it acts on the central nervous system (Kubota *et al.*, 2007). The close association between adiponectin levels and obesity linked metabolic dysfunction has been confirmed through various clinical observations: first, plasma adiponectin concentration inversely associated with visceral fat accumulation (Ryo *et al.*, 2004); second plasma adiponectin concentrations are reduced in patients with type 2 diabetes; and third, elevated levels of adiponectin are correlated with a lower risk for developing type 2 diabetes (Ouchi *et al.*, 2003; Li *et al.*, 2009c).

Expression of adiponectin is also regulated by a proinflammatory mediators such as TNF-α and IL-6 which inhibits the synthesis of adiponectin in adipocyte cell line, this might explain the lower concentration of serum adiponectin in obese individuals, compared with lean individuals (Fasshauer *et al.*, 2003). Mounting evidence suggests that adiponectin interfered with the function of macrophages, contributes to its role in regulating inflammation. Adiponectin abolishes LPS stimulated TNF-α production by macrophages (Yokota *et al.*, 2000), and supresses Toll-like receptor-mediated NF-κB activation in mouse macrophages (Yamaguchi *et al.*, 2005). Moreover, adiponectin induces the production of the anti-inflammatory cytokine IL-10 by human macrophages (Kumada *et al.*, 2004), and inhibits the production of interferon γ by LPS-stimulated human macrophages (Wolf *et al.*, 2004). Also, it has been shown that adiponectin had an anti-inflammatory action on endothelial cells through suppression of TNF-α induced adhesion molecules expression (Ouchi *et al.*, 1999). Furthermore, adiponectin-deficient mice have greater levels of TNF-α gene expression in adipose tissue and TNF-α levels in plasma compare with adiponectin-sufficient mice (Maeda *et al.*, 2002). In contrast, there might be some situations in which adiponectin have proinflammatory effects. In the existence of LPS, high molecular weight adiponectin was shown to increase the synthesis of CXC-chemokine ligand 8 (CXCL8 also known

as IL-8) by human macrophages (Saijo *et al.*, 2005). High molecular weight adiponectin also stimulated the release of IL-6 by human monocytes, while only the low molecular weight isoform had anti-inflammatory effects by reducing the production of IL-6 in response to LPS besides stimulating IL-10 synthesis (Neumeier *et al.*, 2006).

1.5 Resistin and periodontal disease

1.5.1 The discovery, structure and tissue distribution of resistin

Resistin is a recently identified adipocyte-derived hormone that has been shown to play a substantial role in the development of insulin resistance (Steppan *et al.*, 2001a). Resistin was originally identified by three independent groups using a variety of techniques (Holcomb *et al.*, 2000; Kim *et al.*, 2001; Steppan *et al.*, 2001a). A study by Steppan et al.(2001) attempted to screen for genes that are induced during fat cell differentiation but down-regulated in mature adipocytes exposed to thiazolidinediones (TZDs) led to the discovery of a polypeptide, the investigators named resistin (for resistance to insulin) (Steppan *et al.*, 2001a).

Resistin is one amongst a family of three proteins, known as resistin-like molecules (RELMs), which share a conserved pattern of 11 cystien residues at the C-terminal end of the structure (Steppan *et al.*, 2001b).

Recently a new gene family called FIZZ (found in inflammatory zone) has been identified as part of an investigation of molecules associated with allergic

inflammation and airway hyper-responsiveness (AWH) (Holcomb *et al.*, 2000). The described novel gene family comprises of 3 murine genes and two human homologs (mFIZZ1, mFIZZ2, mFIZZ3, hFIZZ1 and hFIZZ3). Interestingly, it was found that mFIZZ3 polypeptide was uniquely expressed in white adipose tissue (Holcomb *et al.*, 2000). Current nomenclature refers to the protein as resistin (Steppan and Lazar, 2002), but adipocyte-specific secreted factor (ADSF) (Kim *et al.*, 2001) and found in inflammatory zone (FIZZ3) (Holcomb *et al.*, 2000) are used synonymously.

Resistin is a 12.5 kDa cysteine rich peptide with a mature sequence consisting of 108 amino acids in humans and 114 amino acids in mice. It includes a 17-amino acid signal peptide, a variable region of 37 amino acids, and a conserved C terminus (Steppan *et al.*, 2001b; Strausberg *et al.*, 2002; Ghosh *et al.*, 2003). The human resistin gene (*Retn*) is sited on chromosome 19, while the mouse resistin gene is located on chromosome 8. The human and mouse resistins share 46.7% analogy at the genomic DNA level, 64.4% sequence similarity at the mRNA levels, and 59% identity at the amino acids levels (Ghosh *et al.*, 2003). It has been postulated that the mature protein constituted of oligomerized resistin molecules that circulates in human peripheral blood in several different low molecular weight and high molecular weight isoforms (Gerber *et al.*, 2005). However, it has been proposed that the inflammatory influence of resistin is independent of its conformation (Silswal *et al.*, 2005), because both the oligomeric and dimeric forms of resistin are able to activate TNF-α and IL-12 in macrophages and monocytes (Silswal *et al.*, 2005).

Adipocytes are the main source of resistin in rodents (Kim *et al.*, 2001; Steppan *et al.*, 2001a; Rajala *et al.*, 2004). Its gene expression and protein secretion is significantly down-regulated by anti-diabetic drugs (TZDs) (Steppan *et al.*, 2001a).

On the other hand, it was found that human resistin is mainly synthesized by non-adipocytes inflammatory resident cells such as peripheral blood mononuclear cells (PBMCs), bone marrow cells, monocytes and most predominantly by macrophages (Savage *et al.*, 2001; McTernan *et al.*, 2002a; Fain *et al.*, 2003; Kaser *et al.*, 2003; Patel *et al.*, 2003; Curat *et al.*, 2006; Jung *et al.*, 2006; Nagaev *et al.*, 2006; Kunnari *et al.*, 2009). Interestingly, the proportion of adipose tissue macrophages is found to be increased with obesity and this may explain the enhanced production of this hormone in obese individuals (Curat *et al.*, 2006). In effect, mounting evidence has identified neutrophils as dominant sources of resistin at the site of inflammation and even systemically during severe bacterial infections (Bostrom *et al.*, 2009; Johansson *et al.*, 2009; Kunnari *et al.*, 2009). In addition, human resistin is expressed in inflamed, fibrotic and damaged liver tissues and also in pancreatic islets (Minn *et al.*, 2003; Bertolani *et al.*, 2006). Paradoxically, some studies have stated that human pre-adipocytes can express resistin, while mature fat cells can not (Janke *et al.*, 2002; Fain *et al.*, 2003). Other studies revealed that resistin can be synthesized by mature human adipocytes (Degawa-Yamauchi *et al.*, 2003).

An attempt have been made by Szalowska et al (2009) to assess the expression of resistin in organs involved in regulation of total body energy metabolism such as liver and adipose tissues. It has been shown that resistin gene and protein expression was significantly higher in liver compared to adipose tissues which may suggest that resistin has other roles in addition to that of adipokine in humans (Szalowska *et al.*, 2009).

It was suggested that resistin maybe one link between obesity, insulin resistance and diabetes in rodents (Steppan *et al.*, 2001a). For instance, the circulating resistin levels were found to be increased in diet induced and genetic forms of obesity in mice and

this was neutralized by administration of anti-resistin which in turn improves blood glucose and insulin action (Steppan *et al.*, 2001a). In addition, impairment of glucose tolerance and insulin action can be obtained by recombinant resistin administration in normal mice (Steppan *et al.*, 2001a). Moreover, the ability of anti-diabetic drugs (TZDs) to down-regulate resistin level support the potential role of resistin in as a link between obesity and diabetes (Steppan *et al.*, 2001a).

A causal role of resistin in glucose homeostasis was demonstrated through animal model studies with altered serum resistin levels in which resistin gene deleted mice showed low blood glucose level after fasting due to reduced hepatic glucose production. This suggests that resistin may play a role in mediating hyperglycaemia associated with obesity (Banerjee *et al.*, 2004).

Several attempts have been made to clarify the importance of resistin in humans. However, the exact role of resistin in human disease is still controversial. Many studies have revealed that elevated human resistin levels were associated with obesity, insulin resistance or diabetes (Azuma *et al.*, 2003; Lu *et al.*, 2006; Tokuyama *et al.*, 2007; Heidemann *et al.*, 2008; Hivert *et al.*, 2008; Chen *et al.*, 2009; Li *et al.*, 2009b). On the other hand, these results differ from some published studies which failed to find this kind of association (Savage *et al.*, 2001; Lee *et al.*, 2003; Heilbronn *et al.*, 2004; Farvid *et al.*, 2005; Dominguez Coello *et al.*, 2008). Interestingly, serum resistin levels were significantly increased in patients with rheumatoid arthritis (Migita *et al.*, 2006) and this may be explained by the fact that resistin acts as an inflammatogenic cytokine have a potent proinflammatory properties as it triggers the release of TNF-α, IL-1 and IL-6 (Bokarewa *et al.*, 2005). Moreover, proinflammatory cytokines such as IL-1, IL-6 and TNF-α have the ability to upregulate resistin mRNA expression in human PBMCs (Kaser *et al.*, 2003; Kunnari

et al., 2009). In addition, LPS dramatically increases resistin expression in human PBMCs (Lu *et al.*, 2002; Kaser *et al.*, 2003; Kunnari *et al.*, 2009) and adipose (Lu *et al.*, 2002; Anderson *et al.*, 2007).

In summary, there is still an on-going debate regarding the precise role of human resistin in obesity, insulin resistance and the development of diabetes.

So far, no previous study has investigated the role of resistin in cross-susceptibility between periodontal disease and diabetes, therefore our research aimed to shine new light on this subject.

1.5.2 Resistin in immunity and inflammation

1.5.2.1 Resistin regulation in immunity and inflammation

Although resistin was firstly postulated to contribute to insulin resistance, it is becoming increasingly difficult to ignore its importance in triggering a proinflammatory state both in vitro as well as in vivo (Bokarewa *et al.*, 2005). It has been shown that resistin expression was up-regulated during the stimulated differentiation of monocytes to macrophages, which potentially indicates a possible role of resistin in macrophage function (Patel *et al.*, 2003).

Furthermore, proinflammatory cytokines such as TNF-α, IL-1 and IL-6 or endotoxin (LPS) can significantly up-regulate the expression of resistin in human macrophages, PBMCs and neutrophils indicating a role of resistin in the inflammatory process (Kaser *et al.*, 2003; Lehrke *et al.*, 2004; Bokarewa *et al.*, 2005; Kunnari *et al.*, 2009;

Shyu *et al.*, 2009). By contrast, in adipocytes and pre-adipocytes, TNF-α treatment markedly reduce resistin mRNA expression and secretion (Fasshauer *et al.*, 2001; Li *et al.*, 2003). Intriguingly, LPS induced resistin gene and protein up-regulation in a variety of blood cells (e.g. rat white blood cells, human PBMCs, human primary macrophages, U937 monocytes, human primary neutrophil) and adipocytes, both in vivo and in vitro (Lu *et al.*, 2002; Lehrke *et al.*, 2004; Kusminski *et al.*, 2007; Kunnari *et al.*, 2009) and the activation of NF-κB is essential prerequisite for LPS induction of resistin in human macrophages, with MAPK activation boosting the magnitude of the response (Lehrke *et al.*, 2004). Also, it has been shown that a dramatic elevation in the circulating resistin levels occurs as a result of endotoxin administration to human volunteers (Lehrke *et al.*, 2004). On contrary, other studies demonstrated that lipopolysaccharide (LPS) have no impact on resistin expression neither in human liver and adipose tissues nor in human U937 monocytes (Yang *et al.*, 2003; Szalowska *et al.*, 2009). Recently, it has been reported that the highly leukotoxic strains of *Aggregatibacter actinomycetemcomitans* induces the release of high levels of resistin in culture supernatants of human neutrophils (Furugen *et al.*, 2011).

Intriguingly, it has been demonstrated that both high glucose and insulin have a substantial impact on resistin expression in human monocytes (Stan *et al.*, 2011). In effect, exposure of human monocytes to high glucose induced a significant up-regulation of resistin on both protein and gene levels, via mechanisms involving MAPKs and transcription factor NF-κB. In addition, insulin decreased high glucose-induced resistin expression (Stan *et al.*, 2011). In 2007, Hu and co-workers demonstrated that CRP could significantly increase resistin mRNA expression and protein secretion in cultured human PBMC, and in a dose-and-time dependent manner (Hu *et al.*, 2007b).

In summary, the regulation of resistin by these regulators is intriguing, however, the underlying physiological and pathophysiological significance remain to be determined.

1.5.2.2 Resistin action in immunity and inflammation

Although, resistin has been thought of as a key factor contribute to insulin resistance, there is increasing body of evidence indicating that it may also be involved in inflammation and immunity. Resistin, per se, serves as a pro-inflammatory mediator. As such, treatment of human PBMCs with recombinant resistin drive to a remarkable up-regulation of gene expression for IL-6, IL-1β and TNF-α, and resistin itself, illustrating that resistin stimulate a positive feedback mechanism on its own expression. The proinflammatory properties of resistin were revoked by NF-κB inhibitor implying the significance of NF-κB signalling pathway for resistin-incited inflammation (Bokarewa *et al.*, 2005). Also, in both human and murine macrophages, resistin boosted the secretion of pro-inflammatory cytokines, IL-12 and TNF-α, and was able to provoke the nuclear translocation of NF-κB transcription factor (Silswal *et al.*, 2005). Notably, resistin-induced TNF-α up-regulation was significantly reduced in the presence of NF-κB inhibitor (Silswal *et al.*, 2005). Similarly, Nagaev et al (2006) have shown that the exposure of white adipose tissue cultures and PBMCs to human resistin induces the inflammatory cytokines IL-6, IL-8 and TNF-α (Nagaev *et al.*, 2006) and that the same induction was found in adipocytes in response to resistin stimulation (Kusminski *et al.*, 2007).

Indeed, the resistin induced inflammation is mediated by NF-κB signalling pathway. This was explained by the marked suppression of resistin proinflammatory activity

following the addition of NF-κB inhibitor to the PBMCs culture (Bokarewa *et al.*, 2005). Up to the present there has been only one published paper where the authors reveal that resistin competes with lipopolysaccharide for binding to TLR4 receptor in human myeloid cells and epithelial cells (Tarkowski *et al.*, 2010). The activation of TLR initiates a cascade of intracellular events resulting in alterations in transcription and signalling pathways including NFκB signalling, hence, antibody blocking of TLR4 was used to manifest that the binding of resistin to human leukocytes and cytokine production by PBMCs in response to resistin induction were inhibited. Likewise, resistin binding was detected in TLR4-transfected human epithelial kidney cell line HEK293, but not with myeloid differentiation factor 2/CD14-transfected, TLR2-transfected or HEK null cells. Since TLR4 bind to exogenous bacterial and viral structures and modulates the protective inflammatory responses of the host, the authors appraised the role of intracellular signalling pathways in resistin-elicited proinflammatory effects in PBMCs. Cells were pre-treated with inhibitors specific for NFκB and MAPKs and then stimulated with resistin. Inhibition of NFκB and MAPKs drove blockage of resistin-induced expression of IL-6, IL-1β and TNF-α at both mRNA and protein levels. These results point out that the proinflammatory intracellular signals induced by resistin are mediated via NFκB and MAPKs signalling mechanisms and are possibly initiated by resistin binding to the TLR4 (Tarkowski *et al.*, 2010). Resistin also suppressed chemotaxis of neutrophils and reduced the oxidative burst provoked by *Escherichia coli* (*E.coli*) and by phorbol myristate acetate (PMA), but it did not impact on neutrophils phagocytosis (Cohen *et al.*, 2008; Cohen and Horl, 2009). On the other hand, resistin induced CD4-positive cell chemotaxis in a concentration-dependent manner (Walcher *et al.*, 2010). Furthermore, It has been suggested that resistin decreases the antigen-uptake process

and the endocytic capacity of lipoteichoic acid (LTA)-stimulated dendritic cells, and thereby interferes with the effectiveness of immune responses induced by Gram positive bacterial infection in human dendritic cells (Son *et al.*, 2008). Incubation of RAW264.7 mouse macrophage cells with resistin resulted in an up-regulation in COX-2 expression, a key enzyme regulating the production of prostaglandin, through NF-κB route. Also an up-regulation in NF-κB subunit p65 was recorded in response to resistin stimulation indicating that resistin possibly activates NF-κB via up-regulation of p65 (Zhang *et al.*, 2010a). In effect, it has been shown that resistin stimulates the expression of cytokines and chemokines in human articular chondrocytes (Zhang *et al.*, 2010b). Resistin has been revealed to stimulate the gene expression of 20 tested cytokines and chemokines in chondrocytes from both normal and osteoarthritic cartilage, these encompassed TNF-α, IL-1β, IL-1α, CCL2, CCL3, CCL3L1, CCL4, CCL5, CCL8, CXCL1, CXCL2, and CXCL3 (Zhang *et al.*, 2010b). It has been suggested that NFκB and C/EBPβ signalling pathways contributes to the resistin-induced up-regulation of these cytokines and chemokines in chondrocytes in response to resistin stimulation (Zhang *et al.*, 2010b). Furthermore, treatment of mouse cartilage cultures with recombinant resistin provoked proinflammatory cytokines and PGE(2) production (Lee *et al.*, 2009). In 3T3-L1 adipocytes, resistin enhanced the production of TNF-α, IL-6 and monocyte chemoattractant protein 1 (MCP-1) (Fu *et al.*, 2006). Furthermore, treatment of cultured human stellate cells with recombinant resistin resulted in an enhanced expression of MCP-1 and IL-8 through activation of Ca^{2+}/NFκB dependent pathway (Bertolani *et al.*, 2006). In mice, resistin remarkably boosted hepatic inflammation and necrosis in LPS-stimulated liver damage (Beier *et al.*, 2008). This impact of resistin was possibly mediated

through activation of mechanisms comprising the coagulation cascade and fibrin accumulation (Beier *et al.*, 2008).

Mounting evidence suggests that the pathogenesis of atherosclerosis is significantly linked with resistin-stimulated inflammatory process. It has conclusively been shown that resistin provokes the expression of VCAM-1, ICAM-1 and MCP-1, and concomitantly decreases TNF-receptor associated factor-3 (TRAF-3) expression on endothelial cells (Verma *et al.*, 2003; Kawanami *et al.*, 2004). It has been reported that resistin up-regulates both the P-selectin and fractalkine (fk) gene and protein expression in human endothelial cells, and the resulting augmented monocytes adhesion by a mechanisms involving a rise in NADPH oxidase activity and reactive oxygen species (ROS) production and activation of NF-κB and AP-1 (Manduteanu *et al.*, 2009a; Manduteanu *et al.*, 2009b; Pirvulescu *et al.*, 2011). Moreover, elevated concentrations of resistin released in conditional media from epicardial adipose tissue of patients with acute coronary syndrome greatly affect *in vitro* endothelial function by significantly increasing endothelial cell permeability. These results suggest that epicardial adipose tissue-produced resistin is a pivotal stimulator of endothelial damage via the stimulation of hyper-permeability in human umbilical vein endothelial cells (HUVECs) (Langheim *et al.*, 2010). Recently, *in vitro* study has shown that resistin caused a remarkable increase in monocyte adhesion to HUVECs and also up-regulated the expression of ICAM-1 and VCAM-1 by endothelial cells (Hsu *et al.*, 2010). Also, resistin enhanced monocyte infiltration into collagen by direct chemoattractive effect as well as by augmenting migration toward monocyte chemoattractant protein-1 (Cho *et al.*, 2011). In human coronary artery endothelial cells (HCAECs), resistin provoked both endothelial proliferation and migration, and markedly up-regulate the gene expression of vascular endothelial growth factor

receptors (VEGFR-1 and VEGFR-2) and matrix metalloproteinases (MMP-1 MMP-2) at both gene and protein levels (Mu *et al.*, 2006), and resistin also up-regulates tissue factor expression in these cells and thereby potentially contributing to atherothrombosis (Calabro *et al.*, 2011). In addition, treating human vascular smooth muscle cells (VSMCs) with recombinant human resistin stimulated MMP-2 and MMP-9 protein and gene expression and induced smooth muscle migration in vitro (Ding *et al.*, 2011).

Overall, these observations provide the evidence that resistin was unquestionably involved in inflammation, although more studies are needed to clarify its precise role in these conditions.

1.5.2.3 Role of resistin in inflammation-related diseases

There is a large volume of published studies describing the important role of resistin in chronic inflammatory disease. In inflammatory bowel disease, it has been revealed that circulating resistin levels were raised and directly associated with white blood cell count, CRP levels and disease activity (Konrad *et al.*, 2007). Elevated circulating resistin levels were also recorded in patients with chronic pancreatitis indicating its influence on pancreatic fibrosis development (Adrych *et al.*, 2009). In patients with systemic lupus erythematosus, comparable serum resistin levels to those in controls were recorded (Almehed *et al.*, 2008). Nonetheless, circulating resistin levels were clearly associated with general inflammation, renal disease, treatment with glucocorticoids, and bone loss in Systemic lupus erythematosus patients (Almehed *et al.*, 2008).

Interestingly, resistin levels correlated positively with levels of inflammatory markers including CRP, TNF-α receptor 2 and IL-6 in both healthy subjects with family history of atherosclerosis and in middle aged and older Chinese population (Reilly *et al.*, 2005; Qi *et al.*, 2008). By contrast, in another study, serum resistin levels were not significantly correlated with TNF-α and IL-6 in elderly Japanese (Furugen *et al.*, 2008).

Recently, several groups have directed their efforts toward the elucidation of the pro-inflammatory influence of resistin in the pathogenesis of arthritis. Healthy mice injected intra-articularly with recombinant mouse resistin in the knee joints developed arthritis compared with mice injected with albumin (Bokarewa *et al.*, 2005). These mice revealed infiltration of synovial tissue with leukocytes associated with hyperatrophy of synovial lining layer and panus formation (Bokarewa *et al.*, 2005). In human, synovial fluid from patients with rheumatoid arthritis revealed significantly higher level of resistin compared with control samples. Furthermore, resistin level in synovial fluid of rheumatoid arthritis patients was positively correlated with synovial leukocyte count and IL-6 synovial fluid level (Bokarewa *et al.*, 2005; Senolt *et al.*, 2007). However, contradictory results on circulating resistin levels in rheumatoid arthritis patients have been reported. While some studies revealed unaltered resistin serum levels in rheumatoid arthritis patients compared to healthy counterparts (Bokarewa *et al.*, 2005; Otero *et al.*, 2006; Forsblad d'Elia *et al.*, 2008), Migita et al (2006) identified greater serum resistin levels, associated with rheumatoid arthritis disease activity markers, CRP, TNF-α and erythrocyte sedimentation rate (Migita *et al.*, 2006).

Furthermore, severe streptococcal infectious conditions such as toxic shock syndrome and necrotizing fasciitis are characterized by hyperresistinemia in circulation as well

as at the local site of infection (Johansson *et al.*, 2009). There is a plethora of pleotropic diseases which are associated with elevated circulating resistin levels, some of them are listed in Table 1.1.

Taken together, although elevated levels of resistin have been correlated with these inflammation-related diseases, further studies should be conducted to clarify the exact role of resistin in these pathophysiological conditions.

Table 1.1 Summary of diseases that have been associated with increased circulating resistin levels

Type of disease	Author	Resistin levels (ng/ml)	P value
Inflammatory arthritis	(Kontunen *et al.*, 2011)	17.4 (case)	<0.001
		10.8 (control)	
Blood hypertension	(Thomopoulos *et al.*, 2011)	11.9 (7.8-16.8) (case)	<0.008
		6.8 (4-9) (control)	
Obesity	(Azuma *et al.*, 2003)	24.6± 12.9 (case)	<0.01
		12.8 ±8.3 (control)	
Non-alcoholic fatty liver disease	(Pagano *et al.*, 2006)	5.87±0.49 (case)	<0.01
		4.3±0.2 (control)	
Alcoholic acute pancreatitis	(Daniel *et al.*, 2010)	12.9±6.38 (case)	<0.05
		4.06±2.63 (control)	
Chronic kidney disease	(Kawamura *et al.*, 2010)	9.5 (normal&CKD1)	<0.001
		10.2 (CKD level 2)	
		11.8 (CKD level 3)	
		21.1 (CKD level 4)	
Ankylosing spondylitis	(Kocabas *et al.*, 2012)	1.58±0.52 (case)	<0.01
		1.13±0.46 (control)	
Acute appendicitis	(Kisacik *et al.*, 2012)*	26.3±11.9 (case)	<0.001
		13.8±5.7 (control)	
Critically ill patients with/without sepsis	(Koch *et al.*, 2009)	18 (3.22-50) (all ICU)	<0.001
		4.7 (2.2-12.7) (control)	
		24.2 (3.22-50) (sepsis)	
		10.5 (3.33-41.1) (Non-sepsis)	
Bahçet's disease	(Kim *et al.*, 2010)	36.8±29.7 (case)	<0.001
		19.5±8.3 (control)	
Postburn insulin dysfunction	(Duffy *et al.*, 2009)	31.04 (case)	<0.05
		11.02 (control)	

*The unit for resistin levels (U/L)

1.5.3 Resistin in obesity, insulin resistance and diabetes

1.5.3.1 Biological mechanisms of resistin regulation and resistin action

Notwithstanding that resistin was first reported as a factor implicating in the development of insulin resistance and diabetes in humans, controversy is still persistent with regard the precise role it plays in obesity, insulin resistance and the pathogenesis of type 2 diabetes mellitus. When resistin was first described by Steppan and co-workers in 2001, a number of momentous discoveries were stated. First, resistin levels were elevated in genetic and diet-induced forms of obesity in rodents. In addition, administration of anti-resistin antibody augmented insulin sensitivity in obese and insulin-resistant animals. Also, treatment with recombinant resistin impaired glucose tolerance and insulin action in healthy mice. And lastly, resistin administration impaired insulin-induced glucose up-take in adipocytes. From these findings, it was concluded that resistin plays a pivotal role in obesity and insulin resistance in the diabetic mouse model (Steppan *et al.*, 2001a), however, to which extent these observations can be applied to human studies is difficult to be determined.

Recently, an abundance of evidence has emerged corroborating the pivotal role of resistin in obesity and insulin resistance. It has been demonstrated that resistin is expressed in human hepatocytes and hepatic tissues and provokes insulin resistance (Sheng *et al.*, 2008). Furthermore, resistin mRNA levels were easily detectable in human PBMCs and revealed to be higher in female patients with type 2 diabetes compared to healthy women, suggesting that resistin might contribute to the pathogenesis of human type 2 diabetes (Tsiotra *et al.*, 2008). Resistin is potentially

implicated in sensing the nutritional status as its mRNA level is reducing during fasting and elevating after food consumption (Kim *et al.*, 2001; Steppan *et al.*, 2001a; Nogueiras *et al.*, 2003; Valsamakis *et al.*, 2004). Intriguingly, a remarkable induction of resistin was found during 3T3-L1 and pre-adipocyte differentiation into adipocytes, therefore, this protein was considered to be adipose sensor through its function as a feedback regulator of adipogenesis (Holcomb *et al.*, 2000; Kim *et al.*, 2001; Liu *et al.*, 2008a). Furthermore, some studies have revealed that resistin gene expression was significantly up-regulated by glucose and mediators acknowledged to increase plasma glucose levels such as glucocorticoids (Haugen *et al.*, 2001; Shojima *et al.*, 2002; Stan *et al.*, 2011). It has been reported that resistin mRNA and protein levels decrease in parallel to glucose and insulin during fasting and are restored after re-feeding. In effect, both adipose resistin expression and serum resistin levels are enhanced in response to hyperinsulinemia and hyperglycemia (Rajala *et al.*, 2004). Numerous studies have investigated the effects of insulin in the regulation of resistin production, but the results were rather incompatible. Kim et al (2001) demonstrated that resistin mRNA expression in adipose tissue of streptozotocin-diabetic mice was significantly up-regulated upon insulin administration (Kim *et al.*, 2001). Similarly, insulin instigated resistin protein secretion in a concentration-dependant manner in adipocytes (McTernan *et al.*, 2003). Conversely, other studies have shown that insulin administration caused inhibition in resistin gene expression (Haugen *et al.*, 2001; Shojima *et al.*, 2002; Kawashima *et al.*, 2003; Liu *et al.*, 2008a). Moreover, the ability of anti-diabetic drugs thiazolidinediones (TZDs); which lower blood glucose by activating Peroxisome proliferator-activated receptor γ (PPARγ); to modulate resistin expression has been demonstrated in a number of studies. Some studies showed a significant down-regulation of resistin gene expression in response to TDZs

treatment (Haugen *et al.*, 2001; Steppan *et al.*, 2001a; Shojima *et al.*, 2002; McTernan *et al.*, 2003). On contrary, an up-regulation of resistin gene expression in response to TZDs treatment has been reported in several other studies (Way *et al.*, 2001; Fukui and Motojima, 2002). Accordingly, the modulation of resistin levels is apparently unrelated to the anti-diabetic effects of TZDs.

One of the most conspicuous biological influences of resistin which has been widely studied is the regulation of glucose homeostasis and insulin sensitivity. Some studies reported impaired glucose tolerance and insulin action in response to resistin as well as a positive association of elevated circulating resistin levels with hyperinsulinemia and hyperglycemia (Steppan *et al.*, 2001a; Rajala *et al.*, 2004), nevertheless, these findings are not without debate. A data from several sources have identified the reduced resistin mRNA expression in adipose tissue of rodent model with obesity associated with impaired insulin sensitivity (Juan *et al.*, 2001; Way *et al.*, 2001; Fukui and Motojima, 2002; Milan *et al.*, 2002; Maebuchi *et al.*, 2003; Haluzik *et al.*, 2006). Furthermore, remarkable attenuation in glucose uptake was detected in differentiated pre-adipocytes treated with recombinant resistin (McTernan *et al.*, 2003).

One of the prominent influences of resistin is the induction of insulin resistance in rodent pancreatic islets. This could be attributable to the action of resistin in reducing the expression of insulin receptor on both gene and protein levels. Notably, low concentration of resistin enhanced pancreatic beta-cell viability. However, resistin did not influence insulin secretion (Brown *et al.*, 2007). In effect, resistin induces insulin resistance in pancreatic islet β-cell at least partially via up-regulation of SOCS-3 and decrease of Akt phosphorylation and impairs glucose-induced insulin secretion (Nakata *et al.*, 2007). It has been hypothesised that resistin serve as a putative regulator of insulin in diurnal feeding/fasting rhythm via a negative feed back

regulation of its action (Oliver *et al.*, 2006). Moreover, in vivo study done by Liu et al (2008) have shown that over-expressing resistin in mice by intramuscular injection of a recombinant eukaryotic expression vector encoding resistin gene increases serum glucose levels, and might be responsible for insulin resistance (Liu *et al.*, 2008c).

The liver is the chief metabolic organ of glucose metabolism. Hepatic insulin resistance is deemed to be the principal cause leading to the development of type 2 diabetes mellitus (Taniguchi *et al.*, 2005). Muse et al (2004) demonstrated that the plasma resistin level is increased after high-fat feeding in rodents, and that this increase is the main cause of hepatic insulin resistance (Muse *et al.*, 2004). Data from several animal studies have spotlighted the ability of resistin to stimulate glucose production and provoke hepatic insulin resistance after both acute and chronic administration (Steppan *et al.*, 2001a; Rajala *et al.*, 2003; Banerjee *et al.*, 2004; Rangwala *et al.*, 2004). It has also been shown that the upregulation of human resistin inhibited significantly insulin-stimulated glucose uptake and glycogen synthesis in HepG2 cells (Sheng *et al.*, 2008). In 2007, Muse and co-workers published a paper in which they demonstrated that the central (hypothalamus) administration of resistin stimulated glucose production and diminished hepatic insulin action on glucose homeostasis independent of circulating levels of glucoregulatory hormones. Reciprocally, central antagonism of resistin action remarkably reduced the ability of circulating resistin to augment glucose production in the presence of physiological hyperinsulinemia. Furthermore, following central infusion of resistin, hepatic gene expression analysis revealed an increase in IL-6, TNF-α and SOCS-3 mRNA which was partly due to centrally mediated actions of resistin. Thus, it is plausible that the central resistin action can contribute to hyperglycaemia in type 2 diabetes mellitus (Muse *et al.*, 2007). Similarly, central infusion of resistin in normal mice enhanced

endogenous hepatic glucose production, consistent with induction of hepatic insulin resistance. Centrally administered resistin also supressed insulin mediated phosphorylation of Akt, enhanced the expression of glucose-6-phosphatase; the enzyme regulating glucose output in the liver; and induced the expression of proinflammatory cytokines and SOCS-3 in liver. Central administration of resistin was associated with neuronal activation in the nuclei and enhanced neuropeptide Y (NPY) expression in the hypothalamus which is considered to be an important regulator of hepatic insulin sensitivity (Singhal *et al.*, 2007). Strikingly, resistin has also been shown to provoke insulin resistance in HepG2 cells partially through the induction of SOCS-3 expression and the inhibition of Akt phosphorylation via an AMPK-independent mechanism. Resistin also enhances hepatic glucose production by increasing the expression of genes encoding the hepatic gluconeogenic enzymes glucose-6-phosphatase (G6Pase) and PEPCK, which are mediated by AMPK (Zhou *et al.*, 2007; Luo *et al.*, 2009). Also, treatment of rat hepatoma cell line H2IIE with resistin provoked insulin resistance but did not influence glucose output. Indeed, resistin diminished various actions of insulin including insulin-induced glycogen synthesis and phosphorylation of IRS, protein kinase B/Akt, as well as GSK-3β. On the other hand, resistin exposure remarkably stimulates the gene and protein expression of SOCS-3 which may contribute to the resistin-mediated suppression of insulin signalling in H2IIE hepatocytes (Liu *et al.*, 2008b).

The effect of resistin on the glycogen metabolism in the liver was discussed thoroughly by Yang et al (2009). A reduction in insulin-stimulated glycogen content was detected in hepatocytes exposed to resistin in the presence of insulin. Also, treatment of hepatocytes with resistin reduced insulin receptor expression and glycogen synthase (GS) activity and increased glycogen phosphorylase (GP) activity.

In other words, a high concentration of resistin decreases the sensitivity of the liver to insulin, enhancing glycogenolysis (by boosting GP activity) and reducing glyconeogenesis (by minimizing the level of insulin receptor and minimizing GS activity), which causes a reduction in the glycogen content of the cells and an elevated blood glucose levels. Hyperglycemia stimulates pancreatic beta cells to release insulin and give rise to hyperinsulinemia. Hyperinsulinemia then drives to insulin resistance in insulin target tissues such as liver (Yang *et al.*, 2009). A recent study by Park et al (2011) involved generating a mouse model of humanized resistin expression to explore the impact of human resistin on glucose homeostasis in inflammatory state. In the generated mouse model, the circulating resistin levels was within normal human range, and similar to human, was elevated in response to lipopolysaccharides. Human resistin was incited in response to inflammation. Resistin reduced endotoxemia-stimulated hypoglycaemia by provoking insulin resistance in liver and adipose tissues, and boosted hepatic insulin resistance by aggravating inflammatory responses in chronic endotoxemia. Thus, in this mouse model resistin regulates glucose homeostasis under inflammatory state (Park *et al.*, 2011).

The most pivotal site for insulin-stimulated glucose uptake is skeletal muscle, and this could be attributable to its mass. Numerous studies have demonstrated that resistin impairs insulin-stimulated glucose uptake in L6 muscle cells by a mechanism including diminished plasma membrane GLUT4 translocation (Palanivel *et al.*, 2006; Fan *et al.*, 2007; Jorgensen *et al.*, 2009), or by reducing the intrinsic activity of cell surface glucose transporter (Moon *et al.*, 2003) and thereby inducing skeletal muscle insulin resistance.

An attempt has been made by Li et al (2010) to explore the influences of resistin on skeletal glucose metabolism, and was performed by constructing a recombinant

plasmid which express resistin and transfecting it into C2C12 monocytes. It has been shown that resistin diminished insulin-induced glucose uptake in C2C12 monocytes by inhibiting the expression of GLUT4 gene, still had no observable impacts on glucose oxidation or glycogen synthesis (Li *et al.*, 2010).

Summarising the currently available evidence, the pro-diabetic effects of resistin could be attributable to one of the following mechanisms: (1) stimulation of hepatic glucose production presumably via reduced activity of AMP-activated protein kinase and enhanced expression of gluconeogenic enzymes in liver (Banerjee *et al.*, 2004); (2) enhancement of free fatty acid release from adipose tissue (Pravenec *et al.*, 2006); (3) blockage of insulin signal transduction pathways (Sheng *et al.*, 2008); (4) reductions of the intrinsic activity of cell surface glucose transporters (Moon *et al.*, 2003); (5) inhibition of GLUT4 translocation (Palanivel *et al.*, 2006) or its gene expression (Fu *et al.*, 2006); and (6) induction of suppressor of cytokine signalling 3 (SOCS-3), a known inhibitor of insulin signalling (Steppan *et al.*, 2005). Nonetheless, identification of the receptor system for resistin and their downstream signalling pathways is required for complete evaluation of the role of resistin system in human physiology.

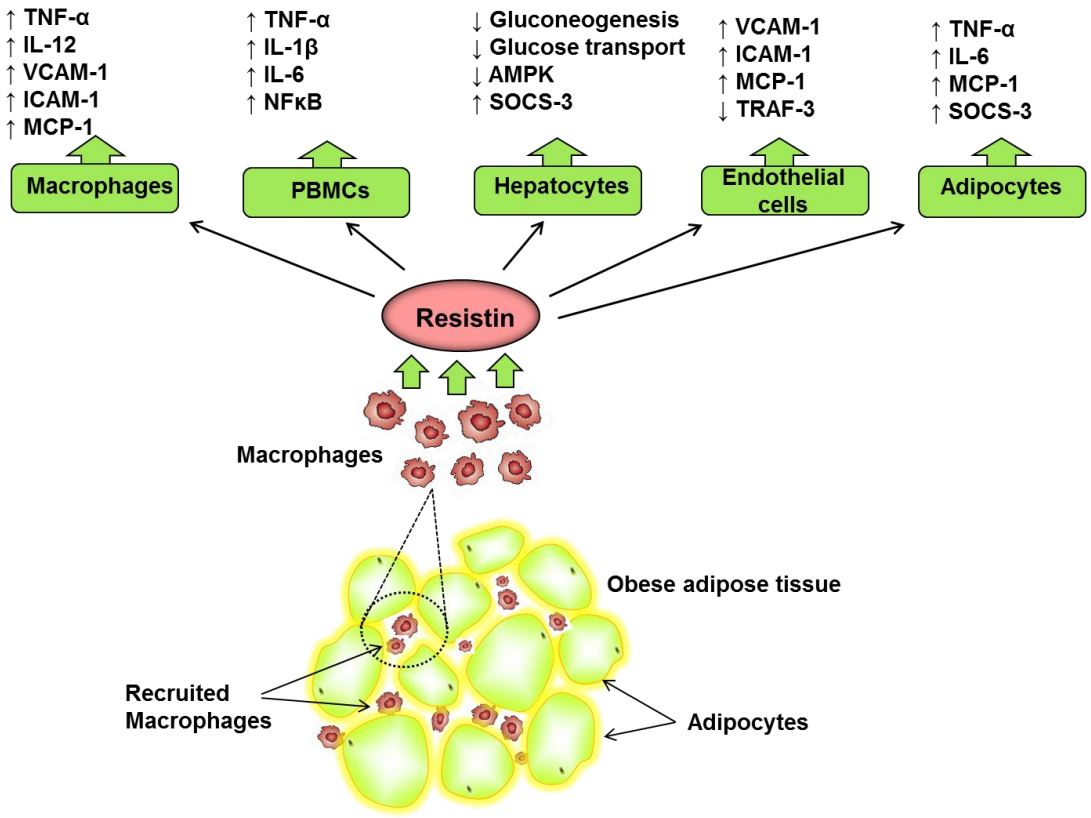


Figure 1.4 The cellular source and the potential effect of human resistin on different cell types

Weight gain is associated with an expansion of adipose tissue accompanied with recruitment of macrophages within these tissues. These macrophages might contribute to local and systemic inflammation, insulin resistance and cardiac pathology via the synthesis of resistin, which might stimulate multiple cell types to regulate various mediators.

1.5.3.2 Clinical evidence for resistin in human obesity, insulin resistance and diabetes

A considerable amount of literature has been published on the contribution of resistin in obesity, insulin resistance and diabetes in human. Although some studies revealed an association links resistin with obesity, insulin resistance or diabetes in humans (Azuma *et al.*, 2003; Silha *et al.*, 2003; Lu *et al.*, 2006; Osawa *et al.*, 2007; Tokuyama *et al.*, 2007; Heidemann *et al.*, 2008; Hivert *et al.*, 2008; Chen *et al.*, 2009; Li *et al.*, 2009b). Other published studies (Savage *et al.*, 2001; Lee *et al.*, 2003; Heilbronn *et al.*, 2004; Farvid *et al.*, 2005; Utzschneider *et al.*, 2005; Dominguez Coello *et al.*, 2008) failed to find this kind of association. Ultimately, there has been little agreement on what is the actual role of resistin in those human pathologies.

Emerging evidence suggests that diabetes is accompanied by changes in serum resistin levels. For instance, one recent study investigated the relationship between circulating resistin levels and diabetes and found that for both women and men, resistin levels were significantly higher in case patients than in control subjects (Chen *et al.*, 2009).

In 2005, Hasegawa and co-workers have investigated the significance of resistin in the pathophysiology of diabetes. A significant increase in resistin levels was recorded in patients with type 2 diabetes compared with non-diabetic subjects. However, no correlation was observed between serum resistin levels and markers of insulin resistance, obesity or hyperlipidaemia (Hasegawa *et al.*, 2005).

Lau and Muniandy (2011) proposed a novel adiponectin-resistin (AR) index, by taking into consideration both adiponectin and resistin levels to produce a better indicator of the metabolic homeostasis and metabolic disorders. Moreover, by

integrating the AR index into an existing insulin resistance index, a novel insulin resistance index (IR_{AR}) was proposed to provide improved diagnostic biomarkers of insulin sensitivity. In this case control study, anthropometric clinical and metabolic parameters encompassing fasting serum total adiponectin and resistin levels were measured. It has been conclusively shown that the AR index was more robustly correlated with increased risk of T2DM and metabolic syndrome (MS) than hypoadiponectinemia and hyperresistinemia alone. Notably, hyperresistinemia was correlated with increased risk of T2DM and MS in Malaysian men. Also, resistin levels were significantly greater in T2DM (without MS) as compared to healthy individuals (Lau and Muniandy, 2011).

A number of studies have found that serum resistin level was significantly higher in obese individuals than in lean subjects (Azuma *et al.*, 2003; Al-Harithy and Al-Ghamdi, 2005). Conversely, other studies have failed to find a significant difference in serum resistin levels between lean healthy and obese individuals (Lee *et al.*, 2003; Silha *et al.*, 2003; Utzschneider *et al.*, 2005).

It has been shown that serum resistin levels were positively correlated with BMI, waist circumference, WHR, HOMA-IR index, serum insulin, plasma glucose and HbA1c levels (Azuma *et al.*, 2003; Al-Harithy and Al-Ghamdi, 2005; Utzschneider *et al.*, 2005; Lu *et al.*, 2006; Mojiminiyi and Abdella, 2007; Norata *et al.*, 2007; Osawa *et al.*, 2007; Li *et al.*, 2009b; Lau and Muniandy, 2011). On the other hand, a negative or no association between resistin levels and BMI, waist circumference, waist-to-hip ratio, HOMA-IR, fasting plasma glucose or insulin levels was reported by other studies (Lee *et al.*, 2003; Heilbronn *et al.*, 2004; Pagano *et al.*, 2006; Won *et al.*, 2009).

The results of the various efforts to determine the circulating levels of resistin and its association with T2DM, insulin resistance and obesity were conflicting, with some, but not all studies demonstrating a significant correlation between serum resistin levels and T2DM, insulin resistance and obesity. Different explanations could account to these discrepancies including different demographic of study groups, the low number of patients enrolled in different studies, and the use of different assay methods. To date, there is substantial evidence regarding the role of resistin in diabetes, IR and obesity (see Table 1.1 and 1.2).

Table 1.2 Summary of studies examining resistin levels in T2DM, insulin resistance and obesity

Author	Study population	Resistin levels (ng/ml) mean±SD	Main findings
(Chen *et al.*, 2009)	T2DM women 359	13.0±1.9	In both women and men T2DM patients had significantly higher levels of resistin than controls Resistin levels were higher in women than men in case subjects and control
	Control women 359	10.8 ±1.7	
	T2DM men 170	10.9±2.0	
	Control men 170	9.6±1.8	
(Heilbronn *et al.*, 2004)	Non-obese 38	4.1±1.7	Serum resistin concentrations were not different among the three groups
	Obese 12	4.2±1.6	
	Obese with T2DM 22	3.7±1.2	
(Al-Harithy and Al-Ghamdi, 2005)	Diab women 44	19.42± 3.60	Fasting serum resistin showed a significant increase from lean to overweight/obese non-diabetics to diabetic women
	OW/OB non-diab 21	16.29±2.29	
	24 lean women	11.59±2.08	
(Hasegawa *et al.*, 2005)	T2DM 111	24.7±2.6	Resistin levels were increased significantly in T2DM patients compared to non-diabetic controls
	Without T2DM 98	15.0±1.2	
(Lu *et al.*, 2006)	Obese diab 30	24.05±9.07	The levels of plasma resistin were significantly increased in diabetes compared to control and in obese diabetics compared to non-obese diabetics
	Non-obese diab 30	18.64±4.65	
	Healthy controls 28	14.16±5.25	
(Won *et al.*, 2009)	With metabolic syndrome 54	8.5±3.6	The levels of plasma resistin were not significantly increased in subjects with metabolic syndrome (MetS) compared to those without MetS Also, Resistin levels were significantly higher in women than men . Plasma resistin not associated with markers of IR, obesity
	Without metabolic syndrome 222	8.3±4.3	

Table 1.3 Summary of studies examining resistin levels in T2DM, insulin resistance and obesity (continued)

Author	Study population	Resistin levels (ng/ml) mean±SD	Main findings
(Norata *et al.*, 2007)	With MetS Without MetS Women 641 Men 449	4.85±2.30 4.15±2.24 4.2±2.5 4.5±2.2	Plasma resistin levels were significantly increased in the presence of MetS Plasma resistin levels were significantly higher in men compared to women
(Dominguez Coello *et al.*, 2008)	T2DM 71 Without T2DM 642 Obese 225 Non-obese 488	3.1±0.2 3.2±0.1 3.1±0.1 3.2±0.1	no significant differences were found in serum resistin levels between subjects with and without T2DM and also in subjects with and without obesity Resistin was inversely associated with insulin resistance and obesity
(Hivert *et al.*, 2008)	With MetS 983 Without MetS 1373	13.6±1.50 12.8±1.49	Plasma resistin levels were significantly increased in the presence of MetS also increased levels of resistin are associated with insulin resistance in subjects at high or low diabetes risk
(Utzschneider *et al.*, 2005)	lean,insulin sensit 53 lean,insulin resist 67 obese,insulin resist 48	5.36±0.3 5.70±0.4 5.94±0.4	No significant difference was recorded in resistin levels between the three groups Resistin is unlikely to be a major mediator of IR or the MetS
(Azuma *et al.*, 2003)	Ob/OW nondiabetic 64 Lean subjects 15	24.58±12.93 12.83±8.30	Serum resistin was significantly higher in obese than in lean Also, resistin is a possible candidate in human insulin resistance
(Kuzmicki *et al.*, 2009)	women with GDM 81 women with NGT 82 Healthy non-pregnant women 25	Median 21.9 Median 19.03 Median 14.8	Resistin levels were significantly higher in women with gestational diabetes than in normal glucose tolerance, and in non-pregnant women

1.5.4 Resistin in periodontal disease

Although a considerable amount of studies have reported an unambiguous association among obesity, type 2 diabetes and periodontal disease (Soskolne and Klinger, 2001; Mealey and Oates, 2006; Preshaw *et al.*, 2007; Saito and Shimazaki, 2007; Preshaw, 2008b), few studies have attempted to examine the possible relationship between periodontal condition and serum levels of resistin (Furugen et al., 2008; Saito et al., 2008).

There is no doubt that adipokines influence insulin resistance and play a role in inflammation and immune responses (Fantuzzi, 2005). However, in human the role of resistin in the development of insulin resistance is still debated. Macrophages and neutrophils are the main source of resistin in human (Patel *et al.*, 2003; Bostrom *et al.*, 2009). Human resistin acts as a proinflammatory molecule and stimulates the production and release of TNF-α and IL-12 (Silswal *et al.*, 2005). Also, inflammatory endotoxins induce resistin in human macrophages via a cascade involving the release of inflammatory cytokines such as TNF-α and IL-6 (Lehrke *et al.*, 2004). Consequently, resistin is thought to be linked to inflammatory process (Bokarewa et al., 2005; Fantuzzi, 2005). Furthermore, the modulating function of resistin in inflammation (Bokarewa et al., 2005) suggesting a probable role in the development of periodontal disease.

Furugen et al. (2008) investigated the relationship between periodontal conditions and serum levels of resistin and adiponectin in elderly Japanese (Furugen *et al.*, 2008). It has been demonstrated that both circulating resistin levels and total leukocytes and neutrophil counts are significantly elevated in subjects with periodontitis when compared with controls (Furugen *et al.*, 2008). Also, resistin levels were significantly

correlated with bleeding on probing and average probing pocket depth but not average attachment loss, suggesting an association of resistin with inflammatory variables rather than periodontal destruction. Furthermore, a significant association of increased serum resistin with periodontal condition was revealed independent with sex, smoking, fasting glucose and BMI. Intriguingly, periodontitis patients with at least one tooth with a probing depth greater than 6 mm have a two fold higher serum resistin levels than subjects without periodontitis (Furugen et al., 2008). A second study by Saito et al (2008) confirms the findings of the previous study, as it showed that having periodontitis was significantly associated with increased serum resistin levels in middle aged Japanese women. This study has reported that women with periodontitis have a three fold greater serum resistin levels than periodontally healthy women (Saito *et al.*, 2008). On the other hand, a recent study aimed to determine the serum resistin levels in periodontally healthy and chronic periodontitis subjects and also, to determine the effect of nonsurgical periodontal therapy on its levels, failed to demonstrate any significant difference in serum resistin levels between the cases and the controls. Additionally, the reduction in serum resistin levels following non-surgical periodontal therapy did not show any statistical significance (Devanoorkar *et al.*, 2012). Furthermore, a pilot study performed by Davies *et al* (2011) to investigate glycaemic control and systemic levels of inflammatory mediators, lipids and adipokines in patients with aggressive periodontitis, provided no evidence to suggest that serum levels of resistin altered in non-diabetic patients with aggressive periodontitis compared with periodontally healthy control subjects (Davies *et al.*, 2011). While the occurrence of hyperresistinemia in subjects with T2DM and periodontal disease is well-established, the knowledge regarding the potential role of resistin in linking between these two disorders is unexplored. Accordingly, the

present project will investigate the presumptive role of resistin in mediating the relationship between type 2 diabetes and periodontal disease.

1.6 Aims

1. To investigate the possible relationship between resistin in saliva and GCF and periodontal disease in patients with and without T2DM.
2. To investigate the possible relationship between serum resistin and periodontal disease in patients with and without T2DM
3. To investigate the effect of LPS and IL-1β on resistin expression and secretion in monocytes, macrophages and oral keratinocytes
4. To investigate the effect of resistin on cytokine expression in monocytes in vitro

The following experiments were conducted to investigate the aims of the present study:

1. Determination of serum, saliva and GCF concentrations of resistin in periodontal disease with and without T2DM, before and after treatment of periodontitis (Chapter 3 and Chapter 4).
2. Stimulation of OKF6 (oral keratinocytes) with IL-1β and investigation of resistin mRNA expression and protein secretion (Chapter 5).
3. Stimulation of THP-1 monocytes/macrophages, U937 monocyte/macrophages and primary human monocytes with different concentrations of LPS and investigation of resistin mRNA expression and protein secretion (Chapter 5).

4. Stimulation of THP-1 monocytes with resistin, and characterization/ investigation of differential cytokine/chemokine mRNA expression (Chapter 6).

Chapter 2 Materials and Methods

2.1 Cell culture

All experiments were carried out within an Astecair Biological Safety Cabinet Class II BHA Series (Astec Environmental Systems Limited, UK). All sterilized plasticware such as tissue culture flasks, pipette tips and plastic tubes were obtained from Greiner Bio-one (Stonehouse, UK). Unless specified otherwise, all laboratory media and reagents were purchased from Sigma-Aldrich (Poole, UK). All equipment used for experiments were sprayed with 70% ethanol for sterilization purposes. The cells were incubated over the course of the growing and experimental period in an incubator set to 37 ^{o}C, 5% CO_2 (Model MCO-17/20 AIC Sanyo, Loughbourgh, UK). The incubator was cleaned on a monthly basis to help prevent infection of the cultures. The cells were stored in liquid nitrogen in liquid nitrogen storage tank.

2.1.1 THP-1 monocytes

The term THP-1 has come to be used to refer to a human monocytic cell line that was originally derived from the blood of a boy with acute monocytic leukaemia (Tsuchiya *et al.*, 1980). Since then THP-1 cells have been used as a monocyte cell model in immunology research. THP-1 cell lines are non-adherent cells; indeed, it is in a pro-monocyte state during culture.

THP-1 monocytes were purchased as frozen vials from the European Collection of Cell Cultures (Salisbury, UK). The cells vials were shaken carefully in a water bath at 37^{o}C to allow cells to thaw swiftly, and then 4-5 x 10^6 cells were conveyed to a 75 cm^2 tissue culture flask. Cell culture medium (RPMI 1640 medium, supplemented with FCS (10% v/v), L-glutamine (2mM), penicillin (100 U/ml) and streptomycin (100 μg/ml)) were added gradually to the cells, then the cells were maintained at 37^{o}C and 5% CO_2. On the following day,

medium was substituted entirely by a fresh medium. The cells were spun for 5 minutes at 2000 rpm, 20^{o}C in a centrifuge, then supernatant was discarded and the cell pellet was re-suspended in new medium.

THP-1 cultures were maintained at a concentration of 3-8 x 10^5 cells/ml in cell culture medium at 37^{o}C, 5% CO_2. Every two days the medium was replaced. The cells were counted at regular intervals under a microscope using a haemocytometer (Bright-Line, Improved Neubauer, Hausser Scientific, PA, USA) in order to ensure optimum cells concentration. To prepare the hemacytometer, the mirror-like polished surface was carefully cleaned with lens paper and ethanol. The coverslip was also be cleaned. The coverslip was placed over the counting surface prior to adding the cell suspension mixture. 10 µl of the cell suspension was mixed with 10 µl of trypan blue solution. Then, 10 µl of the mixture was introduced into the edge of the V-shaped chamber under the coverslip using 10 µl pipette tip, and the area under the coverslip was allowed to be filled with the cell suspension mixture filled by capillary action. The counting chamber was then placed on the microscope stage and the counting grid is brought into focus at low power. Under the microscope, the grid consists of 25 square (each of the squares contains 16 smaller squares). The counting grid is bordered by 3 lines. Cells were counted at the four large corner squares and the one in the middle. The number of the cells per ml of cell suspension was counted as follows:

No. of cells in 5 square x 5 x 2 (dilution factor) x 10,000 (because 10 µl used).

Cell passaging or splitting was performed to maintain cells in exponential growth, alive and growing under cultured conditions for extended periods of time. In order to ensure optimal cell density in culture (which is 3-8 x 10^5 cell/ml), the cells were passaged whenever their density exceeded 8 x 10^5 cell/ml. Therefore, the cells were passaged once every week. After reviving, cells were passaged at least one time before being used for an experiment. Passage nine is the maximum passage which was used for any experiment.

During routine culture, cell viability was monitored on a regular basis using trypan blue exclusion. Trypan blue is a blue dye which only enters a damaged cell membrane, and thereby staining only dead cells. A 10µl cell suspension was diluted 1:1 (v/v) with a trypan blue solution. The number of dead cells (blue stained) and viable cells (unstained) was determined using a haemocytometer. The number of viable cells was considered as 100% and the percent of the dead cells was deducted. During routine cell culture cell viability was found to be >99%.

To enable continuous culture, THP-1 monocytes were frozen in liquid nitrogen. 4-5 x 10^6 cells/ml were resuspended in freezing medium (RPMI-1640 medium supplemented with FCS (20 % v/v), L-glutamine (2mM) and 10% glycerol). Cells were conveyed to cryovials and kept in a freezing container (Nalgene, Cryo 1 ^oC, Hereford, UK) with propan-2-ol (VWR International, Poole, UK) at -80^oC in a freezer over night. On the next day, the cells were stored in liquid nitrogen.

2.1.1.1 Vitamin D_3 treatment of THP-1 monocytes

Unless stated otherwise, THP-1 monocytes (1 x 10^6 cells/ml) were treated with 0.1 µM Vitamin D_3 (1α, 25-dihydroxy-vitamin D_3, Calbiochem, Merck Chemicals, Nottingham, UK) for 24 or 48 hours previous to use in stimulation experiments. Moreover, unless otherwise specified, all experiments were performed as two replicates, and on three independent occasions.

As a consequence of treatment with vitamin D_3, it appears that THP-1 pro-monocyte cells differentiate along the monocytic lineage to mature monocyte-like cells which is similar to the natural phenotype of primary human monocytes (Kitchens *et al.*, 1992; Schwende *et al.*, 1996). Once monocytic THP-1 cells mature they become adherent to the plate and, show an upregulation in the expression of the monocyte marker CD14 (Foster *et al.*, 2005).

2.1.1.2 PMA treatment of THP-1 monocytes

In order to differentiate THP-1 monocytes into macrophages , PMA (phorbol 12-myristate 13-acetate, Source BioScience LifeSciences, Nottingham, UK) at a final concentration of 50 ng/ml was used to treat 1 x 10^6 THP-1 monocytes (Schwende *et al.*, 1996). This treatment was continued for 5 days, during which, the medium were renewed on alternate days, and changed with a fresh medium before the experiment was performed.

2.1.2 U937 monocytes

The term U937 is generally understood to mean a human hematopoietic cell line that was originally derived from the a patient with diffuse histiocytic lymphoma (Sundstrom and Nilsson, 1976). Since then U937 have made important contributions to many disciplines such as immunology and cancer research. U937 cell lines are immature cells of myelomonocytic lineage (Sundstrom and Nilsson, 1976). These cells are non-adherent during culture.

U937 cells were a kind gift from Dr. Lee Borthwick, Respiratory Research Group, Institute of Cellular Medicine, Newcastle University, UK. The cell vials were shaken carefully in a water bath at 37^{o}C to allow cells to thaw swiftly, and then a 5 ml of pre-warmed medium (RPMI 1640 medium, supplemented with FCS (10% v/v), L-glutamine (2mM), penicillin (100 U/ml) and streptomycin (100 μg/ml)) were added gradually to the 4-5 x 10^6 cells. Afterwards, the cells were spun for 4 minutes at 250 g, 20^{o}C in a centrifuge, and then the cell pellet was re-suspended in a fresh medium at a concentration of 0.5 x 10^6 cells/ml. Only after 48 hours the medium was substituted entirely by a fresh medium. The cells were spun for 4 minutes at 1300 rpm, 20^{o}C in a centrifuge, then supernatant was discarded and the cell pellet was re-suspended in new medium. Cell culture was continued as described in 2.1.2.2.

U937 monocytes were maintained in culture at a concentration of 3-8 x 10^5 cells/ml in cell culture medium at 37^{o}C, 5% CO_2 and the medium was changed three times a week. The cells

were counted at regular intervals under a microscope using a haemocytometer (see Section 2.1.1 above) in order to ensure optimum cell concentration recommended by manufacturer's instructions (3-8 x 10^5 cells/ml). Consequently, the cells were passaged steadily once or twice a week. After reviving, cells were passaged at least one time before being used for an experiment. All experiments were conducted from cells in passages from 3-16.

Cell viability for U937 monocytes was determined using the method described for THP-1 monocyte viability, using trypan blue exclusion (see Section 2.1.1 above).

To ensure continuous culture, U937 monocytes were frozen in liquid nitrogen. 4-5 x 10^6 cells/ml were re-suspended in freezing medium (DMEM supplemented with 10% FCS, 10% DMSO). Cells were transferred to cryovials and placed in a feezing container (Nalgene, Cryo 1 ^oC, Hereford, UK) with propan-2-ol (VWR international, Poole, UK) at -80^oC in a Sanyo Ultra Low freezer (MDF-U30865) over night. On the next day, the cells were stored in liquid nitrogen.

2.1.2.1 Vitamin D_3 treatment of U937 monocytes

Treatment of U937 moncytes with Vitamin D_3 was performed using the same protocol that was described for THP-1 monocytes (see Section 2.1.1.1 above).

2.1.2.2 PMA treatment of U937 monocytes

In order to differentiate U937 monocytes into macrophages, PMA (Phorbol 12-Myristate 13-Acetate, Source BioScience LifeSciences) at a final concentration of 50 ng/ml or 100 ng/ml was used to treat 5x10^5 U937 monocytes. For the 50 ng/ml PMA, treatment lasted for 48 hours then the medium was changed before the experiment was performed. For the 100 ng/ml PMA, treatment lasted for 72 hours, during which, the medium were renewed on alternate days and then changed with fresh medium before the experiment was performed.

2.1.3 Isolation and culture of primary human monocytes

Primary human monocytes were prepared from leukocytes (buffy coat) donated by healthy individuals. Buffy coats were obtained from the National Blood Service (Newcastle upon Tyne, UK). Monocytes were isolated using magnetic beads coated with anti-CD14 antibodies for positive or negative monocytes. Each buffy coat was taken from different individual donor for each individual experiment. To date, various methods have been developed and introduced to isolate primary human monocytes, however, in this research monocyte isolation were carried out using the magnetic bead method.

The buffy coat was diluted 1:1 ratio in isolation buffer (phosphate-buffered saline (PBS)/ethylenediamine tetra-acetic acid (EDTA) (1mM), supplemented with 2% FCS), then layered on top of an equal volume of a Histopaque gradient (Sigma) at room temperature. Following centrifugation at 800 g, 20^{o}C for 20 minutes, the buffy coat layer was collected and then diluted into 40ml of isolation buffer (PBS/ EDTA 1mM with 2% FCS). The cells were pelleted at 600g, 4^{o}C for 7 minutes and the supernatant was discarded. Cells were re-suspended in 50 ml isolation buffer and spun for 7 minutes at 250g, 4^{o}C. Cells were re-suspended again in 50 ml isolation buffer and then filtrated through a 30 μm cell mesh. Leukocytes were counted on a haemocytometer, and then centrifuged once more for 5 minutes at 150g, 4^{o}C. Afterwards, leukocytes were re-suspended to a concentration of 1 x 10^{8} cells/ml for positive selection or to a 5 x 10^{7} cells/ml for negative selection of monocytes in RoboSep buffer (StemCell Technologies, Grenoble, France). Subsequently monocytes isolation were achieved using a positive or negative CD14 selection kit (StemCell Technologies) on the fully automated cell separator RoboSep (StemCell Technologies) adopting the manufacturer's instructions. On the RoboSep, the cell suspension was incubated with an antibody mixtures (positive selection: monoclonal CD14; negative selection: monoclonal CD2, CD3, CD16, CD19, CD20, CD56, CD66b, CD123, glycophorin A, F_cR

blocker) and magnetic beads. The beads with the affixed cells were assembled automatically with a magnet and purified monocytes were re-suspended in RoboSep buffer. After isolation, monocytes were counted on a haemocytometer, then re-suspended in cell culture medium (RPMI 1640 medium, supplemented with FCS (10% v/v), L-glutamine (2mM), penicillin (100 U/ml) and streptomycin (100 µg/ml)). Then 4 x 10^6 cells were seeded per well of a 6-well tissue culture plate. Cells in the plate were brooded at 37^{o}C, 5% CO_2 overnight, and afterwards used for stimulation experiments.

2.1.4 Treatment of monocytes with LPS

Ultra pure LPS from both *Porphyromonas gingivalis (P.gingivalis)* and *Escherichia coli (E.coli)* were purchased from Invivogen (via Autogen Bioclear). All the working concentrations of LPS were prepared in cell culture medium and used to stimulate monocytes. In a variety of experiments, THP-1, U937 and primary human monocytes were stimulated with different concentrations of LPS (*P.gingivalis* and *E.coli*) (100ng/ml, 1µg/ml) at different time points (1 hours, 3 hours, 6 hours, 24 hours, 48 hours and 72 hours). Our laboratory has shown that 100 ng/ml E.coli LPS is an optimal concentration used in stimulation experiments (Foster et al., 2005). However, because this concentration was insufficient to drive U937 toward resistin up-regulation a higher concentration (1 µg/ml) was used; which was still lower than that used by a previous study (Yang *et al.*, 2003).

2.1.5 Treatment of THP-1 monocytes with recombinant resistin

Human recombinant resistin (endotoxin concentration below 0.1 ng/µg) was purchased from PeproTech, Inc (London, UK) and reconstituted to a stock solution of 1mg/ml with endotoxin-free water (Sigma) according to manufacturer's instructions. Working dilutions at 50µg/ml were prepared in cell culture medium. Reconstituted resistin was stored at -80^{o}C. A pilot study using different concentrations of resistin (100ng/ml, 250ng/ml and 500ng/ml) was

carried out and the optimal concentration 500ng/ml was chosen for the subsequent stimulation experiments (see Figure 6.1).

2.1.6 OKF6 Oral keratinocytes

OKF6 cells are keratinocyte cell lines derived originally from normal gingival mucosal cells taken from the floor of the mouth. OKF6 cells were obtained from BWH Cell Culture and Microscopy Core, Harvard University. These cells were maintained in Gibco Keratinocyte SFM medium (Invitrogen, UK) augmented with bovine pituitary extract (BPE) at a final concentration of 30 μg/ml, penicillin 100(U/ml), streptomycin (100 μg/ml) diluted in phosphate buffered saline (PBS), epidermal growth factor (EGF) to a final concentration of 0.1 ng/ml, and 0.6 mM calcium chloride ($CaCl_2$). Cells were cultured at 37^oC, 5% CO_2 in 5ml supplemented Gibco Keratinocyte-SFM medium at a density of approximately 0.5 x 10^6 cells per 25cm^2 flask. Fresh medium was added to the cultures every other day until the cells reached 80% confluence which was usually required approximately 5 days.

Since the keratinocytes are adherent cells, and in order to release them from the flask walls, first the cells were washed with 5ml of trypsin/EDTA for just 30 seconds. Then a fresh 5ml aliquot of the same reagent was added to the cells and maintained for 10 minutes at 37^oC and 5% CO_2. Once the cells become non-adherent, the trypsin action was stopped by adding 15 ml of 1:1 DMEM / F12 (Ham mixture) medium supplemented with foetal calf serum (10% v/v) and L-glutamine (2mM). The cells were centrifuged at 2000 rpm for 10 minutes and then re-suspended in fresh keratinocyte medium. Using a haemocytometer, the cells were counted (with the same method as previously described for counting THP-1 and U937 cells) and then seeded in 25 cm^2 flasks. On the following day, the medium was replaced with a 5ml of fresh medium that was then renewed every other day.

To ensure continuous culture, OKF6 keratinocytes were frozen in liquid nitrogen. Typically, 2-3 x 10^6 cells/ml were suspended in keratinocytes medium, then 2X freezing medium (DMEM/F12 (1:1), supplemented with FCS 10% and 20% DMSO) were added to the cells in 1:1 ratio. Cells were transferred to cryovials and placed in a freezing container (Nalgene, Cryo 1s^{o}C, Hereford, UK) with propan-2-ol (VWR international, Poole, UK) at -80^{o}C in a Sanyo Ultra Low freezer (MDF-U30865) over night. On the next day, the cells were stored in liquid nitrogen.

In order to revive the frozen OKF6 cells, the vial of cells was shaken carefully in a water bath at 37^{o}C to allow cells to thaw quickly, and then transfer the thawed cells to a tube and add a quenshing medium (DMEM/F12 (1:1) supplemented with 10% FCS) to it. The cells were then centrifuged for 10 minutes at 2000 rpm and then counted using haemocytometer and re-suspended in fresh keratinocyte medium.

2.1.6.1 Treatment of OKF6 keratinocytes with IL-1β

Human recombinant IL-1β was purchased from R&D Systems (Abingdon, UK). This cytokine was reconstituted according to the manufacturer's instructions as a stock solution at a concentration of 5µg/ml by dissolving the powder in sterile distilled water and stored at -80^{o}C until it was used for experiments. Recombinant IL-1β used in experiments at a concentration of 100pg/ml. This concentration was adopted as previous research in our lab and had found it to be the optimum concentration to stimulate immune responses in these cells (Areibi *et al* Unpublished). Stimulation of the keratinocytes with IL-1β was carried out in individual experiments and repeated two times.

The OKF6 keratinocytes were obtained by trypsinization of the cells as detailed previously (section 2.1.2) and were seeded in duplicate wells (2 x 10^5 cells/well) in three 6 well plates and left to adhere and reach sub-confluence. In order to stimulate the cells, a fresh medium containing IL-1β (100pg/ml) was added (4ml/well) and other cells were incubated with a

plain fresh medium to serve as a control. All treatments and controls were performed in duplicates within two independent cell culture experiments. The cells stimulation period was 4 hours, 24 hours and 48 hours. For extracellular release of resistin supernatants were collected and aliquoted in Eppendorf tubes (1ml), while for assessment of gene expression the cells were scraped and total mRNA was prepared (see section 2.3). Both samples were stored at -80^{o}C until further use.

2.2 Enzyme Linked Immunosorbant Assay (ELISA)

The concentration of resistin and other cytokines in cell culture supernatants were examined using Duoset ELISA kits (R&D systems, UK). However, resistin levels in serum, saliva and GCF were measured using Quantikine ELISA kits (R&D systems). The concentration of MIP-1α in cell culture supernatants were examined using Duoset ELISA kits (R&D systems, UK). The visfatin ELISA was developed from first principles, using individual antibodies rather than a kit. The ELISA technique is built on the antibody sandwich principle. This method involves coating the plate with a analyte-specific capture antibody (primary antibody), removing unbound antibody by washing, blocking all unbound sites, adding antigen (in the standard and samples), adding detection antibody (secondary antibody), adding detection reagent (e.g. streptavidin-HRP) and, lastly, adding a substrate to react with the enzyme and develop a colour in proportion to the amount of bound analyte. Antibody concentrations and standard curve detection range for each ELISA are listed in Table 2.1.

Table 2.1 Antibody working concentrations and standard curve detection ranges for ELISAs

ELISA	Capture antibody	Detection antibody	Standard curve range
Resistin (Quantikine)			10 – 0.16 ng/ml
Resistin (DuoSet kit)	4µg/ml	0.25 µg/ml	2000 – 31.25 pg/ml
MIP-1α	4µg/ml	200 ng/ml	500 – 7.8 pg/ml
TNF-α	4µg/ml	350 ng/ml	1000 – 15.6 pg/ml
Visfatin	2µg/ml	0.8µg/ml	320 – 5 ng/ml
IL-12	4µg/ml	100 ng/ml	2000 – 31.25 pg/ml

For the Duoset ELISAs, the 96-well microtiter plate was coated with 100 µl capture antibody which is diluted in PBS, covered with adhesive strip, and incubated overnight at room temperature. On the next day, the plate was washed three times with wash buffer (0.05% Tween 20 in PBS, v/v), and for complete removal of any remaining washing buffer the plate was inverted and blotted against a clean paper towels. 300 µl of reagent diluent (RD, 1% BSA in PBS, w/v) was added for one hour to block the non-specific binding and then the plate was washed (as detailed above) and the wells were drained. Then 100 µl of standard (in triplicates, prepared with serial dilutions in reagent diluent), samples (in duplicates) and a reagent diluent as a negative control (in triplicates) were added and incubated for two hours (one hour on a shaker at room temperature, and the next hour in incubator at 37^{o}C). The wells were washed again and 100 µl of detection antibody (diluted in reagent diluent) was incubated for 2 hours (on a shaker at room temperature). After washing three times, the ELISA plate was incubated with 100 µl of Streptavidin horse-radish peroxidise (HRP) diluted in reagent diluent (1/200) for 20 minutes. The plate was protected from direct light during this step by wrapping the plate in aluminium foil. The plate was washed again and then 100 µl of tetramethylbenzidine (TMB) substrate solution (solution A and solution B (1:1), v/v) was added to each well and the plate incubated away from direct light for 20 minutes. The reaction was stopped with 50 µl 2N H_2SO_4. The absorbance was read at 450 nm, using a Bio Tek FL 600 microplate fluorescence reader. A reading at 550 nm was subtracted to correct for optical imperfections in the plate. The specific concentrations of samples were computed from the standard curve using a 4-parameter logistic curve fit. The standard curve for each ELISA used in the study are presented in Figure 2.1, Figure 2.2, Figure 2.3, Figure 2.4, Figure 2.5 and Figure 2.6.

To measure resistin levels in clinical samples, a Quantikine resistin ELISA protocol was used. Quantikine kit is designed to eliminate the interference by binding proteins and other

factors in clinical samples; consequently, it was the best assay for the quantitative determination of resistin concentrations in human serum, saliva and GCF. The protocol for the Quantikine kits was slightly different from the commercial DuoSet ELISAs. Briefly, all reagents, samples, and standards were prepared according to the manufacturer's instructions. The Quantikine kit 96 well polystyrene microplate have been already coated (from the manufacturer) with a mouse monoclonal antibody against resistin, for that reason, a 100 µl of assay diluent (buffered protein base) was added directly to the plate and overlaid with 100 µl of standard, control, or samples. The plate was then covered with adhesive strip and incubated for 2 hours at room temperature. Then each well was washed with 400 µl wash buffer using multichannel pipette. This was repeated three times for a total of four washes, after each wash the plate was blotted against a clean paper towel, and at the end of the washing step all the remaining liquid was removed by aspirating each well using aspirator. A 200 µl resistin conjugate (monoclonal antibody against resistin conjugated to horseradish peroxidise) was added and incubated for 2 hours at room temperature. The washing step was performed as detailed previously. Then 200 µl of substrate solution was added to each well and protected from direct light for 30 minutes. The reaction was stopped by adding a 50 µl of stop solution to each well. Determination of the optical density and measurement of protein concentrations was done following the same method used for the other ELISAs (as described before).

Examples of a standard curve for each ELISA are shown in Figure 2.1, Figure 2.2, Figure 2.3, Figure 2.4, Figure 2.5 and Figure 2.6.

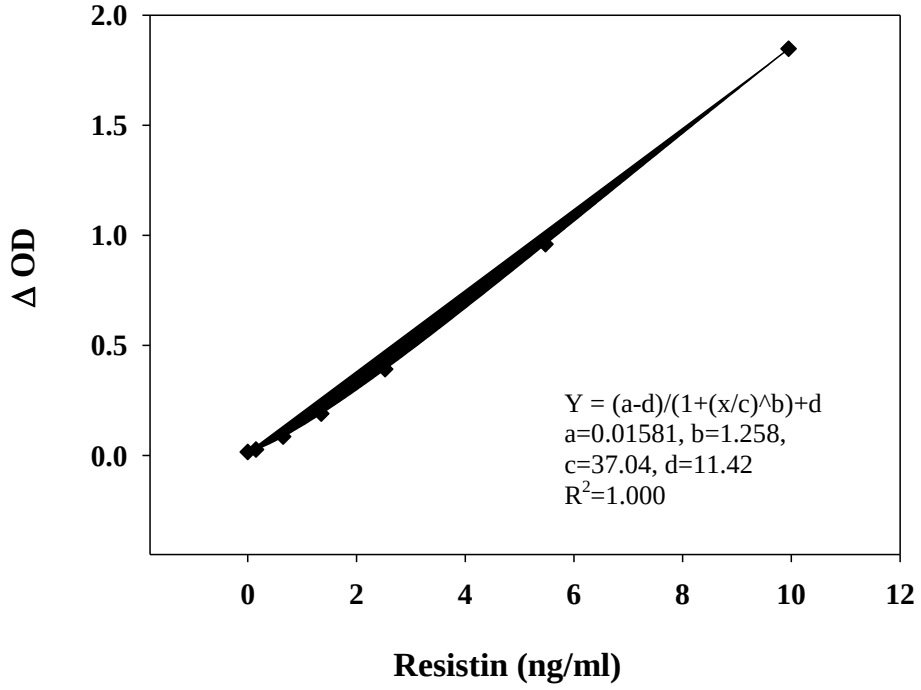


Figure 2.1 Standard curve for the Resistin ELISA (Quantikine)

The unknown concentrations of resistin were calculated using a 4-parameter curve fit, which was created to produce the 7-point standard curve for the resistin ELISA. In the 4-parameter curve fit formula, "a" represents a theoretical response at a concentration equal to zero, "b" the measure of the slope of curve at its inflection point, "c" the value of response at inflection point, "d" the theoretical response at infinite concentration, "x" the concentration, and "y" the response (OD). Consequently, the Δ OD was plotted against different resistin concentrations. Δ OD symbolizes "OD 450 nm – OD 550 nm". 450 nm represents the wavelength in nanometres at which maximum absorption of light by the plate background occurs, while 550 nm represents the wavelength in nanometres at which maximum absorption of light by Resistin occurs. The subtraction of 550 readings from the readings at 450 aims to correct for the optical imperfections in the plate.

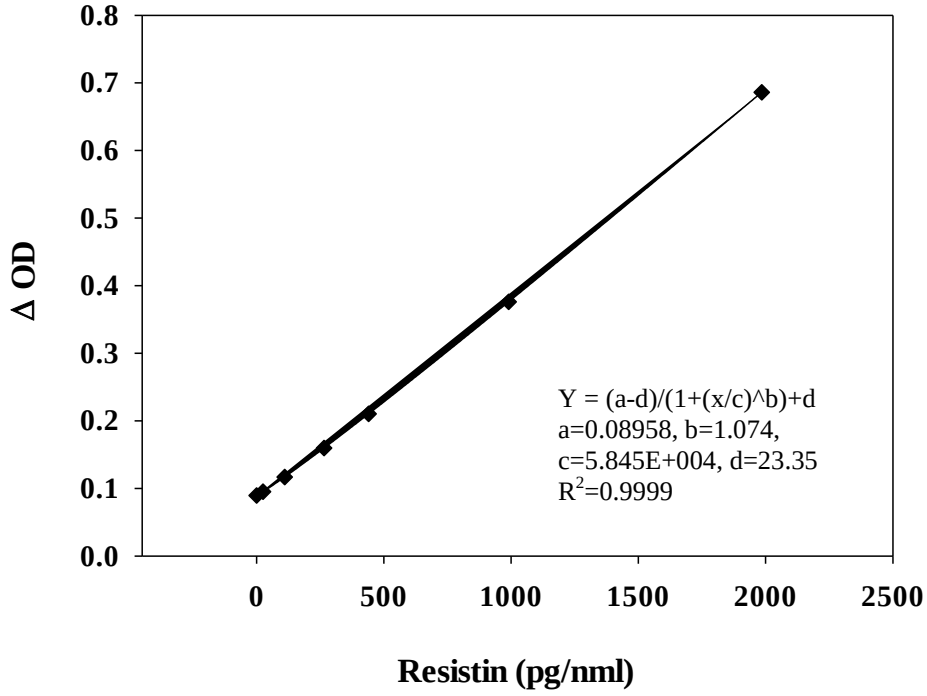


Figure 2.2 Standard curve for the resistin ELISA (Duo Set)

The unknown concentrations of resistin were calculated using a 4-parameter curve fit, which was created to produce the 7-point standard curve for the resistin ELISA. For details see legend to Figure 2.1.

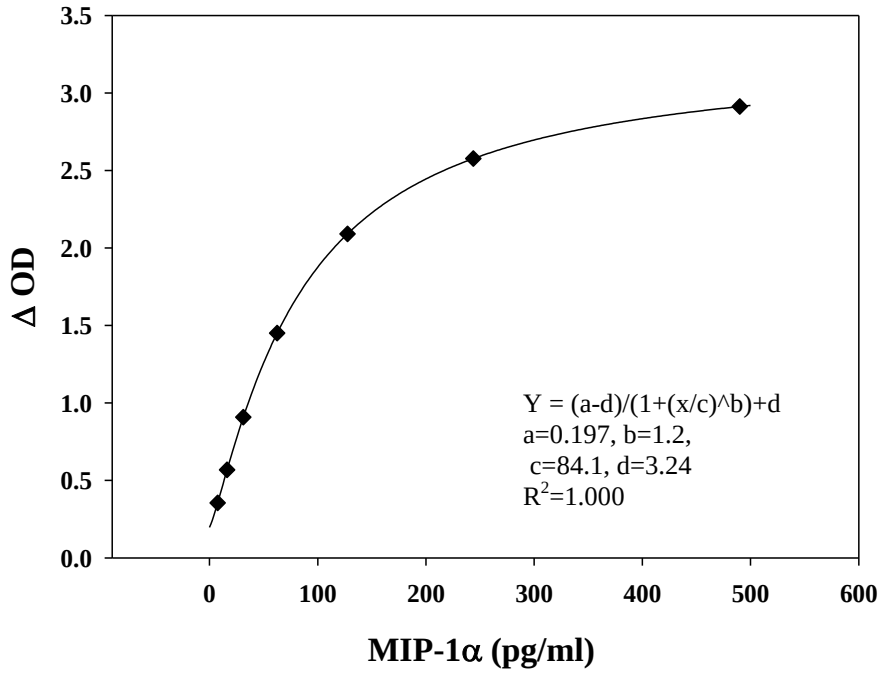


Figure 2.3 Standard curve for the MIP-1α (CCL3) ELISA

The unknown concentrations of MIP-1α were calculated using a 4-parameter curve fit, which was created to produce the 7-point standard curve for the MIP-1α ELISA. For details see legend to Figure 2.1.

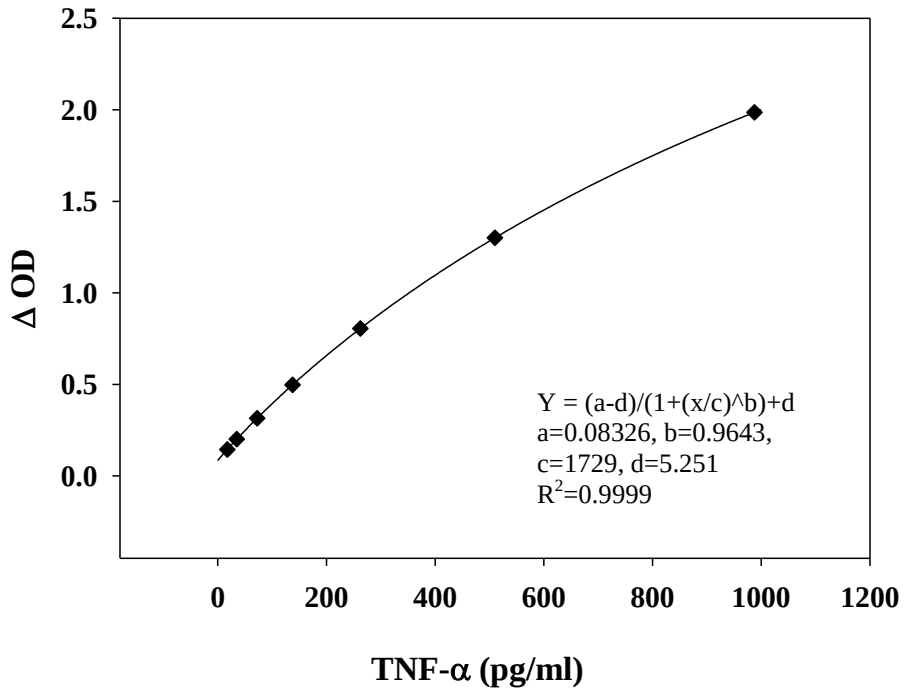


Figure 2.4 Standard curve for the TNF-alpha ELISA

The unknown concentrations of TNF-α were calculated using a 4-parameter curve fit, which was created to produce the 7-point standard curve for TNF-α ELISA. For details see legend to Figure 2.1.

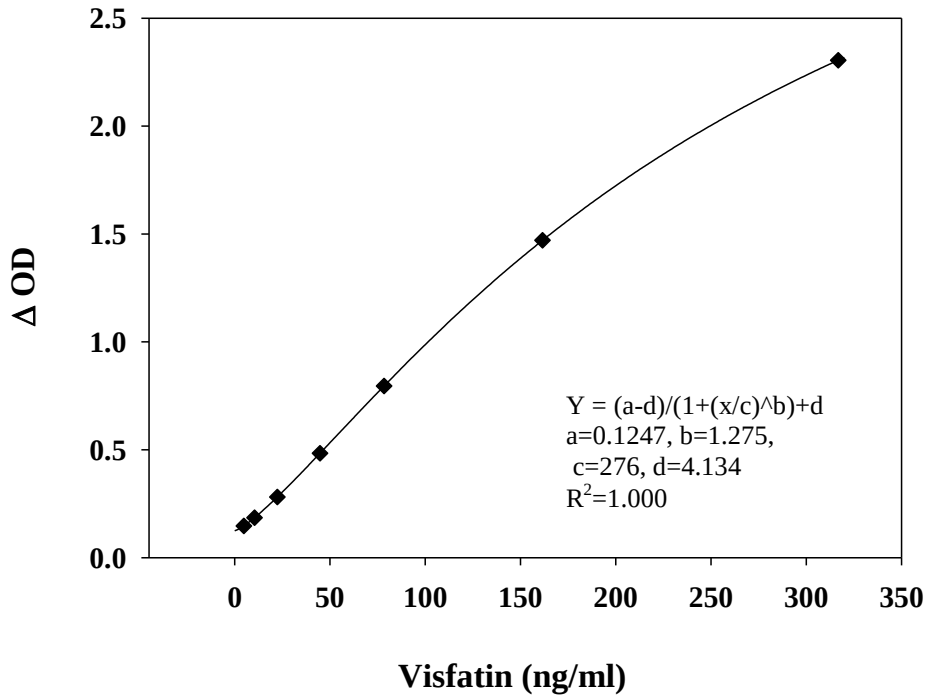


Figure 2.5 Standard curve for the visfatin ELISA

The unknown concentrations of visfatin were calculated using a 4-parameter curve fit, which was created to produce the 7-point standard curve for the visfatin ELISA. For details see legend to Figure 2.1.

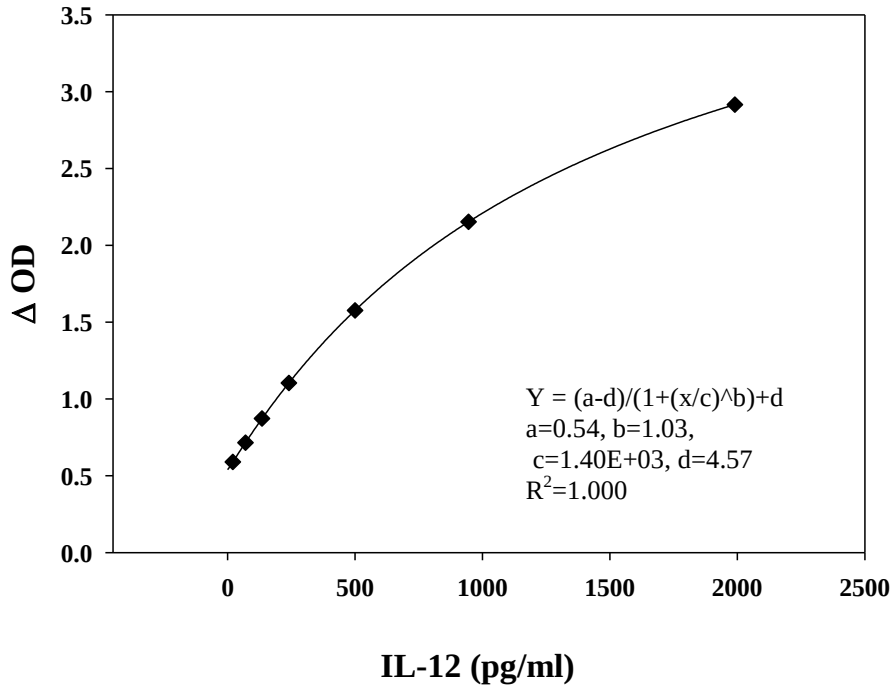


Figure 2.6 Standard curve for IL-12 ELISA

The unknown concentrations of IL-12 were calculated using a 4-parameter curve fit, which was created to produce the 7-point standard curve for the IL-12 ELISA. For details see legend to Figure 2.1.

2.2.1 Resistin ELISA

2.2.1.1 Validation of the resistin Duo Set ELISA for human samples

2.2.1.1.1 Spike recovery assay

The resistin Duo Set ELISA (R&D Systems) was developed for the analysis of cell culture supernatants, but because serum contains many components and factors which may impact the ELISA results, it was necessary to validate the resistin ELISA for the analysis of human serum samples. This was performed by measuring the recovery and linearity for the resistin ELISA. Throughout the study, serum samples were diluted (1:16) in reagent diluent to yield a value within the range of the standard curve. The dilution (1:16) was treated as a new "neat" sample in spike/recovery experiments. In the spike/recovery experiment, a known amount (1200 pg/ml) of recombinant resistin was spiked into a serum sample with a known resistin concentration (the spiked sample), the same amount of recombinant resistin was also spiked into a reagent diluent (spike control), while un-spiked sample represent the neat sample. The spiked, un-spiked samples and the control spike were serially diluted into 1:2, 1:4 and 1:8 dilutions to explore for linearity of the dilutions. The recovery of spiked sample, the control spike and the serial dilutions were calculated as follows:

Spiked sample recovery: $\frac{\text{Spiked sample} - \text{neat sample}}{\text{amount spiked}} \text{ X } 100$

Spiked control recovery: $\frac{\text{Spkied control}}{\text{amount spiked}} \text{ X } 100$

Recovery for 1:2 dilution: $\frac{\text{spiked sample}}{\text{1:2 spiked sample}} \text{ X } 100$

The resulting concentration "recovery of the sample" is then measured to indicate whether a component in the sample interferes in the ELISA. Furthermore, spiked and un-spiked samples were also serially diluted to explore for linearity of the dilutions. According to R&D

Systems recommendations, the spike/recovery should be in the range of 80-120 % to indicate that the assay is suitable for use with the tested sample. The validation assays for resistin have been repeated several times and the results of some of these attempts are shown in tables 2.2. In the first two validation assays the spiked sample recovery was 77 and 87 respectively. The linearity was relatively good for the control spike in both experiments which indicates that there was no error in the preparation of dilutions. However, a poor linearity was observed for the spiked and un-spiked samples. When another validation was attempted using a different reagent diluent (20% FCS instead of 1% PBS) in BSA different results were obtained. Thus, the spike recovery was poor while the linearity was good (Table 2.2).

Table 2.2 Spike/recovery and linearity of resistin Duo Set ELISA for human serum samples (Three independent assays)

Samples	Resistin (pg/ml) 1st Exp	% Recovery 1st Exp	Resistin (pg/ml) 2nd Exp	% Recovery 2nd Exp	Resistin (pg/ml) 3rd Exp	% Recovery 3rd Exp
Spiked sample	1247.4	77	1772.8	86.7	1454.5	65
1:2 spiked sample	1137.6	109.7	1514.7	117	1418.1	102.6
1:4 spiked sample	995.6	125.3	1294.5	137	1451.4	100.2
1:8 spiked sample	992.8	125.7	991.3	178.8	1616.9	90
Neat sample	323.4	…	731.9	…	674.8	…
1:2 neat sample	267.3	121	531.7	137.7	740.5	91.1
1:4 neat sample	220.2	146.9	378	193.6	815.5	82.7
1:8 neat sample	208.1	155.4	613.4	119.3	803.1	84
Spiked control	1111.1	92.6	1118	93.2	992	82.7
1:2 spiked control	1045.4	106.3	1198.2	93.3	996.4	99.6
1:4 spiked control	1033	107.6	1186.6	94.2	1032	96.1
1:8 spiked control	1035.2	107.3	1244.2	89.9	1015.1	97.7

The table illustrates the obtained resistin concentrations in human serum samples and the % recovery. The human serum samples were spiked 1200 pg/ml human recombinant resistin. Spiked controls (reagent diluent) were spiked with the same amount. 2-fold serial dilutions were prepared for spiked samples, neat samples and spiked controls.

The inconsistency in recovery and linearity was a feature of the validation assays carried out for resistin Duo set ELISAs. Consequently, it was decided that the resistin Dou Set ELISA assay could not be reliably used for monitoring resistin concentrations in human serum samples, and its use was restricted to the analysis of cell culture supernatants.

2.2.1.1.2 Intra-and-inter-assay reproducibility and assay sensitivity

In order to assess the reproducibility of the resistin Duo Set ELISA assays, both the intra-and- inter assay coefficient of variability (CV) was measured. Intra-assay reproducibility for resistin Duo set ELISA for serum samples was assessed in 8 replicates in one assay, while the inter-assay reproducibility for the resistin Duo set ELISA for serum samples was assessed in six independent assays. Results are shown in Table 2.3 and Table 2.4. The intra-and-inter-assay reproducibility was calculated as follows:

$$\frac{\text{SD}}{\text{Mean}} \text{ X } 100$$

Intra- and inter-assay precision for resistin Duo set ELISA for serum samples was 7% and 11% respectively. The 11% inter-assay CV expressed an acceptable plate to plate consistency and a high repeatability of resistin Duo set ELISAs for human serum samples.

The assay sensitivity for the resistin Duo set ELISA was determined by adding two standard deviations to the mean optical density (OD) value of the zero standard replicates, and calculating the corresponding concentration in the 4 parameter curve fit using “Sigma plot” solve function. The minimum detectable concentration for resistin was 60.4pg/ml.

Table 2.3 Intra-assay reproducibility of Resistin Duo set ELISAs for human serum samples

Replicate Number	Serum Resistin (pg/ml)
1	1085.1
2	1267.3
3	1185.6
4	1173.9
5	1345.7
6	1181.7
7	1286.9
8	1314.3
Mean	1230.1
Standard Deviation	87.5
Intra-assay variation (%)	7.1

Human serum sample was analysed in replicates for resistin in one assay and intra-assay variation was calculated.

Table 2.4 Inter-assay reproducibility of Resistin Duo set ELISAs for human serum samples

Assay Number	Serum Resistin (pg/ml)
I	607.6
II	731.9
III	674.8
IV	784.1
V	822
VI	787.7
Mean	734.7
Standard Deviation	80.8
Intra-assay variation (%)	11

Human serum sample was analysed for resistin in six independent assays and the inter-assay variation was calculated.

2.2.1.2 Resistin Quantikine ELISA for human samples

2.2.1.2.1 Intra- and- inter-assay reproducibility and assay sensitivity

The concentration of two serum samples were tested six times on one plate to determine the intra-assay reproducibility, while the inter-assay reproducibility of resistin Quantikine ELISA for serum samples was assessed in fives independent assays for one sample and four separate assays in a second sample. Results are shown in Table 2.5 and Table 2.6. The intra-and-inter-assay precision was calculated as follows:

$$\frac{\text{SD}}{\text{Mean}} \text{ X } 100$$

Intra-assay variation for resistin Quantikine ELISA for serum samples was 17.2% for the first sample and 10.8% for the second sample. The inter-assay variation was 17.8% for the first sample and 8.9% for the second sample and this expressed a good plate to plate consistency and repeatability of resistin Quantikine ELISAs for human serum samples.

The assay sensitivity for the resistin Quantikine ELISA was determined by adding two standard deviations to the mean optical density (OD) value of the zero standard replicates, and calculating the corresponding concentration in the 4 parameter curve fit using “Sigma plot” solve function. The minimum detectable concentration for resistin was 0.014ng/ml.

Table 2.5 Intra-assay reproducibility of Resistin Quantikine ELISAs for human serum samples

Replicate Number	Serum Resistin (ng/ml) Sample I	Serum Resistin (ng/ml) Sample II
1	5.7	8.9
2	6.5	11.9
3	4.3	11.8
4	6.3	11.8
5	4.8	10.5
6	6.7	11.9
Mean	5.7	11.1
Standard Deviation	0.98	1.2
Intra-assay variation (%)	17.2	10.8

Two human serum samples were analysed in six replicates for resistin in one assay and intra-assay variation was calculated.

Table 2.6 Inter-assay reproducibility of resistin Quantikine ELISAs for human serum samples

Assay Number	Serum Resistin (ng/ml) Sample I	Serum Resistin (ng/ml) Sample II
1	6.3	5.6
2	3.6	6
3	4.8	5.6
4	4.9	4.9
5	4.6	4.9
6	4.6	…
Mean	4.8	5. 4
Standard Deviation	0.9	0.5
Inter-assay variation (%)	17.8	8.9

Two human serum samples were analysed “one in five and the second in six” independent assays and inter-assay variation was calculated.

The concentration of two saliva samples were tested eight times on one plate to determine the intra-assay reproducibility, while the inter-assay reproducibility of resistin Quantikine ELISA for saliva samples was assessed for two samples in fives independent assays. Results are shown in Table 2.7 and Table 2.8. The intra-and-inter-assay precision was calculated as follows:

$$\frac{\text{SD}}{\text{Mean}} \text{ X } 100$$

Intra-assay variation for resistin Quantikine ELISA for saliva samples was 6.5% for the first sample and 6.9% for the second sample. The inter-assay variation was 9.1% for the first sample and 7.0% for the second sample and this expressed a good plate to plate consistency and repeatability of resistin Quantikine ELISAs for human saliva samples.

The assay sensitivity for the resistin Quantikine ELISA was determined by adding two standard deviations to the mean optical density (OD) value of the zero standard replicates, and calculating the corresponding concentration in the 4 parameter curve fit using “Sigma plot” solve function. The minimum detectable concentration for resistin was 0.09 ng/ml.

Table 2.7 Intra-assay reproducibility of Resistin Quantikine ELISAs for human saliva samples

Replicate Number	Saliva Resistin (ng/ml) Sample I	Saliva Resistin (ng/ml) Sample II
1	3.9	4.2
2	4.4	4.3
3	4.6	4.3
4	4.5	4.6
5	4.2	3.9
6	4.1	4.1
7	4.2	4.0
8	3.8	4.7
Mean	4.2	4.3
Standard Deviation	0.3	0.3
Intra-assay variation (%)	6.5	6.9

Two human saliva samples were analysed in eight replicates for resistin in one assay and intra-assay variation was calculated.

Table 2.8 Inter-assay reproducibility of Resistin Quantikine ELISAs for human saliva samples

Assay Number	Saliva Resistin (ng/ml) Sample I	Saliva Resistin (ng/ml) Sample II
1	15.4	3.4
2	14.1	2.9
3	14.4	3.4
4	17.9	3.4
5	14.1	3.2
Mean	15.2	3.3
Standard Deviation	1.4	0.2
Inter-assay variation (%)	9.1	7.0

Two human saliva samples were analysed in five independent assays and inter-assay variation was calculated.

2.2.2 Development and characterization of specific ELISA for visfatin

Because of the limited availability and prohibitive prices of commercial ELISA kits for visfatin, it was decided to develop a visfatin ELISA from first principles. We have used a monoclonal anti-human/mouse PBEF1 antibody (capture antibody) (R&D Systems), a recombinant visfatin (Enzo Life Sciences, UK) and a biotinylated anti-human PBEF antibody (detection antibody) (R&D Systems). In addition 1/200 streptavidin-HRP (R&D Systems) and a stabilized hydrogen peroxide substrate solution were used as a detection system and then 2N H_2SO_4 as a stop solution.

2.2.2.1 Optimization of concentrations and conditions

The development of visfatin ELISA requires an optimization of the assay through the use of different concentrations of capture antibody (CAb), detection antibody (DAb) and recombinant visfatin standard. The reason for the optimization of the assay is to create an assay capable of detecting the lowest concentrations of the protein in the samples. The assay optimization was carried out through a series of grid experiments in which different conditions and concentrations have been used. In this experiment, one capture antibody concentration (4 µg/ml), four detection antibody concentrations (400, 800, 1600, 3200 ng/ml) and three concentrations for the standard (10, 20, 40 ng/ml) were used. The use of lower concentration of detection antibody (400ng/ml) gave a poor signal (the highest signal equal to 0.27 dOD) which lead us to exclude the use of this concentration in the next trials. On the other hand, the highest signal obtained by the use of the other three concentrations of detection antibody (800, 1600, 3200 ng/ml) was relatively comparable (the highest signal was equal to 0.39, 0.51 and 0.68 dOD respectively). Hence we concluded that the 800ng/ml is the optimal concentration for detection antibody because it gave a relatively comparable signal to the higher concentrations and saving material in the same time.

As a result of these trials, a standard curve was produced with the optimum conditions and concentrations of capture antibody, detection antibody and standards, determined using a capture antibody concentration of 2µg/ml and a detection antibody concentration of 800ng/ml. A serial twofold dilution of the recombinant human visfatin starting from 320ng/ml down to 5ng/ml was performed. Trial and error resulted in the following changes to the standard protocol which gave optimal standard curve for the visfatin ELISA: increasing the number of washes between reagents to five, aspirating each well after each wash, incubating the standard for 30 minutes on a shaker followed by 1.5 hours incubation (at 37^{o}C), incubating the detection antibody for two hours (at 37^{o}C) and incubating the streptavidin-HRP and the substrate solution for 30 minutes. The standard curve for the visfatin ELISA was shown in figure 2.5. The sensitivity of the ELISA was calculated as 3 standard deviations above the mean value of the zero standards, and the minimum detectable dose of visfatin (the sensitivity) in the experiment was 0.17ng/ml.

2.2.2.2 Spike/recovery assay

Serum contains many components and factors which may affect the ELISA. Hence, it was necessary to validate the visfatin ELISA for the analysis of serum samples. This was performed by measuring the recovery and linearity measurements for visfatin ELISA in spike/recovery experiment. In this experiment a known amount of recombinant visfatin (100ng/ml) was spiked into serum sample with a known visfatin concentration (the spiked sample), the same amount of recombinant visfatin was also spiked into a reagent diluent (spike control), while the un-spiked sample represented the neat sample. The spiked, un-spiked samples and the control spike were serially diluted into 1:2, 1:4 and 1:8 dilutions to explore for linearity of the dilutions. The recovery of spiked sample, the control spike and the serial dilutions were calculated as follows:

Spiked sample recovery: $\frac{\text{Spiked sample} - \text{neat sample}}{\text{amount spiked}}$ X 100

Spiked control recovery: $\frac{\text{Spiked control}}{\text{amount spiked}}$ X 100

Recovery for 1:2 dilution: $\frac{\text{Spiked sample}}{\text{1:2 spiked sample}}$ X 100

According to R&D Systems recommendations, the spike/recovery should be in the range of 80-120%. In addition the recovery values for the control spike should be within 80-120%. As illustrated in Table 2.9, the percentage of the spiked sample recovery for visfatin was only 2.5% which indicates a poor recovery. Furthermore, vast discrepancies have been found in the percentage recovery for the control spike, spiked sample and un-spiked sample dilutions which indicates a poor linearity. Also, the reproducibility of the assay was poor because we could not repeat a consistent standard curve in all ELISAs. Hence, this assay was not used to analyse cell culture samples or clinical samples. On the other hand, the commercial ELISA kits for visfatin were not widely available and were prohibitively expensive.

Table 2.9 Spike/recovery and linearity of the visfatin ELISA for human serum samples

Sample	Visfatin (ng/ml)	% Recovery
Spiked sample	507.255	2.5
1:2 spiked sample	551.695	92
1:4 spiked sample	507.705	100
1:8 spiked sample	382.19	132.7
Neat sample	504.78	----
1:2 neat sample	498.495	101.3
1:4 neat sample	445.885	113.2
1:8 neat sample	303.29	166.4
Spiked control	73.537	73.5
1:2 spiked control	37.252	197.4
1:4 spiked control	13.402	548.7
1:8 spiked control	7.1802	1024.2

The table illustrates the visfatin concentrations in human serum samples and the % recovery. The human serum samples were spiked 100 ng/ml human recombinant visfatin. Spiked controls (reagent diluent) were spiked with the same amount. 2-fold serial dilutions were prepared for spiked samples, neat samples and spiked controls.

2.3 RNA analysis

2.3.1 RNA extraction

In order to isolate total RNA from the THP-1, U937, primary monocytes, and keratinocytes the GenElute^{TM} Mammalian Total RNA Miniprep kit (Sigma) was used according to the manufacturer's instructions. Cells were lysed in 350µl/well lysis buffer comprising of β-mercaptoethanol and lysis solution in 1:100 (v/v) mixture, filtered through a GenElute filtration column (blue column inside a 2 ml receiving tube). The tubes were centrifuged at 13000 rpm for 2 minutes at room temperature (Biofuge Pico, Heraeus, DJB labcare). Then the filtration column was discarded and the lysate was stored at -80^{o}C pending further processing. Once samples all the lysates from an individual experiment had been collected and stored, RNA was prepared from the various lysates in a single procedure. Thus, 350µl ethanol (70%) was added to the filtered lysate (kept on ice), and the mixture was transferred into a GenElute binding column (red column inserted in a 2ml collection tube) and centrifuged for 15 seconds at 12000 rpm. The filtrate was discarded and 500µl of wash solution I was added to the column which was then centrifuged for 15 seconds. The columns were placed into 2ml collection tubes then wash solution II (500µl) was added into the column and centrifuged for 15 seconds. A second wash with 500µl wash solution II was carried out and centrifuged at 13000 rpm for 2 minutes. The columns were then transferred into a new collection tubes. Subsequently the RNA was eluted from the column with elution solution (30µl). Finally, the concentration of RNA in each sample was determined on a spectrophotometer (see below) and reserved at -80^{o}C pending further processing.

2.3.2 RNA quantification

A NanoDrop spectrophotometer ND-1000 (Nanodrop Technologies Inc., USA) was used to quantify RNA samples. The machine measured a ratio for absorbance at 260 and 280 nm and

RNA was considered to be acceptably pure if the ratio was 1.8-2.0. The RNA concentrations were computed in 1µl of each sample in ng/µl.

2.3.3 Complementary DNA (cDNA) synthesis by reverse Transcription

In order to transcribe the extracted RNA into cDNA, a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Warrington, UK) was used according to the manufacturer's instructions. The cDNA was generated from 1µg of total RNA. A reaction mixture was prepared on ice and included the following reagents (the volumes quoted are those for a single reverse transcription reaction):

2µl 10X reverse transcription buffer

0.8µl 10X Random Primers

2µl 25X dNTP mix

1µl Multiscribe Reverse Transcriptase

1µl RNase Inhibitor

3.2µl Nuclease-free H_2O

First, 6.8µl of the reaction mixture was added to the PCR tubes, and then a total RNA (equivalent to 1µg) was added with nuclease-free water (13.2µl RNA volume). The reverse transcription was carried out by placing the tubes in thermocycler (Gene Amp PCR System 9700, Applied Biosystems, Warrington, UK) for 10 minutes at 25^{o}C, 120 minutes at 37^{o}C, and 5 seconds at 85^{o}C. The cDNA was preserved at 4^{o}C pending further processing.

2.3.4 Reverse Transcriptase-Polymerase Chain Reaction for resistin

The mRNA expression of resistin gene was analysed using a conventional PCR method. The resistin primer used in this study was purchased from Sigma. The primer sequences design

was quoted from a paper (Silswal *et al.*, 2005). The oligonucleotides sequences for resistin gene primers used for the assay were as follows:

Forward: 5`-CGAGATCTATGAAAGCTCTCTGTCTCCTCCTCG

Reverse: 5`-GGAATTCCCTCAGGGCTGCACACGACA

A PCR analysis was carried out using the complementary DNA (cDNA). In order to confirm successful reverse transcription and PCR, β_2-microglobulin was used as a control gene. The sequences for oligonucleotides of β_2-microglobulin gene primers were 5`acccccactgaaaaagatga (forward) and 5`atcttcaaacctccatgatg (reverse) with a melting temperature (Tm) of 60^{o}C.

The PCR assay was carried out in 25µl volumes. The reaction mix was prepared on ice and included the following:

12.5µl BioMix^{TM} red (Bioline, UK)

1.25µl of each primer

7.5µl nuclease-free H_2O

A reaction Mix of 22.5µl was added to each well in the PCR multiwall plate, then 2.5µl of cDNA (or water as a negative control) was added per well, then on completion of the thermal cycle which was conducted for 35 cycles (annealing temperature 69^{o}C) the samples were analysed on 3% agarose gel.

2.3.5 PCR product analysis

In order to analyse PCR samples, 3% agarose gel was used. Agarose gel was prepared by adding 0.9g of agarose (Sigma) to 30ml 1xTAE buffer (0.4 M Tris base, 50mM EDTA and 1.14% (v/v) acetic acid). Using the microwave oven, the agarose mixture was dissolved by heat, and then 5µl of ethidium bromide (0.5mg/ml) was added to the cool agarose. The

resulting mixture was gently mixed by shaking the flask manually and then poured into a cast with a 10 well comb, and left to set. Once the gel was set, the comb was removed to create lanes in the gel. Following this, the gel was transferred into a hybaid electrophoresis tank filled with 1XTAE buffer. Then a 5µl of Hyperladdaer marker IV containinng nine different sized nucleotides from 100 to 1000 bp was carefully injected into the first lane. Also, 10µl of each sample was added in the other lanes, and the gel was run for 40-50 minutes at 100 volts using a Bio-Rad power PAC 300 which allowed the ethidium bromide in the gel to interpolate into the DNA and fluoresces under the UV light. The ultraviolet transilluminator (UVP Life Sciences) was used to visualize the DNA bands and the Image Store 5000 system (UVP Life Sciences) was used to take the photographs.

2.3.6 Quantitative Real Time RT-PCR

In traditional PCR, DNA is detected at the final phase or endpoint of PCR reaction. On the other hand, real time PCR measures DNA amplification during the exponential phase of the reaction, which is the optimal point for analysing data. In this study, TaqMan Probes were used in real time PCR to measure the quantitative differences in mRNA expression levels. The TaqMan probes are a combination of a fluorescent probe and the forward and reverse PCR primers for the gene of interest. The probe has a high energy dye termed a reporter (FAM-6) at the 5` end a non-fluorescent quencher dye at the 3` end attached, which suppresses the reporter dye emission unless activated. During DNA amplification, the probe is cleaved by the 5`-3` nuclease activity of the DNA polymerase, resulting in separation of the quencher dye from the reporter dye. The fluorescent emission of the reporter increases and recorded with a detection system. The probe attaches itself only to the DNA between the forward and reverse primer sequences; hence unspecific fluorescence does not occur during the replication process. A positive reaction in the real time PCR assay is detected by accumulation of the fluorescent signal. The Ct value represents the cycle threshold at which

an increase in the fluorescence signal is detectable exceeding the background levels. The Ct values are inversely proportional to the amount of target RNA in each sample, thus, the fewer cycles taken to reach the threshold, the greater the amount of target RNA in the sample. In order to determine the relative amount of cDNA for the target gene, the cDNA of a reference gene of the same sample was amplified at the same time. The reference gene is expressed at a constant level and relative fold changes in the mRNA level were calculated with the comparative Ct ($2^{-\delta\delta Ct}$) method (Livak and Schmittgen, 2001) as follows:

$\delta Ct = Ct_{(target\ gene)} - Ct_{(reference\ gene)}$

$\delta\delta Ct = \delta Ct_{(stimulation)} - Ct_{(control)}$

$2^{-\delta\delta Ct}$

For quantification of cDNA, TaqMan Gene Expression assays (Applied Biosystems) with a Real time PCR kit (Sensi MixedT, Quantace, London) were used. The assays were carried out according to the manufacturer's instructions. Each sample was analysed in duplicate and nuclease-free water was used as a negative control.

For PCR amplification, a master mix was prepared on ice and included the following:

12.5µl 2X SensiMix

1.25µl Taqman probe

8.75µl Nuclease-free water

Then 2.5µl of cDNA for each sample was added. In order to run the assay, a real time thermal cycler (ABI Prism, 7000 Sequence Detection System, Applied Biosystems) was set at the following cycle:

50^{o}C 2 minutes ¬ 95^{o}C 10 minutes – 40 X │95 ^{o}C 15 seconds ¬ 60^{o}C 1 minute │

In order to calculate the relative fold changes between stimulations, the comparative Ct approach ($2^{-\delta\delta Ct}$)was used.

2.3.7 Gene Expression Assays (Cytokines Low Density Arrays)

To characterize different genes expressed by THP-1 monocytes in response to resistin stimulation, ready-made Taqman low-density arrays (TLDAs) (Applied Biosystems) were used. The expression profiling using these arrays was based on real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). Ready-made Taqman low-density arrays embraced pre-designed primers and Taqman probes to appraise from one to four cDNA samples generated from total RNA samples in a two step RT-PCR. These arrays contained primers and probes for 96 different cytokine related genes which modulate inflammatory process (see Table 2.10, Table 2.11 and Table 2.12). Each array plate incorporate eight samples loading ports and 2 µl of each 20 µl cDNA reaction was loaded into each port of the array. GAPDH was used as endogenous control. Amplification and real time analysis of cDNA samples loaded onto TLDLAs were carried out by employing 7900HT real time PCR machine (Applied Biosystems). The results were interpreted using SDS software.

Table 2.10 Primers and probes for the quantification of gene expression using ready-made Taqman low-density arrays (TLDAs)

Mediators	Primers and probes assay ID
18S	Hs99999901_s1
ACE	Hs00174179_m1
ACTB	Hs99999903_m1
AGTR1	Hs00241341_m1
AGTR2	Hs00169126_m1
BAX	Hs00180269_m1
BCL2	Hs00153350_m1
BCL2L1	Hs00169141_m1
C3	Hs00163811_m1
CCL19	Hs00171149_m1
CCL2	Hs00234140_m1
CCL3	Hs00234142_m1
CCL5	Hs00174575_m1
CCR2	Hs00174150_m1
CCR4	Hs99999919_m1
CCR5	Hs00152917_m1
CCR7	Hs00171054_m1
CD19	Hs00174333_m1
CD28	Hs00174796_m1
CD34	Hs00156373_m1
CD38	Hs00233552_m1
CD3E	Hs00167894_m1
CD4	Hs00181217_m1
CD40	Hs00374176_m1
CD40LG	Hs00163934_m1
CD68	Hs00154355_m1
CD80	Hs00175478_m1
CD86	Hs00199349_m1
CD8A	Hs00233520_m1
COL4A5	Hs00166712_m1
CSF1	Hs00174164_m1
CSF2	Hs00171266_m1
CSF3	Hs00357085_g1
CTLA4	Hs00175480_m1
CXCL10	Hs00171042_m1
CXCL11	Hs00171138_m1
CXCR3	Hs00171041_m1
CYP1A2	Hs00167927_m1
CYP7A1	Hs00167982_m1
ECE1	Hs00154837_m1
EDN1	Hs00174961_m1
FAS	Hs00163653_m1
FASLG	Hs00181225_m1
FN1	Hs00365052_m1
GAPDH	Hs99999905_m1
GNLY	Hs00246266_m1

Table 2.11 Primers and probes for the quantification of gene expression using Taqman low-density arrays (TLDAs) (continued)

Mediators	Primers and probe assay ID
GUSB	Hs99999908_m1
GZMB	Hs00188051_m1
HLA-DRA	Hs00219575_m1
HLA-DRB1	Hs99999917_m1
HMOX1	Hs00157965_m1
ICAM1	Hs00164932_m1
ICOS	Hs00359999_m1
IFNG	Hs00174143_m1
IKBKB	Hs00395088_m1
IL10	Hs00174086_m1
IL12A	Hs00168405_m1
IL12B	Hs00233688_m1
IL13	Hs00174379_m1
IL15	Hs00174106_m1
IL17	Hs00174383_m1
IL18	Hs00155517_m1
IL1A	Hs00174092_m1
IL1B	Hs00174097_m1
IL2	Hs00174114_m1
IL2RA	Hs00166229_m1
IL3	Hs00174117_m1
IL4	Hs00174122_m1
IL5	Hs00174200_m1
IL6	Hs00174131_m1
IL7	Hs00174202_m1
IL8	Hs00174103_m1
IL9	Hs00174125_m1
LRP2	Hs00189742_m1
LTA	Hs00236874_m1
MYH6	Hs00411908_m1
NFKB2	Hs00174517_m1
NOS2A	Hs00167248_m1
PGK1	Hs99999906_m1
PRF1	Hs00169473_m1
PTGS2	Hs00153133_m1
PTPRC	Hs00365634_g1
REN	Hs00166915_m1
RPL3L	Hs00192564_m1
SELE	Hs00174057_m1
SELP	Hs00174583_m1
SKI	Hs00161707_m1
SMAD3	Hs00232219_m1
SMAD7	Hs00178696_m1
STAT3	Hs00234174_m1
TBX21	Hs00203436_m1

Table 2.12 Primers and probes for the quantification of gene expression using Taqman low-density arrays (TLDAs) (continued)

Mediators	Primers and probe assay ID
TFRC	Hs99999911_m1
TGFB1	Hs00171257_m1
TNF	Hs00174128_m1
TNFRSF18	Hs00188346_m1
VEGF	Hs00173626_m1

2.4 Study population

The measurement of resistin levels in clinical samples from T2DM and non-diabetic control subjects was explored. Two groups of subjects were recruited, namely diabetic and non diabetics. The first group were recruited from the GP practices and secondary care diabetes clinics (Newcastle, UK). On the other hand, the non-diabetic controls were invited from the staff of the Dental school, Newcastle University or from consultant clinics at Newcastle Dental Hospital. The clinical aspect of this study was conducted by a team led by Prof. Philip Preshaw and which included Dr Rebecca Wassall (Lecturer in Restorative Dentistry), Susan Bissett (Research Dental Hygienist), Hannah Fraser (Research Dental Hygienist), Kerry Stone (research dental Nurse), (School of Dental Sciences, Newcastle University and Newcastle Dental Hospital) who were responsible for the recruitment, enrolment, screening, diagnosis and treatment of patients. All participants were examined in a periodontitis/diabetic clinic at School of Dental Sciences, Newcastle University. This study was reviewed and approved by the Sunderland Research Ethics Committee (ref 06/Q0904/8), and each study participants provided written informed consent before participating in this study.

The study sample consisted of 184 subjects of whom 101 were diabetics and 83 were non-diabetic controls. All of the participants were aged between 30 and 55 years old, male or female, with minimum of 20 natural teeth and in a good general health. Subjects were excluded from the study if they were pregnant, had any bleeding disorder, were taking drugs that provoke gingival hyperplasia, were taking immunosuppressant drugs, had any condition requiring prophylactic antibiotics before dental management, or if they had scaling or root planning in the last six months. The general characteristics of the study population are outlined in table 4.1. There was no significant difference between the T2DM and non-

diabetic control groups regarding the number of subjects, smoking status and age. The participants were aged 30-55 years age.

2.4.1 Clinical screening and periodontal treatment

All participants were assessed clinically at the first visit (pre-treatment screening). A full periodontal examination included plaque index, modified gingival index, probing depths (PD), clinical attachment loss (CAL), bleeding on probing (BOP) and recession was performed. The University of North Carolina (UNC) 15 probe (Dentsply, Addlestone, UK) was used for doing measurements. Radiographs were secured as clinically indicated, and clinical and radiographical examinations were used to assert the periodontal diagnosis.

In accordance with the method proposed by the 2005 European Workshop on Periodontology and the 2007 Centre for Disease Control and Prevention-American Academy of Periodontology collaboration (Tonetti and Claffey, 2005; Page and Eke, 2007), the periodontal diagnosis was performed based on specific diagnostic criteria illustrated on Table 2.13.

Inter- and intra-examiner reproducibility for the measurements of PD, recession, plaque and gingival inflammation were performed. The two clinicians responsible for carrying out the periodontal examination underwent calibration training to appraise examiner reproducibility. Each clinician independently made the first estimation to provide measurements of mGI, PI, PD and recession at 6 sites per tooth. Then, after 30 minute break, measurements were repeated for each examiner. During the repeated assessments, the examiners were blind to both the first measurements and the measurements of the other examiner. A variety of patients were used to appraise reproducibility, including those with healthy periodontal tissues, gingivitis and periodontitis, in order to reflect the diversity of patients.

PESA (periodontal epithelium surface area) quantifies the surface area of pocket epithelium that includes both healthy and inflamed pocket epithelium. However, PISA (periodontal inflamed surface area) was calculated to represent the surface area of bleeding pocket epithelium which quantifies the amount of inflamed periodontal tissue and thereby the

inflammatory burden posed by periodontitis. In order to calculate PESA (mm^2) and PISA (mm^2) for each subject, a previously published Microsoft excel spread sheet was used (Nesse *et al.*, 2008). First of all, CAL and recession values at 6 sites per tooth were entered, from which PESA value was calculated for each tooth. Then the PESA for every tooth was multiplied by the proportion of sites around the tooth that was affected by BOP, which provide the PISA for that particular tooth. The sum of all individual PISAs around individual teeth is calculated which equivalent to the total PISA within a patient's mouth.

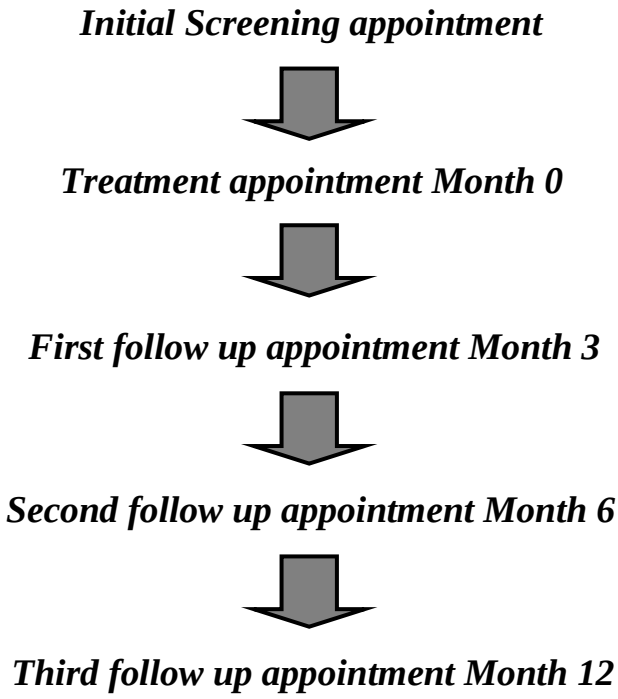
Table 2.13 Diagnostic criteria for periodontal status

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

The table demonstrates the case definitions used in the current study to determine periodontitis, gingivitis, and healthy periodontium.

Furthermore, at screening, serum, saliva and GCF samples were collected for each participant. Then non-surgical periodontal treatment was performed for patients with periodontal disease, including oral hygiene instructions and a full mouth instrumentation approach (Quirynen *et al.*, 2000). Afterward, the patients were followed up at 3 (month 3), 6 (month 6) and 12 months (month 12), with another clinical examination, serum, saliva and GCF samples collection were carried out and periodontal treatment was performed as necessary. Treatment of gingivitis patients included oral hygiene instructions and a full mouth prophylaxis at the time of screening. An overview of the study deeds are shown in Figure 2.7.

Figure 2.7 The study overview



At the screening appointment consent application, demographic data, history of smoking and diabetes, physical examination, clinical periodontal examination and collection of serum, saliva and GCF samples were performed. The first periodontal treatment was done in the treatment appointment within 2 weeks during initial screening. Follow up appointments (at 3, 6, and 12 months) were carried out for participants with periodontal disease, and at which further serum, saliva and GCF samples were obtained.

2.4.2 GCF collection

The GCF samples were collected at initial screening appointment, month 3, 6 and 12 from 4 teeth per patient. The samples were obtained from the mesiobuccal aspect of the four first molars. If the first molar was missing in a quadrant, the second molar was selected, then the second premolar, then the first premolar, then the canine. To collect the samples, the site was isolated with the use of cotton rolls and a saliva ejector to evade contamination with saliva. Also, a curette was used to remove the supra-gingival plaque prior to sampling, and the tooth was dried with air. Next, a PerioPaper strip was carefully inserted into the sulcus and kept for 30 seconds. A calibrated Periotron 6000 machine was used to determine GCF volume. Then the PerioPaper strip was immediately conveyed into an individual sterile cryovial containing 150µl PBS and left on ice, then transported to the laboratory where it was stored in -80^{o}C until analysed. In order to elute GCF from the PerioPaper strip, the samples were thawed and 50 µl of 1% PBS in BSA was added to it, centrifuged for 60 minutes at 300 rpm at 4^{o}C using Sigma 3K10 centrifuges. Afterward, a second centrifugation was done for 2 minutes at 12000 rpm at 4^{o}C. After the elution, the GCF samples were ready for analysis.

2.4.3 Saliva Collection

Saliva sample was taken pre-treatment from all participants at the screening appointment and at month 3, month 6 and month 12 after periodontal treatment for patients with periodontitis.

To obtain saliva sample and with the patient sat upright in the dental chair, 10 ml of sterile saline was splashed with a syringe onto the upper gingival margins. The patient was requested to retain the liquid and move it around for 30 seconds, and then expectorate into a polystyrene cup. This expectorated saliva wash was transposed into sterile 15ml centrifuge tubes (Sarstedt, Leicester, UK). The saliva wash samples were left on ice, and then transferred to the laboratory within 20 minutes. The samples were centrifuge for 15 min at 1500 g, 4 ºC. Then, the fluid was transferred and aliquoted into four labelled 0.5ml micro tubes, to be frozen at -80ºC pending for analysis.

2.4.4 Serum collection

The serum samples were collected at initial screening appointment, month 3, 6 and 12. Non-fasting blood samples were obtained by venous puncture. First part of the blood sample was sent to the Clinical Biochemistry Laboratory of the Royal Victoria Infirmary (Newcastle upon Tyne) for HbA1c, hsCRP, Cholesterol, HDL, LDL, and triglycerides analysis. The second part was reserved for measurement of serum resistin concentrations. Within 1 hour, blood samples were centrifuged for 15 minutes at 1500g and 4^{o}C, the serum was collected, and preserved at -80^{o}C pending further investigations by ELISA.

In an attempt to combat potential causes of specimen variation and loss of sensitivity and reproducibility of analysis due to sample degradation on storage, a variety of methods were used including storing all samples (serum, saliva and GCF) at -80 ºC freezer rather than -20 ºC freezer. Also, in the current study the freeze/thaw cycle have been avoided as possible by distributing each sample into multiple aliquots. Potentially, doing trial storage experiments to assess recovering standard concentrations of mediator from stored samples over a relatively long period of time is the best way to ascertain the extent to which the analysis is sensitive and reproducible. However, it is noteworthy that the trial storage experiments were not performed in the current study.

2.5 Statistical analysis

Statistical analysis was performed in SPSS 19.0. Graphs were created in Sigma plot 11.0 or Microsoft Excel 2007. Box and scatter plots were created in SPSS 19.0.

2.5.1 Analysis of clinical data

Clinical data includes demographic data such as ethnicity, clinical biochemistry data such as HbA1c, clinical periodontal data such as probing depth, as well as levels of local and systemic mediators, including resistin.

Clinical data were presented as box plots, scatter plots or tables. All variables were assessed for normality and homogeneity of variance using Shapiro-Wilk testing for normal distribution and Levene testing for homogeneity of variance. Where there was no evidence to reject normality, means and standard deviations (SD) of these parametric variables were calculated. Where the assumption of normality was rejected, medians and interquartile ranges (IQR) of these non-parametric variables were calculated. Non-parametric data were analysed with Kruskal-Wallis or Mann-Whitney U test. One-way analysis of variance (ANOVA) and Student's t-test were applied for parametric data. Discrete variables were analysed using Chi-squared tests. Longitudinal non-parametric data were analysed with the Friedman test and Wilcoxon Mann-Whitney test. At each time-point, for subjects with periodontitis only, data were analysed using Mann-Whitney tests for non-parametric data. P-values were corrected for multiple comparisons with the Bonferroni-Holm test. A p-value of < 0.05 was considered significant. Spearman correlation analysis was used to determine possible associations between pairs of parameters. Spearman p values were considered to be significant when $p < 0.05$.

2.5.2 Analysis of cell culture data

Cell culture data (or *in vitro* data) represents all data obtained from ELISA assays and real-time RT-PCR assays, which were carried out on samples (e.g. supernatants, and RNA) created via cell culture experiments.

Unless otherwise stated, *in vitro* data were presented as the means ± S.D. of the results from three independent experiments. Shapiro-Wilk testing for normal distribution and Levene testing for homogeneity of variance were performed. Parametric data were analysed with ANOVA or Student's t-test. Non-parametric data were analysed with Kruskal-Wallis or Mann-Whitney U test. P-values were corrected for multiple comparisons with the Bonferroni-Holm test. A p-value of < 0.05 was considered significant. Statistical analysis of Real time RT-PCR data was performed on δCt values as described by (Yuan *et al.*, 2006).

Chapter 3 Investigation of salivary and GCF resistin concentrations in patients with periodontal disease with and without type 2 diabetes mellitus

3.1 Introduction

People with diabetes suffer increased prevalence and severity of periodontal destruction as compared to systemically healthy individuals (Duarte *et al.*, 2007; Graves *et al.*, 2007; Acharya *et al.*, 2010). In effect, pertinent studies have revealed a bidirectional relationship between periodontal disease and diabetes (Mealey, 2006; Mirza *et al.*, 2010; Preshaw *et al.*, 2012). The exacerbation of the inflammatory response in the periodontal tissues of subjects with diabetes is potentially the reason behind the increased risk of periodontal disease in those cohorts (Lalla *et al.*, 2007; Nishimura *et al.*, 2007; Venza *et al.*, 2010). On the other hand, inflammatory mediators produced in the periodontium may gain access to the circulation, and these mediators can inhibit the signalling pathways downstream of insulin receptors, jeopardizing their function, thereby leading to resistance to insulin (Wellen and Hotamisligil, 2005; Gomes *et al.*, 2006; Lamster *et al.*, 2008; Acharya *et al.*, 2010). Resistin is a protein with proinflammatory and immunomodulatory properties that is expressed both systemically and locally at the site of inflammation (Bokarewa *et al.*, 2005; Silswal *et al.*, 2005; Nagaev *et al.*, 2006; Bostrom *et al.*, 2008). A plethora of evidence is available demonstrating an association between higher circulating levels of resistin and the development of T2DM (Hasegawa *et al.*, 2005; Lu *et al.*, 2006; Al-Sari *et al.*, 2007; Chen *et al.*, 2009; Gharibeh *et al.*, 2010; Lau and Muniandy, 2011).

In the pathogenesis of periodontal disease, the significance of the host inflammatory response to the microbial challenge and the production of inflammatory mediators are well established (Graves, 2008; Preshaw and Taylor, 2011). Emerging research has reported elevated resistin levels in serum and GCF samples from periodontitis subjects compared to health (Furugen *et al.*, 2008; Saito *et al.*, 2008; Hiroshima *et al.*, 2012). The importance of T2DM as a risk factor for periodontitis is thought to be linked mainly to the nature and intensity of the inflammatory response in the periodontal tissues (Duarte *et al.*, 2007; Graves *et al.*, 2007; Venza *et al.*, 2010). Clearly, it is difficult to quantify the inflammatory burden represented by periodontitis using clinical periodontal measures only, as these parameters merely provide a qualitative assessment of the inflammatory response. Therefore, the analysis of inflammatory mediators in oral fluids such as GCF and saliva could be used as a reliable method to quantitatively assess the host response in periodontal disease (Lamster and Ahlo, 2007; Giannobile *et al.*, 2009). The analysis of inflammatory mediators in whole saliva potentially provides a comprehensive measure of oral inflammatory burden, including periodontitis (Kaufman and Lamster, 2002; Giannobile *et al.*, 2009). Whole saliva represents a combination of oral fluids that originate from secretions of the salivary glands, as well as gingival fluid, sloughed epithelial cells, bacteria and food debris (Lamster and Ahlo, 2007). On the other hand, GCF is an inflammatory exudate collected from the gingival crevice or at the orifice of the crevice, and the fluid volume and constituents reflect a quantitative measure for the inflammatory response in that area (Lamster and Ahlo, 2007). GCF contains substances derived from serum, the cellular response in the periodontium, and contributions from the gingival crevice (Lamster and Ahlo, 2007). Although ample studies have revealed direct biological influences of various mediators on

periodontal destruction in diabetic subjects, the mechanisms are not fully comprehended and still remain debatable. To my knowledge, there are no previous studies which have investigated resistin in saliva or investigated the saliva levels of resistin in periodontal disease subjects with or without T2DM. Additionally, only one study has confirmed the existence of resistin in the GCF in subjects with and without periodontitis and diabetes mellitus-related periodontitis (Hiroshima *et al.*, 2012). Therefore, the aim of this study was to investigate the role of salivary resistin as a potential local biomarker in periodontal disease subjects with and without T2DM. Furthermore, the salivary resistin levels were further evaluated at 3 months, 6 months and 12 months after non-surgical periodontal treatment. Moreover, the possible relationship of salivary resistin with anthropometric and metabolic parameters, clinical periodontal parameters, inflammatory cytokines in saliva and resistin in serum was examined. Finally, a pilot study was also undertaken to evaluate resistin levels in GCF in periodontal disease subjects with and without T2DM, and to explore the possible relationship of GCF resistin with saliva and serum resistin, clinical periodontal parameters and GCF cytokine levels. To sum up, in this study we hypothesize that the concentration of resistin in saliva results from both the diabetes and periodontal disease, reflective of inflammation in the oral cavity (oral inflammatory burden).

3.2 Results

3.2.1 Analysis of pre-treatment salivary resistin concentrations in type 2 diabetic patients and non-diabetic controls with or without periodontal disease

As shown in Table 3.1 and Figure 3.1, the pre-treatment salivary resistin concentrations [median (IQR)] in the T2DM group and non-diabetic control group were [3.71 (1.89-8.17) ng/ml] and [3.63 (1.3-10.97) ng/ml], respectively. The statistical analysis revealed no significant differences in salivary resistin levels between subjects with T2DM and the non-diabetic group.

Table 3.2 and Figure 3.2 present salivary resistin concentrations before periodontal treatment following further categorisation of subjects based on their periodontal diagnosis. When considering resistin levels in saliva, there were no significant differences found in any category of periodontal disease between patients with T2DM and the non-diabetic subjects. In subjects with T2DM, salivary resistin levels [median (IQR)] were significantly higher in periodontitis patients [6.94 (2.94-14.14) ng/ml], compared to those with healthy periodontal tissues [2.35 (1.52-3.24) ng/ml] ($p<0.001$). Similarly, in subjects with T2DM, saliva levels of resistin [median (IQR)] were significantly higher in periodontitis patients [6.94 (2.94-14.14) ng/ml], compared to those with gingivitis [2.93 (1.61-4.99) ng/ml] ($p<0.001$). However, in subjects with T2DM, no significant differences in the salivary resistin levels were seen in those subjects with gingivitis [2.93 (1.61-4.99) ng/ml] compared to those with healthy periodontal tissues [2.35 (1.52-3.24) ng/ml]. In non-diabetic subjects, saliva resistin levels [median (IQR)] were significantly higher in periodontitis patients [8.27 (3.59-15.32) ng/ml], compared to those with healthy periodontal tissues [1.53 (0.79-

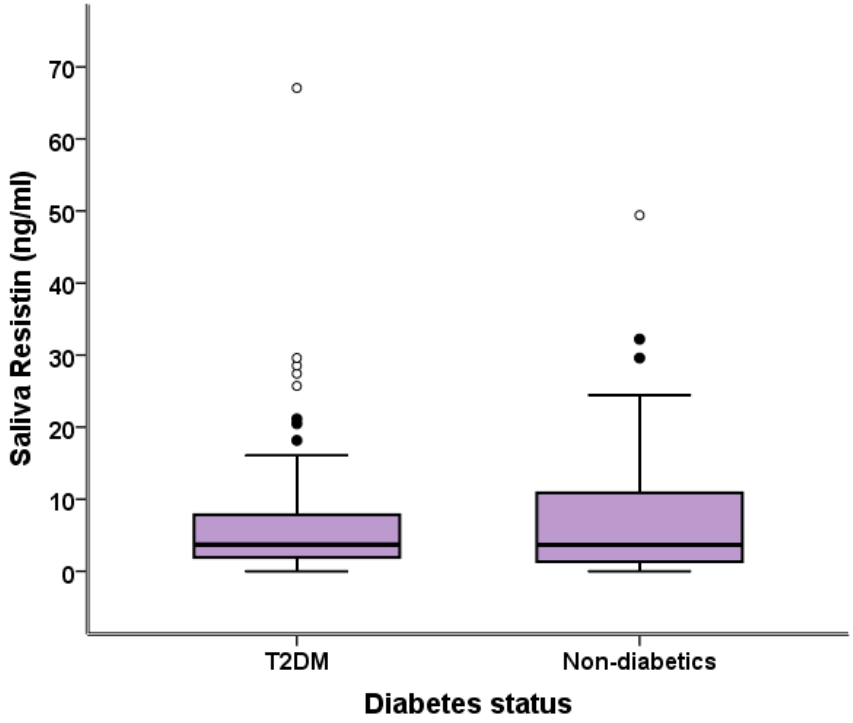
2.57) ng/ml] ($p<0.001$). Likewise, in non-diabetic subjects, saliva levels of resistin [median (IQR)] were significantly higher in periodontitis patients [8.27 (3.59-15.32) ng/ml], compared to those with gingivitis [1.21 (0.39-3.67) ng/ml] ($p<0.001$). However, in non-diabetic subjects, no significant differences in the salivary resistin levels were seen in those with gingivitis [1.21 (0.39-3.67) ng/ml] compared to those with healthy periodontal tissues [1.53 (0.79-2.57) ng/ml]. Therefore, for both patients with T2DM and non-diabetic subjects, resistin levels in saliva increased as the periodontal status worsens (Table 3.2 and Figure 3.2).

Table 3.1 Pre-treatment salivary concentrations of resistin in patients with T2DM and non-diabetic controls

	Diabetic subjects (n=101)	Non-diabetic subjects (n=82)	p-value
Resistin (ng/ml)	3.71 (1.89-8.17)	3.63 (1.3-10.97)	NS

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

Figure 3.1 Pre-treatment salivary concentrations of resistin in patients with T2DM and non-diabetic subjects



Boxplots of pre-treatment salivary levels of resistin in 101 T2DM and 82 non-diabetic subjects. Statistics: Mann Whitney-U test. ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the boundaries.

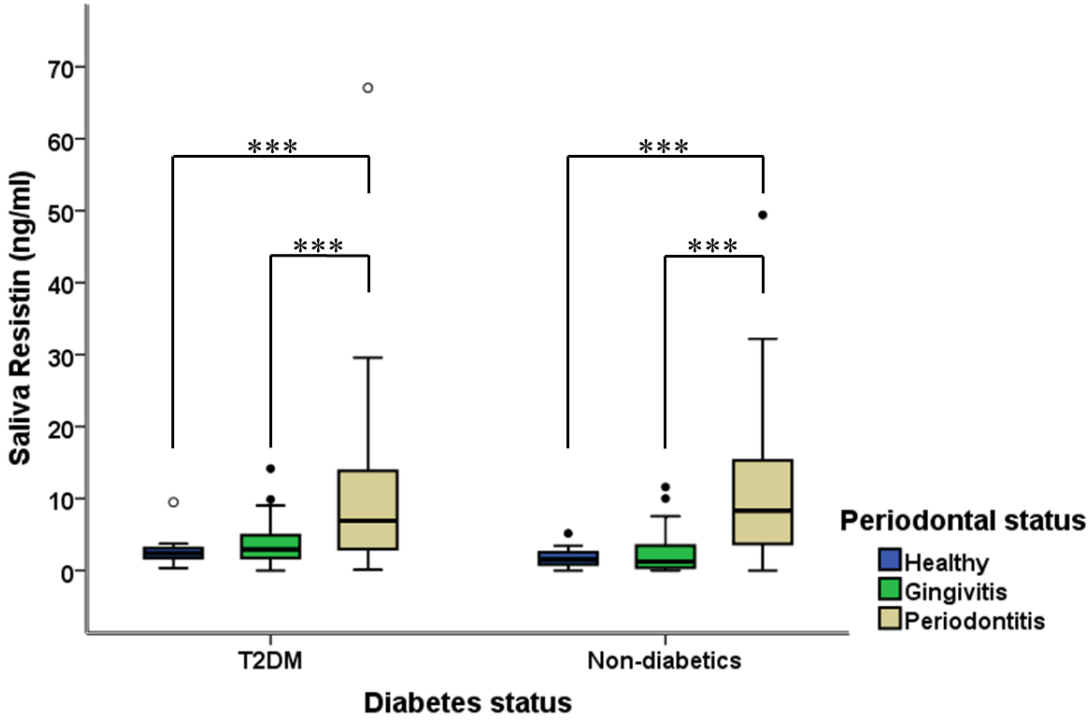
Table 3.2 Pre-treatment salivary resistin data comparing groups based on diabetic status and periodontal diagnosis

	Diabetic subjects (n=101)			Non-diabetic subjects (n=82)			p-value
	Healthy (n=14)	Gingivitis (n=39)	Periodontitis (n=48)	Healthy (n=16)	Gingivitis (n=19)	Periodontitis (n=47)	
Resistin (ng/ml)	2.35 (1.52-3.24)[¶]	2.93 (1.61-4.99)[†]	6.94 (2.94-14.14)	1.53 (0.79-2.57)[¶]	1.21 (0.39-3.67)[†]	8.27 (3.59-15.32)	[¶,†] <0.001

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

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

Figure 3.2 Pre-treatment salivary levels of resistin comparing groups based on diabetic status and periodontal diagnosis



Boxplots of pre-treatment salivary resistin data in 101 T2DM subjects (healthy periodontal tissues n=14, gingivitis n=39, periodontitis n=48) and 82 non-diabetic subjects (healthy periodontal tissues n=16, gingivitis n=19, periodontitis n=47)**.** Statistics: Kruskal-Wallis with Mann Whitney-U post hoc test *<0.05, **p<0.01, ***p<0.001 (according to periodontal status within T2DM or non-diabetic group); § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups within the corresponding periodontal status). ○ outlier more that 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

3.2.2 Exploration of the changes in salivary resistin data following non-surgical periodontal management

Table 3.3 and Figure 3.3 summarise the salivary resistin levels following non-surgical periodontal management for patients with T2DM and periodontitis and non-diabetic patients with periodontitis.

When considering salivary resistin levels, no significant differences were found between patients with T2DM and non-diabetic subjects at any of the four time points. When compared to pre-treatment levels [6.84 (2.1-10.99) ng/ml], salivary resistin levels in subjects with T2DM showed significant reductions following non-surgical periodontal management at 3 months [4.58 (2.17-9.22) ng/ml] ($p<0.05$), 6 months [3.71 (1.91-8.42) ng/ml] ($p<0.01$) and 12 months [2.66 (1.63-8.05) ng/ml] ($p<0.05$). Similarly, compared to pre-treatment levels [11.38 (3.75-16.6) ng/ml], salivary resistin levels in non-diabetic subjects showed significant reductions following non-surgical periodontal management at 3 months [6.38 (4.04-10.66) ng/ml] ($p<0.05$), and 6 months [4.95 (0.45-8.02) ng/ml] ($p<0.01$). Moreover, compared to pre-treatment levels [11.38 (3.75-16.6) ng/ml], salivary resistin levels in non-diabetic subjects showed apparent reduction at month 12 [4.73 (0.61-13.45) ng/ml], however, this difference failed to reach statistical significance (Table 3.3 and Figure 3.3).

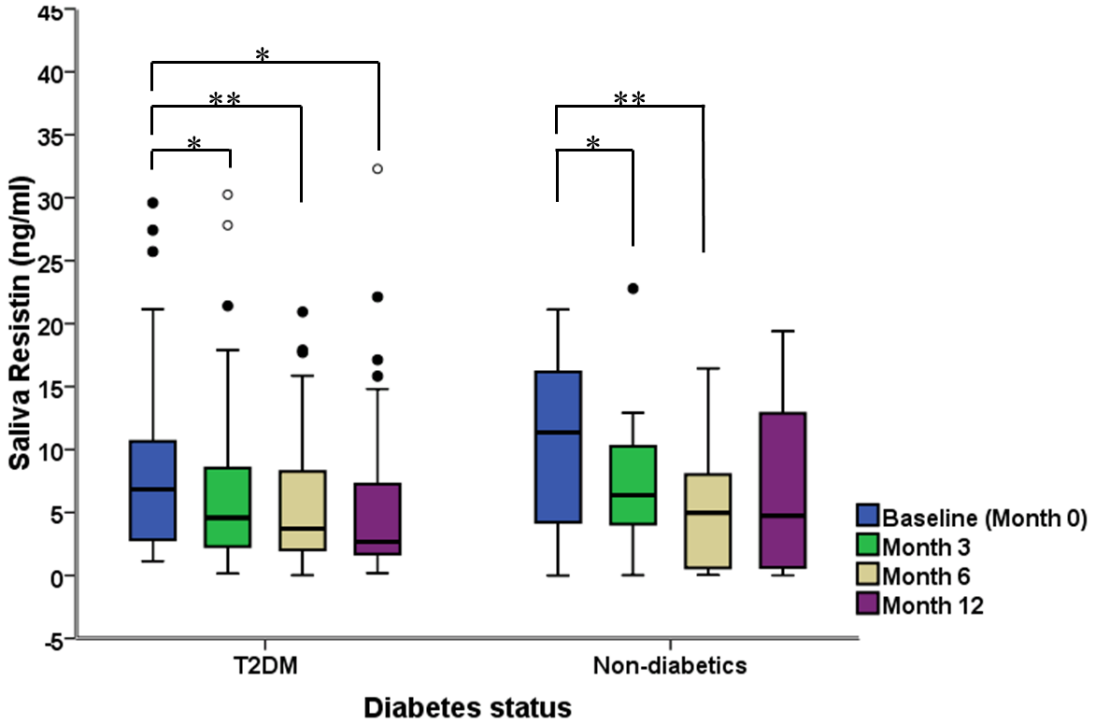
Table 3.3 Salivary resistin data in subjects with periodontitis pre- and post non-surgical periodontal management for both patients with T2DM and non-diabetic subjects

	Pre-treatment	Month 3	Month 6	Month 12	P-value
T2DM	(n=48)	(n=37)	(n=36)	(n=21)	
Resistin (ng/ml)	6.84 (2.1-10.99)	4.58 (2.17-9.22)$^{\$}$	3.71 (1.91-8.42)$^{\#}$	2.66 (1.63-8.05)¶	$^{\$, \P}$ <0.05, $^{\#}$ <0.01
Non-diabetics	(n=47)	(n=28)	(n=19)	(n=18)	
Resistin (ng/ml)	11.38 (3.75-16.6)	6.38 (4.04-10.66)$^{\$}$	4.95 (0.45-8.02)$^{\#}$	4.73 (0.61-13.45)	$^{\$}$ <0.05, $^{\#}$ <0.01

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

$^{\$}$ indicates a comparison within rows between pre-treatment and month 3 within either diabetes or non-diabetes groups
$^{\#}$ indicates a comparison within rows between pre-treatment and month 6 within either diabetes or non-diabetes groups
¶ indicates a comparison within rows between pre-treatment and month 12 within either diabetes or non-diabetes groups

Figure 3.3 Salivary resistin levels in subjects with periodontitis pre- and post non-surgical periodontal management for both T2DM and non-diabetic subjects



Boxplots of salivary resistin levels pre- and post non surgical periodontal management in subjects with periodontitis in both T2DM subjects (pre-treatment n=48, month 3 n=37, month 6 n=36, month 12 n=21) and non-diabetic subjects (pre-treatment n=47, month 3 n=28, month 6 n=19, month 12 n=18)**.** Statistics: Friedman test with Wilcoxon post hoc test *<0.05, **p<0.01, ***p<0.001 (according to time within T2DM or non-diabetic group); Mann-Whitney U test (according to T2DM versus non-diabetic group at each time point ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

3.2.1 Exploration of the relationship between salivary resistin levels and anthropometric clinical and metabolic parameters

To elucidate whether the salivary resistin is associated with systemic inflammation, glycaemic control, or risk factors for developing T2DM, the relationships between pre-treatment levels of resistin (ng/ml) in saliva and hsCRP (mg/L), HbA1c (%) and BMI (kg/m^2) were explored using Spearman's correlation test. Correlations were undertaken for the whole study population (n=183). A series of scatter plots were used to graphically display correlations of hsCRP (mg/L), HbA1c (%) and BMI (kg/m^2) with resistin levels in saliva.

Spearman's correlations between salivary resistin levels and hsCRP (mg/L), HbA1c (%) and BMI (kg/m^2) are shown in Figure 3.4, Figure 3.5 and Figure 3.6, respectively. Levels of salivary resistin were significantly and positively correlated with hsCRP (Spearman's $\rho=0.16$, $p<0.05$). However, there was no significant correlation between saliva resistin levels with HbA1c (%) and BMI.

Figure 3.4 The relationship of salivary resistin with hsCRP

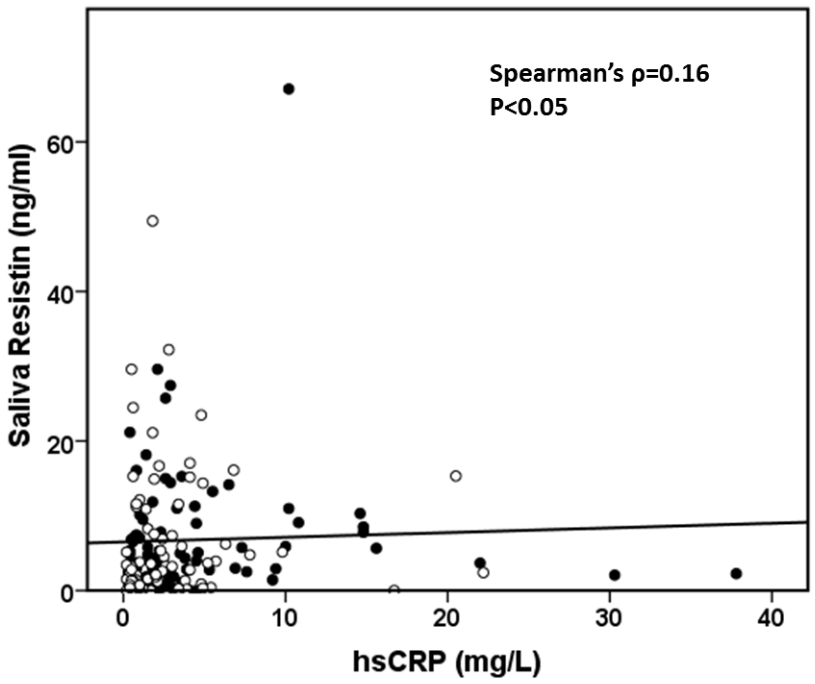


Figure shows Spearman correlation of salivary resistin concentrations with hsCRP in all subjects (n=183). Levels of hsCRP in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.16, P<0.05).

Figure 3.5 The relationship between salivary resistin with HbA1c

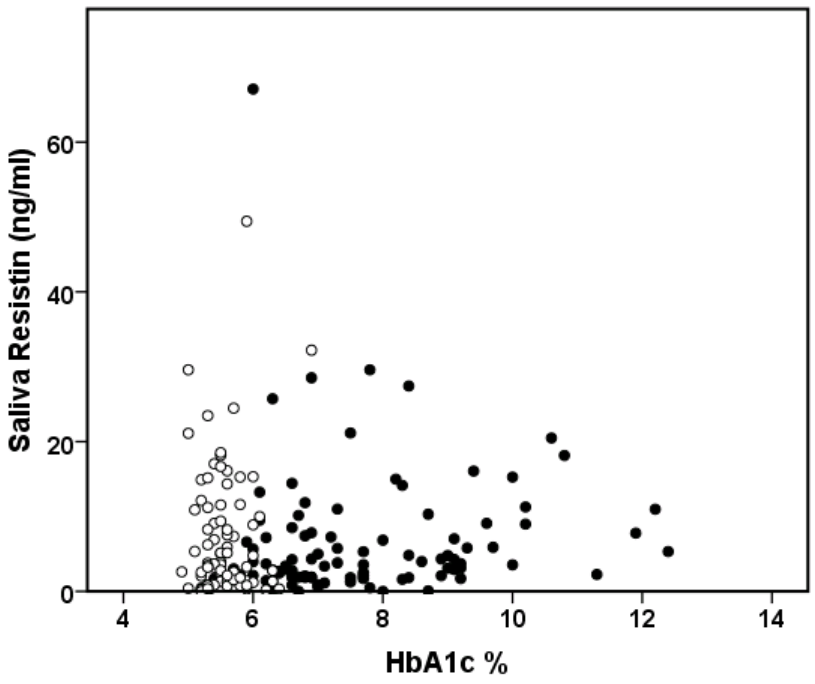


Figure shows Spearman correlation of salivary resistin concentrations with percentage of HbA1c in all subjects (n=183). Percentage of HbA1c in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated.

Figure 3.6 The relationship of salivary resistin with BMI

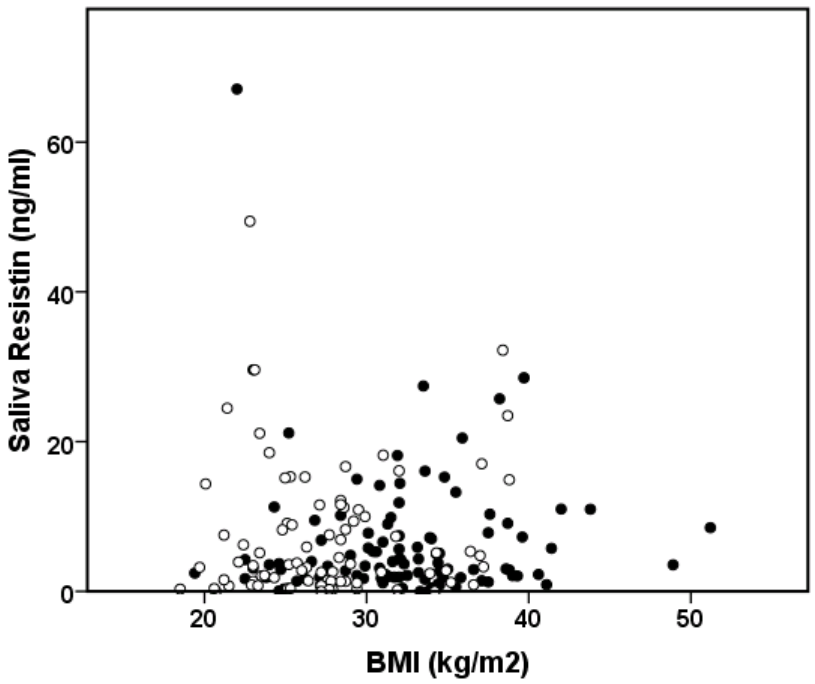


Figure shows Spearman correlation of salivary resistin concentrations with BMI in all subjects (n=183). BMI (kg/m^2) in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated.

3.2.2 Exploration of the relationship between salivary resistin levels and clinical parameters of periodontal disease

Pre-treatment levels of salivary resistin were investigated for correlations with clinical periodontal parameters including BOP%, mean PD (mm), mean LOA (mm), and PISA (mm). Correlations were undertaken for the whole study population (n=183). A series of scatter plots were used to graphically display correlations of BOP%, mean PD (mm), mean LOA (mm), and PISA (mm) with resistin levels (ng/ml) in saliva. Levels of salivary resistin were significantly and positively correlated with percentage BOP (ρ=0.476, p<0.001), mean PD (mm) (ρ=0.594, p<0.001), mean LOA (mm) (ρ=0.598, p<0.001), and PISA (mm) (ρ=0.573, p<0.001) (Figure 3.7, Figure 3.8, Figure 3.9 and Figure 3.10).

Figure 3.7 The relationship of salivary resistin with %BOP

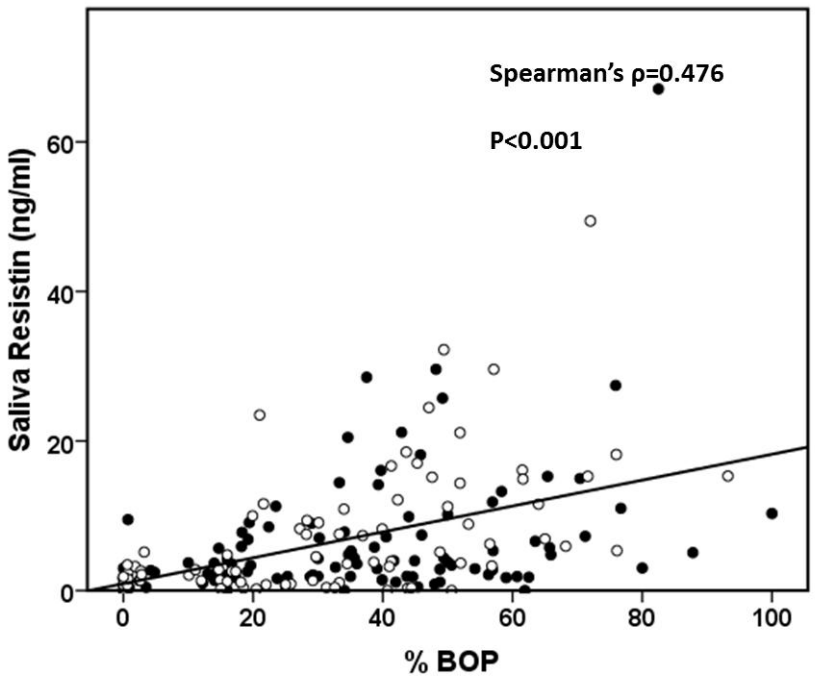


Figure shows Spearman correlation of salivary resistin concentrations with %BOP in all subjects (n=183). Percentage of BOP in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.476, P<0.001).

Figure 3.8 The relationship of salivary resistin with mean PD

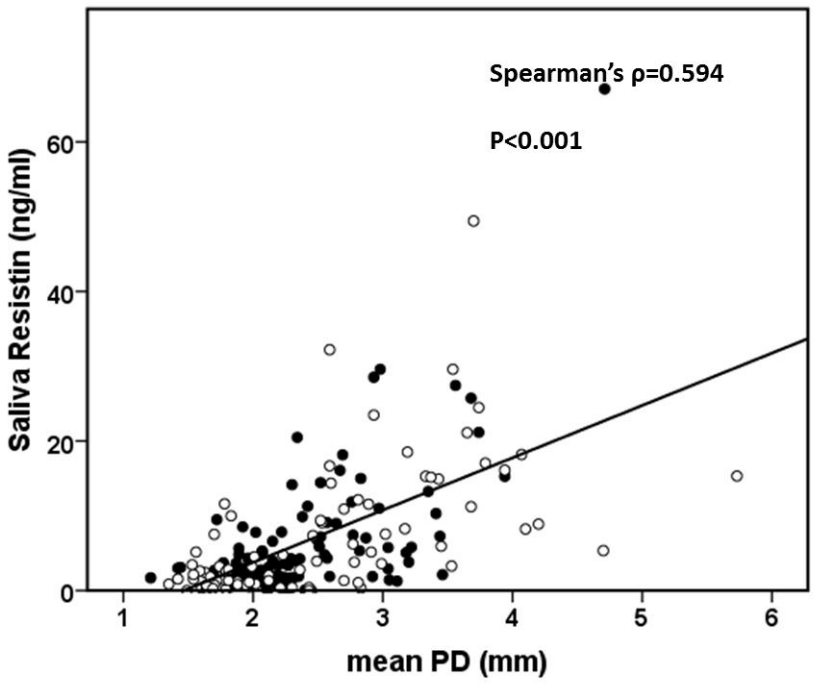


Figure shows Spearman correlation of salivary resistin concentrations with mean PD in all subjects (n=183). Mean PD in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.594, P<0.001).

Figure 3.9 The relationship of salivary resistin with mean LOA

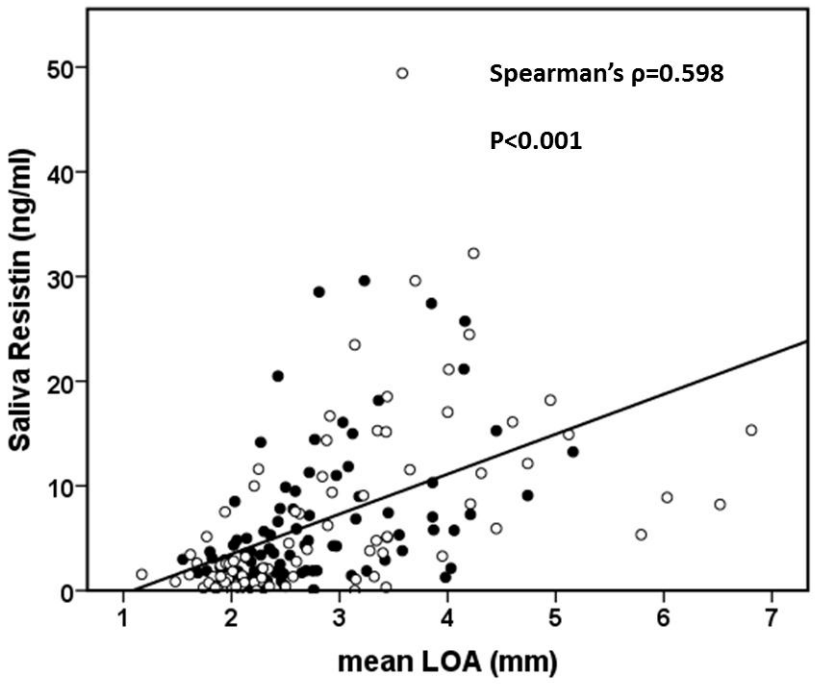


Figure shows Spearman correlation of salivary resistin concentrations with mean LOA in all subjects (n=183). Mean LOA in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.598, P<0.001).

Figure 3.10 The relationship between salivary resistin with PISA

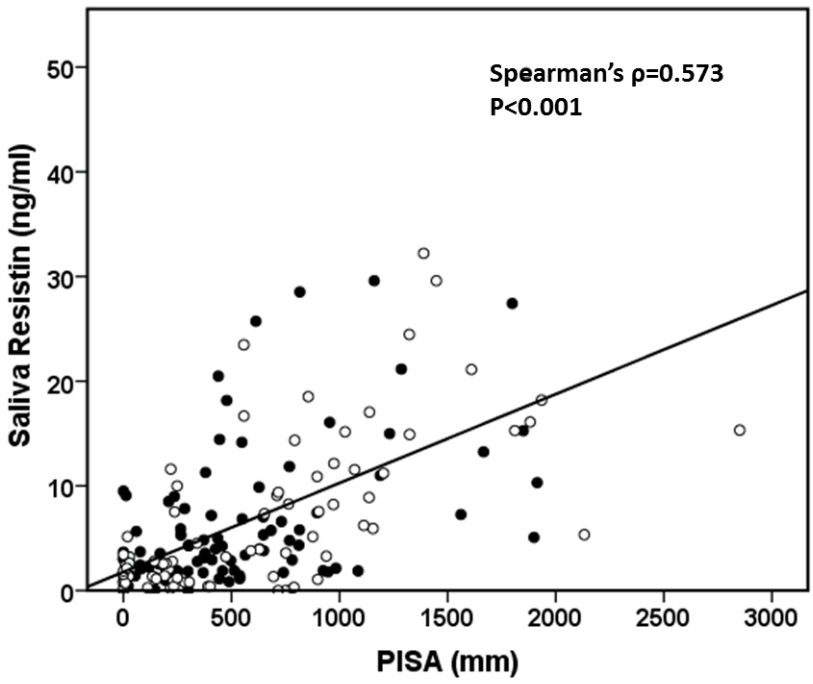


Figure shows Spearman correlation of salivary resistin concentrations with PISA in all subjects (n=183). PISA (mm) in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.573, P<0.001).

3.2.3 Exploration of the relationship between salivary resistin levels and the levels of inflammatory cytokines in saliva

Pre-treatment levels of salivary resistin were investigated for correlations with the pre-treatment levels of salivary TNF-α (pg/ml), salivary IL-1β (pg/ml), salivary IL-6 (pg/ml), salivary IFN-γ (pg/ml), and serum resistin (ng/ml). Correlations were undertaken for the whole study population (n=183). A series of scatter plots were used to graphically display correlations of salivary TNF-α (pg/ml), salivary IL-1β (pg/ml), salivary IL-6 (pg/ml), salivary IFN-γ (pg/ml) and serum resistin (ng/ml) with resistin levels (ng/ml) in saliva. Levels of salivary resistin were significantly and positively correlated with salivary IL-1β (pg/ml) (ρ=0.284, p<0.001), salivary IL-6 (pg/ml) (ρ=0.195, p<0.01), and serum resistin (ng/ml) (ρ=0.19, p<0.01). However, there was no significant correlation between resistin in saliva with salivary levels of TNF-α (pg/ml) and IFN-γ (pg/ml). (See Figure 3.11, Figure 3.12, Figure 3.13, Figure 3.14 and Figure 3.15).

Figure 3.11 The relationship of salivary resistin with TNF-α level in saliva

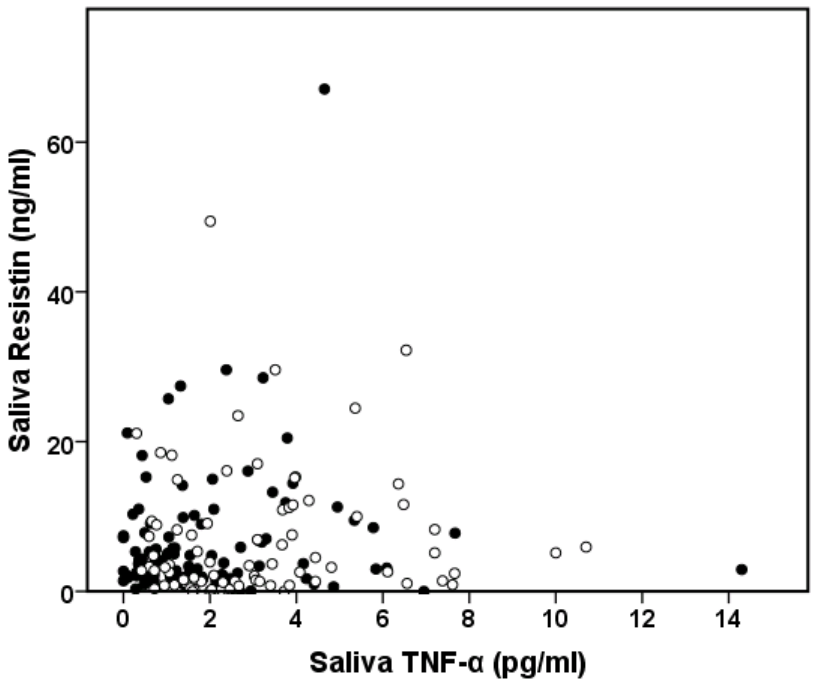


Figure shows Spearman correlation of salivary resistin concentrations with salivary TNF-α in all subjects (n=183). Levels of salivary TNF-α in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated.

Figure 3.12 The relationship between salivary resistin and IL-1β levels in saliva

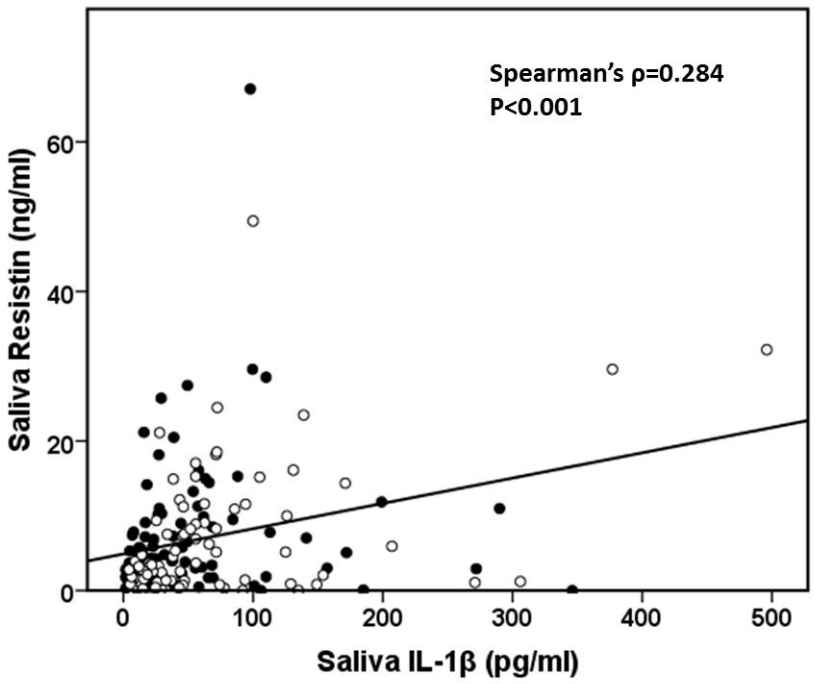


Figure shows Spearman correlation of salivary resistin concentrations with salivary IL-1β in all subjects (n=183). Levels of salivary IL-1β in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.284, P<0.001).

Figure 3.13 The relationship between salivary resistin and IL-6 levels in saliva

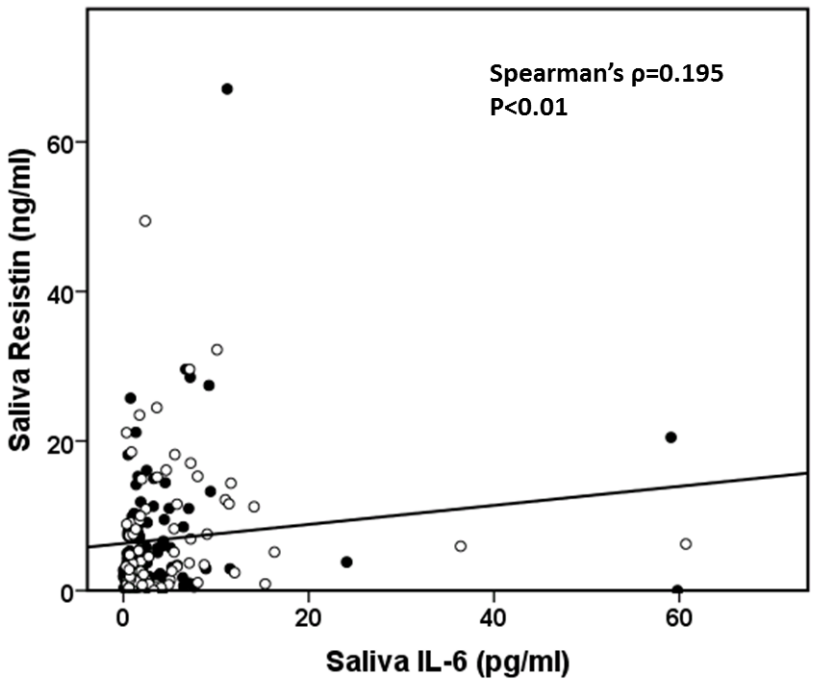


Figure shows Spearman correlation of salivary resistin concentrations with salivary IL-6 in all subjects (n=183). Levels of salivary IL-6 in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.195, P<0.01).

Figure 3.14 The relationship of salivary resistin and IFN-γ levels in saliva

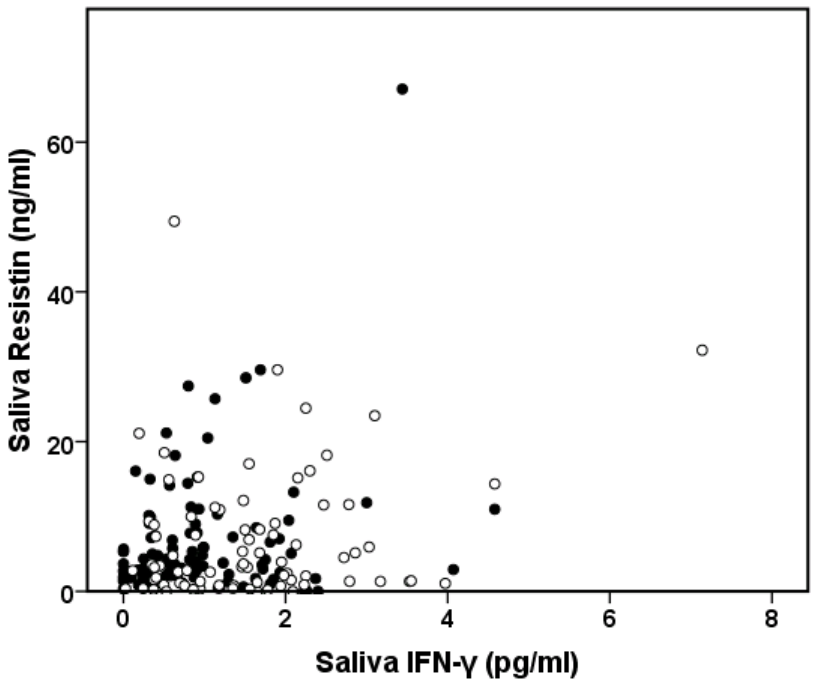


Figure shows Spearman correlation of salivary resistin concentrations with salivary IFN-γ in all subjects (n=183). Levels of salivary IFN-γ in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated.

Figure 3.15 The relationship between salivary resistin and resistin levels in serum

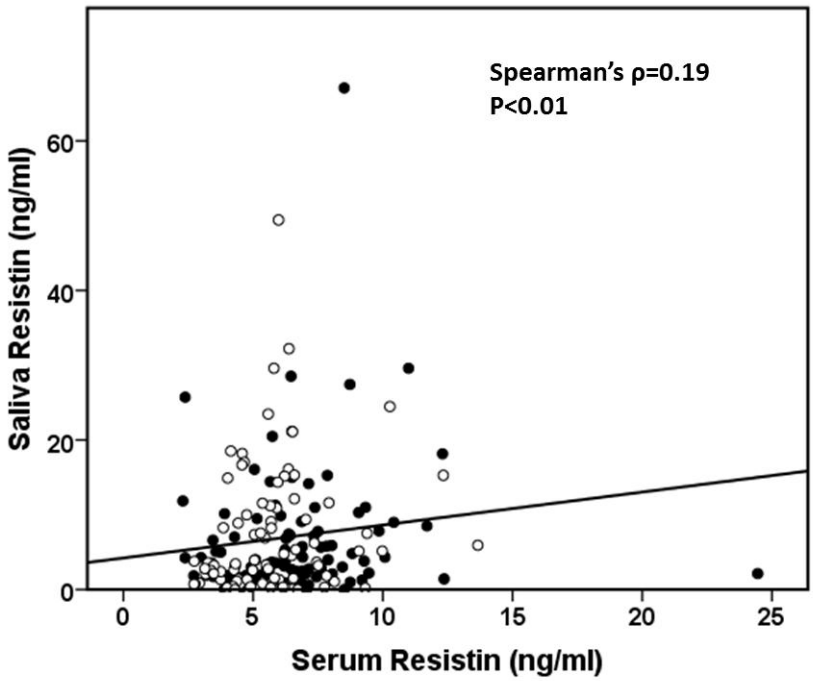


Figure shows Spearman correlation of salivary resistin concentrations with serum resistin in all subjects (n=183). Levels of serum resistin in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.19, P<0.01).

3.2.4 Analysis of pre-treatment GCF resistin levels in T2DM and non-diabetic subjects with or without periodontal disease

Table 3.4 and Figure 3.16 shows that before periodontal treatment, no significant differences in the GCF levels of resistin were found in subjects with T2DM [2.81 (0.99-5.44) ng/ml] compared to non-diabetic subjects [2.77 (0.87-6.25) ng/ml]. However, caution must be used when interpreting these results because of the small sample size.

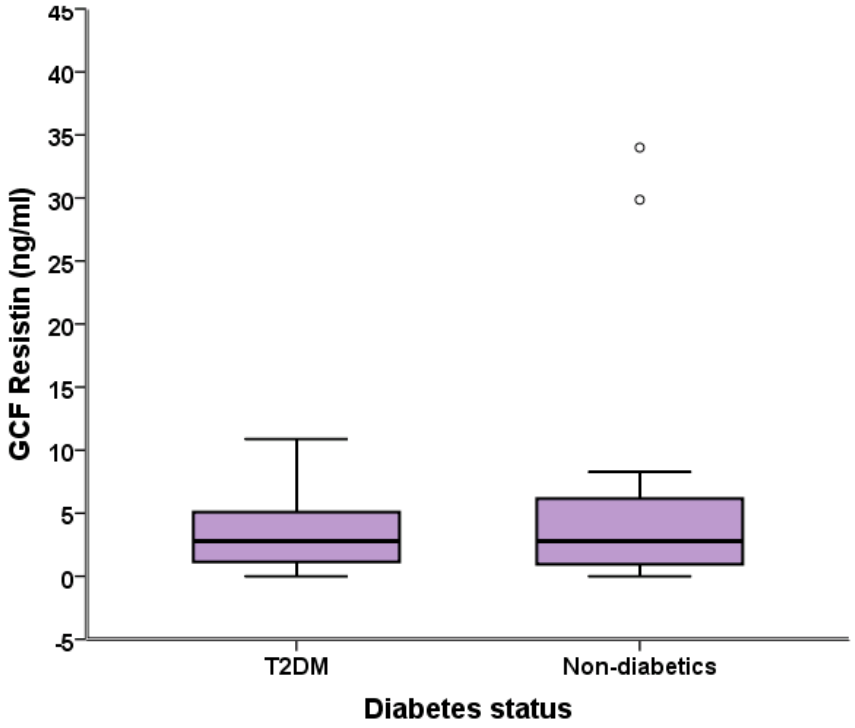
Table 3.5 and Figure 3.17 present GCF resistin levels before periodontal treatment following further categorisation of subjects based on their periodontal diagnosis. When considering resistin levels in GCF, there were no significant differences found in any periodontal category between patients with T2DM and non-diabetic subjects. In T2DM subjects, resistin levels in GCF appeared to be higher in gingivitis patients [4.22 (0.68-8.45) ng/ml] compared to those with healthy periodontal tissues [2.17 (0.0-5.84) ng/ml] and periodontitis [2.81 (1.14-3.39) ng/ml], however, these differences failed to reach statistical significance. In non-diabetic subjects, resistin levels in GCF appeared to be higher in periodontitis patients [6.38 (1.69-24.46) ng/ml] compared to those with healthy periodontal tissues [1.41 (0.89-3.15) and gingivitis [0.94 (0.13-4.07) ng/ml], however, again these differences failed to reach statistical significance. Nevertheless, caution must be used when interpreting these results as data were limited by small sample size.

Table 3.4 Pre-treatment GCF resistin concentrations in T2DM patients and non-diabetic subjects

	Diabetic subjects (n=21)	Non-diabetic subjects (n=19)	p-value
Resistin (ng/ml)	2.81 (0.99-5.44)	2.77 (0.87-6.25)	NS

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

Figure 3.16 Pre-treatment GCF concentrations of resistin in patients with T2DM and non-diabetic subjects



Boxplots of pre-treatment GCF levels of resistin in 21 T2DM and 19 non-diabetic subjects. Statistics: Mann Whitney-U test ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the boundaries.

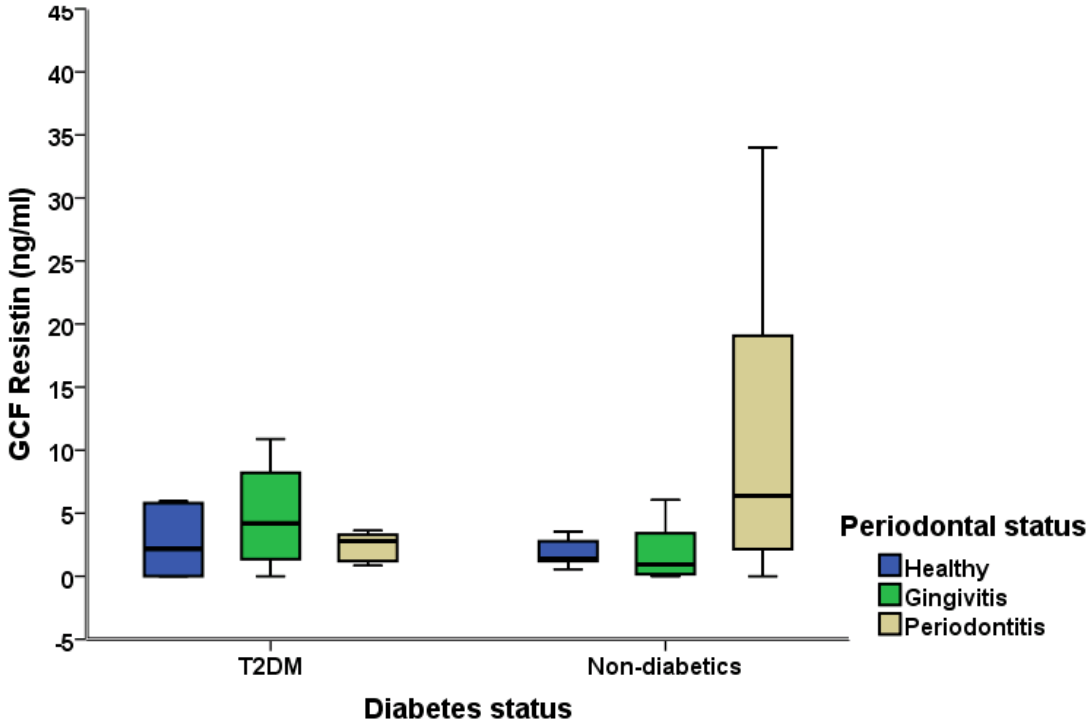
Table 3.5 Pre-treatment GCF resistin data comparing groups based on diabetic status and periodontal diagnosis

	Diabetic subjects (n=21)			Non-diabetic subjects (n=19)			p-value
	Healthy (n=6)	Gingivitis (n=8)	Periodontitis (n=7)	Healthy (n=5)	Gingivitis (n=6)	Periodontitis (n=8)	
Resistin (ng/ml)	2.17 (0.0-5.84)	4.22 (0.68-8.45)	2.81 (1.14-3.39)	1.41 (0.89-3.15)	0.94 (0.13-4.07)	6.38 (1.69-24.46)	NS

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

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

Figure 3.17 Pre-treatment GCF levels of resistin comparing groups based on diabetic status and periodontal diagnosis



Boxplots of pre-treatment GCF resistin data in 21 T2DM subjects (healthy periodontal tissues n=6, gingivitis n=8, periodontitis n=7) and 19 non-diabetic subjects (healthy periodontal tissues n=5, gingivitis n=6, periodontitis n=8)**.** Statistics: Kruskal-Wallis with Mann Whitney-U post hoc test (according to periodontal status within T2DM or non-diabetic group); (T2DM versus non-diabetic groups within the corresponding periodontal status). ○ outlier more that 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

3.2.5 Exploration of the relationship of resistin levels in GCF with salivary and serum resistin levels, clinical periodontal parameters and GCF cytokine levels

Pre-treatment levels of GCF resistin were investigated for correlations with resistin levels in saliva and serum, clinical periodontal parameters and GCF cytokine levels. Spearman's correlations (correlation coefficient and P-values) between GCF resistin levels and resistin levels in saliva and serum, clinical periodontal parameters and GCF cytokines levels are presented in Table 3.6, Table 3.7 and Table 3.8.

Interestingly, levels of GCF resistin were significantly and positively correlated with salivary resistin levels (Spearman's ρ=0.422, p<0.01). On the other hand, no significant association were found between GCF resistin levels and resistin levels in serum (Spearman's ρ=0.076, p>0.05) (Table 3.6).

When considering the correlation between GCF resistin levels and the clinical periodontal parameters, significant positive correlations were demonstrated between resistin levels in GCF and %BOP (Spearman's ρ=0.435, p<0.01), mean PD (Spearman's ρ=0.347, p<0.05), and PISA (Spearman's ρ=0.49, p<0.01). However, no significant associations were found between GCF resistin levels and mean LOA (Spearman's ρ=0.295, p>0.05) (Table 3.7).

When considering the correlation between GCF resistin levels and cytokine levels in GCF, significant positive correlations were demonstrated between resistin levels in GCF and GCF levels of TNF-α (Spearman's ρ=0.525, p<0.001), IL-1β (Spearman's ρ=0.462, p<0.01), IL-6 (Spearman's ρ=0.563, p<0.001) and IFN-γ (Spearman's ρ=0.526, p<0.001) (Table 3.8).

Table 3.6 Correlations between GCF resistin levels and resistin levels in saliva and serum

	Correlation Coefficient	P-value
Saliva Resistin	0.422	0.007
Serum Resistin	0.076	0.644

Table 3.7 Correlations between GCF resistin levels and clinical periodontal parameters

Clinical parameters	Correlation Coefficient	P-value
BOP (%)	0.435	0.005
Mean PD (mm)	0.347	0.028
Mean LOA (mm)	0.295	0.065
PISA (mm^2)	0.49	0.002

Table 3.8 Correlations between GCF resistin levels and cytokine levels in GCF

GCF cytokines	Correlation Coefficient	P-value
TNF-α	0.525	0.001
IL-1β	0.462	0.003
IL-6	0.563	0.000
IFN-γ	0.526	0.000

Tables show Spearman rank correlation coefficients

3.3 Discussion

Substantial evidence has linked periodontal disease with type 2 diabetes, but studies focusing on the possible role of resistin in this interrelationship are limited. It is widely accepted that diabetes represents an established hyper-inflammatory state, and chronic inflammation has been deemed as an underlying cause for insulin resistance, with resulting hyperglycaemia (Dandona *et al.*, 2003; Sjoholm and Nystrom, 2006). Particularly, mediators generated during the inflammatory process can suppress the signalling downstream of the insulin receptor, thereby resulting ultimately in insulin resistance (Wellen and Hotamisligil, 2005). Periodontal disease is considered as a chronic local oral infection that triggers a local and systemic inflammatory response (Ebersole and Cappelli, 2000). Numerous studies have suggested that periodontitis may induce and perpetuate a chronic systemic hyper-inflammatory state (Loos *et al.*, 2000; Noack *et al.*, 2001; D'Aiuto *et al.*, 2004). Resistin is a proinflammatory cytokine which can be produced by macrophages, monocytes and neutrophils during the tissue inflammatory response to various stimuli including the microbial challenge (Lehrke *et al.*, 2004; Bokarewa *et al.*, 2005; Furugen *et al.*, 2011).

An abundance of studies have recently deliberated the potential diagnostic properties of saliva. Emerging research is highlighting the importance of saliva not only as a tool help diagnose oral diseases, but also as a body's "mirror" that could be used to monitor general health and in the diagnosis of systemic conditions (Wong, 2006; Hu *et al.*, 2007a; Giannobile *et al.*, 2009). Currently, the assessment of clinical parameters of tissue destruction and signs of tissue inflammation are the primary methods for clinical diagnosis of periodontal diseases. Although these measures are easy to use to determine the periodontal status of patients, the time and expertise

required for a full periodontal examination presents serious limitations to epidemiological surveys. Efficient and convenient methods for screening patients would be particularly useful for investigations designed to uncover risk factors and risk indicators of periodontal diseases with a large sample size. Saliva could be utilized as a useful, non-invasive diagnostic body fluid to monitor biomarkers released during disease initiation and progression (Kaufman and Lamster, 2000; Taba *et al.*, 2005; Giannobile *et al.*, 2009). Identification of particular salivary biomarkers associated with periodontal disease extent and severity could have substantial influence on the diagnosis and monitoring of periodontal diseases. A plethora of evidence has identified GCF as the primary source of cytokines in whole saliva (Ruhl *et al.*, 2004; Rudrakshi *et al.*, 2011). It has been demonstrated that TNF-α, IL-1α, IL-6 and IL-8 were present in whole saliva at concentrations significantly higher than in major salivary gland secretions, which suggested that the detected cytokines in whole saliva did not come from the secretions of major salivary glands, and that GCF was the likely source of these cytokines (Wozniak *et al.*, 2002; Ruhl *et al.*, 2004). Potentially, whole saliva contains GCF from all periodontal sites providing a useful assessment of periodontal disease status.

In reviewing the literature, no published research was found investigating salivary resistin levels in subjects with healthy periodontal tissues, gingivitis and periodontitis with or without T2DM. The present study showed for the first time the existence of resistin in saliva and compared resistin levels among saliva samples from periodontally healthy, gingivitis and periodontitis subjects with and without T2DM. The present study demonstrated that salivary resistin levels are elevated in subjects with periodontitis as compared to periodontally healthy controls and gingivitis subjects in both T2DM and non-diabetic groups. On the other hand, salivary resistin

levels showed no significant difference between T2DM and non-diabetic groups irrespective of periodontal status. Interestingly, the saliva resistin levels in periodontitis patients decreased after 3, 6 and 12 months of non-surgical periodontal management in both T2DM and non-diabetic groups. Moreover, the present study revealed that the levels of salivary resistin are significantly and positively correlated with hsCRP, saliva IL-6, saliva IL-1β and serum resistin. Additionally, salivary resistin also positively correlated with %BOP, mean PD, mean LOA and PISA. Furthermore, a pilot study was also undertaken to investigate resistin levels in GCF samples from subjects with healthy periodontal tissues, gingivitis and periodontitis with or without T2DM. Resistin levels in GCF showed no significant difference between T2DM and non-diabetic groups irrespective of periodontal status. In the non-diabetic group, periodontitis subjects appear to have a trend of higher GCF resistin levels than periodontally healthy subjects. Finally, the present study revealed that the levels of GCF resistin are significantly and positively correlated with saliva resistin, GCF TNF-α, GCF IL-1β, GCF IL-6 and GCF INF-γ. Additionally, GCF resistin also positively correlated with %BOP, mean PD, and PISA.

Very little was found in the literature on the question of the existence of resistin in saliva. One previous study detected resistin in saliva and found that salivary resistin levels were significantly higher ($p=0.001$) in patients with primary Sjögren's syndrome compared to controls, suggesting a strong association of resistin with local inflammation in patients with primary Sjögren's syndrome (Bostrom *et al.*, 2008). Another study aimed to suggest methods of determining resistin, adiponectin and visfatin levels in saliva, and to evaluate their correlations with serum levels in healthy individuals. This study reported that salivary resistin levels in healthy subjects were

1.69 (0.73-6.55) ng/ml, and correlated positively with serum resistin levels (Mamali *et al.*, 2012).

To the best of our knowledge, this is the first study to measure resistin concentrations in saliva in both healthy and periodontally diseased subjects with or without T2DM. In the present study, no significant difference was found between the T2DM [3.71 (1.89-8.17) ng/ml] and non-diabetic control group [3.63 (1.3-10.97) ng/ml] irrespective of periodontal status. In general, therefore, it seems that diabetes had no impact on salivary resistin levels, and a possible explanation for this might be that resistin in saliva originates from immune cells in the local periodontal tissues rather than the body adipose tissues, and therefore salivary resistin was not associated with systemic inflammation represented by diabetes. Interestingly, following further categorization of subjects based on their periodontal diagnosis, the current study demonstrated that subjects with T2DM and periodontitis have significantly higher levels of resistin in saliva [6.94 (2.94-14.14) ng/ml] compared to T2DM subjects with gingivitis [2.93 (1.61-4.99) ng/ml] and T2DM subjects with healthy periodontal tissues [2.35 (1.52-3.24) ng/ml]. The same pattern was replicated in the non-diabetic group, in which periodontitis subjects have significantly higher levels of resistin in saliva [8.27 (3.59-15.32) ng/ml] compared to those with gingivitis [1.21 (0.39-3.67) ng/ml] and those with healthy periodontal tissues [1.53 (0.79-2.57) ng/ml]. Hence, it could conceivably be hypothesised that elevated salivary resistin is associated with the local inflammation in periodontitis, and that patients with extensive periodontal inflammation have the highest resistin levels. One of the potential sources of resistin in saliva is the cellular response in the periodontium, this could be explained by the fact that GCF traverses through inflamed periodontal tissues *en route* to the sulcus, and mediators (such as resistin) are gathered from the surrounding areas and are

subsequently eluted into whole saliva. It may be that the immune cells in the periodontium stimulated by the persistent microbial challenge produce high levels of resistin, which in turn congregates with other contributions from periodontium to form the gingival exudate that flows through the gingival sulcus into the oral fluid. It can thus be suggested that the elevated levels of resistin in saliva reflect the intensity of local inflammation in the periodontium. Interestingly, the levels of salivary resistin in periodontally healthy non-diabetic subjects in the current study [1.53 (0.79-2.57) ng/ml] were comparable to those in normal healthy individuals [1.69 (0.73-6.55) ng/ml] in a study performed by (Mamali *et al.*, 2012), and this was in spite of the difference in the saliva collection techniques implemented in the two studies. Most studies that have investigated the role of resistin in periodontitis have only focussed on the levels of resistin in serum and GCF. An increased serum resistin level associated with periodontitis was reported in two previous studies (Furugen *et al.*, 2008; Saito *et al.*, 2008). Additionally, Hiroshima *et al*. (2012) reported that GCF resistin levels from patients with periodontitis or diabetes-related periodontitis were significantly higher than those of healthy subjects (Hiroshima *et al.*, 2012). Although no previous study has investigated salivary resistin levels in periodontitis subjects with or without T2DM, the salivary levels of other inflammatory cytokines have been explored in periodontitis patients in a number of recent studies (Miller *et al.*, 2006; Gursoy *et al.*, 2009; Teles *et al.*, 2009; Costa *et al.*, 2010). It has been suggested that salivary levels of IL-1β and IL-6 were significantly higher in subjects with periodontitis compared to those without periodontitis (Miller *et al.*, 2006; Gursoy *et al.*, 2009; Costa *et al.*, 2010). On the other hand, Teles *et al*. (2009) quantified a number of inflammatory cytokines in saliva using a multiple bead immunoassay, and found no significant differences between periodontitis and periodontally healthy

groups with regards to levels of TNF-α, IL-1β, IL-6 and IFN-γ (Teles *et al.*, 2009). This inconsistency between these studies might be attributed to the different levels of disease in the various study populations. Presently, the published research data investigating the role of inflammatory cytokines in saliva in subjects with T2DM and periodontitis are very limited. Costa *et al*. (2010) showed no significant difference in salivary IL-6 levels when subjects with T2DM with periodontitis (n=24) were compared to non-diabetic subjects with periodontitis (n=24) (Costa *et al.*, 2010).

Intriguingly, saliva samples used in the current study were saliva wash rather than whole saliva, which was obtained by oral rinse using 10ml of saline. This consequently diluted the saliva sample and made the actual volume of whole saliva in each sample unknown. Therefore, it is interesting to note that the current study was able to identify a significant difference in salivary resistin levels without any detectable variability in the data, and this substantiates the validity of investigating resistin levels in saliva.

Overall, data from the current study confirm that salivary levels of resistin increase significantly with the development of periodontitis. This would support the possibility of selecting salivary resistin as a biomarker that may reflect periodontal status. The current study was the first research investigating salivary resistin levels in both healthy and periodontally diseased subjects with or without T2DM, thus further investigations on the current topic are therefore recommended to confirm these findings.

No published data were found in the literature on the association of salivary levels of resistin with the degree of obesity, glycaemic control, systemic inflammation, salivary levels of inflammatory cytokines, and clinical periodontal parameters. The results of the current study did not show any significant correlation of salivary resistin levels

with BMI and HbA1c. On the other hand, salivary resistin levels were found to be significantly and positively correlated with hsCRP (Spearman's $\rho=0.16$, $p<0.05$), albeit this correlation was relatively weak (Cohen, 1988). These findings suggest that salivary resistin levels do not appear related to BMI and glycaemic control, and are only weakly correlated with systemic inflammation represented by hsCRP. Within the current literature, there is no published study exploring the correlation between salivary cytokines levels and resistin levels in saliva. The current study demonstrated a significant positive correlation between salivary levels of IL-1β (Spearman's $\rho=0.284$, $p<0.001$), and IL-6 (Spearman's $\rho=0.195$, $p<0.01$), and the salivary resistin levels. On the other hand, no significant correlation was found between salivary resistin levels and the levels of TNF-α and IFN-γ in saliva. Interestingly, salivary resistin levels correlated significantly and positively with resistin levels in serum (Spearman's $\rho=0.19$, $p<0.01$), albeit this correlation was relatively small (Cohen, 1988). This finding corroborates the findings of Mamali *et al* (2012), who reported a significant correlation of salivary resistin levels with resistin levels in serum (Spearman's $\rho=0.441$, $p<0.01$) in healthy individuals (Mamali *et al.*, 2012).

In reviewing the literature, no data were found on the relationship between the levels of resistin in saliva and the clinical periodontal parameters. In the current study, significant positive correlations were determined between %BOP (Spearman's $\rho=0.476$, $p<0.001$), mean PD (Spearman's $\rho=0.594$, $p<0.001$), mean LOA (Spearman's $\rho=0.598$, $p<0.001$), and PISA (Spearman's $\rho=0.573$, $p<0.001$) and salivary levels of resistin. These findings suggest that as the clinical periodontal measurements increase, the levels of resistin in saliva also increase. It can therefore be assumed that the elevated resistin concentration in saliva may be one of the host-

response components associated with the clinical manifestations of periodontal disease.

In spite of the substantial evidence that testifies to the importance of saliva in providing adjunctive information in the diagnosis and monitoring of periodontal diseases (Miller *et al.,* 2006; Miller *et al.,* 2010), there are few published longitudinal studies evaluating the influence of non-surgical periodontal management on the levels of inflammatory mediators in saliva (Sexton *et al.,* 2011). Numerous studies have suggested that periodontal treatment, which reduces the intraoral bacterial bioburden and decreases periodontal inflammation, can have a significant effect on systemic inflammatory status (Christgau *et al.,* 1998; Correa *et al.,* 2008; Goncalves *et al.,* 2008; O'Connell *et al.,* 2008). It has been reported that periodontal treatment is associated with improved glycaemic control in subjects with both diabetes and periodontal diseases (Nishimura *et al.,* 2007). To the best of our knowledge, this is the first study to explore the changes in salivary resistin levels following non-surgical periodontal management. In the current study, in subjects with T2DM, compared to pre-treatment levels [6.84 (2.1-10.99) ng/ml], salivary resistin levels showed significant reductions following non-surgical periodontal management at 3 months [4.58 (2.17-9.22) ng/ml], 6 months [3.71 (1.91-8.42) ng/ml] and 12 months [2.66 (1.63-8.05) ng/ml]. Likewise, in non-diabetic subjects, compared to pre-treatment levels [11.38 (3.75-16.6) ng/ml], salivary resistin levels showed significant reductions following non-surgical periodontal management at 3 months [6.38 (4.04-10.66) ng/ml] and 6 months [4.95 (0.45-8.02) ng/ml]. Additionally, in non-diabetic subjects compared to pre-treatment levels [11.38 (3.75-16.6) ng/ml], saliva resistin showed an apparent reduction at month 12 [4.73 (0.61-13.45) ng/ml], but the difference did not reach the level of statistical significance. Although there are currently no published

longitudinal data investigating the impact of non-surgical periodontal management on levels of resistin in saliva, it is encouraging to compare our data with research that explored the changes in levels of other salivary inflammatory mediators in response to non-surgical periodontal management. Sexton *et al* (2011) reported significant reductions in salivary levels of TNF-α and IL-1β at 4 months in subjects receiving oral hygiene instruction alone, and in subjects receiving non-surgical periodontal management (Sexton *et al.*, 2011). According to the present findings, no significant differences in salivary resistin levels were found between T2DM patients and non-diabetic subjects at any of the four time points. Overall, this study is the first to examine the role of salivary resistin for monitoring periodontal status in a longitudinal study design. In the present study, patients with T2DM and chronic periodontitis exhibited similar reductions in salivary resistin levels after periodontal therapy compared to their systemically healthy counterparts. These findings provide further support for the hypothesis that although salivary resistin levels are not related to diabetic status, they reflected periodontal disease severity and response to therapy and this in turn suggests a potential utility of salivary resistin levels for monitoring periodontal disease status.

Gingival crevicular fluid contains many constituents derived from the serum, the cellular response in the periodontium, degraded components of periodontal tissues, contributions from the gingival crevice and bacterial products (Ozmeric, 2004; Taba *et al.*, 2005; Lamster and Ahlo, 2007; Newman *et al.*, 2012). Resistin in GCF is possibly derived from PBMCs, macrophages and neutrophils in periodontal tissues and blood. In the current study, a pilot investigation was undertaken to measure resistin levels in GCF in both healthy and periodontally diseased subjects with or without T2DM. The current study demonstrated no significant difference in the GCF

levels of resistin in subjects with T2DM [2.81 (0.99-5.44) ng/ml] compared to non-diabetic subjects [2.77 (0.87-6.25) ng/ml]. When considering resistin levels in GCF following further categorisation of subjects based on their periodontal diagnosis there were no significant differences found in any periodontal status category between T2DM and non-diabetic subjects. In T2DM subjects, resistin levels in GCF appeared to be higher in gingivitis patients [4.22 (0.68-8.45) ng/ml] compared to those with healthy periodontal tissues [2.17 (0.0-5.84) ng/ml] and periodontitis [2.81 (1.14-3.39) ng/ml], however, these differences failed to reach statistical significance. In non-diabetic subjects, resistin levels in GCF appeared to be higher in periodontitis patients [6.38 (1.69-24.46) ng/ml] compared to those with healthy periodontal tissues [1.41 (0.89-3.15) and gingivitis [0.94 (0.13-4.07) ng/ml], however, again these differences failed to reach statistical significance. Very little has been reported in the literature on the levels of resistin in GCF. Only one published study has investigated the existence of resistin in GCF, and found that resistin levels in GCF samples from patients with periodontitis or diabetes mellitus-related periodontitis were significantly higher than those of healthy subjects (Hiroshima *et al.*, 2012). The findings of the current study do not support the previous research and this rather contradictory result may be due to the lack of adequate sample size in the present study, as the numbers of GCF samples available for analysis were very limited. Accordingly, with such a small sample size, these data must be interpreted with caution.

The correlations of GCF resistin levels with resistin levels in saliva and serum, GCF levels of inflammatory cytokines, and clinical periodontal parameters were investigated in the current study. The results of this study indicated that the levels of GCF resistin were significantly and positively correlated with salivary resistin levels (Spearman's $\rho=0.422$, $p<0.01$). On the other hand, no significant association was

found between GCF resistin levels and resistin levels in serum (Spearman's $\rho=0.076$, $p>0.05$). In the present study, GCF resistin levels were found to correlate significantly and positively with GCF levels of TNF-α (Spearman's $\rho=0.525$, $p<0.001$), IL-1β (Spearman's $\rho=0.462$, $p<0.01$), IL-6 (Spearman's $\rho=0.563$, $p<0.001$) and IFN-γ (Spearman's $\rho=0.526$, $p<0.001$). It is interesting to note that when considering the correlation between GCF resistin levels and the clinical periodontal parameters, significant positive correlations were demonstrated between resistin levels in GCF and %BOP (Spearman's $\rho=0.435$, $p<0.01$), mean PD (Spearman's $\rho=0.347$, $p<0.05$), and PISA (Spearman's $\rho=0.49$, $p<0.01$). It is encouraging to compare this result with that found by Hiroshima *et al* (2012) who found a significant correlation between resistin levels in GCF and the gingival index score (Hiroshima *et al.*, 2012). On the other hand, in the current study no significant association were found between GCF resistin levels and mean LOA (Spearman's $\rho=0.295$, $p>0.05$). Intriguingly, the association of GCF resistin levels with saliva and serum resistin levels, GCF levels of inflammatory cytokines, and the main clinical periodontal parameters have not previously been described.

In conclusion, the present study demonstrated for the first time that salivary resistin levels were significantly elevated in subjects with periodontitis compared to gingivitis and periodontally healthy subjects in both T2DM and non-diabetic groups. Specifically, resistin levels in saliva were associated with periodontal disease, but not diabetes mellitus. The levels of salivary resistin correlated significantly and positively with hsCRP, serum resistin, salivary levels of IL-1β and IL-6 and the four main clinical periodontal parameters (i.e. %BOP, mean PD, mean LOA and PISA). Interestingly, in both diabetic and non-diabetic subjects, significant reductions in salivary resistin levels were seen at 3, 6 and 12 months after non-surgical periodontal

management. With regard to the resistin levels in GCF and within the limitations of the pilot study, no significant differences were found in GCF levels of resistin between the three periodontal categories in both T2DM and non-diabetic groups. Finally, GCF resistin levels were correlated significantly and positively with salivary resistin, GCF levels of TNF-α, IL-1β, IL-6 and IFN-γ, %BOP, mean PD and PISA. Thus, this study suggested that salivary resistin might be involved in the tissue destruction process in the periodontium. Additionally, resistin in saliva can be considered as a possible biomarker which reflects the inflammatory activity in periodontal disease. Although further studies may be necessary to confirm the efficacy of measuring salivary resistin levels in the prediction, diagnosis and management of periodontal disease, our study certainly highlights the potential for salivary resistin to move one step closer to becoming an established biomarker for periodontal disease. Therefore, addressing the clinical implications and pathological mechanisms of salivary and GCF resistin in periodontal disease progression are warranted.

Chapter 4 Investigation of serum resistin in periodontal disease patients with and without type 2 diabetes mellitus

4.1 Introduction

Periodontal diseases and diabetes are both common complex chronic diseases for which there is considerable evidence for a bidirectional relationship (Preshaw *et al.*, 2012). Periodontitis has been described as the sixth complication of diabetes (Loe, 1993). It has long been accepted that the prevalence, severity and progression of periodontal diseases are higher in diabetic patients when compared with non-diabetic subjects (Mealey and Oates, 2006; Preshaw, 2008b; Taylor and Borgnakke, 2008), which confirm the notion of diabetes as a major risk factor for periodontitis (Kinane and Bouchard, 2008). It is becoming increasingly difficult to ignore the importance of glycaemic control as a key determinant for the increased risk of periodontitis in diabetic individuals. On the other hand, periodontal diseases have a significant negative impact on the glycaemic control in diabetes (i.e. having periodontitis increases the risk of poor glycaemic control over time) (Taylor *et al.*, 1996). In conclusion, it is clear from a large volume of published studies that diabetes is a major risk factor for periodontal disease, and that the risk for periodontitis is greater if glycaemic control is poor.

Resistin is a cytokine which regulates pleiotropic activities in various biological processes including insulin resistance, inflammation and immunity. Elevated systemic resistin levels were reported to be positively associated with several chronic

diseases including inflammatory bowel disease (Konrad *et al.*, 2007), chronic kidney disease (Kawamura *et al.*, 2010), rheumatoid arthritis (Migita *et al.*, 2006), diabetes (Chen *et al.*, 2009; Lau and Muniandy, 2011) and periodontal disease (Furugen *et al.*, 2008; Saito *et al.*, 2008). The existence of resistin was confirmed in GCF from chronic periodontitis patients, patients with diabetes and periodontitis, and healthy subjects, and this cytokine was significantly elevated in patients with periodontitis or diabetes mellitus-related periodontitis when compared with healthy subjects (Hiroshima *et al.*, 2012). *In vitro*, Hiroshima *et al* (2012) demonstrated that LPS from *P. gingivalis* enhanced resistin release from human neutrophils (Hiroshima *et al.*, 2012). In effect, studies focusing on the levels of serum resistin in periodontitis patients are limited. Recent studies failed to provide any evidence to suggest that serum levels of resistin are altered in periodontitis patients compared with periodontally healthy control subjects (Davies *et al.*, 2011; Devanoorkar *et al.*, 2012). However, in 2008, two studies documented that the serum resistin concentration of elderly Japanese people with periodontitis was significantly higher than that of healthy subjects and that the serum resistin level was associated with bleeding on probing "a clinical marker of periodontal inflammation" (Furugen *et al.*, 2008; Saito *et al.*, 2008). The aforementioned studies have provided important insights about a potential role of resistin in periodontal disease.

In diabetes, the levels of serum resistin were found to be elevated in gestational diabetes (Chen *et al.*, 2007; Vitoratos *et al.*, 2011) and type 2 diabetes patients (Hasegawa *et al.*, 2005; Lu *et al.*, 2006; Chen *et al.*, 2009; Lau and Muniandy, 2011) compared to subjects without diabetes. Current evidence documented that serum resistin levels were positively correlated with HOMA-IR index (the surrogate measure for assessing insulin resistance and β-cell function), serum insulin, plasma glucose

and whole blood HbA1c levels in a case control study (Lau and Muniandy, 2011). Furthermore, multivariate analysis revealed that circulating resistin levels were positively correlated with CRP in treatment naive diabetes obese patients (de Luis et al., 2010). The relationship between resistin and BMI is controversial. Some articles, reported that in humans resistin levels correlate with BMI (Li *et al.*, 2009b; Lau and Muniandy, 2011), while other investigations failed to observe any correlation of BMI with resistin levels (Pagano *et al.*, 2006; Won *et al.*, 2009). These provide compelling evidence that hyper-resistinemia may be an important determinant for increased risk of type 2 diabetes.

Comprehensive evaluation of the literature indicates that hyper-resistinemia occurs in both periodontal disease and type 2 diabetes. However, to my knowledge, there are no published studies which have investigated the serum levels of resistin in type 2 diabetes subjects with periodontal disease. Therefore, the aim of this study was to investigate the role of serum resistin as a potential systemic biomarker in periodontal disease subjects with and without type 2 diabetes. Finally, the possible relationship of serum resistin levels with anthropometric clinical and metabolic parameters of diabetes, as well as with clinical parameters of periodontal disease and inflammatory cytokine levels in serum were also examined.

4.2 Results

4.2.1 Characteristics of the study population

The study population composed of two main groups; T2DM and non-diabetic controls. T2DM subjects were recruited from both secondary care databases, and databases held within primary care settings, such as general medical practices. The non-diabetic subjects were recruited from either those patients referred from general dental practice to the restorative department within the Newcastle Dental Hospital or patients attending student treatment clinics within the Newcastle Dental Hospital. All subjects underwent full mouth clinical periodontal measurements, in addition to the physical examination. Diabetes history, smoking history and demographic data were recorded for all participants. Serum, saliva and GCF samples were collected from all subjects to be used subsequently for both clinical biochemistry, cytokines and adipokines analysis.

The characteristics of the T2DM patients and non-diabetic subjects are summed up in Table 4.1. There were no significant differences between the groups in age, gender, ethnicity, smoking status, pack years smoked (relevant to current and ex-smokers only), and diastolic blood pressure. This reflects an appropriate matching in the present study for the T2DM and non-diabetic groups in terms of age, gender, ethnicity, and smoking status. In both T2DM and non-diabetic groups, a 9% prevalence of current smokers was recorded.

Subjects with T2DM had significantly higher systolic blood pressure [146.9 (±21.2) mmHg] compared to non-diabetic subjects [136.6 (±18.9) mmHg]. Likewise, case patients with T2DM had significantly higher values for BMI [32 (29-35) kg/m^2] than

non-diabetic group [27.2 (23-29) kg/m^2]. Moreover, it is apparent from Table 4.1 that the proportions of T2DM and non-diabetic subjects in each BMI rank were significantly different, with the T2DM group including a higher percentage of obese (47%) and morbidly obese (24%) subjects compared to the non-diabetic group in which the corresponding proportions were (10.8%) and (10.8%) respectively.

Table 4.1 Baseline characteristics of T2DM and non-diabetic groups

	T2DM patients (n=101)	Non-diabetic patients (n=83)	p-value
Gender (n (%))			
Male	67 (66.3%)	50 (60.2%)	NS
Age (years)	49 (45-53)	48 (43-54)	NS
Ethnicity (n (%))			
Caucasian	96 (95%)	83 (100%)	
Black	1 (1%)	0 (0%)	NS
Asian	4 (4%)	0 (0%)	
Smoking			
Status (n (%))			
Current	9 (8.9%)	9 (10.8%)	
Ex	37 (36.6%)	25 (30.1%)	NS
Never	55 (54.5%)	49 (59%)	
Pack years *	16 (10-38.8)	15 (4.9-23.8)	NS
Systolic blood pressure (mmHg)	146.9 (±21.2)	136.64 (±18.9)	**0.001**
Diastolic blood pressure (mmHg)	81 (74-90)	81 (74-88)	NS
BMI (kg/m^2)	32 (29-35)	27.2 (23-29)	**<0.001**
Status (n(%))			
normal weight	12 (12%)	27 (32.5%)	
overweight	16 (16%)	38 (45.8%)	**<0.001**
obese	47 (47%)	9 (10.8)	
morbidly obese	24 (24%)	9 (10.8)	

P-values were determined using chi-squared test for discrete variables, Mann-Whitney U tests for continuous non-parametric variables (age, pack years, diastolic blood pressure and BMI) and independent t-test for continuous parametric variables (systolic blood pressure). * Applicable only to current and ex-smokers (n=45 diabetic subjects, n=33 non-diabetics subjects). Means (±SD) are presented for parametric data and medians (IQR) are presented for non-parametric data.

4.2.2 Baseline clinical biochemistry parameters

The baseline clinical biochemistry data for the controls and subjects with T2DM are outlined in Table 4.2. Among these parameters, hsCRP alone showed no significant difference between the T2DM and non-diabetic controls. In contrast, there were a significant difference between the T2DM and non-diabetic groups for HbA1c, triglycerides, HDL, non-HDL, and total cholesterol. Patients with diabetes had significantly lower levels of HDL [1.2 (1.0-1.4) mmol/L], non-HDL [3.2 (2.7-3.9) mmol/L], and total cholesterol [4.4 (3.9-5.3) mmol/L] compared to subjects without diabetes for whom the corresponding values were [1.5 (1.2-1.8) mmol/L] for HDL, [4.1 (3.4-4.6) mmol/L] for non- HDL and [5.5 (5.0-6.1) mmol/L] for total cholesterol. As anticipated, HbA1c was significantly higher ($p<0.001$) in diabetic subjects [7.2 (6.5-8.9) %] compared to the non-diabetic controls [5.5 (5.3-5.7) %]. Although a higher levels of hsCRP was recorded in T2DM patients [2.3 (0.9-4.5) mg/L] compared to subjects without diabetes [1.9 (0.8-3.9) mg/L], this difference was not statistically significant.

Table 4.2 Baseline clinical biochemistry data comparing subjects with and without T2DM

	T2DM patients (n=101)	Non-diabetic patients (n=83)	p-value
HbA1c (%)	7.2 (6.5-8.9)	5.5 (5.3-5.7)	<0.001
Triglycerides (mmol/L)	2.3 (1.5-3.5)	1.6 (1.1-2.4)	<0.001
HDL (mmol/L)	1.2 (1.0-1.4)	1.5 (1.2-1.8)	<0.001
Non-HDL (mmol/L)	3.2 (2.7-3.9)	4.1 (3.4-4.6)	<0.001
Total cholesterol (mmol/L)	4.4 (3.9-5.3)	5.5 (5.0-6.1)	<0.001
hsCRP (mg/L)*	2.3 (0.9-4.5)	1.9 (0.8-3.9)	NS

P-values determined using Mann Whitney-U tests as all variables are continuous non-parametric. Median and IQR presented for this non-parametric data. *(92 T2DM and 72 non-diabetic subjects).

4.2.3 Pre-treatment clinical parameters of periodontal disease

Table 4.3 outlines the basic clinical periodontal data for T2DM patients and the non-diabetic control group before periodontal therapy. Of note, when comparing the diabetic and non-diabetic groups there was a tendency for increased BOP in diabetic subjects [35.4 (18.2-49.9) %] compared to non-diabetic subjects [30.1 (14.9-48.8) %], however, this difference was not statistically significant. Furthermore, no significant difference was recorded between the T2DM patients and non-diabetic control group in terms of mean probing depth, mean loss of attachment, and the periodontal inflamed surface area.

Table 4.4 provides the clinical periodontal baseline data following classification of the study population according to periodontal diagnosis. When appraising bleeding on probing, statistical tests revealed that %BOP was significantly higher in diabetic patients with healthy periodontal tissues [4.5 (0.7-13.1) %] and gingivitis [35.1 (25-44.9) %] compared to non-diabetic subjects with healthy periodontal tissues [0.65 (0-2.6) %] and gingivitis [22 (17.3-32.6) %]. Furthermore, the comparison was also performed between the three periodontal categories (healthy periodontal tissues, gingivitis and periodontitis) within the T2DM and non-diabetic groups. In the T2DM group, %BOP was significantly higher in those with gingivitis [35.1 (25-44.9) %] in comparison to those with healthy periodontal tissues [4.5 (0.7-13.1) %] ($p<0.001$). Likewise, the %BOP was significantly higher in those with periodontitis [46 (30-60.7) %] in comparison to those with healthy periodontal tissues [4.5 (0.7-13.1) %] ($p<0.001$). Moreover, the %BOP was significantly higher in those with periodontitis [46 (30-60.7) %] in comparison to those with gingivitis [35.1 (25-44.9) %] ($p<0.05$). In the non-diabetic group, the %BOP was significantly higher in those with gingivitis

[22 (17.3-32.6) %] in comparison to those with healthy periodontal tissues [0.65 (0-2.6) %] ($p<0.001$). Likewise, the %BOP was significantly higher in those with periodontitis [42.95 (29.5-56.7) %] in comparison to those with healthy periodontal tissues [0. 65 (0-2.6) %] ($p<0.001$). Moreover, the %BOP was significantly higher in those with periodontitis [42.95 (29.5-56.7) %] in comparison to those with gingivitis [22 (17.3-32.6) %] ($p<0.01$). The aforementioned results considering %BOP are presented in Table 4.4 and Figure 4.1.

When appraising probing depth, statistical analysis showed that the mean PD was significantly higher in diabetic patients with healthy periodontal tissues [1.75 (1.6-1.8) mm] and gingivitis [2.09 (1.96-2.2) mm] compared to non-diabetic subjects with healthy periodontal tissues [1.61 (1.5-1.7) mm] and gingivitis [1.9 (1.8-2.1) mm]. Furthermore, a comparison was performed between the three periodontal categories (healthy periodontal tissues, gingivitis and periodontitis) within the T2DM and non-diabetic groups. In the T2DM group, the mean PD was significantly higher in those with gingivitis [2.09 (1.96-2.2) mm] in comparison to those healthy periodontal tissues [1.75 (1.6-1.8) mm] ($p<0.001$). Likewise, the mean PD was significantly higher in those with periodontitis [2.82 (2.4-3.2) mm] in comparison to those with healthy periodontal tissues [1.75 (1.6-1.8) mm] ($p<0.001$). Moreover, the mean PD was significantly higher in those with periodontitis [2.82 (2.4-3.2) mm] in comparison to those with gingivitis [2.09 (1.96-2.2) mm] ($p<0.001$). In the non-diabetic group, the mean PD was significantly higher in those with gingivitis [1.9 (1.8-2.1) mm] in comparison to those with healthy periodontal tissues [1.6 (1.5-1.7) mm] ($p<0.001$). Likewise, the mean PD was significantly higher in those with periodontitis [2.9 (2.5-3.5) mm] in comparison to those with healthy periodontal tissues [1.6 (1.5-1.7) mm] ($p<0.001$). Furthermore, the mean PD was significantly higher in those with

periodontitis [2.9 (2.5-3.5) mm] in comparison to those with gingivitis [1.9 (1.8-2.1) mm] ($p<0.001$). The aforementioned results considering mean PD are presented in Table 4.4 and Figure 4.2.

When appraising loss of attachment, statistical analysis showed that the mean LOA was significantly higher in diabetic patients with gingivitis [2.3 (2.1-2.4) mm] compared to non-diabetic subjects with gingivitis [2.1 (2.0-2.3) mm]. Furthermore, the comparison was done between the three periodontal categories (healthy periodontal tissues, gingivitis and periodontitis) within T2DM and non-diabetic groups. In T2DM group, mean LOA was significantly higher in those with gingivitis [2.3 (2.1-2.4) mm] in comparison to those healthy periodontal tissues [1.9 (1.8-2.1) mm] ($p<0.001$). Likewise, mean LOA was significantly higher in those with periodontitis [3.1 (2.8-3.9) mm] in comparison to those with healthy periodontal tissues [1.9 (1.8-2.1) mm] ($p<0.001$). Moreover, mean LOA was significantly higher in those with periodontitis [3.1 (2.8-3.9) mm] in comparison to those with gingivitis [2.3 (2.1-2.4) mm] ($p<0.001$). In the non-diabetic group, mean LOA was significantly higher in those with gingivitis [2.1 (2.0-2.3) mm] in comparison to those with healthy periodontal tissues [1.8 (1.6-2.0) mm] ($p<0.001$). Likewise, mean LOA was significantly higher in those with periodontitis [3.4 (2.9-4.2) mm] in comparison to those with healthy periodontal tissues [1.8 (1.6-2.0) mm] ($p<0.001$). Moreover, mean LOA was significantly higher in those with periodontitis [3.4 (2.9-4.2) mm] in comparison to those with gingivitis [2.1 (2.0-2.3) mm] ($p<0.001$). The aforementioned results considering mean LOA are presented in Table 4.4.

When appraising the periodontal surface area which was deemed inflamed owing to the presence of bleeding on probing, statistical tests revealed no significant differences in PISA (periodontal inflamed surface area) between T2DM and non-

diabetic groups. Nonetheless, the comparison was done between the three periodontal categories (healthy periodontal tissues, gingivitis and periodontitis) within the T2DM and non-diabetic groups. In the T2DM group, the PISA was significantly higher in those with gingivitis [342.4 (238.4-492.7) mm^2] in comparison to those healthy periodontal tissues [24.7 (0.0-89.8) mm^2] ($p<0.001$). Likewise, the PISA was significantly higher in those with periodontitis [739.7 (456.5-1085.5) mm^2] in comparison to those with healthy periodontal tissues [24.7 (0.0-89.8) mm^2] ($p<0.001$). Moreover, the PISA was significantly higher in those with periodontitis [739.7 (456.5-1085.5) mm2] in comparison to those with gingivitis [342.4 (238.4-492.7) mm^2] ($p<0.001$). In the non-diabetic group, the PISA was significantly higher in those with gingivitis [242.6 (195.6-353.3) mm^2] in comparison to those with healthy periodontal tissues [4.5 (0.0-23.3) mm^2] ($p<0.001$). Likewise, the PISA was significantly higher in those with periodontitis [898.8 (702.2-1262.7) mm^2] in comparison to those with healthy periodontal tissues [4.5 (0.0-23.3) mm^2] ($p<0.001$). Moreover, the PISA was significantly higher in those with periodontitis [898.8 (702.2-1262.7) mm^2] in comparison to those with gingivitis [242.6 (195.6-353.3) mm^2] ($p<0.001$). The aforementioned results considering the PISA are presented in Table 4.1 and Figure 4.3. In summary, the results of clinical analysis confirm previous findings with regard to periodontal parameters in subjects with or without T2DM.

Table 4.3 Pre-treatment clinical periodontal parameters comparing subjects with and without T2DM

	Diabetic subjects (n=101)	Non-diabetic subjects (n=83)	p-value
Month 0 BOP (%)	35.4 (18.23-49.9)	30.1 (14.9-48.8)	NS
Month 0 mean PD (mm)	2.3 (2.0-2.8)	2.4 (1.8-3.1)	NS
Month 0 mean LOA (mm)	2.5 (2.2-3.1)	2.6 (2.0-3.5)	NS
Month 0 PISA (mm^2)	431.3 (201.1-746.8)	557.3 (151.7-971.2)	NS

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

Table 4.4 Pre-treatment clinical periodontal parameters comparing groups based on diabetic status and periodontal diagnosis

	Diabetic subjects (n=101)			Non-diabetic subjects (n=83)			p-value
	Healthy (n=14)	Gingivitis (n=39)	Periodontitis (n=47)	Healthy (n=16)	Gingivitis (n=19)	Periodontitis (n=48)	
Month 0 BOP (%)	4.5 (0.7-13.1) $^{\#,\P,\$1}$	35.1 (25-44.9) $^{\dagger1,\$2}$	46 (30-60.7)	0.65 (0-2.6) $^{\#,\P}$	22 (17.3-32.6) ^{†2}	42.95 (29.5-56.7)	$^{\#,\P}$< 0.001, ^{†1}< 0.05 $^{\dagger2,\$1}$< 0.01, $^{,\$2}$< 0.05
Month 0 mean PD (mm)	1.75 (1.6-1.8) $^{\#,\P,\$}$	2.1 (1.96-2.2) $^{\dagger,\$2}$	2.8 (2.4-3.2)	1.6 (1.5-1.7) $^{\#,\P}$	1.9 (1.8-2.1) †	2.9 (2.5-3.5)	$^{\#,\P,\dagger}$< 0.001 $^{\$}$< 0.05, $^{,\$2}$< 0.01
Month 0 mean LOA (mm)	1.9 (1.8-2.1) $^{\#,\P}$	2.3 (2.1-2.4) $^{\dagger,\$}$	3.1 (2.8-3.9)	1.8 (1.6-2.0) $^{\#,\P}$	2.1 (2.0-2.3) †	3.4 (2.9-4.2)	$^{\#,\P,\dagger}$< 0.001 $^{\$}$< 0.05
Month 0 PISA (mm^2)	24.7 (0.0-89.8) $^{\#,\P}$	342.4 (238.4-492.7) †	739.7 (456.5-1085.5)	4.5 (0.0-23.3) $^{\#,\P}$	242.6 (195.6-353.3) †	898.8 (702.2-1262.7)	$^{\#,\dagger,\P}\leq$ 0.001

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

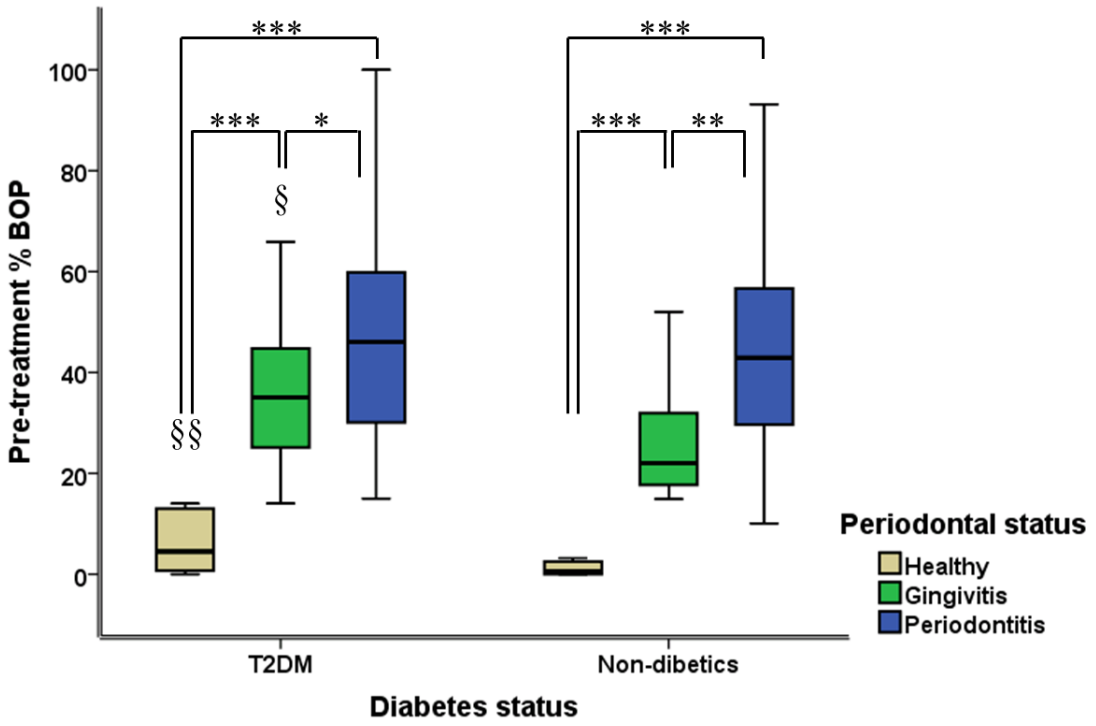
$ indicates a comparison within rows between diabetics and non-diabetic group with the same periodontal diagnosis

# indicates a comparison within rows between periodontally healthy and gingivitis groups within either diabetes or non-diabetes groups

¶ indicates a comparison within rows between periodontally healthy and periodontitis groups within either diabetes or non-diabetes groups

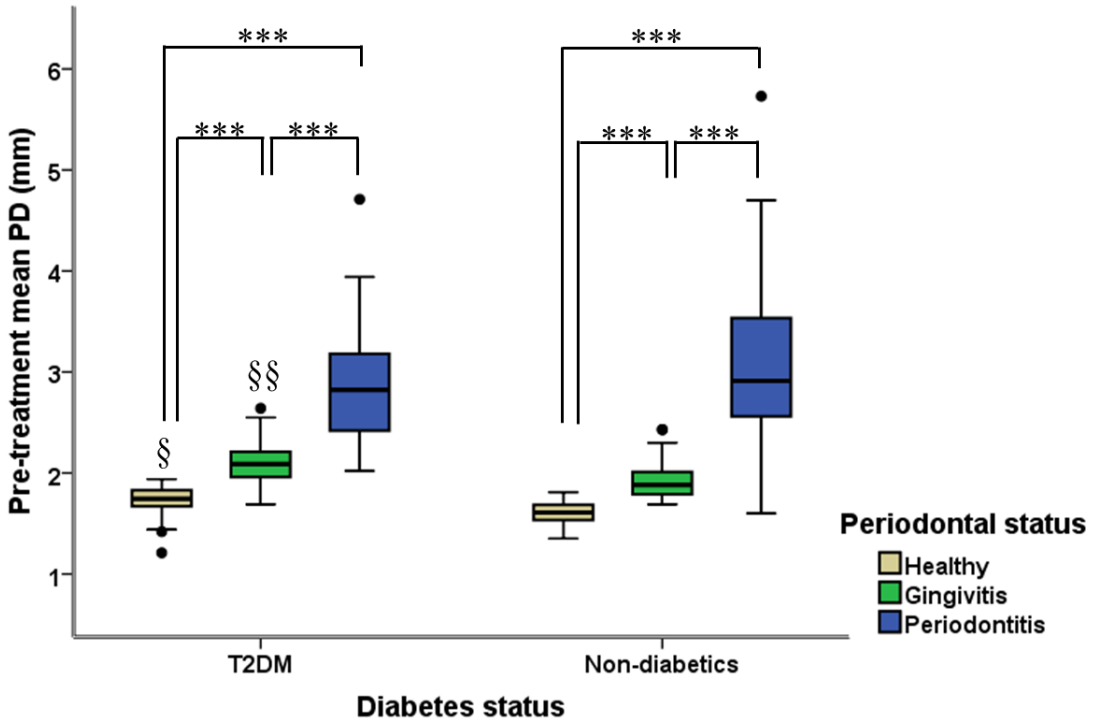
† indicates a comparison within rows between gingivitis and periodontitis groups within either diabetes or non-diabetes groups

Figure 4.1 Baseline mean % BOP data comparing groups based on diabetic status and periodontal diagnosis



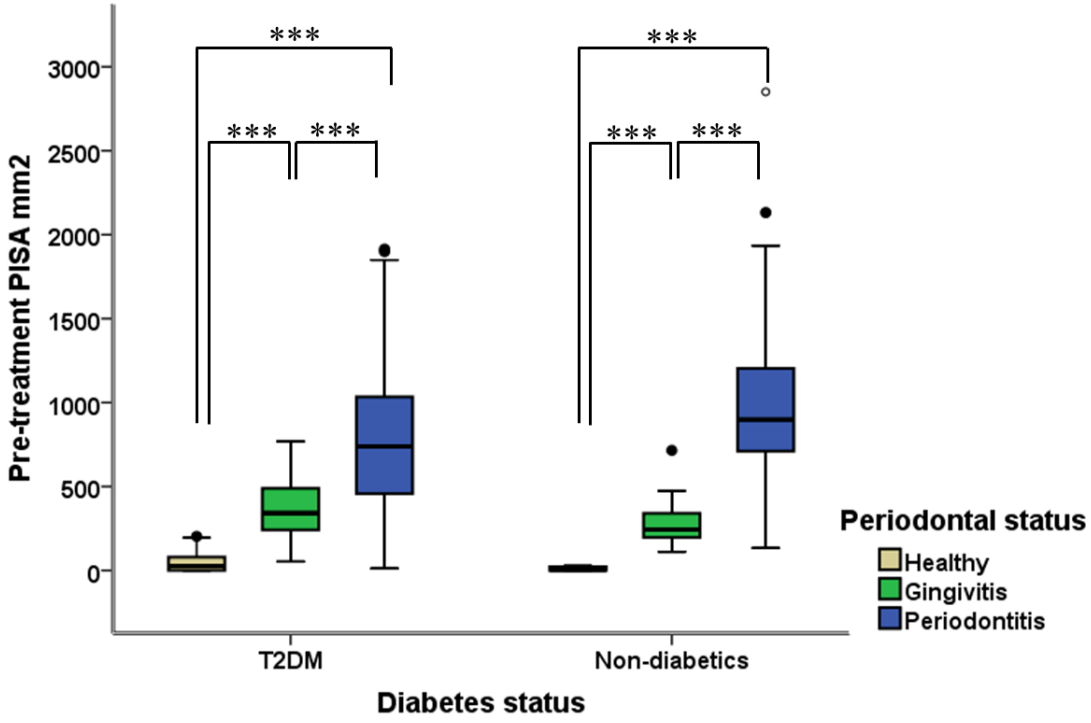
Boxplots of baseline % BOP data in 100 T2DM subjects (healthy periodontal tissues n=14, gingivitis n=39, periodontitis n=47) and 83 non-diabetic subjects (healthy periodontal tissues n=16, gingivitis n=19, periodontitis n=48)**.** Statistics: Kruskal-Wallis with Mann Whitney-U post hoc test *<0.05, **p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

Figure 4.2 Baseline mean probing depth data comparing groups based on diabetic status and periodontal diagnosis



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

Figure 4.3 Baseline periodontal inflamed surface area data comparing groups based on diabetic status and periodontal diagnosis



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

4.2.4 Analysis of baseline serum resistin concentrations in T2DM patients and non-diabetic controls with and without periodontal disease

As shown in Table 4.5 and Figure 4.4, the baseline serum resistin concentrations [median (IQR)] in the T2DM group and the non-diabetic control group were [6.42 (4.99-7.85) ng/ml] and [5.58 (4.32-6.55) ng/ml], respectively. The statistical analysis demonstrated that serum resistin levels were significantly higher in T2DM group than of those in non-diabetic control subjects (p <0.01).

Table 4.6 and Figure 4.5 illustrate serum resistin concentrations before periodontal treatment following further categorisation of subjects based on their periodontal diagnosis. Serum resistin levels appeared higher in diabetic subjects with healthy periodontal tissues [5.98 (4.83-7.13) ng/ml] compared to non-diabetic subjects with healthy periodontal tissues [4.3 (3.48-5.96) ng/ml]. However, the Bonferroni-Holm correction of p-values for multiple comparisons placed the critical p-value at 0.019, therefore, this difference was not statistically significant and might be considered as a trend (p=0.025). Additionally, there were no significant differences found in gingivitis or periodontitis between T2DM and non-diabetic subjects.

Furthermore, a comparison was done between the three periodontal categories (healthy periodontal tissues, gingivitis and periodontitis) within T2DM and non-diabetic groups. In the T2DM group, serum levels of resistin [median (IQR)] were not significantly different in those with healthy periodontal tissues [5.98 (4.83-7.13) ng/ml], gingivitis [6.83 (5.1-7.93) ng/ml], and periodontitis [6.42 (4.26-7.95) ng/ml]. In the non-diabetic group, serum resistin levels appeared higher in subjects with periodontitis [5.75 (4.6-6.59) ng/ml] compared to those with healthy periodontal

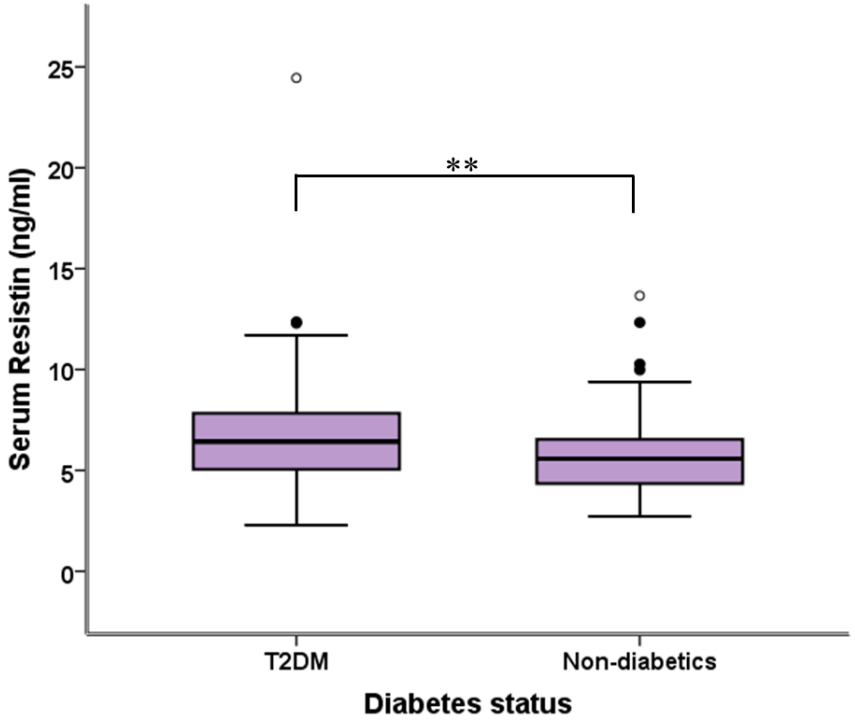
tissues [4.3 (3.48-5.96) ng/ml]. However, the Bonferroni-Holm correction of p-values for multiple comparisons placed the critical p-value at 0.03, therefore, this difference was not statistically significant and might be considered as a trend (p=0.036). Additionally, statistical analysis showed no significant difference in serum resistin levels between subjects with healthy periodontal tissues [4.3 (3.48-5.96) ng/ml] and those with gingivitis [4.98 (4.37-7.45) ng/ml]. Similarly, no significant difference was found between subjects with gingivitis [4.98 (4.37-7.45) ng/ml] and those with periodontitis [5.75 (4.6-6.59) ng/ml]. The aforementioned results considering resistin levels according to periodontal status in T2DM subjects and non-diabetic controls are presented in Table 4.6 and Figure 4.5.

Table 4.5 Baseline serum resistin concentrations in T2DM patients and non-diabetic subjects

	Diabetic subjects (n=97)	Non-diabetic subjects (n=83)	p-value
Resistin (ng/ml)	6.42 (4.99-7.85)	5.58 (4.32-6.55)	**<0.01**

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

Figure 4.4 Baseline serum concentrations of resistin in patients with T2DM and non-diabetic subjects



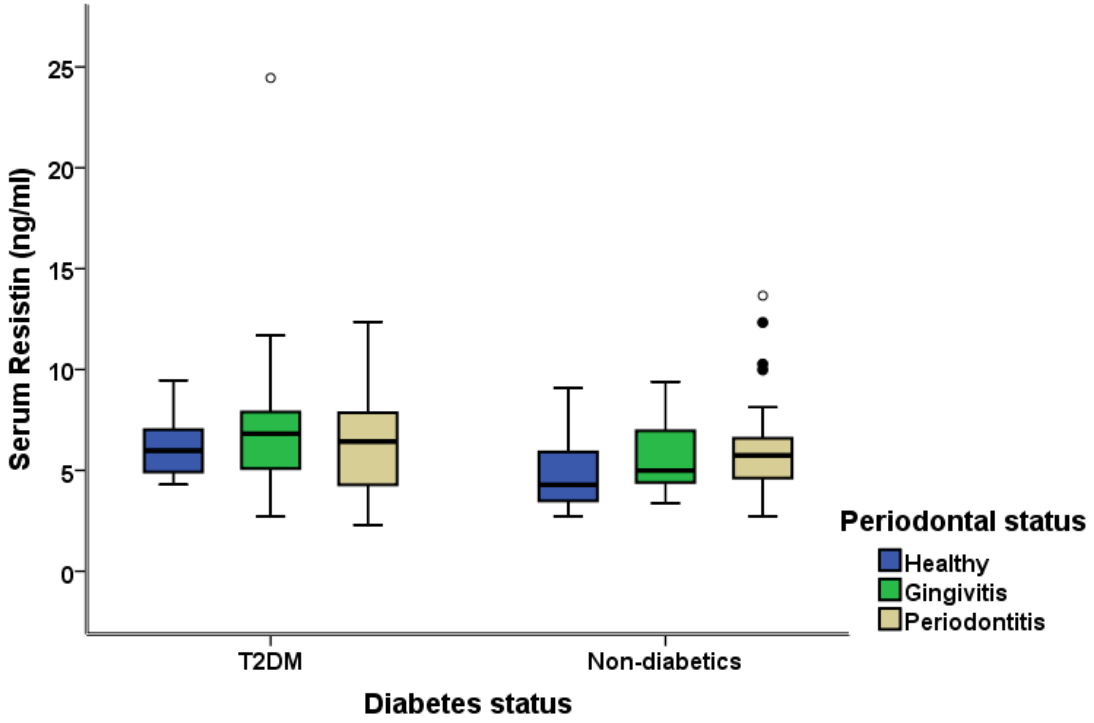
Boxplots of pre-treatment serum levels of Resistin in 97 T2DM and 83 non-diabetic subjects. Statistics: Mann Whitney-U test * $p<0.05$, ** $p<0.01$, *** $p<0.001$. ○ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the boundaries.

Table 4.6 Pre-treatment serum resistin comparing groups based on diabetic status and periodontal diagnosis

	Diabetic subjects (n=101)			Non-diabetic subjects (n=83)			p-value
	Healthy (n=14)	Gingivitis (n=38)	Periodontitis (n=45)	Healthy (n=16)	Gingivitis (n=19)	Periodontitis (n=48)	
Resistin (ng/ml)	5.98 (4.83-7.13)	6.83 (5.1-7.93)	6.42 (4.26-7.95)	4.3 (3.48-5.96)	4.98 (4.37-7.45)	5.75 (4.6-6.59)	NS

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

Figure 4.5 Pre-treatment serum levels of resistin comparing groups based diabetic status and periodontal diagnosis



Boxplots of pre-treatment serum resistin data in 97 T2DM subjects (healthy periodontal tissues n=14, gingivitis n=38, periodontitis n=45) and 83 non-diabetic subjects (healthy periodontal tissues n=16, gingivitis n=19, periodontitis n=48)**.** Statistics: Kruskal-Wallis with Mann Whitney-U post hoc test *<0.05, **p<0.01, ***p<0.001 (according to periodontal status within T2DM or non-diabetic group); § p<0.05, §§ p<0.01, §§§p<0.001 (T2DM versus non-diabetic groups within the corresponding periodontal status). ○ outlier more that 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

4.2.5 The relationship between serum resistin levels and anthropometric clinical and metabolic parameters

To elucidate whether the serum resistin levels are associated with glycaemic control, systemic inflammation, or risk factors for developing T2DM, the relationship between baseline serum resistin levels and HbA1c, hsCRP, BMI, total cholesterol, triglycerides, HDL, or non-HDL were explored using the Spearman's correlation test. Blood samples taken from T2DM patients and non-diabetic controls were analysed for HbA1c, hsCRP, total cholesterol, triglycerides, HDL, and non-HDL at the Haematology and Clinical Biochemistry labs of the Royal Victoria Infirmary (Newcastle). Correlations of HbA1c, hsCRP, BMI, total cholesterol, triglycerides, HDL, and non-HDL with serum resistin were performed for the whole study population (n=180), and were displayed using a series of scatter plots (Figure 4.6 to Figure 4.12).

Spearman correlations between serum resistin levels and HbA1c, hsCRP, BMI, total cholesterol, triglycerides, HDL, and non-HDL are shown in Figure 4.6, Figure 4.7, Figure 4.8, Figure 4.9, Figure 4.10, Figure 4.11 and Figure 4.12, respectively. Levels of serum resistin were significantly and positively correlated with HbA1c (Spearman's $\rho=0.21$, $p<0.01$), hsCRP (Spearman's $\rho=0.21$, $p<0.01$), BMI (Spearman's $\rho=0.17$, $p<0.05$). However, there was no significant correlation between serum resistin and total cholesterol, triglycerides, HDL, and non-HDL.

Figure 4.6 The relationship of serum resistin with HbA1c

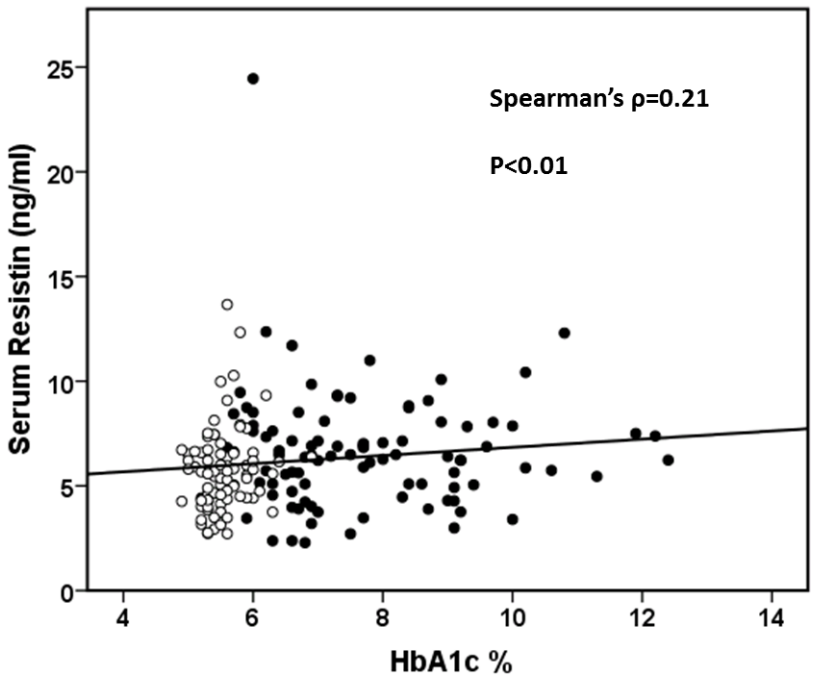


Figure shows Spearman correlation of serum resistin concentrations with percentage of HbA1c in all subjects (n=180). Percentage of HbA1c in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.21, P<0.01).

Figure 4.7 The relationship of serum resistin with hsCRP

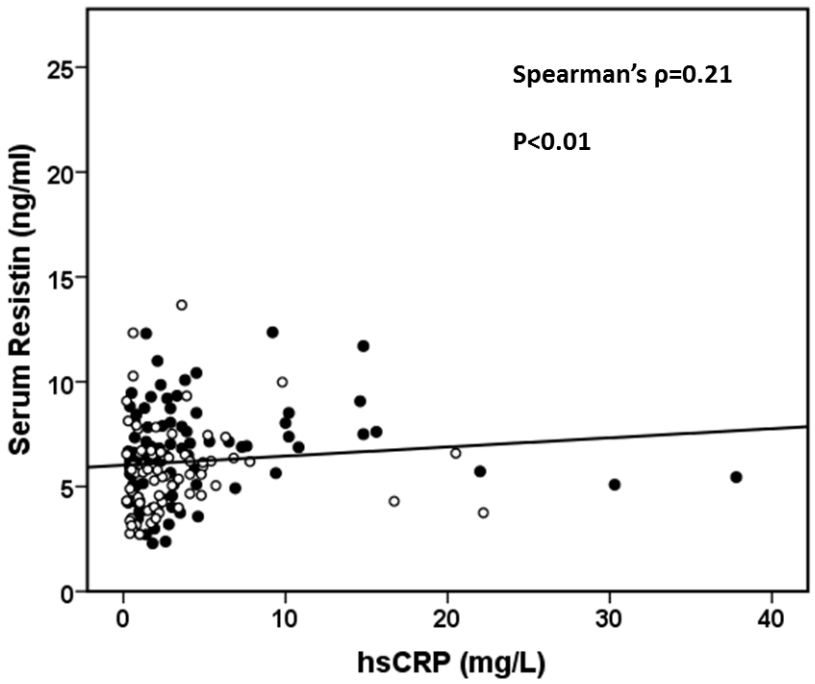


Figure shows Spearman correlation of serum resistin concentrations with hsCRP levels in all subjects (n=180). Levels of hsCRP in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.21, P<0.01).

Figure 4.8 The relationship of serum resistin with BMI

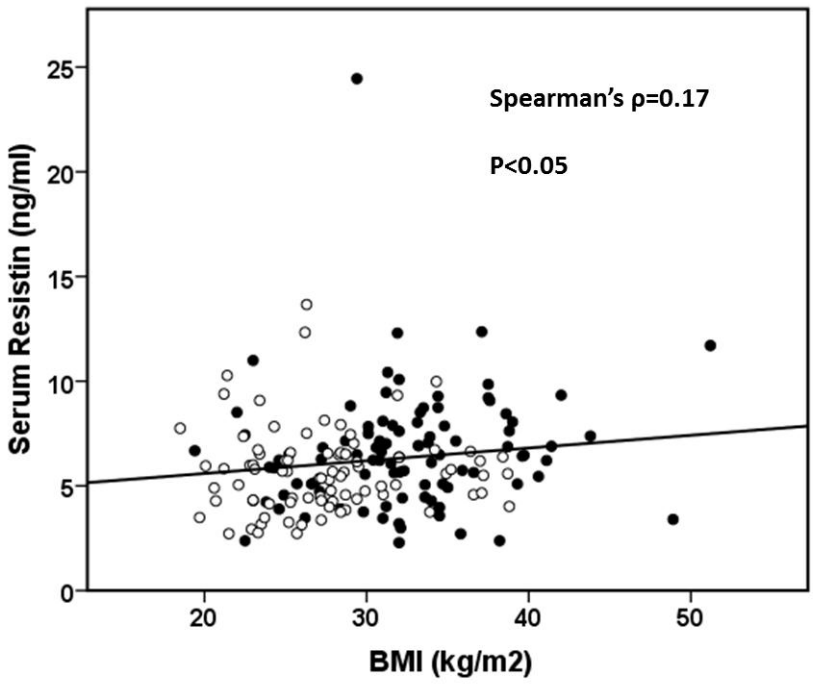


Figure shows Spearman correlation of serum resistin concentrations with BMI in all subjects (n=180). BMI in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.17, P<0.05).

Figure 4.9 The relationship of serum resistin with total cholesterol

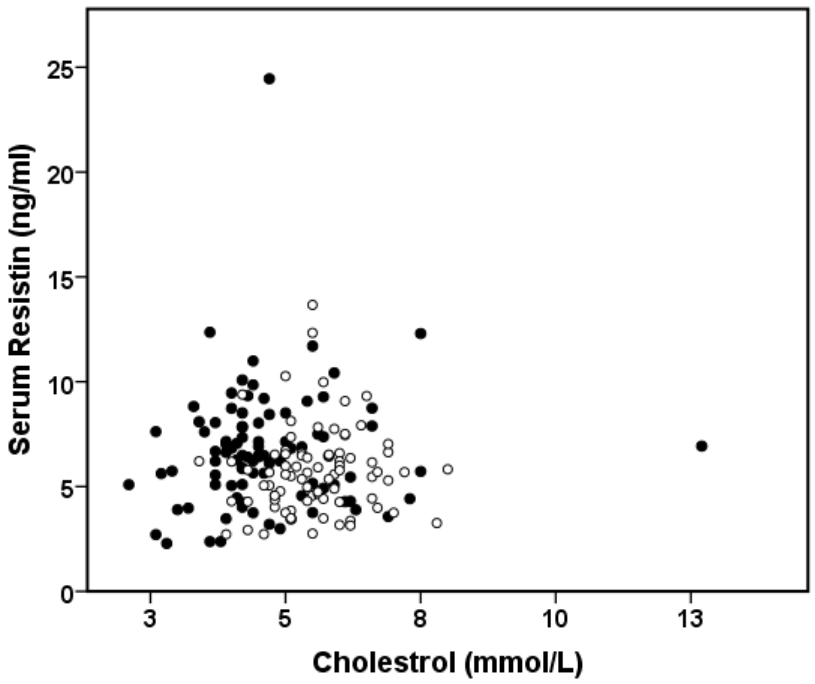


Figure shows Spearman correlation of serum resistin concentrations with total cholesterol in all subjects (n=180). Levels of total cholesterol in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated.

Figure 4.10 The relationship of serum resistin with triglycerides

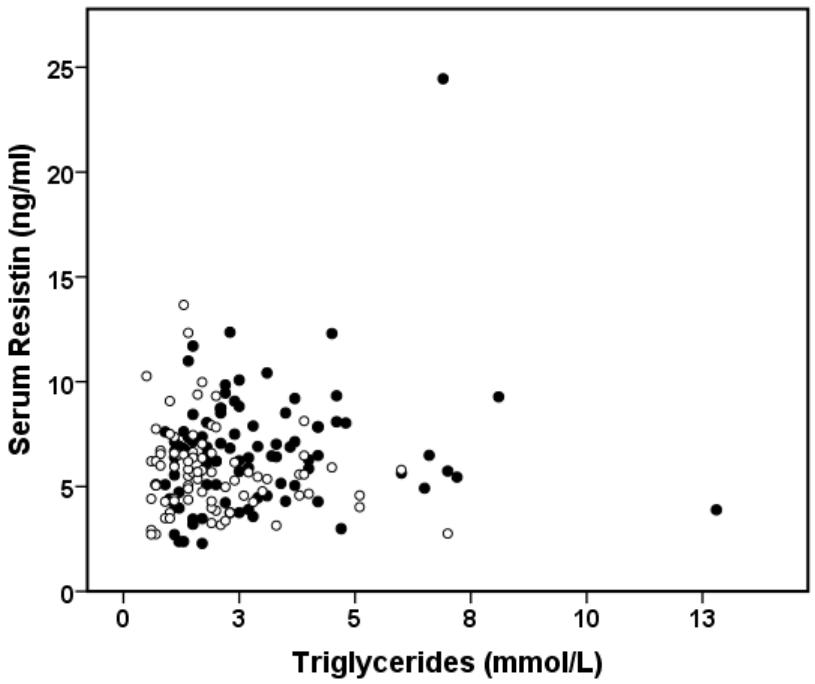


Figure shows Spearman correlation of serum resistin concentrations with triglycerides in all subjects (n=180). Levels of triglycerides in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated.

Figure 4.11 The relationship of serum resistin with HDL

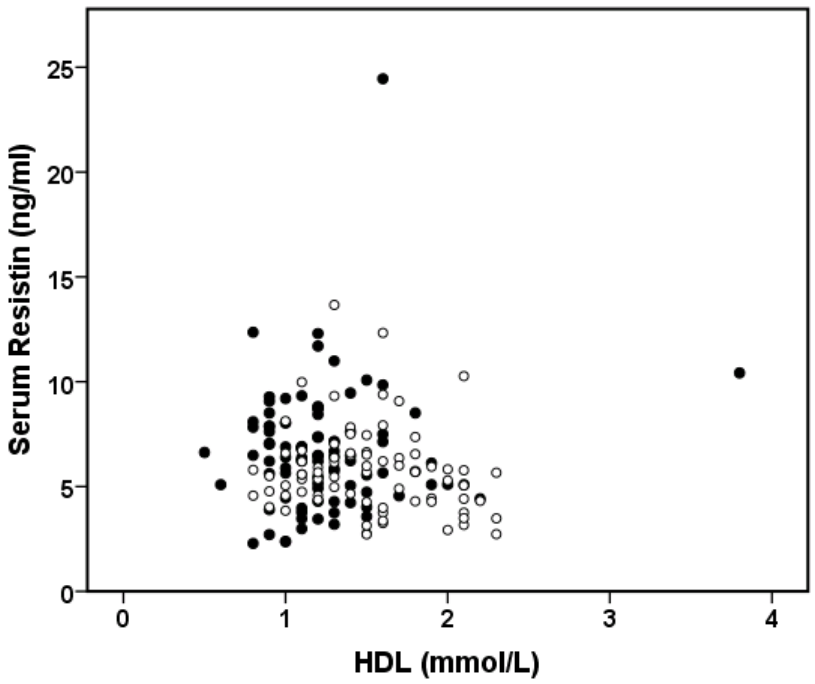


Figure shows Spearman correlation of serum resistin concentrations with HDL in all subjects (n=180). Levels of HDL in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated.

Figure 4.12 The relationship of serum resistin with non-HDL

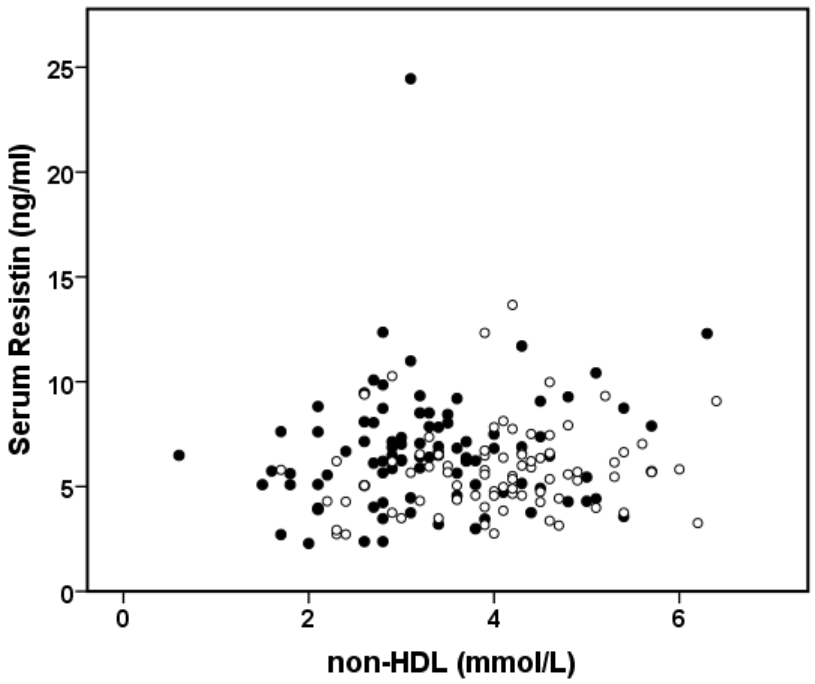


Figure shows Spearman correlation of serum resistin concentrations with non-HDL in all subjects (n=180). Levels of non-HDL in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated.

4.2.6 The relationship between serum resistin levels and clinical parameters of periodontal disease

Pre-treatment levels of serum resistin were investigated for correlations with the basic clinical periodontal parameters including BOP, mean PD, mean LOA and PISA. Correlations were undertaken for the whole study population (n=180). A series of scatter plots were used to graphically represent correlations of BOP, mean PD, mean LOA, and PISA with serum resistin (Figure 4.13, Figure 4.14, Figure 4.15 and Figure 4.16).

Levels of serum resistin were significantly and positively correlated with percentage BOP (Spearman's ρ=0.22, p <0.01), mean PD (Spearman's ρ=0.18, p <0.05), and PISA (Spearman's ρ=0.17, p <0.05). However, there was no significant correlation between serum resistin and mean LOA (Figure 4.13, Figure 4.14, Figure 4.15 and Figure 4.16).

Figure 4.13 The relationship of serum resistin with %BOP

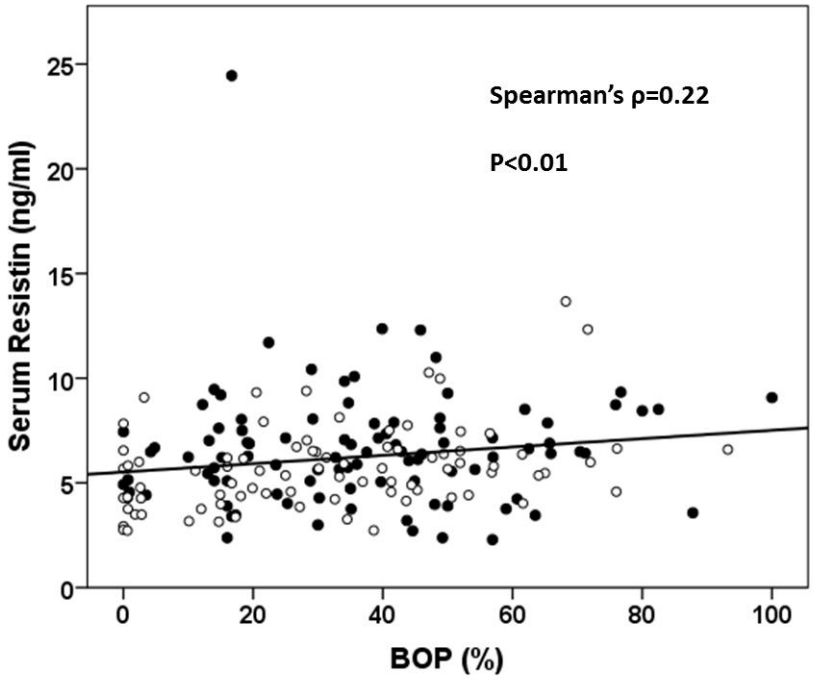


Figure shows Spearman correlation of serum resistin concentrations with %BOP in all subjects (n=180). Percentage of BOP in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.22, P<0.01).

Figure 4.14 The relationship of serum resistin with mean probing depth

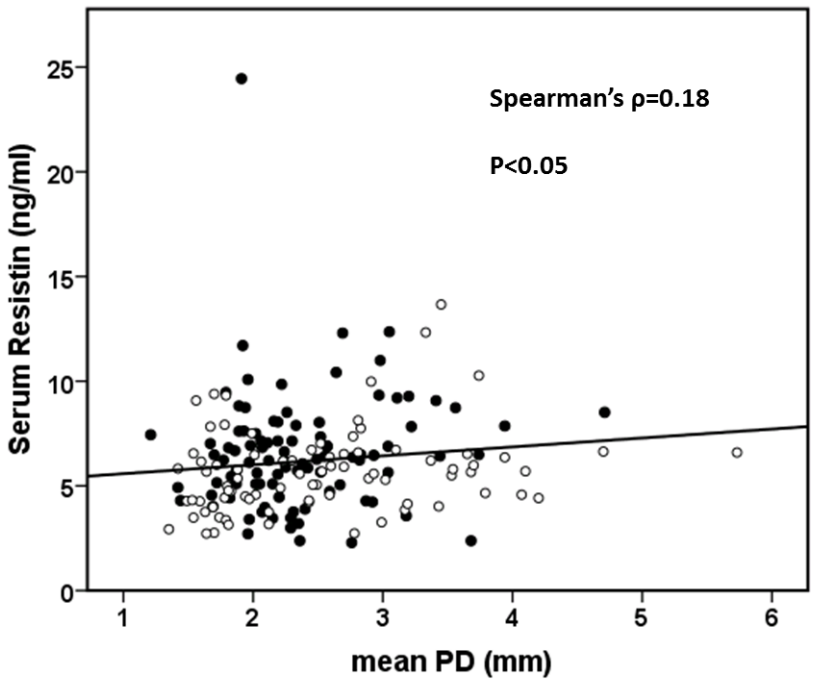


Figure shows Spearman correlation of serum resistin concentrations with mean PD in all subjects (n=180). Mean PD in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.18, P<0.05).

Figure 4.15 The relationship of serum resistin with mean LOA

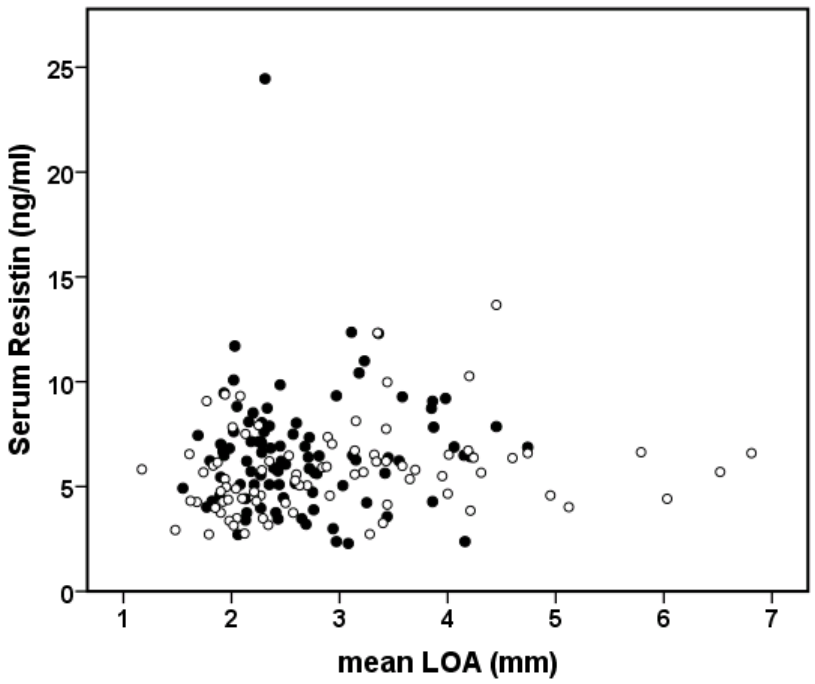


Figure shows Spearman correlation of serum resistin concentrations with mean LOA in all subjects (n=180). Mean LOA in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated.

Figure 4.16 The relationship of serum resistin with PISA

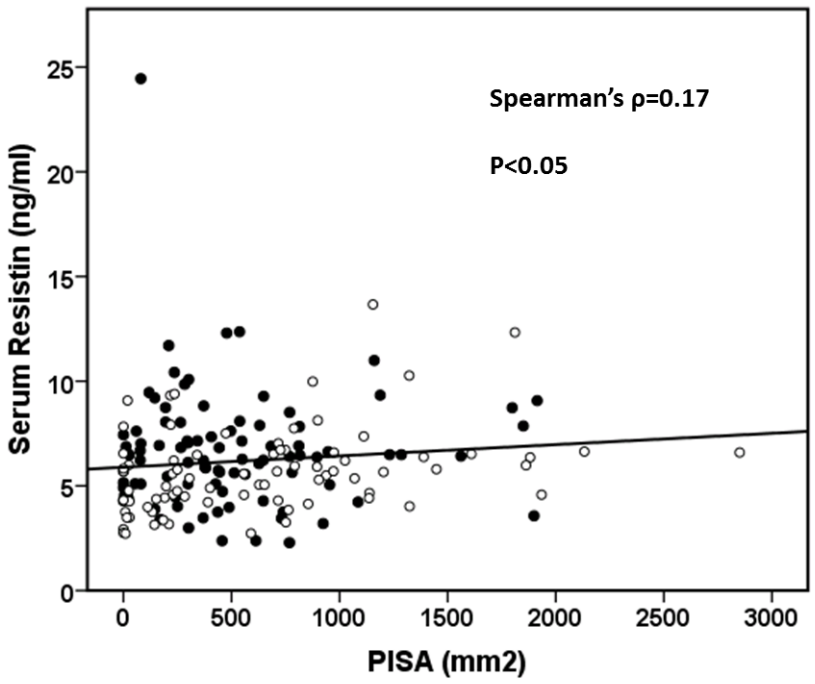


Figure shows Spearman correlation of serum resistin concentrations with PISA in all subjects (n=180). PISA in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.17, P<0.05).

4.2.7 The relationship between serum resistin levels and inflammatory cytokines in serum

Pre-treatment levels of resistin in serum were investigated for correlations with the baseline serum levels of TNF-α, IL-1β, IL-6 and IFN-γ. Correlations were undertaken for the whole study population. A series of scatter plots were used to graphically represent correlations of serum TNF-α, serum IL-1β, serum IL-6 and serum IFN-γ with resistin levels in serum (Figure 4.17, Figure 4.18, Figure 4.19 and Figure 4.20).

Levels of serum resistin were significantly and positively correlated with serum TNF-α (Spearman's $\rho=0.25$, $p<0.01$), serum IL-6 (Spearman's $\rho=0.32$, $p<0.001$), and serum IFN-γ (Spearman's $\rho=0.22$, $p<0.01$). However, there was no significant correlation between serum resistin and serum IL-1β (Figure 4.17, Figure 4.18, Figure 4.19 and Figure 4.20).

Figure 4.17 The relationship of serum resistin with TNF-α in serum

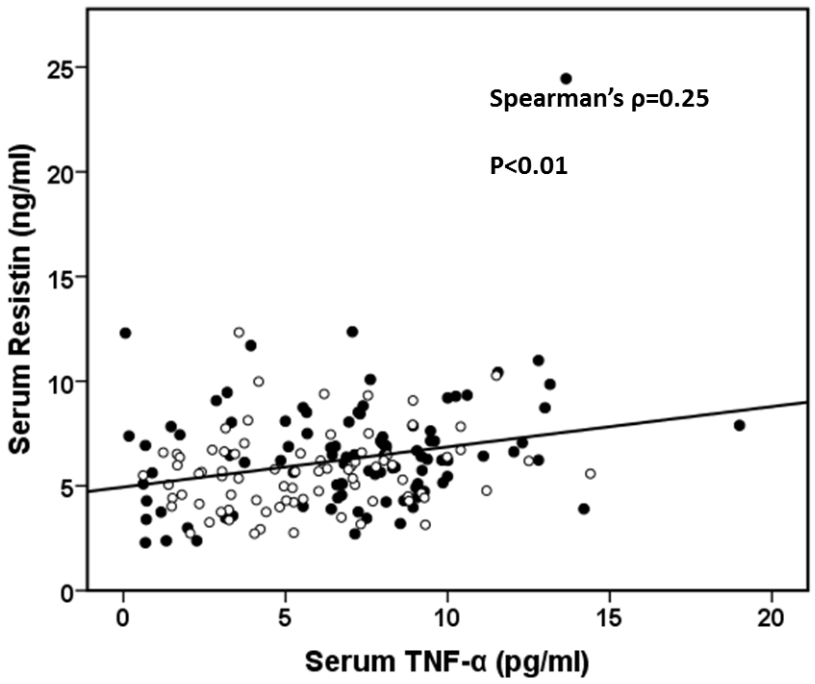


Figure shows Spearman correlation of serum resistin concentrations with serum TNF-α in all subjects (n=180). Levels of serum TNF-α in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.25, P<0.01).

Figure 4.18 The relationship of serum resistin with IL-1β in serum

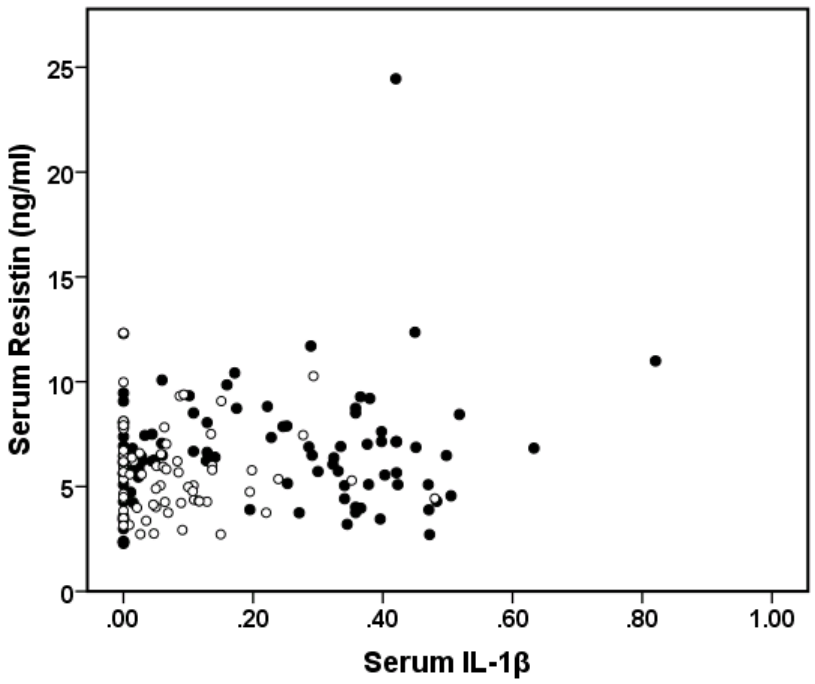


Figure shows Spearman correlation of serum resistin concentrations with serum IL-1β in all subjects (n=180). Levels of serum IL-1β in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated.

Figure 4.19 The relationship of serum resistin with IL-6 in serum

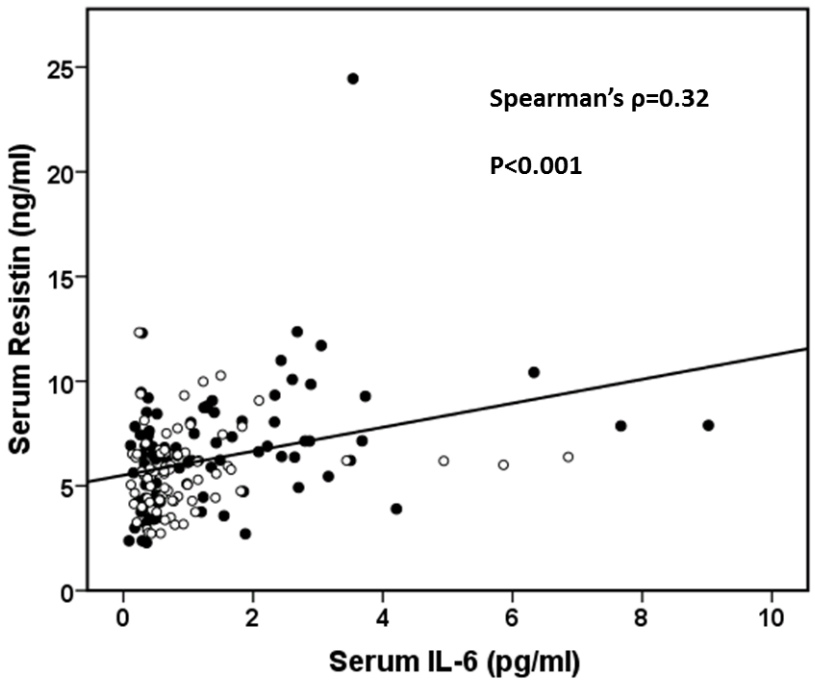


Figure shows Spearman correlation of serum resistin concentrations with serum IL-6 in all subjects (n=180). Levels of serum IL-6 in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.32, P<0.001).

Figure 4.20 The relationship of serum resistin with IFN-γ in serum

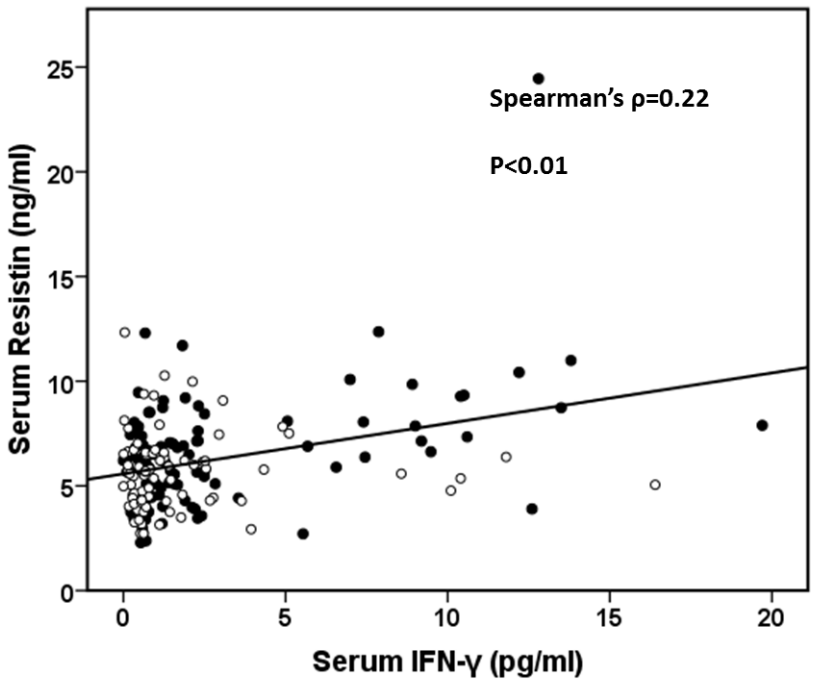


Figure shows Spearman correlation of serum resistin concentrations with serum IFN-γ in all subjects (n=180). Levels of serum IFN-γ in subjects with T2DM (●) and non-diabetic subjects (o) are illustrated. The addition of a trend-line demonstrates the presence of a significant correlation, (Spearman's ρ=0.22, P<0.01).

4.3 Discussion

A strong relationship between periodontal disease and T2DM has been reported in the literature (Mirza *et al.*, 2010; Preshaw *et al.*, 2012). There is substantial evidence that diabetes is a risk factor for periodontal disease (Kinane and Bouchard, 2008). Additionally, the influence of periodontitis as a risk factor for diabetes is documented (Taylor, 2001). Studies by several investigators suggested that inflammation is a key player in the link between periodontal disease and diabetes (Dandona *et al.*, 2004; Loos, 2005; Stumvoll *et al.*, 2005; Santos *et al.*, 2010). Numerous cytokines and adipokines have been shown to be associated with T2DM and with periodontal disease (Salvi *et al.*, 1998; Nishimura *et al.*, 2003; Karthikeyan and Pradeep, 2007; Bertoni *et al.*, 2010; Duarte *et al.*, 2010; Pradeep *et al.*, 2011a). Because of its link with inflammation and insulin resistance and its potential association with periodontal disease and diabetes, resistin has been tagged as a potential useful marker for both diabetes and periodontal disease. Supporting this theory, subjects with T2DM or periodontal disease tend to have higher serum resistin levels than their healthy counterparts (Furugen *et al.*, 2008; Chen *et al.*, 2009). However, the correlation between T2DM and serum resistin in humans remains controversial, supported by some studies (Hasegawa *et al.*, 2005; Lu *et al.*, 2006; Chen *et al.*, 2009) and not in others (Heilbronn *et al.*, 2004; Dominguez Coello *et al.*, 2008). For instance, a recent study by Mirza et al (2012) reported no significant difference in serum resistin levels between diabetes and non-diabetes groups (Mirza *et al.*, 2012).

The demographic and baseline biochemistry findings of the current study are presented in Table 4.1 and Table 4.2. An association between metabolic syndromes and abdominal obesity, dyslipidemia, inflammation, insulin resistance or diabetes and

increased risk of developing cardiovascular disease has been reported (Despres and Lemieux, 2006). An abundance of evidence has demonstrated that all five single components of metabolic syndrome (BMI, blood pressure, serum HDL-cholesterol, serum triglycerides and impaired fasting glucose) predicted the occurrence of T2DM (Hanson *et al.*, 2002; Cheung *et al.*, 2007; Sattar *et al.*, 2008; Laaksonen *et al.*, 2010). It is well established that obesity is a dominant risk factor for T2DM (Chan *et al.*, 1994), with BMI being a strong risk predictor of T2DM (Wang *et al.*, 2005). Kahn et al (2006) reported that obesity is a principal cause for developing insulin resistance (Kahn *et al.*, 2006). In the current study, patients with T2DM had significantly higher BMI [32 (29-35) kg/m^2] compared to non-diabetic controls [27.2 (23-29) kg/m2]. Moreover, the T2DM group contained a higher proportion of obese (47%) and morbidly obese (24%) compared to (10.8%) and (10.8%) in the non-diabetic group, respectively. This also fits with previous research which showed that patients with T2DM had significantly higher values of BMI than control subjects (Chen *et al.*, 2009; Mirza *et al.*, 2012).

Previous research has demonstrated that diabetes and hypertension share common pathways such as obesity, inflammation and insulin resistance (Cheung and Li, 2012). Indeed, both diabetes and hypertension represent the end result of the metabolic syndrome, for that reason they may develop one after the other in the same individual (Cheung *et al.*, 2008). In the present study, systolic blood pressure was significantly higher in subjects with T2DM compared to subjects without diabetes. This finding was consistent with those of Lau and Muniandy (2011) who reported a significant elevation in systolic blood pressure in T2DM patients with and without metabolic syndrome compared to healthy controls (Lau and Muniandy, 2011).

The risk of diabetic complications is substantially correlated with hyperglycaemia in T2DM patients. However, intensive glucose-control measures decrease the HbA1c levels which in turn considerably reduce the risk for developing diabetes complications (UKPDS, 1998). There are ample reports which endorse the use of HbA1c as a diagnostic test for diabetes with HbA1c≥6.5 recommended as the cut-off point for diagnosing diabetes (Cavagnolli *et al.*, 2012; Kim *et al.*, 2012; Phillips, 2012). In agreement with previous research (Hasegawa *et al.*, 2005; Chen *et al.*, 2009; Dag *et al.*, 2009; Lau and Muniandy, 2011; Mirza *et al.*, 2012), the current findings showed that glycated haemoglobin levels were significantly higher in diabetic patients [7.2 (6.5-8.9) %] compared to non-diabetic subjects [5.5 (5.3-5.7) %], suggesting a deterioration in glycaemic control in diabetic patients which may predispose those subjects to diabetic complications such as periodontitis. Interestingly, the present study showed significantly higher levels of triglycerides and lower levels of HDL cholesterol in diabetic subjects compared to non-diabetic controls. This finding is in agreement with previous studies which demonstrated a high prevalence of elevated triglycerides and low HDL cholesterol in individuals with diabetes (Siegel *et al.*, 1996; Bell *et al.*, 2011; Mirza *et al.*, 2012). There is evidence which indicates that non-HDL cholesterol, which can be readily calculated from routine lipid indices by subtracting HDL cholesterol from total cholesterol, is a superior risk predictor for incident type 2 diabetes (Ley *et al.*, 2012) and also for cardiovascular disease in subjects with diabetes (Lu *et al.*, 2003). In the present study diabetic subjects had significantly lower levels of both non-HDL cholesterol and total cholesterol compared to those without diabetes. It is difficult to explain this result, but it might be related to the regular dietary advice directed to T2DM patients in the UK to optimise blood lipid profiles and the use of lipid lowering therapy to decrease

cardiovascular disease risk in those patients according to the UK management guidelines for T2DM (NICE, 2008). In contrast to the present findings, a previous study reported significantly higher levels of non-HDL cholesterol and total cholesterol in incident type 2 diabetes compared to non-diabetic controls (Ley *et al.*, 2012). Another study demonstrated a trend for increased levels of total cholesterol in subjects with T2DM compared to non-diabetic subjects (Mirza *et al.*, 2012). On the other hand, Kardesler et al (2010) found no significant differences in total cholesterol, triglycerides and low density lipoprotein levels between patients with T2DM and non-diabetic subjects (Kardesler *et al.*, 2010).

Very little has been reported in the literature regarding levels of hsCRP in subjects with T2DM and periodontitis (Correa *et al.*, 2010; Kardesler *et al.*, 2010). One study demonstrated comparable levels of hsCRP in patients with T2DM and non-diabetic subjects (Kardesler *et al.*, 2010). However, Mirza et al (2012) reported significantly higher levels of hsCRP in participants with diabetes compared to those subjects without diabetes (Mirza *et al.*, 2012). An abundance of evidence demonstrated that elevation of CRP concentrations is an independent predictive parameter of type 2 diabetes mellitus (Barzilay *et al.*, 2001; Pradhan *et al.*, 2001; Freeman *et al.*, 2002; Thorand *et al.*, 2003). In the present study, no significant difference was found in levels of hsCRP in patients with T2DM [2.3 (0.9-4.5) mg/L] compared to non-diabetic subjects [1.9 (0.8-3.9) mg/L].

Considerable epidemiological evidence relating to the association between diabetes and periodontal disease supports the concept of increased prevalence and severity of periodontal disease in patients with type 2 diabetes compared to non-diabetic subjects (Shlossman *et al.*, 1990; Sandberg *et al.*, 2000; Mattout *et al.*, 2006; Moles, 2006; Novak *et al.*, 2008; Susanto *et al.*, 2011). More recently, longitudinal interventional

studies appraising the impacts of periodontal treatment on clinical periodontal response indicated that clinical periodontal measurements (e.g. PD, CAL, BOP, and PI) were comparable at baseline in subjects with and without diabetes (Dag *et al.*, 2009; Kardesler *et al.*, 2010; Chen *et al.*, 2012). On the other hand, Correa et al (2008) have demonstrated that people with diabetes had significantly worse clinical periodontal parameters (i.e. greater percentage of sites with PI, BOP, higher median PD and CAL) than the control group at baseline (Correa *et al.*, 2008). In the current study, no significant difference was found in %BOP, mean PD, mean LOA and PISA between patients with T2DM and non-diabetic subjects. Additionally, when data were further categorised based on periodontal status, no significant difference was found in the mean PD for diabetic subjects with periodontitis [2.8 (2.4-3.2) mm] compared to non-diabetic subjects with periodontitis [2.9 (2.5-3.5) mm]. However, the mean PD values were significantly higher in diabetic subjects with healthy periodontal tissues [1.75 (1.6-1.8) mm] and gingivitis [2.1 (1.96-2.2) mm] when compared to non- diabetic subjects with healthy periodontal tissues [1.6 (1.5-1.7) mm] and gingivitis [1.9 (1.8-2.1) mm]. Intriguingly, the mean PD values found in the present study were similar to those reported in some former research (Kiran *et al.*, 2005; Dag *et al.*, 2009; Chen *et al.*, 2012), nevertheless lower than those exhibited by others (Correa *et al.*, 2008; Kardesler *et al.*, 2010; Auyeung *et al.*, 2012), suggesting that the extent of periodontal disease may be dissimilar between different studies which make direct comparisons between studies difficult.

In the current study, analysis of the data with regard to gingival inflammation showed significantly higher %BOP in subjects with T2DM and healthy periodontal tissues [4.5 (0.7-13.1) %] or gingivitis [35.1 (25-44.9) %] compared to non-diabetic subjects with healthy periodontal tissues [0.65 (0.0-2.6) %] or gingivitis [22 (17.3-32.6) %].

These results corroborate the findings of a great deal of the previous work in this field which reported significantly higher levels of gingival inflammation in subjects with T2DM compared to non-diabetic control (Lu and Yang, 2004; Campus *et al.*, 2005; Mattout *et al.*, 2006; Correa *et al.*, 2008; Pan *et al.*, 2010; Susanto *et al.*, 2011). The observed increase in gingival inflammation in subjects with T2DM could be attributed to the upregulated systemic inflammation that is recognised to be present in diabetes. The current study also showed a higher levels of %BOP in subjects with T2DM and periodontitis [46 (30-60.7) %] compared to non-diabetic subjects with periodontitis [42.95 (29.5-56.7) %], albeit this difference was not statistically significant. It is difficult to explain this result, but it might be related to the more intense periodontal disease found in non-diabetic subjects compared to T2DM patients which may obscure the existence of high level of gingival inflammation in diabetic patients with periodontitis compared to non- diabetic subjects with periodontitis.

In 2008, Nesse et al published a paper in which they developed a method to quantify the amount of inflamed periodontal tissues and thereby to create a new method to assess the extent of periodontitis which can be easily and broadly applied (Nesse *et al.*, 2008). Accordingly, the PISA parameter was proposed to represent the surface area of bleeding pocket epithelium in square millimeters, and therefore it is supposed that PISA quantifies the inflammatory burden posed by periodontitis (Nesse *et al.*, 2008). In the present study, PISA was able to reflect crucial differences between subjects with different periodontal status. For instance, when comparing subjects within the T2DM group, the PISA was significantly higher in those with gingivitis [342.4 (238.4-492.7) mm^2] compared to those with healthy periodontal tissues [24.7 (0.0-89.8) mm^2] ($p<0.001$), the PISA was significantly higher in those with periodontitis [739.7 (456.5-1085.5) mm^2] compared to those with gingivitis [342.4

(238.4-492.7) mm^2] ($p<0.001$), and the PISA was significantly higher in those with periodontitis [739.7 (456.5-1085.5) mm^2] compared to those with healthy periodontal tissues [24.7 (0.0-89.8) mm^2] ($p<0.001$). Likewise, within the non-diabetic group the PISA was significantly higher in those with gingivitis [242.6 (195.6-353.3) mm^2] compared to those with healthy periodontal tissues [4.5 (0.0-23.3) mm^2] ($p<0.001$), the PISA was significantly higher in those with periodontitis [898.8 (702.2-1262.7) mm^2] compared to those with gingivitis [242.6 (195.6-353.3) mm^2] ($p<0.001$) and the PISA was significantly higher in those with periodontitis [898.8 (702.2-1262.7) mm^2] compared to those with healthy periodontal tissues [4.5 (0.0-23.3) mm^2] ($p<0.001$). Hence, it could conceivably that PISA may quantify the inflammatory burden posed by periodontitis.

A dose-response relationship between control of blood glucose levels over time (HbA1c) and the amount of inflamed periodontal tissue (PISA) in type 2 diabetes has been reported (Nesse et al., 2009). In the current study, PISA was lower in subjects with T2DM and periodontitis [739.7 (456.5-1085.5) mm2] compared to non-diabetic subjects with periodontitis [898.8 (702.2-1262.7) mm^2], but this difference failed to reach statistical significance. This finding was inconsistent with a previous research in which PISA was found to be significantly higher in T2DM subjects when compared to non-diabetic controls ($p= 0.016$) (Susanto *et al.*, 2011).

In reviewing the literature, no published research was found which alluded to the role of resistin in serum in subjects with T2DM and periodontitis. The present study demonstrated that serum resistin concentrations in T2DM patients are significantly higher than non-diabetic controls. In particular, this study showed a trend of elevated serum resistin levels in T2DM patients with healthy periodontal tissues as compared to periodontally healthy non-diabetic controls. Moreover, non-diabetic subjects with

periodontitis seem to have a trend of higher serum resistin concentrations than non-diabetic subjects with healthy periodontal tissues. Finally, the present study revealed that the levels of serum resistin are significantly and positively correlated with HbA1c, hsCRP, BMI, serum TNF-α, serum IL-6 and serum INF-γ. Additionally, serum resistin also positively correlated with %BOP, mean PD, and PISA.

This study produced results which corroborate the findings of a great deal of the previous work in this field. In agreement with previous studies (Al-Harithy and Al-Ghamdi, 2005; Hasegawa *et al.*, 2005; Lu *et al.*, 2006; Chen *et al.*, 2009; Lau and Muniandy, 2011), the results of the present study have shown that higher serum resistin levels were found in T2DM patients when compared with age, sex and smoking status- matched non-diabetic controls.

The mechanisms responsible for the elevation of serum resistin in diabetic patients remain unclear. It has been demonstrated that high glucose significantly increases resistin gene expression and protein production in human monocytes (Stan *et al.*, 2011), and therefore, it is reasonable to suggest that high blood glucose levels in diabetic patients may contribute to the hyper-resistinemia found in those patients. Additionally, the upregulating effect of insulin on resistin expression in mice adipose tissue has been reported by (Kim *et al.*, 2001). Previous research documented that resistin is expressed in pancreatic islets and up-regulated in insulin resistance (Minn *et al.*, 2003). This finding suggests that the increase of serum resistin levels may be a result of beta-cell deterioration, which is common in type 2 diabetes patients (Wajchenberg, 2010).

However, along with the above-mentioned factors, some other phenomena may also be involved in the increase of resistin levels in type 2 diabetes. Cytokines and adipokines are involved in subclinical inflammation accompanied by T2DM

(Fantuzzi, 2005; Bastard *et al.*, 2006). There is growing evidence regarding the role of resistin in inflammatory processes such as rheumatoid arthritis (Bokarewa *et al.*, 2005). Therefore, it can be also possible to consider the increase in the serum level of resistin in T2DM patients as a manifestation of subclinical inflammation. Furthermore, the regulation of glucocorticoids is impaired in type 2 diabetes patients (Bruehl *et al.*, 2007). Since glucocorticoid treatment enhances resistin levels in serum (Almehed *et al.*, 2008) and upregulates resistin gene levels in adipocytes (Haugen *et al.*, 2001), they could also be responsible for this phenomenon, however it is important to mention that the subjects in the current study were not receiving glucocorticoid treatment.

While hyperglycaemia appears likely to play a role in the increased serum levels of resistin in T2DM patients, conflicting findings were previously reported regarding the relationship between serum resistin levels and HbA1c or plasma glucose levels (Al-Harithy and Al-Ghamdi, 2005; Mojiminiyi and Abdella, 2007; Tokuyama *et al.*, 2007; Chen *et al.*, 2009; Lau and Muniandy, 2011). Several recently published studies, measuring resistin levels and various metabolic parameters in subjects with or without type 2 diabetes and undertaking correlation tests, reported no significant correlation between resistin levels with HbA1c and/or fasting glucose levels (Heilbronn *et al.*, 2004; Hasegawa *et al.*, 2005; Mojiminiyi and Abdella, 2007; Tokuyama *et al.*, 2007; Takata *et al.*, 2008; Chen *et al.*, 2009; de Luis *et al.*, 2011; Mirza *et al.*, 2012). Additionally, a study investigating the effect of glucose loading on serum resistin levels in human by using the oral glucose tolerance test has shown that serum resistin levels were significantly decreased at 60 and 120 minutes during the test compared to baseline, with a more pronounced reduction observed in subjects with greater baseline concentrations of resistin (Yamauchi *et al.*, 2008). Moreover, resistin levels

correlated negatively with glucose in pre-pubertal boys (Li *et al.*, 2009b). On the contrary, in recent studies a significant and positive correlation was reported between serum resistin levels with HbA1c and/or fasting glucose, indicating that hyperglycaemia may influence the levels of resistin in serum (Al-Harithy and Al-Ghamdi, 2005; Lu *et al.*, 2006; Lau and Muniandy, 2011). The results of the current study are in accordance with the findings of the latter studies. A significant positive relationship between serum resistin levels and hyperglycaemia reflected by HbA1c was detected. Although the statistical analysis revealed a significant correlation, it is important to note that the correlation between serum resistin levels and HbA1c was relatively weak (spearman's ρ=0.21). Therefore, the biological importance of the correlation is questionable.

C-reactive protein (CRP), a plasma protein synthesized by the liver, represents a sensitive and dynamic systemic biomarker of inflammation (Pepys and Hirschfield, 2003). Interestingly, the production of CRP is part of the non-specific acute-phase response to most forms of inflammation, infection, and tissue damage (Pepys and Hirschfield, 2003). Compelling evidence has demonstrated that elevated CRP levels indicate a high inflammatory state in several disease pathologies such as cardiovascular disease, rheumatoid arthritis, periodontitis and diabetes (Jialal *et al.*, 2004; Nesto, 2004; Emery *et al.*, 2007; Dasanayake, 2009; Mugabo *et al.*, 2010). A cross-sectional study evaluating the association between diabetes and inflammation in patients with diabetes has shown a significant correlation between CRP and resistin levels (Mirza *et al.*, 2012). In the present study, as in some previous studies (Ohmori *et al.*, 2005; Reilly *et al.*, 2005; Kunnari *et al.*, 2006; Qi *et al.*, 2008; de Luis *et al.*, 2011), the levels of serum resistin correlated positively and significantly with levels of hsCRP, and therefore it is reasonable to suggest that the concentration of this hormone

may reflect systemic inflammation. This finding also corroborates the ideas of (Chen *et al.*, 2009), who conducted two prospective type 2 diabetes-control studies and suggested that CRP was significantly correlated with plasma resistin, and this association remained positive even after adjustment for age and BMI. Additionally, in line with this finding, (Mojiminiyi and Abdella, 2007) demonstrated a significant BMI-dependent association of resistin with CRP levels in type 2 diabetes patients, which supported the potential link between resistin and the degree of adiposity. In addition, previous study have shown that CRP is a significant predictor of serum resistin levels and suggested a potential role of resistin as a pro-inflammatory factor (McTernan *et al.*, 2003). However, the findings of the current study do not support a previous study by (Takata *et al.*, 2008), who reported that circulating resistin was not significantly correlated with CRP in subjects with T2DM. Similarly, (Hasegawa *et al.*, 2005) also reported no correlation between CRP adjusted by BMI and resistin.

BMI is a widely used diagnostic measure in the current classification system for obesity (Gomez-Ambrosi *et al.*, 2011). It is deemed a pivotal indicator for the development of diabetes (Arnlov *et al.*, 2011). To date, the relationship between BMI and serum resistin has been controversial (Lee *et al.*, 2003; Janowska *et al.*, 2006; Mojiminiyi and Abdella, 2007; de Luis *et al.*, 2009; de Luis *et al.*, 2011). It has been reported that resistin levels were not correlated with BMI in lean subjects, whereas there was a highly significant positive correlation between resistin and BMI in overweight/obese women with or without diabetes (Al-Harithy and Al-Ghamdi, 2005). Moreover, Chen et al (2009) demonstrated that BMI was not correlated with plasma resistin in men, however it was significantly correlated in women (Chen *et al.*, 2009). Interestingly, a large number of studies failed to find a significant correlation between serum resistin and BMI (Stejskal *et al.*, 2003; Hasegawa *et al.*, 2005; Norata

et al., 2007; Takata *et al.*, 2008; Won *et al.*, 2009; de Luis *et al.*, 2011; Lau and Muniandy, 2011). In the present study, and consistent with a previously published reports (Lu *et al.*, 2006; Mojiminiyi and Abdella, 2007; Tokuyama *et al.*, 2007; de Luis *et al.*, 2010; Thomopoulos *et al.*, 2011), serum resistin levels were significantly and positively correlated with BMI. Therefore, the current study supported the potential link between resistin and the degree of obesity, which probably is partly due to an indirect impact of obesity-induced elevation of inflammatory cytokines such as TNF-α and IL-6, which is produced by adipose tissue (Wisse, 2004). The enhanced proinflammatory cytokines levels can stimulate resistin production (Kaser *et al.*, 2003; Lehrke *et al.*, 2004). Another explanation for the correlation with obesity is that the highest levels of resistin mRNA (Curat *et al.*, 2006) and protein (McTernan *et al.*, 2002b) were detected in human mononuclear cells (e.g. macrophages), a vital source of pro-inflammatory markers. Macrophage infiltration into visceral adipose tissue is pivotal feature of obesity, and the infiltrated macrophages secrete cytokines which induce systemic insulin resistance (Weisberg *et al.*, 2003; Xu *et al.*, 2003). This fact could explain the relationship of resistin levels with obesity and type 2 diabetes that we described in the current study. Taken together, resistin showed a significant correlation with obesity and glycated haemoglobin and elevated levels in type 2 diabetes. Resistin is also correlated with CRP, suggesting that the relationship between obesity and resistin in relation to type 2 diabetes may be via inflammatory mechanisms.

In view of the findings in the current study with regard to the correlations of baseline serum resistin with the hyperglycaemia, degree of obesity, and systemic inflammation, further correlational analysis were investigated to determine whether the other metabolic and inflammatory factors were also associated with serum resistin.

The results of this study did not show any significant correlation of serum resistin with factors related to metabolic syndrome, namely, total cholesterol, triglycerides, HDL and non-HDL. These findings concur with previous reports (Farvid *et al.*, 2005; Reilly *et al.*, 2005; Mojiminiyi and Abdella, 2007; Tokuyama *et al.*, 2007; Won *et al.*, 2009; Thomopoulos *et al.*, 2011) which failed to find significant correlations between circulating resistin levels and dyslipidaemia. However, other studies reported a significant correlation of total cholesterol (Stejskal *et al.*, 2003; Koch *et al.*, 2009), triglycerides (Norata *et al.*, 2007; Qi *et al.*, 2008), and HDL (Stejskal *et al.*, 2003; Qi *et al.*, 2008; Koch *et al.*, 2009; Lau and Muniandy, 2011) with circulating resistin levels. These controversial data may be due to different comorbidities (diabetes mellitus, hypertension), or genetic backgrounds of the participants.

There are a number of published data exploring the relationship between serum resistin and levels of inflammatory cytokines in the serum of subjects with type 2 diabetes. In one study evaluating relationship between resistin concentrations and laboratory markers of inflammation in subjects with type 2 diabetes mellitus, systemic inflammation and healthy subjects, a significant positive correlation was demonstrated between resistin concentrations with the values of IL-6 and TNF-α in the whole population study, however, these correlations was not found when only the T2DM group was considered (Stejskal *et al.*, 2003). In a further study of subjects with metabolic syndrome, the plasma resistin levels were not correlated with IL-6, however, but were significantly and negatively correlated with TNF-α only in subjects without metabolic syndrome (Won *et al.*, 2009). Furthermore, no significant correlation was observed between circulating resistin levels and IL-6 in treatment naïve patients with type 2 diabetes mellitus and obesity (de Luis *et al.*, 2011). On the other hand, in gestational diabetes mellitus, serum resistin correlated significantly

with IL-6 in the case group, but not in the control group (Kuzmicki *et al.*, 2009). In a cross-sectional study assessing the association between diabetes and inflammation in patients with diabetes, a significant positive correlation was found between serum resistin and levels of IL-1β, IL-6, TNF-α, IL8 and leptin (Mirza *et al.*, 2012). In fact, serum resistin correlated significantly with IL-6 in several clinical studies of different comorbidities (Reilly *et al.*, 2005; Qi *et al.*, 2008; Koch *et al.*, 2009; Fargnoli *et al.*, 2010; Kontunen *et al.*, 2011).

The results of the current study show that serum resistin levels correlated positively and significantly with serum levels of TNF-α (ρ=0.25, $P<0.01$), IL-6 (ρ=0.32, $P<0.001$) and IFN-γ (ρ=0.22, $P<0.01$). Although, these results differ from some published studies (Won *et al.*, 2009; de Luis *et al.*, 2011), they are consistent with those of (Stejskal *et al.*, 2003; Mirza *et al.*, 2012). Additionally, no significant correlation between serum resistin levels and serum levels of IL-1β was observed in the present study. This finding is in contrast with those of (Mirza *et al.*, 2012) who found a significant correlation between IL-1β and serum resistin levels. However the significant correlation demonstrated in this one study by (Mirza *et al.*, 2012) was relatively weak (ρ=0.12, $P<0.01$) (Cohen, 1988) and so it is not necessarily surprising that the data from the current study failed to corroborate this finding. In reviewing the literature, no data were found on the association between serum resistin levels and serum levels of IFN-γ.

Taken these findings together, serum resistin concentrations are elevated in T2DM patients. The elevation of serum resistin is associated with HbA1c, BMI, hsCRP, TNF-α, IL-6 and IFN-γ. These data suggest that the relationship between serum resistin and obesity in relation to T2DM may be via inflammatory mechanisms, and re-confirmed the systemic inflammatory state in type 2 diabetic patients. However,

the role of systemic resistin in the progression of periodontitis in subjects with or without type 2 diabetes remains unclear. A number of studies have demonstrated that serum resistin levels are elevated in various inflammation-related diseases (Migita *et al.*, 2006; Konrad *et al.*, 2007; Adrych *et al.*, 2009), including diabetes mellitus (Hasegawa *et al.*, 2005; Chen *et al.*, 2009; Lau and Muniandy, 2011). So far, only four studies have investigated serum resistin levels in periodontal disease (Furugen *et al.*, 2008; Saito *et al.*, 2008; Davies *et al.*, 2011; Devanoorkar *et al.*, 2012), and the results were relatively conflicting.

Previous research has demonstrated an association between higher circulating levels of resistin and the development of periodontal disease, with significantly higher levels of resistin found in subjects with periodontitis compared to periodontally healthy subjects (Furugen *et al.*, 2008; Saito *et al.*, 2008). Within the current literature, there is no published study exploring the role of serum resistin in T2DM associated with the development of periodontal disease. In the present study, when the serum resistin data were categorized according to periodontal status within T2DM and non-diabetic groups, serum resistin levels appeared higher in diabetic subjects with healthy periodontal tissues [5.98 (4.83-7.13) ng/ml] compared to non-diabetic subjects with healthy periodontal tissues [4.3 (3.48-5.96) ng/ml], however, the Bonferroni-Holm correction of p-values for multiple comparisons placed the critical p-value at 0.019, therefore, this difference was not statistically significant and might be considered as a trend (p=0.025). This finding does not support the previous research showing significantly higher circulating resistin levels in subjects with T2DM compared to non-diabetic controls (Hasegawa *et al.*, 2005; Chen *et al.*, 2009; Lau and Muniandy, 2011). The current study showed no significant difference in serum resistin levels between periodontally healthy, gingivitis and periodontitis subjects within the

diabetes group. In the non-diabetic group, serum resistin levels appeared higher in periodontitis subjects [5.75 (4.6-6.59) ng/ml] compared to subjects with healthy periodontal tissue [4.3 (3.48-5.96) ng/ml], however, the Bonferroni-Holm correction of p-values for multiple comparisons placed the critical p-value at 0.03, therefore, this difference was not statistically significant and might be considered as a trend (p=0.036). These results are inconsistent with those of the two previously published studies which suggest that increased serum resistin levels are significantly associated with periodontal condition (Furugen *et al.*, 2008; Saito *et al.*, 2008). In one study evaluating the relationship between serum resistin and periodontal condition in elderly Japanese people with or without periodontitis, a statistically non-significant tendency of increased resistin levels in periodontitis patients (with model 1 criterion; with or without ≥6 mm of probing pocket depth) was reported. Furthermore, periodontitis patients with bleeding on probing (model 2 criterion; 10% of BOP was considered in addition to probing pocket depth) showed a significantly higher concentrations of resistin (Furugen *et al.*, 2008). Similarly, another study exploring circulating resistin levels in women with and without periodontitis demonstrated that serum resistin was significantly higher in the women with periodontitis (Saito *et al.*, 2008). On the contrary, in a pilot study by Davies et al (2011) investigating serum levels of various mediators and adipokines, no significant differences in serum resistin levels were found between aggressive periodontitis patients and periodontally healthy controls (Davies *et al.*, 2011); however, with a small sample size, caution must be applied when interpreting the results of this study. Furthermore, Devanoorkar and co-workers (2012) failed to demonstrate any significant differences in serum resistin levels between the chronic periodontitis patients and periodontally healthy controls (Devanoorkar *et al.*, 2012).

Only one previous study has investigated the relationship of serum resistin levels with clinical periodontal parameters in subjects with or without periodontitis. Furugen et al (2008) found a significant positive correlation between levels of resistin in serum and %BOP (ρ=0.198, p=0.003), but failed to find any significant correlation between average probing pocket depth and average attachment loss with resistin levels in serum (Furugen *et al.*, 2008). Additionally, logistic regression analysis showed that the association of serum resistin with the periodontal condition was promoted only in model 2 in which periodontitis with bleeding and control without bleeding was considered for the selection criteria of periodontal condition (Furugen *et al.*, 2008). To the best of our knowledge, this is the first study to explore the relationship between clinical periodontal parameters and resistin levels in serum in subjects with or without periodontitis within T2DM and non-diabetic groups. The current study reported a significant positive correlation between resistin levels in serum with %BOP (Spearman's ρ=0.22, p<0.01), mean probing depth (Spearman's ρ=0.18, p<0.05) and (for the first time with) PISA (Spearman's ρ=0.17, p<0.05). However, the results of this study did not show any significant correlation of serum resistin levels with mean loss of attachment. In the present study, the significant correlation of serum resistin with %BOP, mean PD and PISA, and the absence of association with LOA may indicate that serum resistin is related to an existing active inflammation in the periodontal tissues.

In conclusion, the present study demonstrated that serum resistin levels were significantly elevated in T2DM patients. Furthermore, in the non-diabetic group, levels of resistin in serum were relatively higher in periodontitis patients compared to periodontally healthy subjects; but this difference was not statistically significant. The levels of serum resistin were positively correlated with HbA1c, hsCRP, BMI, the

serum pro-inflammatory cytokines TNF-α, IL-6, INF-γ, and the clinical periodontal parameters %BOP, mean PD, and PISA. These findings suggest that the relationship between T2DM and resistin may be mediated by obesity-related inflammation, and that the role of resistin in the development of diabetes seems to be closely connected with an intensification of the systemic inflammatory state. Additionally, the association of serum resistin with the clinical periodontal parameters suggests that the inflammatory cells (which are well known to be a major source of resistin in humans) such as monocytes and macrophages in inflamed periodontal tissues may contribute to the elevation of serum resistin levels in non-diabetic periodontitis patients, albeit this elevation was not statistically significant. Further studies with a larger sample size and a comprehensive appraisal of potential confounding factors are necessary to elucidate the role of resistin in the two-way relationship between periodontal disease and T2DM.

Chapter 5 The effect of LPS and IL-1β on resistin expression and secretion in monocytes, macrophages and oral keratinocytes

5.1 Introduction

Periodontal diseases are an inflammatory processes initiated by bacteria of oral micro-flora. These microbes instigate tissue breakdown indirectly by activating host defence cells, which in turn produce and release inflammatory mediators that stimulate the effector mechanisms of connective tissue destruction (Williams, 2008). Epithelial cells, monocytes and macrophages all have important roles in periodontal pathogenesis. The epithelial cells represent the first line to confront the attacking bacteria and therefore it play important role in maintaining periodontal health (Dale, 2002). In effect, the pivotal role of epithelial cells in inflammation and in defence against microbes is attributed to its ability to respond robustly to exogenous factors, by migrating, proliferating and producing various cytokines and proteolytic enzymes (Uitto *et al.*, 2003). Gingival epithelial cells and macrophages have been identified as major sources of IL-1β in periodontium (Sfakianakis *et al.*, 2001). IL-1β, a multifunctional cytokine, provokes the production and expression of several important mediators, which in turn trigger a series of molecular and cellular responses, including tissue inflammation and adaptive immunity. It has been demonstrated that proinflammatory cytokines such as IL-1β have a central role in the development of epithelial inflammation in periodontitis (Barksby *et al.*, 2007).

The induced changes in the epithelium in response to bacterial challenge facilitate both vascular permeability and the recruitment of neutrophils, which represent the most important local defence in fending off periodontal bacteria. The dilated vessels in the microcirculation become engorged with blood, and the leukocytes extravasated and inflammatory infiltrate formed (Kornman *et al.*, 1997). The migrated monocytes differentiate in the tissues into macrophages, which serve to enhance inflammatory reaction as well as initiate the immune response. Intriguingly, the microbial components of periodontal bacteria such as LPS have stimulatory effect on cytokine production by monocytes and macrophages. (Teng, 2006; Liu *et al.*, 2010). In effect, macrophages play a central role in the initial sensing of the microbes. It presents in the periodontium and shelter the requisite system receptors called pattern recognition receptors (PRRs) such as TLR. The substantial importance of monocytes as circulating cells functional in microbe recognition comes from the fact that these cells are precursors of macrophages in tissues (Teng, 2006; Liu *et al.*, 2010).

LPS is one of a group of pathogen associated molecular patterns which are recognised by host cells and incite cytokine responses (Dixon *et al.*, 2004; Jain and Darveau, 2010). Therefore, LPS is a robust activator of the innate immune system, which it accomplished by instigating the Toll like receptor (TLR), a cell surface protein that recognises bacterial products (Dixon *et al.*, 2004). In effect, the LPS receptor is a macromolecular complex composed of TLR4, MD-2 and CD14. This complex is expressed on several cell types, involving immune cells, macrophages and dendritic cells (Schumann *et al.*, 1990; Wright *et al.*, 1990; Shimazu *et al.*, 1999; Dixon *et al.*, 2004; Jain and Darveau, 2010). The LPS of oral bacteria binds to receptors (TLR and CD14) on the surface of cells in the periodontium such as epithelial cells, fibroblasts, neutrophils, dendritic cells and monocytes/macrophages, which in turn instigate the

host cells to produce inflammatory mediators and cytokines and conduced to localized periodontal inflammatory reaction (Page *et al.*, 1997; Slots and Ting, 1999; Nishihara and Koseki, 2004).

In brief, a variety of pro-inflammatory mediators including pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and pro-inflammatory cytokines such as IL-1β activates proinflammatory responses in a wide range of host cells.

Resistin is a 12.5 kDa polypeptide hormone, which was discovered 10 years ago as a fat cell secreted factor that modulate insulin resistance in mice (Steppan *et al.*, 2001a). The principal cells that express resistin in human are monocytes, macrophages and neutrophils (Savage *et al.*, 2001; Patel *et al.*, 2003; Jung *et al.*, 2006; Bostrom *et al.*, 2009; Johansson *et al.*, 2009; Kunnari *et al.*, 2009), which suggest a possible involvement of resistin in inflammatory processes (Nagaev *et al.*, 2006).

Several Studies illustrated that inflammatory stimuli may regulate resistin production in myeloid immune cells but the findings have been conflicting. Also there is no data on the regulation of resistin secretion in epithelial cells. The aim of the experiments in this chapter is therefore to investigate the *in vitro* effect of LPS on the expression and secretion of resistin in in monocytic and macrophagic cell line THP-1, monocytic and macrophagic cell line U937 and in primary monocytes. In addition, this study investigated whether IL-1β stimulates resistin expression and secretion in OKF6 keratinocyte cell line.

5.2 Results

5.2.1 Investigation of the effect of IL-1β on resistin expression and secretion in OKF6 (oral keratinocytes)

Resistin production by OKF6 cells in response to IL-1β was assessed. OKF6 oral keratinocytes (2 x 10^5) were stimulated with human recombinant IL-1β (0.1 ng/ml) for three different time points (4, 24, and 48 hours) as described previously (section 2.1.6.1). Un-stimulated cells served as a control. The cells were collected for RNA extraction, quantification, reverse transcription and RT-PCR analysis as previously described in section 2.3. Tissue culture supernatants for stimulated and un-stimulated cells were collected and assayed for resistin concentrations with ELISA as described in section 2.2.

Figure 5.1 shows that the levels of resistin in control cultures were below detection at the three time points. Also, IL-1β had no effect on resistin secretion in OKF6 oral keratinocytes after 4, 24, and 48 h incubation compared to control. As shown in Figure 5.2, the resistin gene was not expressed by OKF6 oral keratinocytes neither in the control nor in the stimulated cultures at the three time points.

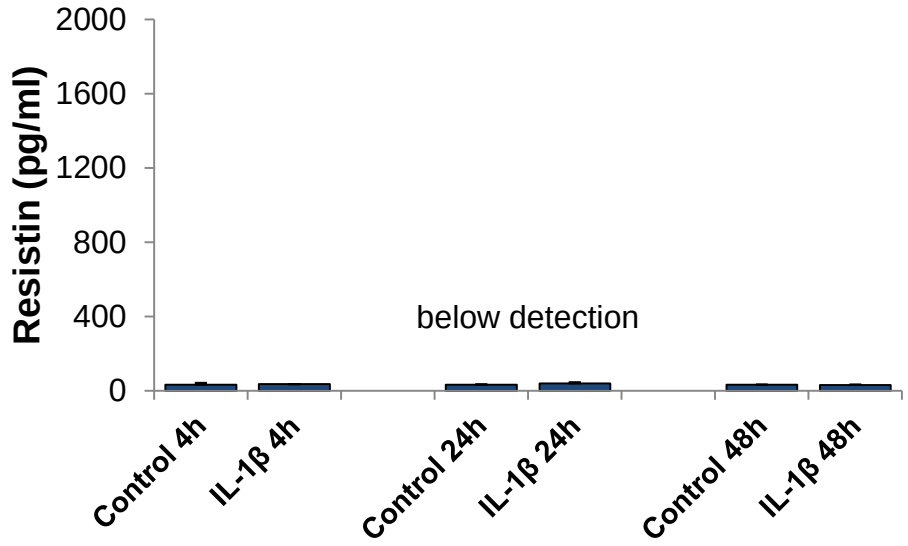


Figure 5.1 The effect of IL-1β on resistin secretion in OKF6 (oral keratinocytes) at three different time points

OKF6 oral keratinocytes (2 x 10^5) were cultured for different periods of time in the absence (control) or presence of IL-β (0.1 ng/ml). Resistin levels in supernatants was measured by ELISA. Each value represents the mean and standard deviation of two independent cell culture experiments (duplicate cultures i.e. n=4 in total).

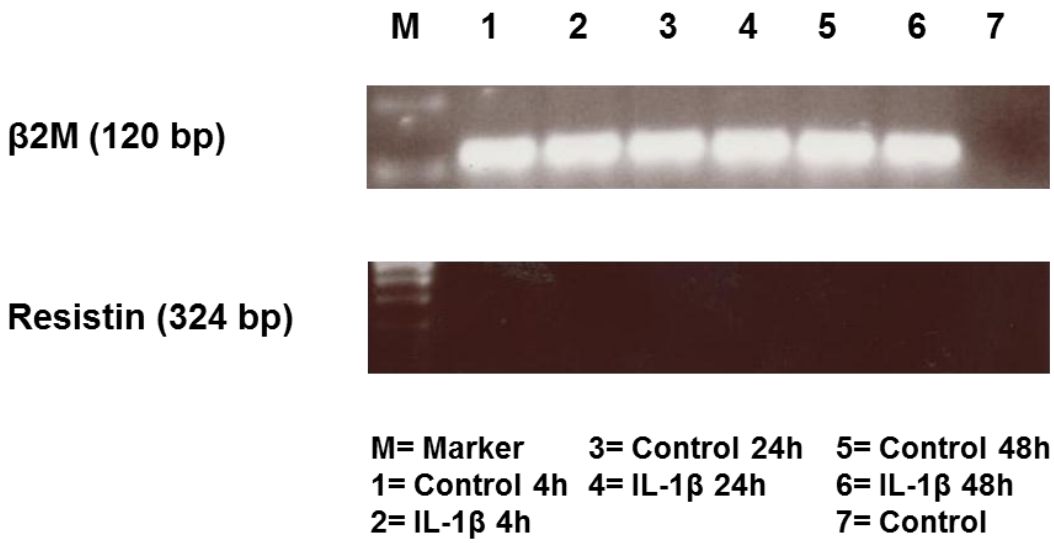


Figure 5.2 The effect of IL-1β on resistin gene expression in OKF6 at three different time points

Sub-confluent OKF6 cells were co-cultured in the absence or presence of (0.1 ng/ml) IL-1β for 4, 24, 48 h. Total cellular RNA was isolated and mRNA expression of resistin and β2 microglobulin were analysed by RT-PCR. The products were analysed on 3% agarose gels and stained with ethidium bromide.

5.2.2 Analysis of the effect of LPS on resistin protein secretion in THP-1 monocytes and macrophages

To study the effect of LPS on resistin release, THP-1 monocytes (2 x 10^6) were stimulated with *P.gingivalis* LPS (100 ng/ml) or *E.coli* LPS (100 ng/ml) for 3 different time points: 6, 24, and 48 h. Un-stimulated cells served as a control. Supernatants were collected and assayed for resistin concentrations with ELISA as described in section 2.2. Figure 5.3 shows that the levels of resistin in control cultures were below detection at the three time points. Also, both *P.gingivalis* and *E.coli* had no effect on resistin secretion in THP-1 monocytes after 6, 24, and 48 h incubation compared to control.

In order to assess the response of THP-1 monocytes to LPS stimulation, supernatants were also assayed for TNF-α concentrations with ELISA as described in section 2.2.

Figure 5.4 shows that *P.gingivalis* and *E.coli* LPS strongly increased TNF-α secretion in THP-1 monocytes after 6, 24, and 48 h incubation compared to control. The levels of TNF-α in control cultures were below detection at the 3 time points. TNF-α concentrations in *P.gingivalis* LPS stimulated cultures were 5157±208 pg/ml, 1533±37 pg/ml, 860±145 pg/ml at 6, 24, and 48 h stimulation, respectively. The up-regulation in TNF-α concentration in response to *E.coli* LPS was relatively lower than that seen in response to *P.gingivalis* LPS. TNF-α concentrations in *E.coli* LPS stimulated cultures were 1799±113 pg/ml, 444±72 pg/ml, 374±24 pg/ml at 6, 24, and 48 h stimulation, respectively.

To examine the effect of LPS on resistin release from THP-1 macrophages (see differentiation in section 2.1.1.2) cells were stimulated with *P.gingivalis* LPS (100 ng/ml) or *E.coli* LPS (100 ng/ml) for 3 different time points 6, 24, and 48 h. Un-

stimulated cells served as a control. Supernatants were collected and assayed for resistin concentrations with ELISA as described in section 2.2. Figure 5.5 illustrates that the levels of resistin in control cultures were below detection at the three time points. Also, both *P.gingivalis* and *E.coli* had no effect on resistin secretion in THP-1 macrophages after 6, 24, and 48 h incubation compared to control.

In order to assess the response of THP-1 macrophages to LPS stimulation, supernatants were also analysed for TNF-α concentrations with ELISA as described in section 2.2. Figure 5.6 shows that LPS remarkably enhanced TNF-α secretion in THP-1 macrophages after 6, 24, and 48 h incubation compared to control. The levels of TNF-α in control cultures were below detection at the 3 time points. TNF-α concentrations in *P.gingivalis* LPS stimulated cultures were 1968±709 pg/ml, 879±111 pg/ml, 689±114 pg/ml at 6, 24, and 48 h stimulation, respectively. The up-regulation in TNF-α concentrations in response to *E.coli* LPS was relatively higher than that seen in response to *P.gingivalis* LPS. TNF-α concentrations in *E.coli* LPS stimulated cultures were 2149±844 pg/ml, 1197±142 pg/ml, 855±46 pg/ml at 6, 24, and 48 h stimulation, respectively.

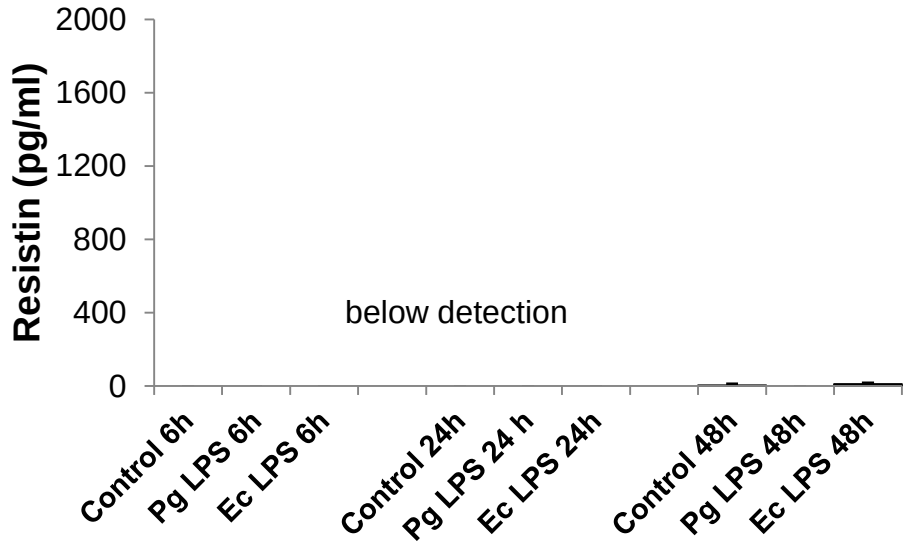


Figure 5.3 The effect of *P.gingivalis* and *E.coli* LPS on resistin secretion in THP-1 monocytes at three different time points

THP-1 (2 x 10^6) were cultured for different periods of time in the absence (control) or presence of *P.gingivalis* LPS (100 ng/ml) or *E.coli* LPS (100 ng/ml). Resistin levels in supernatants was measured by ELISA. Each value represents the mean and standard deviation of a single cell culture experiment (duplicate cultures i.e. n=2 in total).

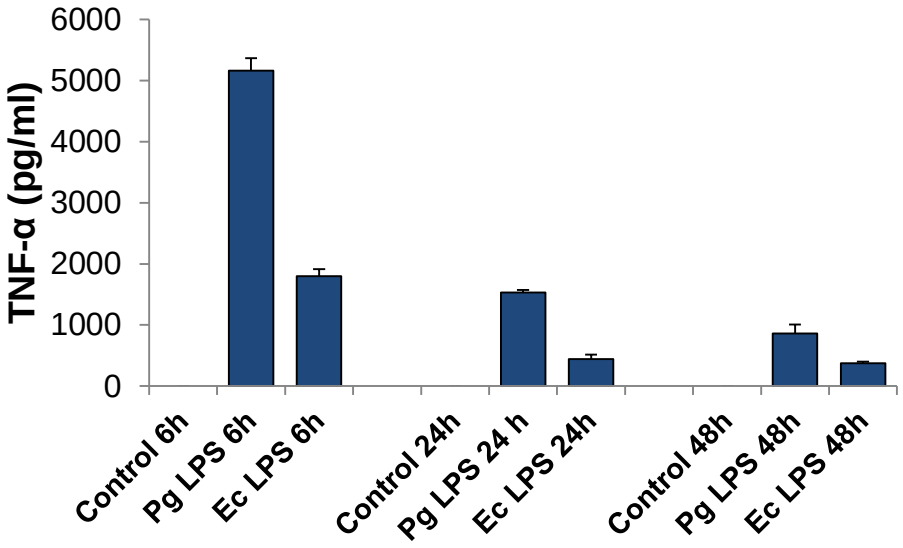


Figure 5.4 The effect of *P.gingivalis* and *E.coli* LPS on TNF-α secretion in THP-1 monocytes at three different time points

THP-1 (2×10^6) were cultured for different periods of time in the absence (control) or presence of *P.gingivalis* LPS (100 ng/ml) or *E.coli* LPS (100 ng/ml). TNF-α levels in supernatants was measured by ELISA. Each value represents the mean and standard deviation of a single cell culture experiment (duplicate cultures i.e. n=2 in total).

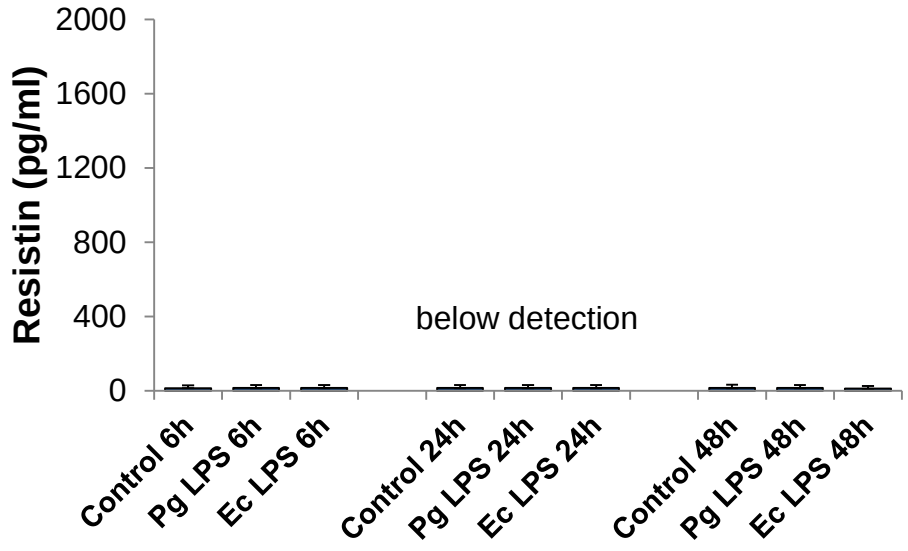


Figure 5.5 The effect of *P.gingivalis* and *E.coli* LPS on resistin secretion in THP-1 macrophages at three different time points

THP-1 cells (2 x 10^6) were differentiated with (50 ng/ml) PMA for 24 h. Then THP-1 macrophages were cultured for different periods of time in the absence (control) or presence of *P.gingivalis* LPS (100 ng/ml) or *E.coli* LPS (100 ng/ml). Resistin levels in supernatants was measured by ELISA. Each value represents the mean and standard deviation of 2 independent cell culture experiments (duplicate cultures i.e. n=4 in total).

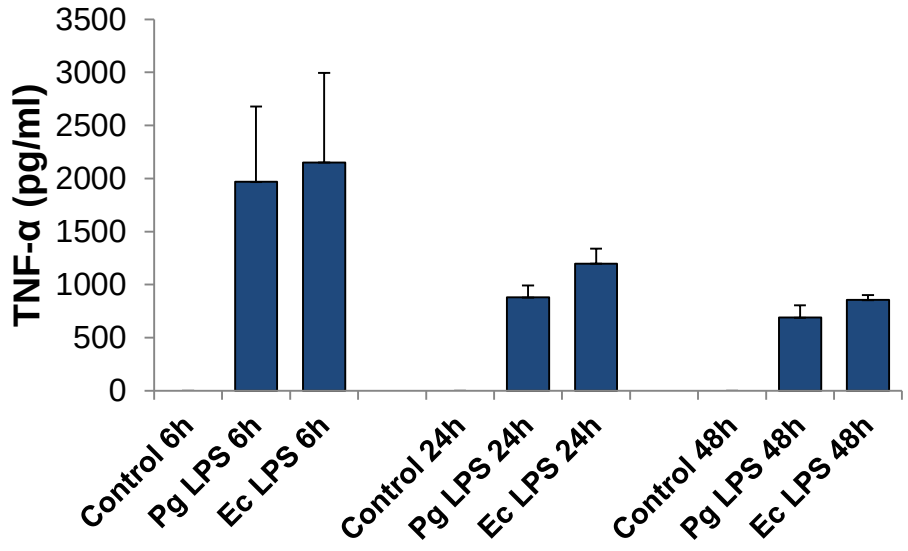


Figure 5.6 The effect of *P.gingivalis* and *E.coli* LPS on TNF- secretion in THP-1 macrophages at three different time points

THP-1 cells (2 x 10^6) were differentiated with (50 ng/ml) PMA for 24 h. Then THP-1 macrophages were cultured for different periods of time in the absence (control) or presence of *P.gingivalis* LPS (100 ng/ml) or *E.coli* LPS (100 ng/ml). TNF-α levels in supernatants was measured by ELISA. Each value represents the mean and standard deviation of 2 independent cell culture experiments (duplicate cultures i.e. n=4 in total).

5.2.3 Analysis of the effect of LPS on resistin expression and secretion in U937 monocytes

To explore the effect of LPS on resistin production at the mRNA and protein levels, U937 monocytes (2 x 10^6) were stimulated with *P.gingivalis* LPS (100 ng/ml) or *E.coli* LPS (100 ng/ml) for 3 different time points 4, 24, and 48 h. Un-stimulated cells served as a control. mRNA was analysed for resistin gene expression using RT-PCR (section 2.3). Supernatants were collected and assayed for resistin concentrations with ELISA as described in section 2.2. As shown in

Figure 5.7, U937 monocytes constitutively expressed resistin mRNA. There is no evidence for an effect of *P.gingivalis* or *E.coli* LPS on resistin mRNA expression in U937 monocytes at 4, 24, and 48 h. Similarly, Figure 5.8 illustrates that U937 monocytes constitutively secreted resistin protein. Stimulation with *P.gingivalis* and *E.coli* LPS did not alter resistin protein levels in tissue culture supernatants at 4, 24, and 48 h.

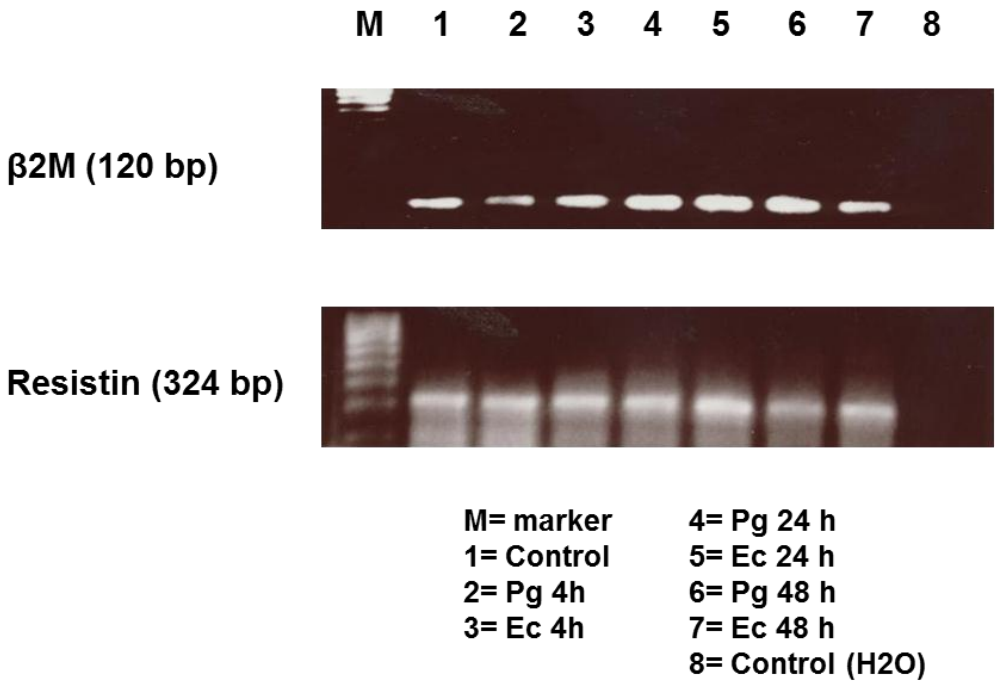


Figure 5.7 The effect of *P.gingivalis* and *E.coli* LPS on resistin expression in U937 monocytes at three different time points

U937 monocytes (2 x 10^6) were co-cultured in the absence or presence of either *P.gingivalis* LPS (100 ng/ml) and *E.coli* LPS (100 ng/ml) for 4, 24, and 48 h. Total RNA was isolated and the mRNA expression of resistin and β2 microglobulin were analysed by RT-PCR. The graph represent single cell culture experiments (duplicate cultures i.e. n=2).

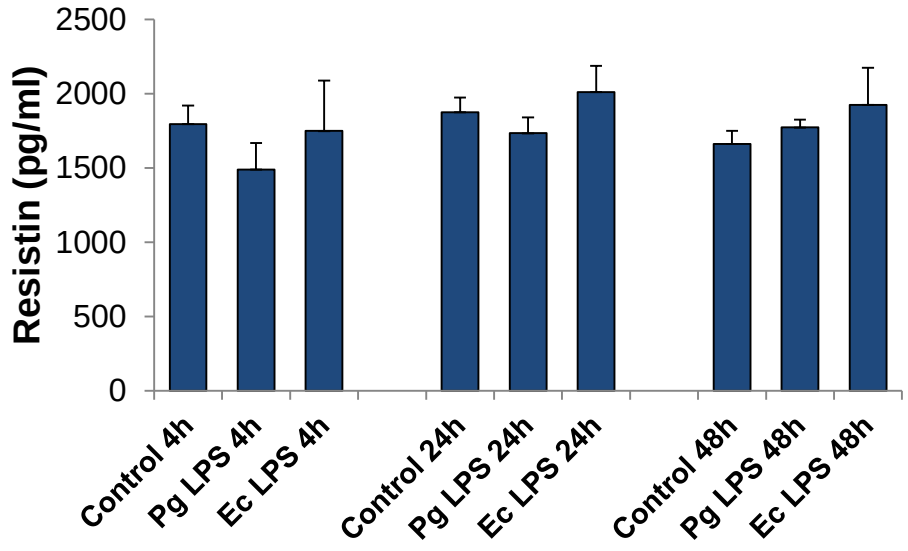


Figure 5.8 The effect of *P.gingivalis* and *E.coli LPS* on resistin secretion in U937 monocytes at three different time points

U937 monocytes (2 x 10^6) were co-cultured in the absence or presence of either *P.gingivalis* LPS (100 ng/ml) and *E.coli* LPS (100 ng/ml) for 4, 24, and 48 h. Resistin levels in supernatants was measured by ELISA and compared with controls. Each value represents the mean and standard deviation of a single cell culture experiment (duplicate cultures i.e. n=2).

5.2.4 Analysis of the effect of LPS on resistin expression and secretion in U937 macrophages

To study the effect of LPS on resistin expression and secretion in U937 macrophages, a series of pilot experiments were initially performed to establish optimal experimental conditions for investigating resistin expression in U937 macrophages.

In order to determine the optimal conditions for differentiation of U937 into macrophages, U937 (2×10^6) were treated with 50 ng/ml PMA for 48 h or 100 ng/ml PMA for 72 h. Then, the resultant macrophages were stimulated with *E.coli* LPS (100 ng/ml) for 24 h. Un-stimulated cells served as a control. Supernatants were collected and assayed for resistin and TNF-α concentration with ELISA as described in section 2.2. The results were derived from single stimulation experiment (pilot experiment in duplicate culture) and therefore no statistical analysis was performed. It is apparent from the data presented in Figure 5.9 that resistin secretion was enhanced in response to *E.coli* LPS in macrophages using both protocols in a comparable manner. Similarly, Figure 5.10 shows a comparable elevation in TNF-α levels in response to *E.coli* LPS in macrophages using both protocols. It was decided to prepare U937 macrophages by incubating U937 monocytes with 50 ng/ml PMA for 48 h in subsequent experiments.

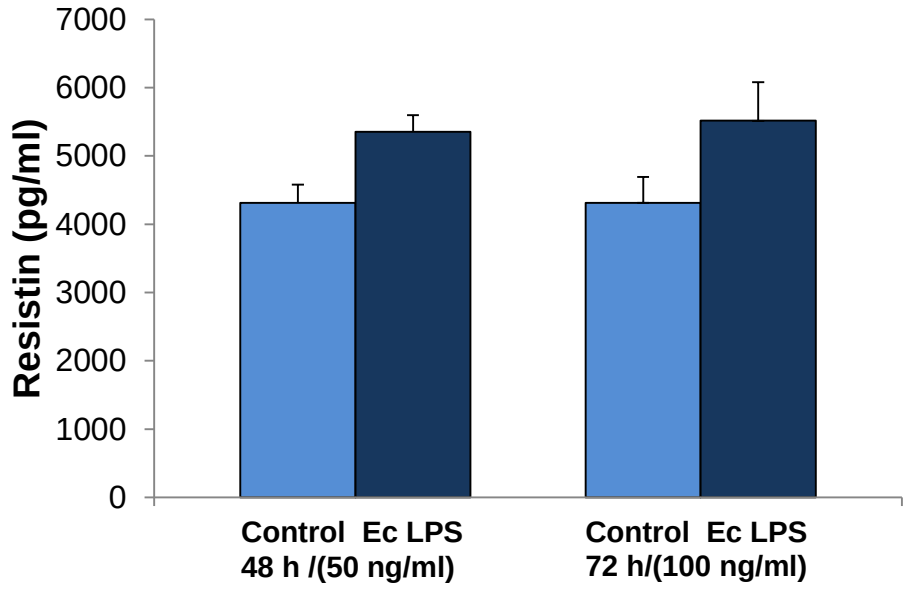


Figure 5.9 The effect of *E.coli* LPS on resistin secretion in U937 monocytes differentiated with 50 ng/ml PMA for 48 h or 100 ng/ml PMA for 72 h

U937 (2 x 10^6) were incubated with 50 ng/ml PMA for 48 h or 100 ng/ml PMA for 72 h, then were cultured in the presence or absence of *E.coli* (100 ng/ml) for 24 h. Resistin levels in supernatants were measured by ELISA and compared with controls. Each value represents the mean and standard deviation of a single cell culture experiment (duplicate numbers i.e. n=2).

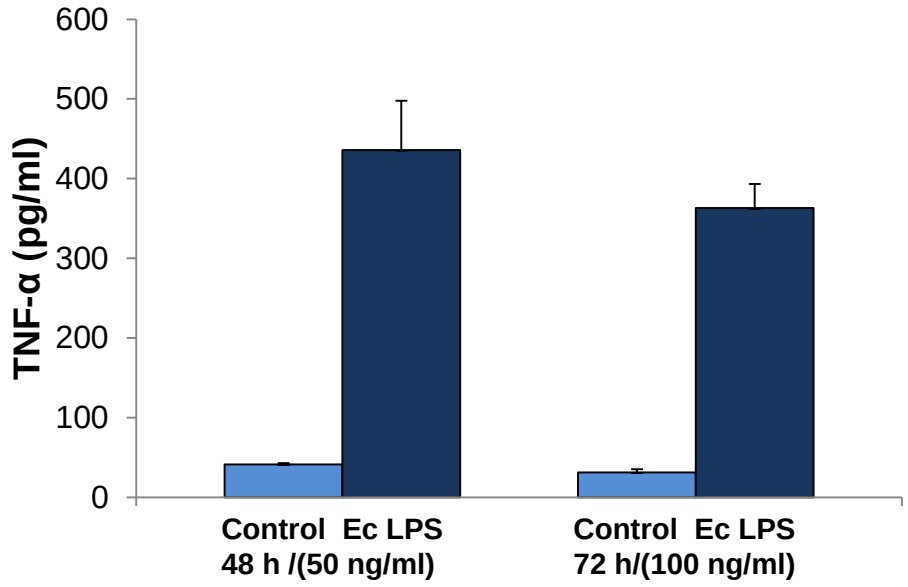


Figure 5.10 The effect of *E.coli* LPS on TNF-α secretion in U937 monocytes differentiated with 50 ng/ml PMA for 48 h or 100 ng/ml PMA for 72 h

U937 (2 x 10^6) were incubated with 50 ng/ml PMA for 48 h or 100 ng/ml PMA for 72 h, then were cultured in the presence or absence of *E.coli* (100 ng/ml) for 24 h. TNF-α levels in supernatants were measured by ELISA and compared with controls. Each value represents the mean and standard deviation of a single cell culture experiment (duplicate numbers i.e. n=2).

In order to determine the optimal time point at which U937 macrophages respond to LPS with the highest resistin up-regulation, two time point experiments were performed. In both experiments, U937 (2×10^6) were treated with 50 ng/ml for 48 h to be differentiated into macrophages, then were stimulated with *E.coli* LPS (100 ng/ml) for different time points 30 min, 1, 3, 6, 24, 48, and 72 h. In the second of these 2 experiments the PMA was washed away by changing the medium two times a day for 4 days before stimulation with LPS, also the 30 min time point was replaced by 24 h time point in the second experiment. Un-stimulated cells served as a control. Supernatants were collected and assayed for resistin and TNF-α concentration with ELISA as described in section 2.2. The results were derived from single stimulation experiment (in duplicate culture) and therefore no statistical analysis was performed.

The results obtain from the first experiment is presented in Figure 5.11 and Figure 5.12. As shown in Figure 5.11, *E.coli* LPS appears to induce increased levels of resistin secretion in U937 macrophages as compared to controls, in particular at longer time points (48 and 72 h). Similarly, Figure 5.12 shows that there is a clear trend of increasing TNF-α secretion levels from U937 macrophages in response to *E.coli* LPS stimulation at all time points except the 30 min. Figure 5.13 presents the results obtained from the second experiment which reveals that *E.coli* LPS seems to provoke enhanced levels of resistin secretion in U937 macrophages as compared to controls, in particular at longer time points. Having no remarkable difference in resistin induction between the two experiments, it was decided to choose the protocol of the first experiment.

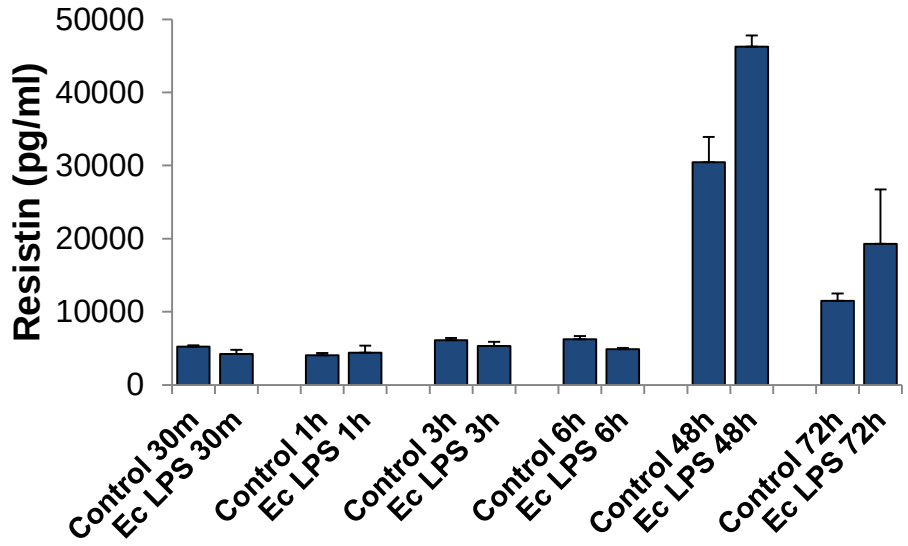


Figure 5.11 The effect of *E.coli* LPS on resistin secretion in U937 macrophages

U937 monocytes (2 x 10^6) were incubated with PMA (50 ng/ml) for 48 h, then were cultured for different times in the presence or absence of *E.coli*. Resistin levels in supernatants were measured by ELISA and compared with controls. Each value represents the mean and standard deviation of a single cell culture experiment (duplicate cultures i.e. n=2 in total).

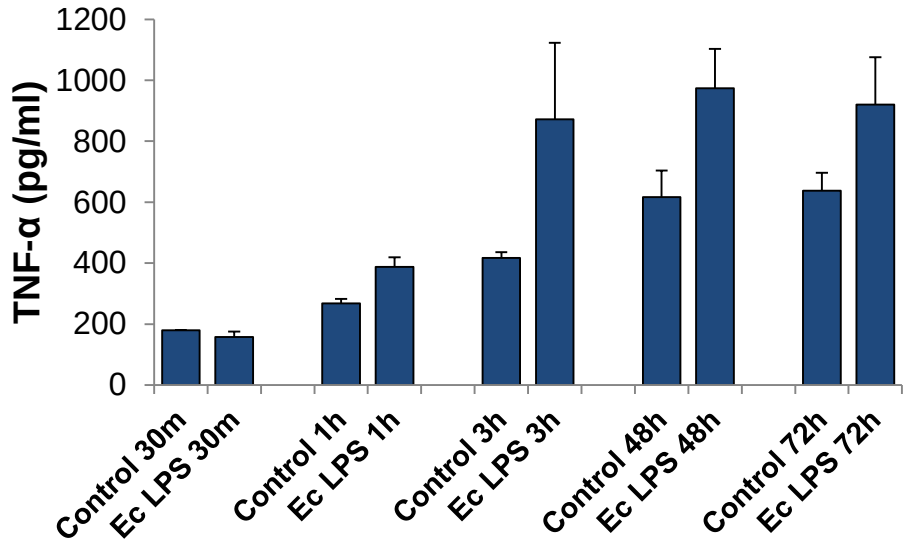


Figure 5.12 The effect of *E.coli* LPS on TNF-α secretion in U937 macrophages

U937 monocytes (2 x 10^6) were incubated with 50 ng/ml PMA for 48 h, then were cultured for different period of times in the presence or absence of *E.coli* (100 ng/ml). Resistin levels in supernatants were measured by ELISA and compared with controls. Each value represents the mean and standard deviation of a single cell culture experiment (duplicate cultures i.e. n=2 in total).

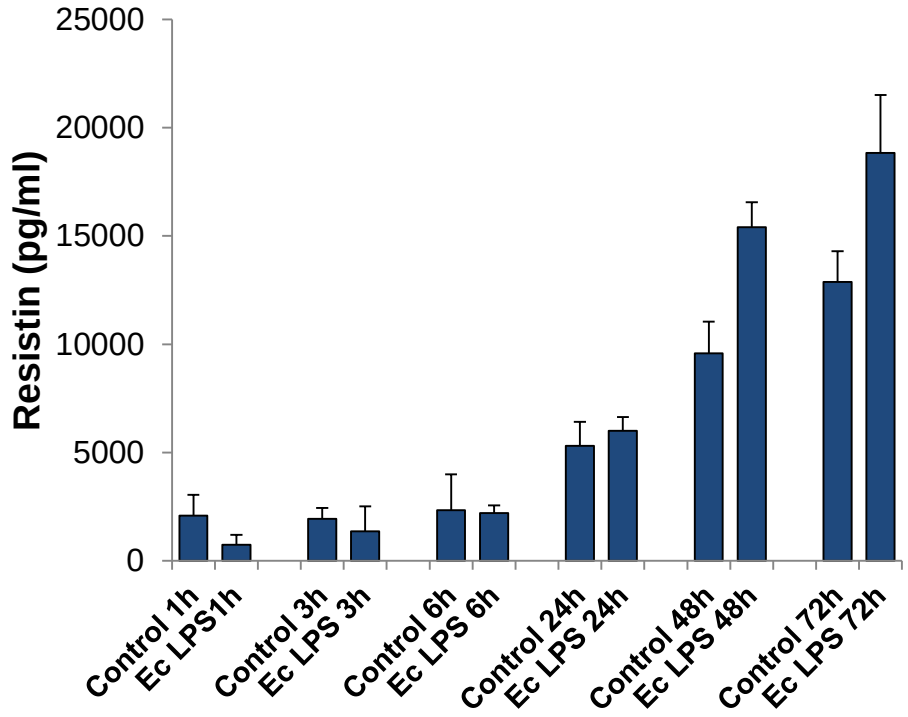


Figure 5.13 The effect of *E.coli* LPS on resistin secretion in U937 macrophages washed free of PMA

U937 monocytes (2 x 10^6) were incubated with PMA (50 ng/ml) for 48 h, PMA were washed away by changing the medium two times a day for 4 days, and then the cells were cultured for different period of times in the presence or absence of *E.coli* (100 ng/ml). Resistin levels in supernatants were measured by ELISA and compared with controls. Each value represents the mean and standard deviation of a single cell culture experiment (duplicate culture i.e. n=2 in total).

To investigate the role of *P.gingivalis* LPS and *E.coli* LPS (100 ng/ml) on resistin production at the mRNA and protein levels, U937 macrophages (2×10^6) were stimulated with *P.gingivalis* and *E.coli* LPS (both at 100 ng/ml) for 48 h. Un-stimulated cells served as the control. Supernatants were collected and assayed for resistin concentrations with ELISA as described in section 2.2. Total cellular RNA was extracted and reverse transcribed to produce cDNA and the amount of resistin mRNA expression was quantified by Real-Time PCR as described in section 2.3.6.

Figure 5.14 shows the results from Real-Time PCR. Compared to control, both *P.gingivalis* and *E.coli* LPS (100 ng/ml) stimulated a very slight up-regulation of resistin mRNA expression, however, this was not statistically significant.

Figure 5.15 shows that both *P.gingivalis* and *E.coli* LPS (100 ng/ml) had no significant effect on resistin secretion in U937 macrophages. In effect, U937 macrophages constitutively secreted resistin and the levels of resistin in supernatants were comparable from both control and stimulated groups. Resistin concentrations in control cultures, *P.gingivalis* LPS and to *E.coli* LPS stimulated cultures (mean ± SD) were; 37709 ± 3812 pg/ml, 38682 ± 1158 pg/ml, 42113 ± 9649 pg/ml, respectively.

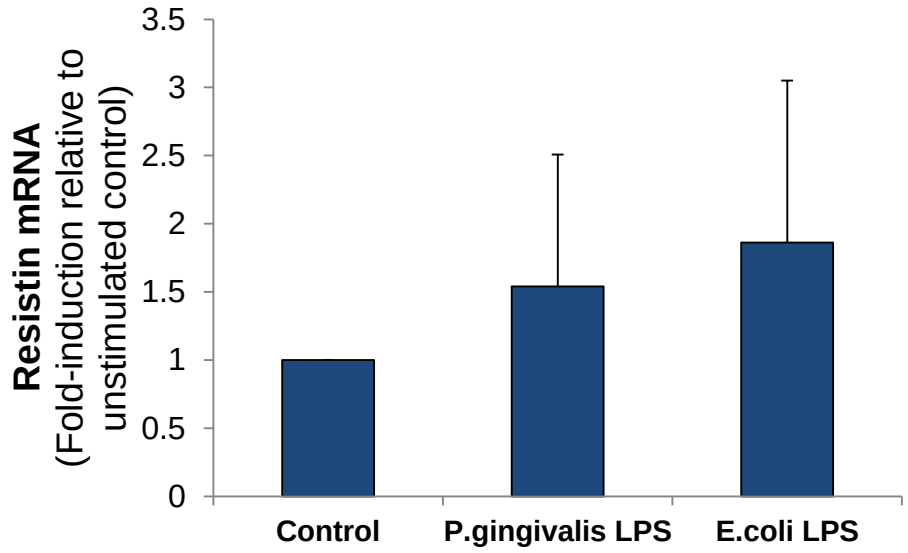


Figure 5.14 The effect of *P.gingivalis* (100 ng/ml) and *E.coli* (100 ng/ml) on resistin mRNA expression in U937 macrophages

U937 macrophages (2 x 10^6) were stimulated with *P.gingivalis* LPS (100 ng/ml) or *E.coli* LPS (100 ng/ml) for 48 h. The amount of resistin mRNA was quantified by real time RT-PCR. The data are expressed as mean fold-induction of 3 independent cell culture experiments (duplicate cultures i.e. n=6 in total). Statistical analysis was performed on δCT values using ANOVA (p = 0.587).

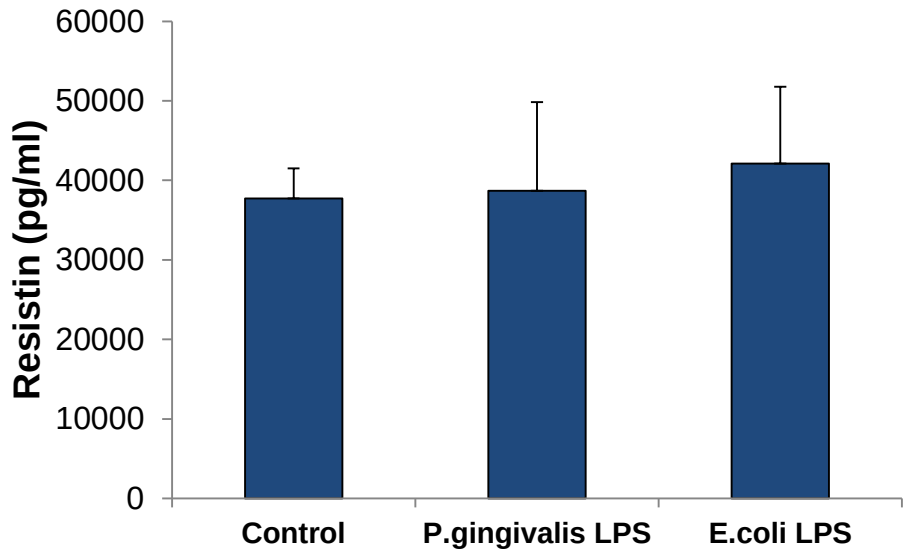


Figure 5.15 The effect of *P.gingivalis* (100 ng/ml) and *E.coli* LPS (100 ng/ml) on resistin secretion in U937 macrophages

U937 macrophages (2 x 10^6) were stimulated with *P.gingivalis* LPS (100 ng/ml) and *E.coli* LPS (100 ng/ml) for 48 h. Resistin Levels in supernatants were measured by ELISA and compared with un-stimulated controls. Each value represents the mean and standard deviation of 3 independent cell culture experiments (duplicate cultures i.e. n=6 in total). Statistics: ANOVA (p = 0.670).

To be able draw a concrete conclusion about whether or not LPS could drive U937 macrophages to enhance resistin production it was important to carry out three independent experiments in which U937 macrophages (2×10^6) were stimulated with either *Pseudomonas aeruginosa* whole cell lysate or *E.coli* LPS (100 ng/ml and 1µg/ml) for two time points 24 h and 48 h. Un-stimulated cells served as a control. Supernatants were collected and assayed for resistin concentrations with ELISA as described in section 2.2.

As shown in Figure 5.16, *P.aeruginosa* whole cell lysate down-regulated resistin secretion from U937 macrophages at both 24 and 48 h.

Figure 5.17 shows that treatment with *E.coli* LPS at a concentration of 100 ng/ml had no effect on resistin secretion in U937 macrophages at both 24 and 48 h.

Figure 5.18 shows that *E.coli* LPS at a concentration of 1µg/ml seems to induce increased levels of resistin secretion in U937 macrophages after 24 and 48 h incubation compared to control.

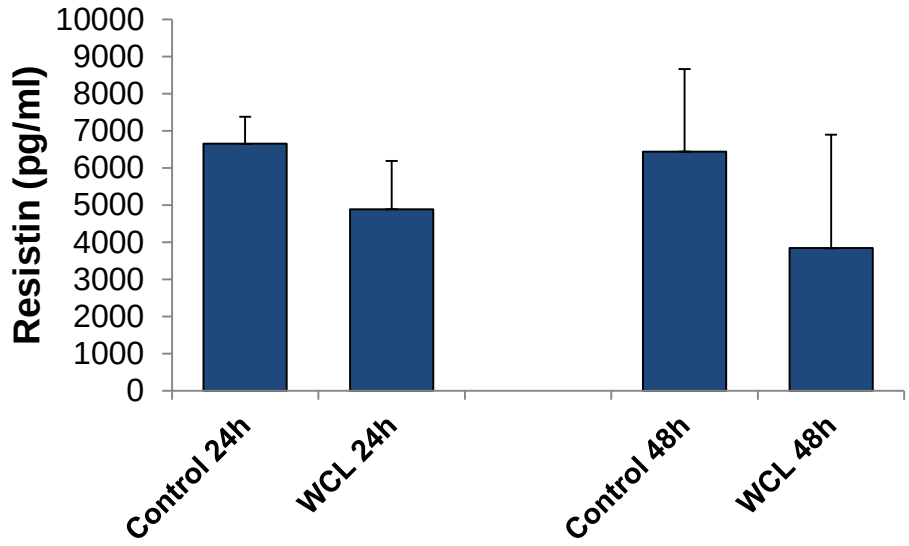


Figure 5.16 The effect of *Pseudomonas aeruginosa* whole cell lysate on resistin secretion in U937 macrophages at 24 h and 48 h

U937 macrophages (2 x 10^6) were stimulated with *Pseudomonas aeruginosa* whole cell lysate for 24 and 48 h. Resistin Levels in supernatants were measured by ELISA and compared with un-stimulated controls. Each value represents the mean and standard deviation of a single experiment (triplicate cultures i.e. n=3 in total).

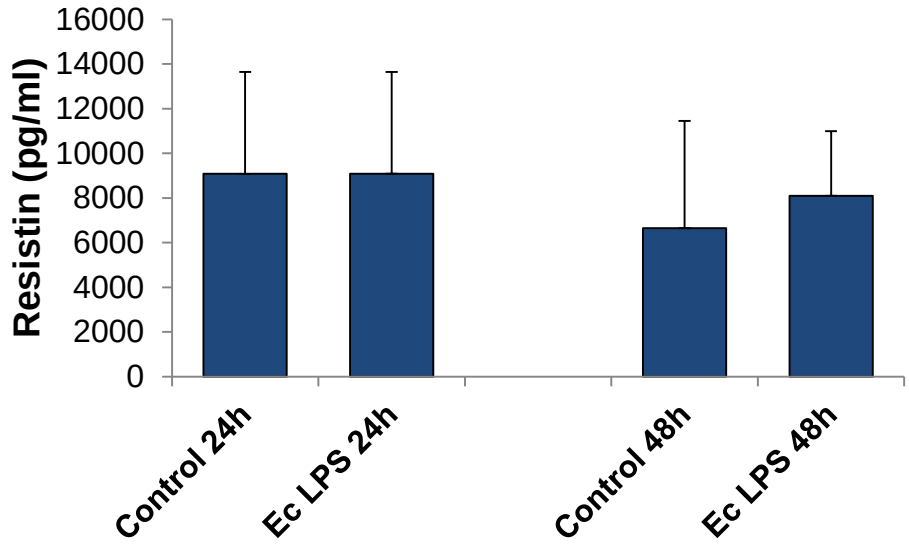


Figure 5.17 The effect of (100 ng/ml) *E.coli* LPS on resistin secretion in U937 macrophages for 24 h and 48 h

U937 macrophages (2 x 10^6) were stimulated with *E.coli* LPS (100 ng/ml) for 24 and 48 h. Resistin Levels in supernatants were measured by ELISA and compared with un-stimulated controls. Each value represents the mean and standard deviation of a single experiment (triplicate cultures i.e. n=3 in total).

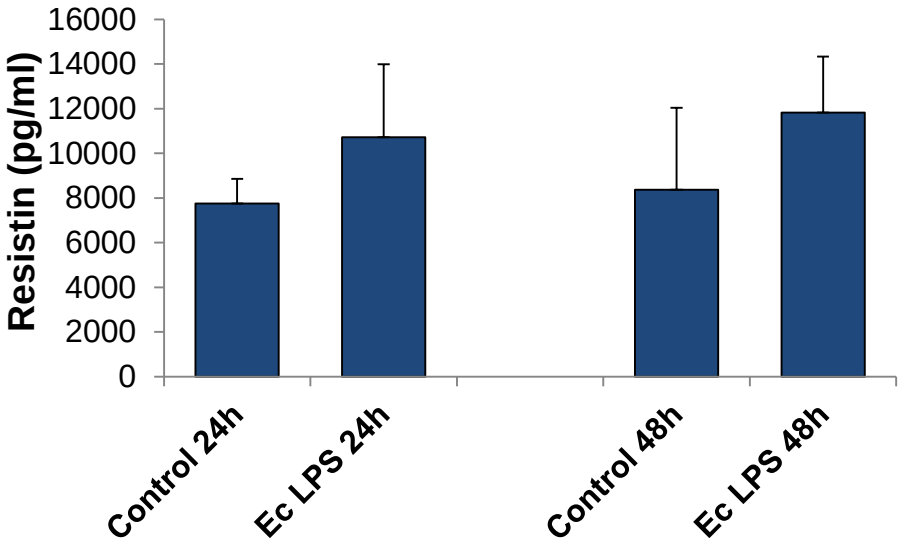


Figure 5.18 The effect of (1 µg/ml) *E.coli* LPS on resistin secretion in U937 macrophages at 24 h and 48 h

U937 macrophages (2 x 10^6) were stimulated with *E.coli* LPS (1µ/ml) for 24 and 48 h. Resistin Levels in supernatants were measured by ELISA and compared with un-stimulated controls. Each value represents the mean and standard deviation of a single experiment (triplicate cultures i.e. n=3 in total).

To evaluate the effect of LPS (1 µg/ml) on resistin production at mRNA and protein levels, U937 macrophages (2 x 10^6) were stimulated with *P.gingivalis* and *E.coli* LPS (1 µg/ml) for 48 h. Un-stimulated cells served as the control. Supernatants were collected and assayed for resistin concentrations with ELISA as described in section 2.2. Total cellular RNA was extracted and reverse transcribed to produce cDNA and the amount of resistin mRNA expression was quantified by Real-Time PCR as described in section 2.3.6.

Figure 5.19 shows that *P.gingivalis* and *E.coli* LPS significantly increased resistin secretion in U937 macrophages after 48 h incubation ($p < 0.05$) compared to control. In controls cultures, the levels of resistin (mean ± SD) were 21580 ± 4541 pg/ml which increased to 33947 ± 9450 pg/ml ($p < 0.05$, compared to control) in *P.gingivalis* LPS stimulated cultures and 32424 ± 8518 pg/ml ($p < 0.05$, compared to control) in *E.coli* LPS stimulated cultures.

Figure 5.20 shows the results from Real-Time PCR. Compared to control, both *P.gingivalis* and *E.coli* LPS (1 µg/ml) significantly up-regulated resistin mRNA expression (2.9 fold and 2.5 fold respectively) at 48 h ($p < 0.05$).

Taken all together, the present study demonstrated that *P.gingivalis* and *E.coli* LPS (1µg/ml) up-regulates resistin production at the mRNA and protein levels in U937 macrophages.

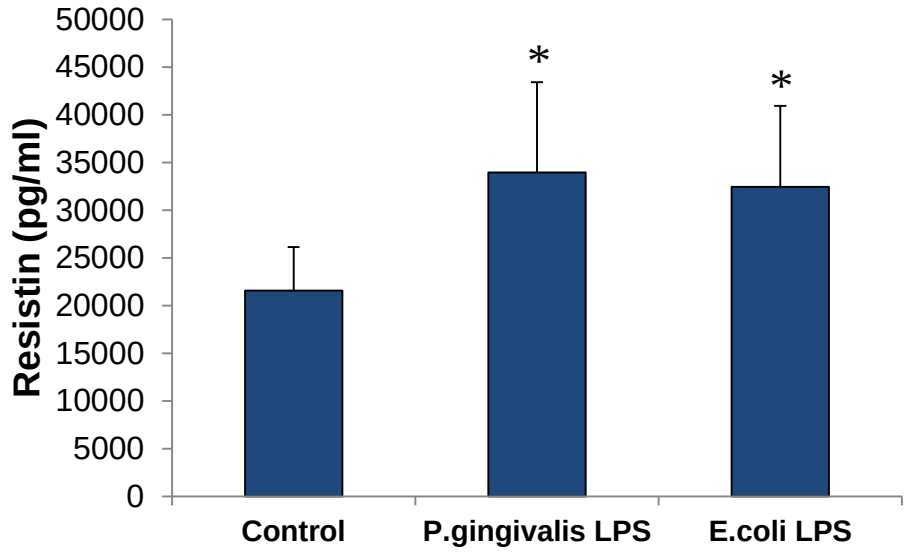


Figure 5.19 Resistin secretion in U937 macrophages is significantly enhanced by *P.gingivalis* and *E.coli* LPS (1 µg/ml) stimulation

U937 macrophages (2 x 10^6) were stimulated with *P.gingivalis* LPS (1µg/ml) and *E.coli* LPS (1µg/ml) for 48 h. Resistin Levels in supernatants were measured by ELISA and compared with un-stimulated controls. Each value represents the mean and standard deviation of 3 independent cell culture experiments (each comprising duplicate cultures i.e. n=6 in total). Statistics: Kruskal-Wallis, Mann-Whitney *U* test *p <0.05 compared to control.

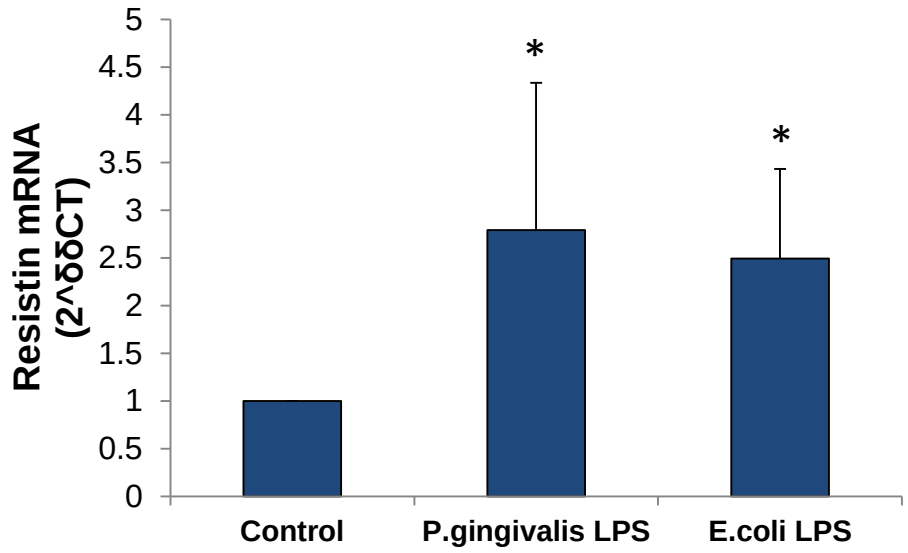


Figure 5.20 Resistin gene expression in U937 macrophages is significantly up-regulated in response to *P.gingivalis* and *E.coli* LPS (1 µg/ml) stimulation

U937 macrophages (2 x 10^6) were stimulated with *P.gingivalis* LPS (1µg/ml) or *E.coli* LPS (1µg/ml) for 48 h. The amount of resistin mRNA was quantified by real time RT-PCR. The data are expressed as mean fold-induction of 3 independent experiments measured in 3 separate occasions (duplicate cultures i.e. n=6 in total). Statistical analysis was performed on δCT values using Kruskal-Wallis, Mann-Whitney *U* test *p <0.05 compared to control.

5.2.5 Analysis of the effect of LPS on resistin expression and secretion in human primary monocytes

To study the effect of LPS (1 µg/ml) on resistin production at mRNA and protein levels, primary human monocytes (4 x 10^6) were stimulated with *P.gingivalis* and *E.coli* LPS (1 µg/ml) for 48 h. Un-stimulated cells served as the control. Supernatants were collected and assayed for resistin concentrations with ELISA as described in section 2.2. Total cellular RNA was extracted and reverse transcribed to produce cDNA and the amount of resistin mRNA expression was quantified by Real-Time PCR as described in section 2.3.6.

Compared to control, both *P.gingivalis* and *E.coli* LPS significantly enhanced resistin release in human primary monocytes (Figure 5.21). The concentration of resistin (mean ± SD) were 228 ± 92 pg/ml for control cultures versus 889 ± 606 pg/ml and 927 ± 153 pg/ml for *P.gingivalis* and *E.coli* LPS stimulated culture, respectively.

Figure 5.22 shows the results from Real-Time PCR. Compared to control, both *P.gingivalis* and *E.coli* LPS (1 µg/ml) significantly up-regulated resistin mRNA expression (15 fold and 54 fold respectively) in human primary monocytes at 48 h ($p < 0.05$).

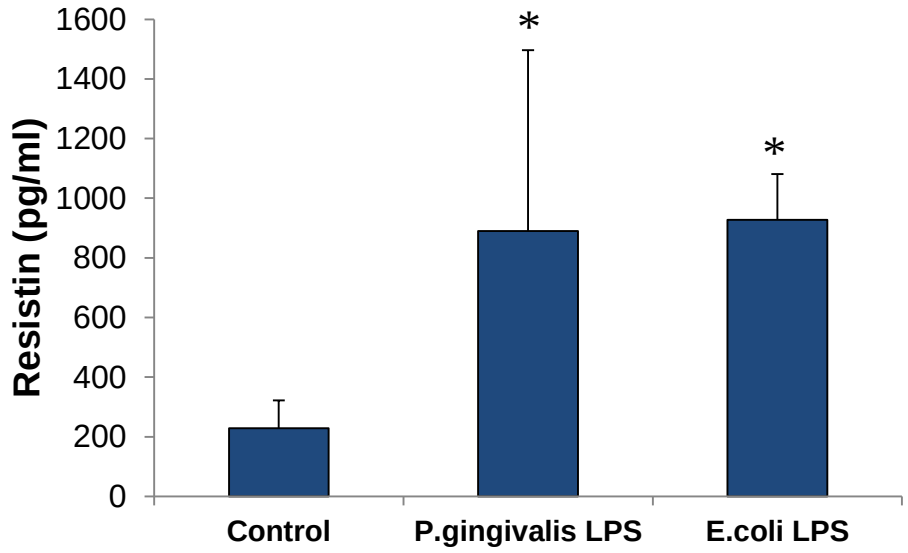


Figure 5.21 Resistin secretion in primary monocytes is significantly enhanced in response to *P.gingivalis* and *E.coli* LPS (1 µg/ml) stimulation

Primary monocytes (4 x 10^6) were stimulated with *P.gingivalis* LPS (1µg/ml) and *E.coli* LPS (1µg/ml) for 48 h. Resistin Levels in supernatants were measured by ELISA and compared with un-stimulated controls. Each value represents the mean and standard deviation of 2 cell culture experiments (each comprising duplicate cultures i.e. n=4 in total). Statistics: Kruskal-Wallis, Mann-Whitney *U* test *p <0.05 compared to control.

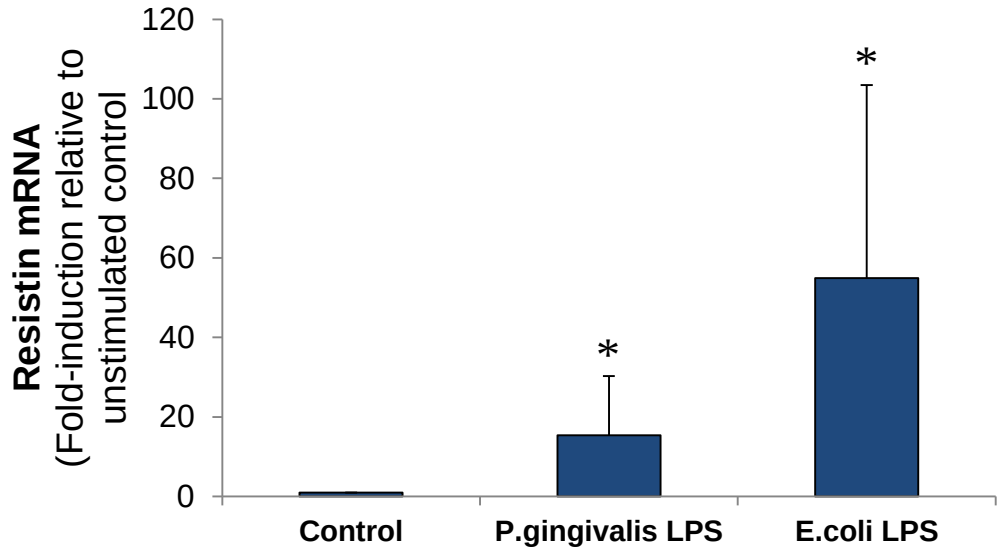


Figure 5.22 Resistin gene expression in human primary monocytes is significantly up-regulated in response to *P.gingivalis* and *E.coli* LPS (1µg/ml) stimulation

Primary monocytes (4 x 10^6) were stimulated with *P.gingivalis* LPS (1µg/ml) or *E.coli* LPS (1µg/ml) for 48 h. The amount of resistin mRNA was quantified by real time RT-PCR. The data are expressed as mean fold-induction of 2 independent cell culture experiments measured in 2 separate occasions (duplicate cultures i.e. n=4 in total). Statistical analysis was performed on δCT values using Kruskal-Wallis, Mann-Whitney *U* test **p* <0.05 compared to control.

5.3 Discussion

Periodontal diseases are bacteria-induced destructive inflammatory processes affecting the tooth-supporting structures and leading to attachment loss, periodontal pocket formation and alveolar bone resorption (Williams, 1990; Williams, 2008). The primary mechanisms involved in the local host response to bacterial aggression in periodontal tissues encompass leukocytes recruitment and release of inflammatory mediators and cytokines (Okada and Murakami, 1998). IL-1β have been identified as an crucial mediator in the pathogenesis of periodontal disease (Sakai *et al.*, 2006). It has also been shown to be raised in GCF and is produced in patients with periodontal disease (Stashenko *et al.*, 1991; Boch *et al.*, 2001; Sakai *et al.*, 2006). Many cell types are responsible for IL-1β production, including lymphocytes, epithelial cells, fibroblasts and monocytes/macrophages (Kang *et al.*, 1996; Sfakianakis *et al.*, 2001). IL-1β instigates a variety of cell types to produce pro-inflammatory mediators such as IL-6, TNF-α, IL-8, IL-1β itself, prostaglandin E2, and MMPs, which mediate tissue destruction in periodontal disease (MacNaul *et al.*, 1990; Boch *et al.*, 2001; Sfakianakis *et al.*, 2001).

Periodontal disease is driven by a variety of bacteria, which have different forms of LPS which robustly activate immunity and in subtly different pathways (Barksby *et al.*, 2009; Hajishengallis, 2009). Lipopolysaccharide (LPS) expresses the virulence factor of Gram-negative bacteria, and plays a pivotal role in the destruction of periodontal tissue driven by these microorganisms. It has been shown that LPS induces a prominent pro-inflammatory cytokine response in several cell types such as epithelial cells, monocytes/macrophages, neutrophils and fibroblasts. This was demonstrated via the up-regulation of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, IL-8, and MCP-1 in response to LPS stimulation (Page and Kornman,

1997; Slots and Ting, 1999; Sandros *et al.*, 2000; Nishihara and Koseki, 2004; Diya *et al.*, 2008).

The present study demonstrated that both *P.gingivalis* and *E.coli* LPS enhances resistin protein secretin and gene expression in human macrophages. In effect, stimulation of human macrophagic cell line U937 with 1 µg/ml *P.gingivalis* and *E.coli* LPS significantly enhanced resistin expression and secretion. However, treatment with *P.gingivalis* and *E.coli* LPS at a concentration of 100 ng/ml had no effect on resistin expression and secretion in human macrophagic cell line U937. In addition, *P.gingivalis* and *E.coli* LPS (1 µg/ml) significantly enhanced resistin expression and secretion in cultured human primary monocytes. This study also demonstrated that human oral epithelial cell line OKF6 neither secreted nor expressed resistin. Also, stimulation of human monocytic/macrophagic cell line THP-1 with LPS did not give rise to resistin release.

It is now well acknowledged that many of non-immune cells in the periodontium such as keratinocytes induce the production of cytokines in response to bacteria and other cytokines (Liu *et al.*, 2010). OKF6 cell lines were originally derived from normal gingival mucosal cells taken from the floor of the mouth, which were engineered to evade senescence and achieve immortality. Two cellular alterations were carried out to immortalize these cells, the over-expression of telomerase and the deletion of $P16^{INK4a}$ regulatory protein (Dickson *et al.*, 2000). The human telomerase reverse transcriptase (hTERT) symbolizes the catalytic protein subunit of the telomerase which is expressed in both germ cells and cancer cells (Meyerson *et al.*, 1997; Nakamura *et al.*, 1997). The introduction of the active component of telomerase (hTERT) into many cell types allows infinite number of cell division and thereby unlimited production of these cells (Bodnar *et al.*, 1998). On the other hand, $P16^{INK4a}$

protein which is important tumour suppressor has been found to be deficient or non-functional in a several human cancers including oral carcinoma (Dickson *et al.*, 2000).

The present study was designed to determine if human oral epithelial cell line OKF6 can produce resistin and be considered one of the cellular sources of this protein, and also to study the effect of IL-1β on resistin expression and secretion by these cells. In reviewing the literature, no data was found on epithelial cells being a source of resistin or on the effect of IL-1β on resistin production by these cells. The current study found that human oral epithelial cell line OKF6 do not appear to express resistin mRNA, or secrete resistin into the culture medium at any of the time points used. Also, IL-1β had no effect on resistin expression and secretion in human oral epithelial cell line OKF6. Prior studies have noted the importance of gingival epithelial cells in cytokines production. Using RT-PCR, Lundqvist and co-workers (1994) reported that gingival epithelial cells freshly isolated from normal and inflamed gingiva expressed IL-1β, IL-6, IL-8 and TNF-α (Lundqvist *et al.*, 1994). In addition, human oral keratinocytes produce IL-6, IL-8 and TNF-α at the protein and mRNA levels (Formanek *et al.*, 1998). It was also reported that IL-1β significantly enhanced the release of pro-inflammatory cytokines such as IL-8, IL-6 and TNF-α (Eskan *et al.*, 2008). Intriguingly, resistin mRNA was significantly up-regulated in response to IL-1β stimulation in PBMC (Kaser *et al.*, 2003). It is difficult to explain the inability of OKF6 to express resistin, but it might be related to the fact that OKF6 are engineered cells (not normal) and the cellular alterations happened in these cells to immortalize them might have changed their natural attributes. Together, these findings suggest that human oral epithelial cell line OKF6 is incapable of specifically inducing resistin expression and secretion.

There is a growing interest in monocytes as circulating cells functional in bacterial recognition as these are the precursors of tissue macrophages that harbour the necessary array of receptors (PRRs) to recognise bacteria (Auffray *et al.*, 2009). Macrophages have been identified as a primary source of human resistin in tissues (Patel *et al.*, 2003; Yang *et al.*, 2003). We have therefore carried out experiments to test whether resistin was produced by human monocytic/macrophagic cell line THP-1, and to study the effect of LPS stimulation on resistin secretion by these cells. The results from this study showed that both human monocytic and macrophagic cell line THP-1 do not appear to secrete resistin protein into the culture medium under any of the conditions and time points used in these experiments. Moreover, neither the stimulation with *P.gingivalis* LPS nor with *E.coli* LPS gave rise to the release of resistin protein. These results are consistent with those of other studies which suggest that human monocytic/macrophagic cell line THP-1 do not seem to express resistin gene or release resistin protein. Previous research exploring the characteristics of human resistin gene and the various cells expressing it have reported that resistin gene was not expressed in THP-1 monocytes/macrophages (Nagaev *et al.*, 2006; Xu *et al.*, 2006; Kunnari *et al.*, 2009). In addition, it was shown that neither stimulation with LPS nor with TNF-α drive the expression of resistin mRNA in THP-1 monocytes (Bokarewa *et al.*, 2005). In parallel, treatment of THP-1 macrophages with recombinant resistin or with glucose containing medium did not give rise to the expression of resistin gene (Rae and Graham, 2006). In order to assess the response of both human monocytic and macrophagic cell line THP-1 to LPS stimulation, TNF-α was measured in the same tissue culture supernatants in which resistin levels were measured. It was interesting to note that *P.gingivalis* and *E.coli* LPS remarkably enhanced TNF-α secretion in culture medium of both human monocytic and

macrophagic cell line THP-1, and under all of the conditions used in these experiments. This also accord with other research which found that TNF-α was up-regulated markedly by both *P.gingivalis* and *E.coli* LPS at gene and protein levels (Lamont and Jenkinson, 1998; Bokarewa *et al.*, 2005; Barksby *et al.*, 2009). The observed inability of monocytic/macrophagic THP-1 cells to express and secrete resistin could be attributed to the fact that THP-1 monocytes/macrophages are myeloid cell lines of blood leukemic origin (i.e. not primary cells) and the validity of these cells to express the natural biological functions and attributes of primary cells has never been fully verified. Besides, how faithfully do these cell lines resemble primary cells is a question to be challenged with carefulness because behavioural pathways in THP-1 cell lines and primary cells may diverges to some degree considering the malignant origin of THP-1cell. It can therefore be assumed that the up-regulation of TNF-α protein in response to LPS demonstrating that both human monocytic and macrophagic cell line THP-1 were responsive to LPS and were incapable of specifically inducing resistin secretion.

As a consequence of incapability of human monocytic/macrophagic cell line THP-1 to induce resistin production, it was necessary to look for another monocytic cell line to be used in the study. Human monocytic/macrophagic cell line U937 was reported to be one of the most widely used models for investigating monocytic differentiation and ensuing biological functions of differentiated cell (Baek *et al.*, 2009). These cells have shown to express resistin in many previous studies (Yang *et al.*, 2003; Xu *et al.*, 2006; Kunnari *et al.*, 2009; Singh *et al.*, 2010) and therefore it was used in the subsequent stimulation experiments. Contrary to THP-1 cells at less mature stage owing to their blood leukemic origin, the origin of U937 cells are histocytic lymphoma and they are arrested at a more advanced stage of differentiation, this fact

may influence the expression of resistin and the response of these cells to stimuli such as LPS (Baek *et al.*, 2009).

The current study was carried out to test whether LPS functions as an inducer of the up-regulation of gene expression and protein production of resistin in human monocytic cell line U937. Our results have shown that U937 monocytic cells constitutively and highly express resistin (gene and protein), and that LPS stimulation (both *P.gingivalis* and *E.coli*) at a concentration of 100 ng/ml was insufficient to induce any further up-regulation in resistin gene and protein at any of the three time point used in this experiment. Although these results differ from a previous published research (Kunnari *et al.*, 2009), they are in agreement with those of Yang et al (2003), who found that resistin is highly expressed in U937 macrophagic cells, and its expression is not up-regulated by LPS at a concentration of 5 µg/ml (Yang *et al.*, 2003). A possible explanation for this preliminary data might be that a very high level of resistin was produced constitutively by these cells and therefore it cannot produce any further in response to stimulation. The inability of LPS to drive U937 monocytic cells to up-regulate resistin may indicate that resistin behave differently from a typical inflammatory mediator in these cells.

As part of the present study, several pilot experiments were conducted to determine the optimal conditions for differentiation of U937 into macrophages to be stimulated later with LPS, and also to determine the optimal time point for LPS stimulation. It is important to note that there was no remarkable difference in differentiation U937 cells into macrophages using PMA 50 ng/ml for 48 h and using PMA 100 ng/ml for 72 h for the same purpose. In both protocols, PMA treatment resulted in a population of adherent macrophage-like cells as well as some non-adherent cell population that had yet to differentiate. The differentiated cells by each protocol were then used for LPS

stimulation. The up-regulation in resistin levels and TNF-α levels in culture medium of the two experiments were comparable suggesting no difference in cells response by using the two protocols. In the following experiments to explore the optimal time point at which LPS drives U937 macrophages for the highest enhancement of resistin production, we found that the up-regulation in resistin (and even TNF-α) levels were remarkable at 48 and 72 h, which entailed the use of 48 h time point in the subsequent experiments. Of note, resistin levels were increased with time even in the control culture, and this might be attributed to cell proliferation that increases the cell numbers at the higher time points.

Prior research has reported the contribution of LPS in the pathogenesis of periodontal disease (Jain and Darveau, 2010) and the role of macrophages as a main source of resistin in tissues (Patel *et al.*, 2003). We have therefore set out experiments to explore whether LPS at a concentration of 100 ng/ml can induce resistin (gene and protein) up-regulation in human macrophagic cell line U937. In effect, the results of this study did not show any significant increase in resistin gene or resistin protein in response to both *P.gingivalis* and *E.coli* LPS stimulation at a concentration of 100 ng/ml, and although a trend of up-regulation in resistin gene was observed in response to LPS, it was not statistically significant. In agreement with this finding, Yang et al (2003) reported that LPS stimulation did not enhance resistin gene expression in U937 macrophages (Yang *et al.*, 2003). The present finding seems also to be consistent with other research which found that LPS stimulation was unable to up-regulate resistin expression and secretion in human liver slices, and the LPS-induced inflammation does not influence resistin protein synthesis in human liver (Szalowska *et al.*, 2009). In contrast to our results, various investigations consistently revealed a significant up-regulation in resistin expression and secretion in response to LPS in a

wide range of cells including U937 monocytes, neutrophils and eosinophils, primary human macrophages and monocytes, human PBMCs, mouse adipocytes, and human adipocytes (Lu *et al.*, 2002; Kaser *et al.*, 2003; Lehrke *et al.*, 2004; Kusminski *et al.*, 2007; Kunnari *et al.*, 2009; Bala *et al.*, 2011).

Baek et al (2009) have reported that the expression of CD14 in U937 cell line was enhanced only after vitamin D3 inducement, however no CD14 induction was observed after differentiation with PMA (Baek *et al.*, 2009). And, because CD14 play an essential role in LPS recognition (Pugin *et al.*, 1994), its expression is certainly indicative of enhanced sensitivity of the cells to LPS stimulation and vice versa. Therefore; in the current study; the inability of LPS (100 ng/ml) to up-regulate resistin in U937 macrophages could be attributed to the proposed lack of CD14 expression in PMA-differentiated U937 cells which diminished the sensitivity of these cells to LPS induction.

Most studies have tended to focus on the effect of individual molecular component such as LPS on the simulation of inflammatory cascades by host cells. Because, the use of whole cell bacteria (which is recognized by numerous pattern recognition molecules) for stimulation may possibly drive a holistic response to all bacterial components, and in such case the identification of individual pathways is difficult to be measured. A plethora of studies have demonstrated that several cytokines/chemokines were up-regulated by host cells following exposure to both live and non-viable *P. gingivalis* (Milward *et al.*, 2007; Taylor, 2010); and clinically this is possibly significant, as the composition of the plaque biofilm, in terms of presence of both live and dead bacterial species, may be critical to periodontal disease progression.

As part of the current study, three pilot experiments were carried out to test the responsiveness of U937 macrophages to different stimuli; *pseudomonas aeruginosa* whole cell lysate, *E.coli* LPS (100 ng/ml and 1 µg/ml) at two time points (24 h and 48 h). Both the whole cell lysate and *E.coli* LPS (100 ng/ml) were unable to induce an up-regulation in resistin release from U937 macrophages. However, only the high concentration of *E.coli* LPS (1 µg/ml) was able to enhance resistin production from these cells. Considering the data from these experiments, it appears that the high concentration (1 µg/ml) is requisite for the LPS to drive U937 macrophages to enhance resistin production.

In the present study, the effect of high concentration of LPS (1 µg/ml) on resistin production was assessed at both protein and mRNA levels. Our results have shown that both, *P.gingivalis* and *E.coli* LPS at a concentration of 1 µg/ml enhance resistin release and expression in both U937 macrophages and primary monocytes as shown by the results from ELISA and Real-Time-PCR analysis. The data are in good agreement with previous reports (Lu *et al.*, 2002; Kaser *et al.*, 2003; Lehrke *et al.*, 2004; Kusminski *et al.*, 2007; Kunnari *et al.*, 2009; Bala *et al.*, 2011) which clearly demonstrated a significant up-regulation in resistin production in response to LPS stimulation in a wide spectrum of cells encompassing U937 monocytes, human primary macrophages and monocytes, human PBMCs, neutrophils and eosinophils, mouse adipocytes, and human adipocytes. Therefore, this study produced results which corroborate the findings of a great deal of the previous work in this field. The enhancement of resistin expression and secretion by LPS might be attributed to the secondary induction by LPS-induced cytokines such as TNF-α and IL-1β.

Together, these results show that a concentration of 100 ng/ml LPS, a dose that has previously been demonstrated to enhance resistin production in PBMC (Kaser *et al.*,

2003), have no effect on resistin mRNA expression and protein secretion in U937 macrophages. Meanwhile, using a high concentration of LPS (1µg/ml) significantly enhance resistin expression and secretion in U937 macrophages. It seems possible that the high LPS concentration helps the LPS signalling in U937 macrophages to bypass the necessity of CD14 component of receptor complex; which is deficient in these cells according to (Baek *et al.,* 2009); and therefore to drive U937 macrophages to up-regulate resistin in response to LPS.

In addition to the pivotal role of LPS in the pathogenesis of periodontal disease (Jain and Darveau, 2010), it has been shown that LPS induce insulin resistance in human (Agwunobi *et al.,* 2000). Moreover, type 2 diabetes is associated with elevated circulating serum LPS levels (Creely *et al.,* 2007). Here we report that resistin expression and secretion is enhanced in human monocytes and macrophages by LPS treatment, suggesting that insulin resistance provoked by LPS might at least be partially mediated by resistin.

In conclusion, the present study supports that both monocytes and macrophages are important source of resistin in human, and showed a strong increase in resistin expression and secretion by LPS stimulation in these cells, also suggest that the concentrations of LPS that are required to elicit such a response are likely to be considerable. This combination of findings provides some support for the conceptual premise that resistin may paly a role in the link between periodontitis and insulin resistance in diabetes.

Chapter 6 The effect of resistin on cytokine expression in monocytes in Vitro

6.1 Introduction

Resistin is a 12.5 kDa cysteine-rich peptide hormone (Steppan *et al.*, 2001b) which is primarily produced by macrophages, PBMCs and bone marrow cells (Fain *et al.*, 2003; Patel *et al.*, 2003). Besides its actions on the regulation of glucose homeostasis and insulin sensitivity (Steppan *et al.*, 2001a), resistin has also been implicated in the modulation of immune and inflammatory processes (Bokarewa *et al.*, 2005; Fantuzzi, 2005). Numerous studies have shown that resistin is a potential regulator of cytokine production. Resistin regulates the production of several proinflammatory cytokines, chemokines and MMPs (Bokarewa *et al.*, 2005; Silswal *et al.*, 2005; Fu *et al.*, 2006; Nagaev *et al.*, 2006; Kusminski *et al.*, 2007; Lee *et al.*, 2009; Zhang *et al.*, 2010b). Also, proinflammatory cytokines (e.g. IL-1β, TNF-α and IL-6) or LPS can enhance resistin production in human macrophages, PBMCs and neutrophils (Kaser *et al.*, 2003; Lehrke *et al.*, 2004; Bokarewa *et al.*, 2005; Kunnari *et al.*, 2009; Shyu *et al.*, 2009). Resistin was found to upregulate the expression and secretion of IL-1β, TNF-α, IL-6, IL-8 and IL-12 in PBMCs and macrophages (Bokarewa *et al.*, 2005; Silswal *et al.*, 2005). Likewise, in recent research human resistin induced the release of multiple cytokines and chemokines from human primary monocytes, these included IL-1α, IL-1β, IL-1Rα, IL-6, IL-7, CCL3/MIP-1α, CCL4/MIP-1β, CXCL8/IL-8, IL-10, CCL2/MCP-1, G-SCF, and TNF-α (Lee *et al.*, 2009). In mouse macrophage cells, resistin enhances the expression of COX-2 (a key enzyme regulating the production of prostaglandins), and also up-regulated p65 (a subunit of NF-κB) at the mRNA and protein level (Zhang *et al.*, 2010a).

Furthermore, resistin regulates the production of mediators and cytokines in non-immune cells such as adipocytes, chondrocytes, endothelial and smooth muscle cells (Kusminski *et al.*, 2007; Zhang *et al.*, 2010; Ding *et al.*, 2011; Hsu *et al.*, 2011). In a recent study, resistin significantly induced the mRNA expression of some 20 proinflammatory cytokines and chemokines in chondrocytes from the preserved area of osteoarthritic cartilage as well as normal human chondrocytes (Zhang *et al.*, 2010b). Several studies indicate that resistin enhances the expression of various molecules in endothelial cells such as ICAM-1 and VCAM-1 (Verma *et al.*, 2003; Kawanami *et al.*, 2004; Hsu *et al.*, 2011), P-Selectin (Manduteanu *et al.*, 2010), fractalkine (Manduteanu *et al.*, 2009a), VEGFR-1, VEGFR-2, MMP-1, MMP-2 (Mu *et al.*, 2006) and tissue factor (TF) (Calabro *et al.*, 2011), suggesting a direct proinflammatory effect of resistin on vascular endothelial cells. These proinflammatory influences of resistin on various cell types, however, are based on a small number of studies and much more information is required to characterize resistin more comprehensively.

Periodontal disease is initiated by periodontal bacteria that trigger an inflammatory cascade which induces host-mediated tissue destruction. The production of different mediators and cytokines are responsible for the induction of inflammation in periodontal tissues. These mediators act to enhance the adhesion molecule on the leukocytes and endothelial cells, which is a crucial phase for leukocytes to leave the vasculature and penetrate into the adjacent tissues. The instigation of primary mediators such as TNF-α and IL-1 provokes the production of secondary mediators including chemokines and cyclooxygenases. This results in exaggeration of inflammatory response, stimulation of enzymes that breakdown connective tissue (Graves and Cochran, 2003). Diabetes is a possible risk factor for periodontal disease (Bascones-Martinez *et al.*, 2011). Serum resistin levels have been observed to be higher in diabetic individuals than in apparently healthy subjects (Tokuyama *et al.*, 2007; Chen *et al.*,

2009; Lau and Muniandy, 2011). Moreover, having periodontitis was significantly associated with elevated serum levels of resistin (Furugen *et al.*, 2008; Saito *et al.*, 2008). A recent study by Hiroshima and co-workers (2012) reported that the resistin level in gingival crevicular fluid from patients with periodontitis or diabetes mellitus-related periodontitis was significantly higher than that of healthy individuals, and that resistin level correlated with the intensity of periodontal inflammation as defined by gingival index score (Hiroshima *et al.*, 2012). Since resistin has been shown to exhibit potent proinflammatory properties; it is tempting to suggest that the overproduction of resistin during type 2 diabetes could influence periodontitis through activation of various cytokines and mediators that are responsible for induction of inflammation in periodontal tissues. Therefore, the aims of the present study were to investigate the *in vitro* effect of resistin on the regulation of cytokine-related genes in the human monocytic cell line THP-1 and to identify genes that are up-regulated in response to resistin stimulation. In addition, this study investigated the effect of resistin on the expression and/or secretion of multiple cytokines and chemokines including MIP-1α, TNF-α, IL-12, CXCL10, IL-1β, and IL-6. This investigation may be helpful in furthering our knowledge of cytokine expression in monocytes in response to resistin stimulation.

6.2 Results

6.2.1 Characterization of differential cytokine mRNA expression in resistin-stimulated THP-1 monocytes

To study the effect of resistin on the regulation of cytokine-related genes in THP-1 monocytes, a pilot dose response experiment was first performed. THP-1 monocytes (4 x 10^6) were stimulated with resistin at 3 different concentrations (100, 250 and 500 ng/ml) for 48 h. Un-stimulated cells served as a control. Supernatants were collected and assayed for TNF-α concentrations with ELISA as described in section 2.2. Figure 6.1 shows that resistin at doses of 100, 250 and 500 ng/ml induced increased TNF-α release in THP-1 monocytes compared to controls. Statistical analysis was not performed due to the limited number of replicates in this pilot experiment. As the most substantial response was obtained at resistin concentration of 500 ng/ml, this concentration was therefore used in all subsequent experiments.

To study the temporal regulation of TNF-α secretion in THP-1 monocytes following resistin treatment and in order to determine the optimal time point for resistin stimulation, kinetic experiments were performed. THP-1 monocytes (4 x 10^6) were stimulated with resistin (500 ng/ml) for 4 different time points; 1 h, 6 h, 24h and 48 h. Un-stimulated cells served as a control. Supernatants were collected and assayed for TNF-α concentrations with ELISA as described in section 2.2. As shown in Figure 6.2 resistin induced increased TNF-α secretion in THP-1 monocytes after 1, 6, 24 and 48 h incubation compared to controls. Again, Statistical analysis was not performed due to the limited number of replicates in this pilot

experiment. As the most pronounced response was obtained at the early time points (1 and 6 h), 2 h was therefore be chosen to be used in all subsequent experiments.

To evaluate the effect of resistin on the regulation of different cytokine-related genes, THP-1 monocytes (4×10^6) were stimulated with resistin (500 ng/ml) for 2 h. Un-stimulated cells served as a control. Total cellular RNA was extracted and reverse transcribed to produce cDNA and the amount of different cytokines mRNA expression was quantified by Taqman low-density arrays (TLDA) as described in section 2.3.7. As can be seen from Table 6.1 twenty-eight of the 96 different genes (see Table 2.10) represented on the arrays were up-regulated by resistin treatment compared with un-stimulated cells. These genes involved those encoding mediators with well-documented and crucial roles in chronic inflammation and pathogenesis of periodontal disease such as IL-1β, IL-6 and TNF-α and also chemokines such as CCL3 (MIP-1α), CXCL10 (IP-10) and CXCL11.

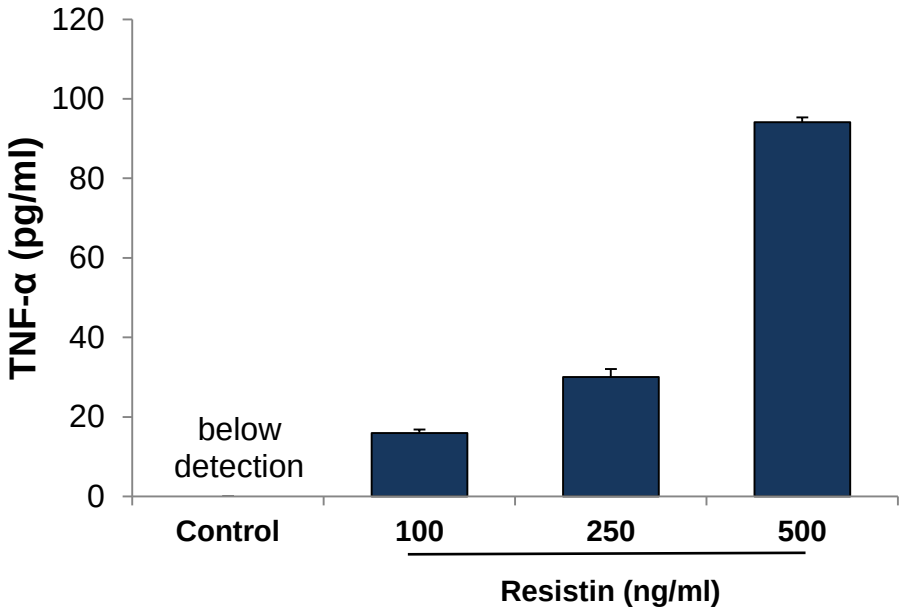


Figure 6.1 Dose response of TNF-α secretion to resistin stimulation by THP-1 monocytes

THP-1 monocytes (4 x 10^6) were stimulated with varying concentrations of resistin. TNF-α levels in supernatants was measured by ELISA and compared with controls. Each value represents the mean and standard deviation of a single experiment (duplicate cultures i.e. n=2).

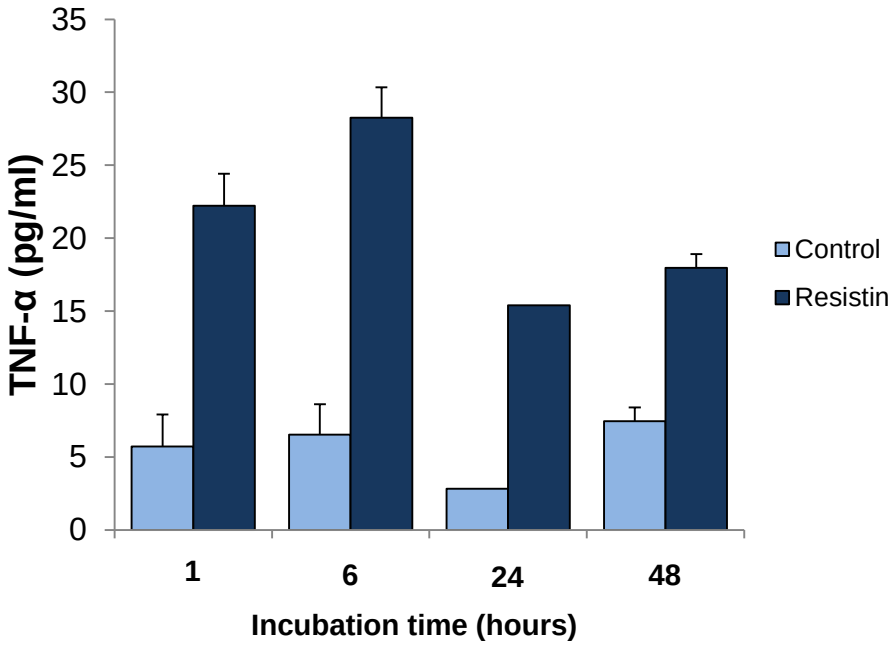


Figure 6.2 Kinetics of TNF-α secretion in response to resistin

THP-1 monocytes (4 x 10^6) were cultured for different periods of time in the presence or absence of resistin (500 ng/ml). TNF-α levels in supernatants was measured by ELISA and compared with controls. Each value represents the mean and standard deviation of a single experiment (duplicate cultures i.e. n=2).

Table 6.1 Immune-regulatory genes those are up-regulated by resistin

Gene	Fold change normalized to 18S gene	Fold change normalized to GAPDH gene
CCL19	14.0	14.9
CCL2	5.6	5.9
CCL3	26.5	28.3
CCR4	1.2	1.3
CD40	2.5	2.7
CD80	5.8	6.2
CSF-1	6.5	6.9
CSF-3	16.6	17.7
CXCL10	161.4	172.3
CXCL11	45.1	48.2
CYP7A1	1.8	1.9
EDN1	28.1	29.9
ICAM-1	19.2	20.5
IL-12β	72.9	77.9
IL-13	2.9	3.0
IL-15	1.7	1.8
IL-1α	39.4	42.1
IL-1β	31.2	33.4
IL-6	21.9	23.5
IL-8	3.7	3.9
LRP-2	1.3	1.4
LTA	2.7	2.9
NFKB-2	6.1	6.5
PTGS-2	5.4	5.8
SMAD-7	1.8	1.9
TBX21	2.4	2.6
TNF-α	5.8	6.2
VEGF	1.4	1.5

THP-1 monocytes were treated with resistin (500 ng/ml) for 2 h. Gene expression was quantified using Taqman low-density arrays by real-time polymerase chain reaction and normalised to either 18S or GAPDH genes. The values shown are the mean fold change compared with untreated cells. These data were obtained from single experiment in duplicate cultures (n=2).

6.2.2 Investigation of the effect resistin on MIP-1α expression and secretion in THP-1 monocytes

MIP-1α (CCL3) was one of the novel mediators which was most strongly upregulated in THP-1 monocytes by resistin and therefore this cytokine was selected for further investigation. To study the effect of resistin on MIP-1α production at mRNA and protein levels, THP-1 monocytes (4×10^6) were stimulated with resistin (500 ng/ml) for 2 h. Un-stimulated cells served as a control. Supernatants were collected and assayed for MIP-1α concentrations with ELISA as described in section 2.2. Total cellular RNA was extracted and reverse transcribed to produce cDNA and the amount of MIP-1α mRNA expression was quantified by Real-Time PCR as described in section 2.3.6.

Figure 6.3 shows the results from Real-Time PCR. Resistin significantly up-regulated MIP-1α mRNA expression (38 fold) in THP-1 monocytes at 2 h ($p < 0.01$) compared to control.

As shown in Figure 6.4, resistin significantly enhanced MIP-1α secretion in THP-1 monocytes after 2 h incubation ($p < 0.001$) compared to control. In control cultures, the levels of MIP-1α (mean ± SD) were 1.6 ± 2.03 pg/ml and increased up to 24.6 ± 7.61 pg/ml in resistin stimulated cultures ($p < 0.001$ compared to control).

Taken all together, the present study demonstrated that resistin not only up-regulates MIP-1α release but also enhances MIP-1α mRNA expression in THP-1 monocytes.

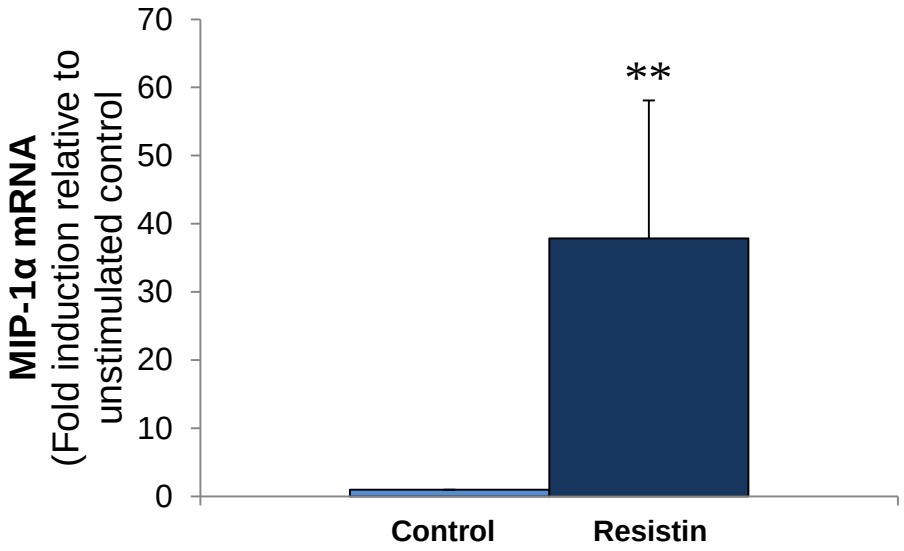


Figure 6.3 Resistin up-regulates MIP-1α mRNA expression in THP-1 monocytes

THP-1 monocytes (4 x 10^6) were stimulated with resistin (500 ng/ml) for 2 h. The MIP-1α mRNA expression was quantified by Real-Time PCR. The data are expressed as mean fold-induction of three independent cell culture experiments (each experiment comprising duplicate cultures i.e. n=6 in total). The mRNA expression was normalized to polymerase II RNA. Statistical analysis was performed on δCt values using Mann-Whitney test. ** $p<0.01$ compared with controls.

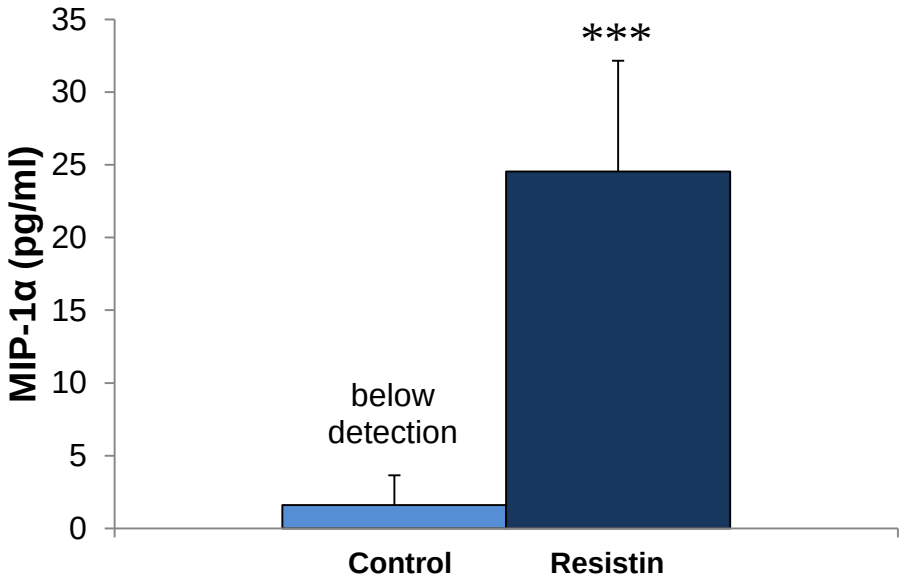


Figure 6.4 The effect of resistin on MIP-1α secretion in THP-1 monocytes

THP-1 monocytes (4 x 10^6) were stimulated with resistin (500 ng/ml) for 2 h. MIP-1α levels in supernatants were measured by ELISA and compared with un-stimulated controls. Each value represent a mean and standard deviation of 3 experiments (each comprising duplicate cultures i.e. n=6 in total). These experiments were carried out on 3 different occasions. Statistics: Student's *t*-test ***p <0.001 compared to control.

6.2.3 Investigation of the effect of resistin on TNF-α expression and secretion in THP-1 monocytes

As expected, the data from the TLDA experiment showed upregulation of TNF-α mRNA by resistin. Therefore, to investigate the role of resistin on TNF-α production at both the mRNA and protein levels, THP-1 monocytes (4×10^6) were stimulated with resistin (500 ng/ml) for 2 h. Un-stimulated cells served as a control. Supernatants were collected and assayed for TNF-α concentrations with ELISA as described in section 2.2. Total cellular RNA was extracted and reverse transcribed to produce cDNA and the amount of TNF-α mRNA expression was quantified by Real-Time PCR as described in section 2.3.6.

Figure 6.5 presents the results from Real-Time PCR. Resistin significantly up-regulated TNF-α mRNA expression (8.7 fold) in THP-1 monocytes at 2 h ($p < 0.01$) compared to control.

As shown in Figure 6.6, resistin significantly increased TNF-α release in THP-1 monocytes after 2 h incubation ($p < 0.01$) compared to control. In control cultures, the levels of TNF-α (mean ± SD) were 10.8 ± 10.78 pg/ml and increased up to 46.5 ± 9.96 pg/ml in resistin stimulated cultures ($p < 0.01$ compared to control).

All in all, the present study demonstrated that resistin not only regulates TNF-α secretion but also enhances TNF-α mRNA expression in THP-1 monocytes.

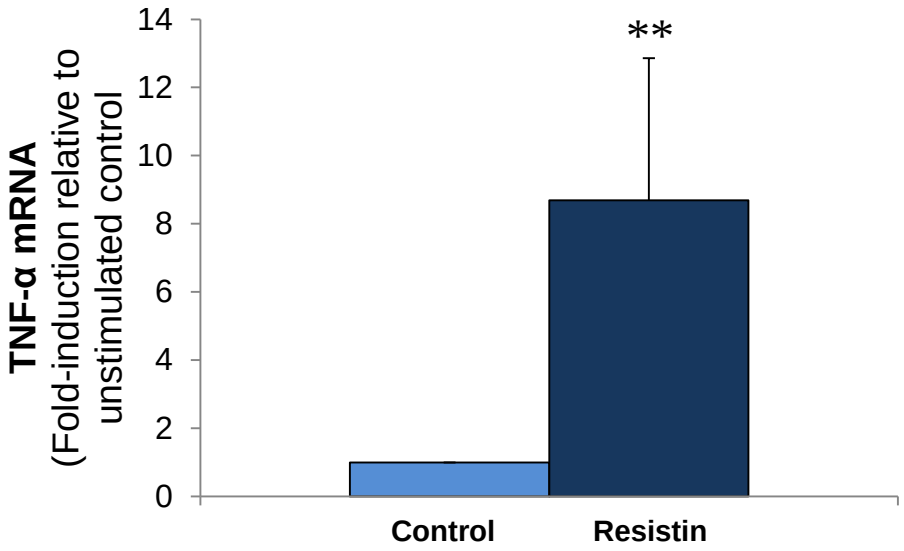


Figure 6.5 Resistin up-regulates TNF-α mRNA expression in THP-1 monocytes

THP-1 monocytes (4 x 10^6) were stimulated with resistin (500 ng/ml) for 2 h. The TNF-α mRNA expression was quantified by Real-Time PCR. The data are expressed as mean fold-induction of 3 independent cell culture experiments (each comprising duplicate cultures i.e. n=6 in total). The mRNA expression was normalized to polymerase II RNA. Statistical analysis was performed on δCt values using Mann-Whitney test. ** P<0.01 compared with controls.

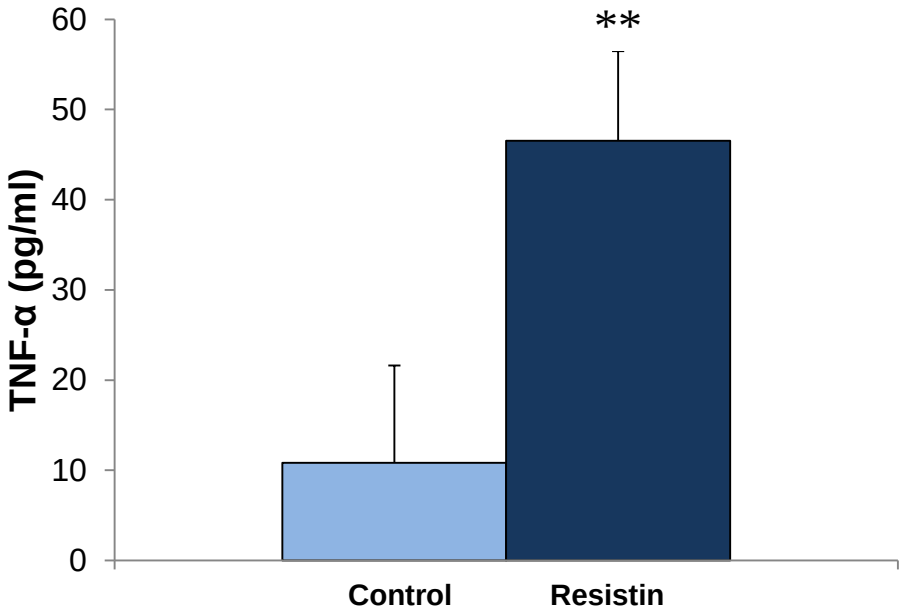


Figure 6.6 The effect of resistin on TNF-α secretion in THP-1 monocytes

THP-1 monocytes (4 x 10^6) were stimulated with resistin (500 ng/ml) for 2 h. TNF-α levels in supernatants were measured by ELISA and compared with un-stimulated controls. Each value represents a mean and standard deviation of 3 experiments (each comprising duplicate cultures i.e. n=6 in total). These experiments were carried out on 3 different occasions. Statistics: Mann-Whitney test. ***p* <0.01 compared to control.

6.2.4 Investigation of the effect of resistin on IL-12 expression and secretion in THP-1 monocytes

IL-12 was another mediator identified as being strongly regulated by resistin in the TLDA experiment. To assess the effect of resistin on the production of IL-12 at mRNA and protein levels, THP-1 monocytes (4×10^6) were stimulated with resistin (500 ng/ml) for 2 h. Un-stimulated cells served as a control. Supernatants were collected and assayed for IL-12 concentrations with ELISA as described in section 2.2. Total cellular RNA was extracted and reverse transcribed to produce cDNA and the amount of IL-12 mRNA expression was quantified by Real-Time PCR as described in section 2.3.6.

Figure 6.7 presents the results from Real-Time PCR. Compared to control, resistin had no significant effect on IL-12 mRNA expression. Although a trend of up-regulation (12.9 fold) can be observed after resistin stimulation, these changes were not statistically significant (p=0.055).

Figure 6.8 shows that the levels of IL-12 in both control and resistin-stimulated cultures were below detection and that resistin had no effect on IL-12 secretion in THP-1 monocytes after 2 h.

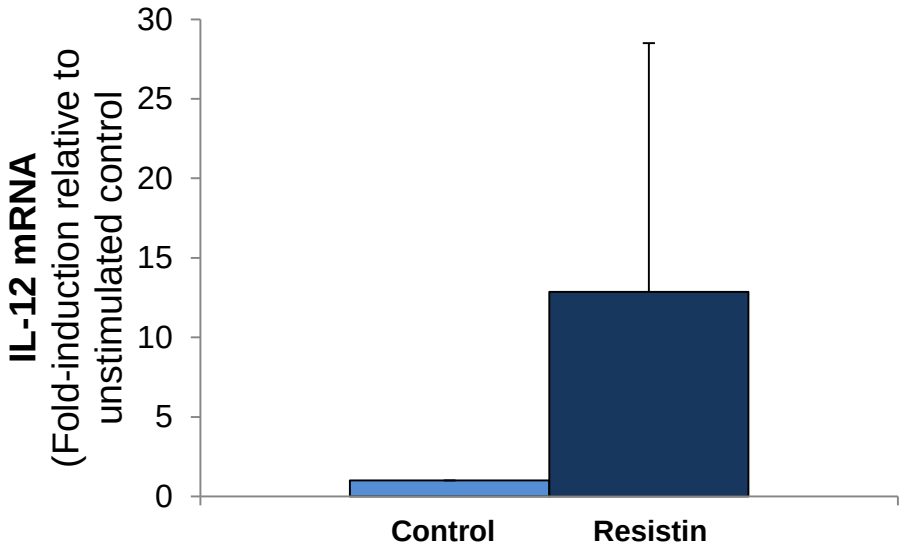


Figure 6.7 The effect of resistin on IL-12 mRNA expression in THP-1 monocytes

THP-1 monocytes (4 x 10^6) were stimulated with resistin (500 ng/ml) for 2 h. The amount of IL-12 mRNA expression was quantified by Real-Time PCR. The data are expressed as mean fold-induction of 3 independent cell culture experiments (each comprising duplicate cultures i.e. n=6 in total). The mRNA expression was normalized to polymerase II RNA.

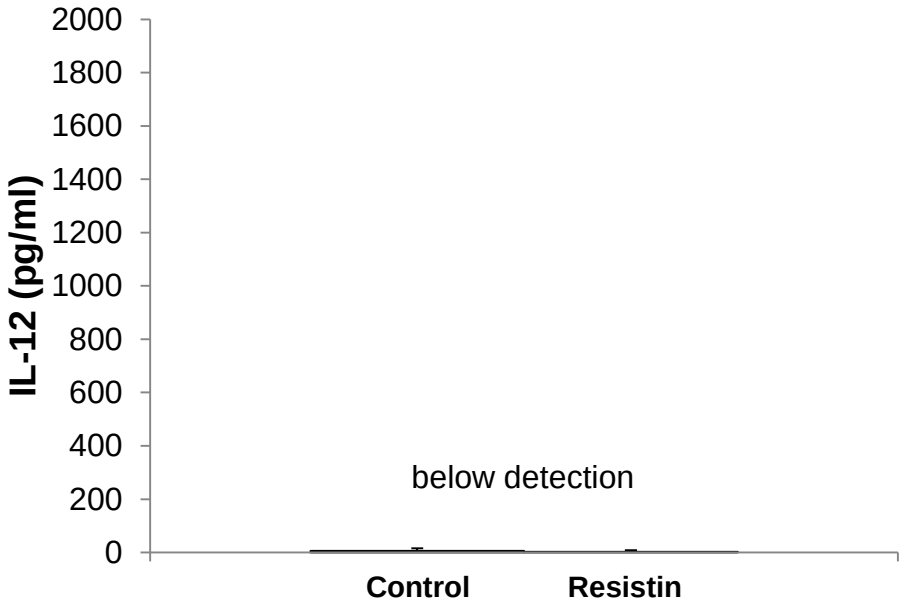


Figure 6.8 The effect of resistin on IL-12 secretion in THP-1 monocytes

THP-1 monocytes (4 x 10^6) were stimulated with resistin (500 ng/ml) for 2 h. IL-12 levels in supernatants were measured by ELISA and compared with un-stimulated controls. Each value represents a mean and standard deviation of 3 experiments (each comprising duplicate cultures i.e. n=6 in total). These experiments were carried out on 3 different occasions.

6.2.5 Investigation of the effect of resistin on CXCL10 expression in THP-1 monocytes

TLDA experiments revealed that CXCL10 mRNA was strongly upregulated in THP-1 monocytes by resistin. To confirm the effect of resistin on the mRNA expression of CXCL10 (IP-10), THP-1 monocytes (4×10^6) were stimulated with resistin (500 ng/ml) for 2 h. Un-stimulated cells served as a control. Total cellular RNA was extracted and reverse transcribed to produce cDNA and the amount of CXCL10 (IP-10) mRNA expression was quantified by Real-Time PCR as described in section 2.3.6.

Figure 6.9 presents the results from Real-Time PCR. Compared to control, resistin significantly up-regulated CXCL10 (IP-10) mRNA expression (77.5 fold) at 2 h ($p < 0.01$).

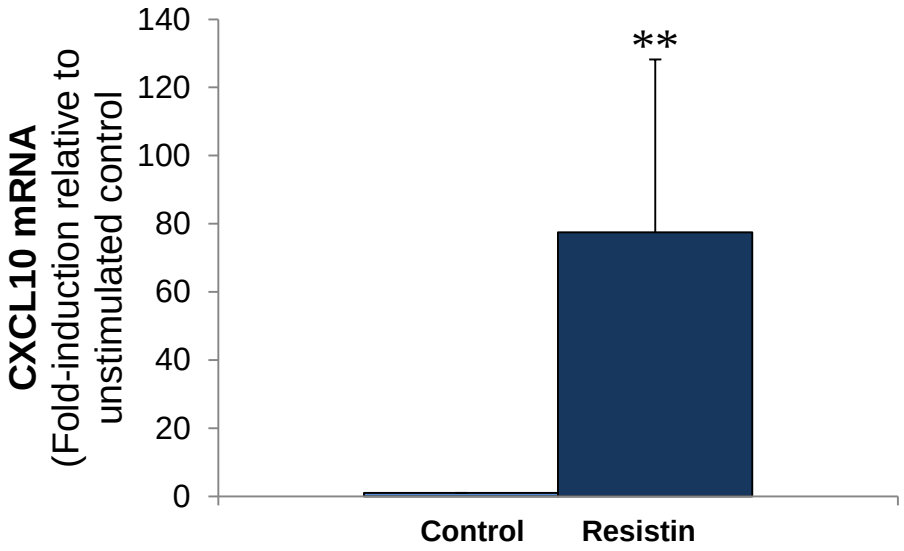


Figure 6.9 The effect of resistin on CXCL10 mRNA expression in THP-1 monocytes

THP-1 monocytes (4 x 10^6) were stimulated with resistin (500 ng/ml) for 2 h. The CXCL10 mRNA expression was quantified by Real-Time PCR. The data are expressed as mean fold-induction of 3 independent cell culture experiments (each comprising duplicate cultures i.e. n=6 in total). The mRNA expression was normalized to polymerase II RNA. Statistical analysis was performed on δCt values using Mann-Whitney test. ** P<0.01 compared with controls.

6.2.6 Investigation of the effect of resistin on IL-1β expression in THP-1 monocytes

To evaluate the effect of resistin on the mRNA expression of IL-1β, THP-1 monocytes (4 x 10^6) were stimulated with resistin (500 ng/ml) for 2 h. Un-stimulated cells served as a control. Total cellular RNA was extracted and reverse transcribed to produce cDNA and the amount of IL-1β mRNA expression was quantified by Real-Time PCR as described in section 2.3.6.

Figure 6.10 shows the results from Real-Time PCR. Compared to control, resistin significantly up-regulated IL-1β mRNA expression (50.05 fold) at 2 h ($p < 0.05$) in THP-1 monocytes.

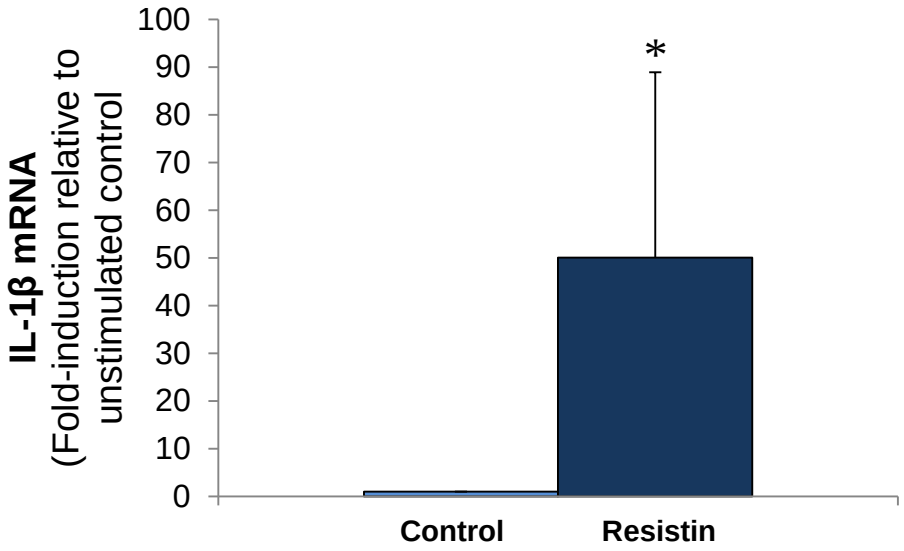


Figure 6.10 The effect of resistin on IL-1β mRNA expression in THP-1 monocytes

THP-1 monocytes (4 x 10^6) were stimulated with resistin (500 ng/ml) for 2 h. The IL-1β mRNA expression was quantified by Real-Time PCR. The data are expressed as mean fold-induction of 3 independent cell culture experiments (each comprising duplicate cultures i.e. n=6 in total). The mRNA expression was normalized to polymerase II RNA. Statistical analysis was performed on δCt values using Mann-Whitney test. * $P<0.05$ compared with controls.

6.2.7 Investigation of the effect of resistin on IL-6 mRNA expression in THP-1 monocytes

To assess the effect of resistin on the mRNA expression of IL-6, THP-1 monocytes (4×10^6) were stimulated with resistin (500 ng/ml) for 2 h. Un-stimulated cells served as a control. Total cellular RNA was extracted and reverse transcribed to produce cDNA and the amount of IL-6 mRNA expression was quantified by Real-Time PCR as described in section 2.3.6.

Figure 6.11 shows the results from Real-Time PCR. Compared to control, resistin significantly up-regulated IL-6 mRNA expression (26.9 fold) at 2 h ($p < 0.01$) in THP-1 monocytes.

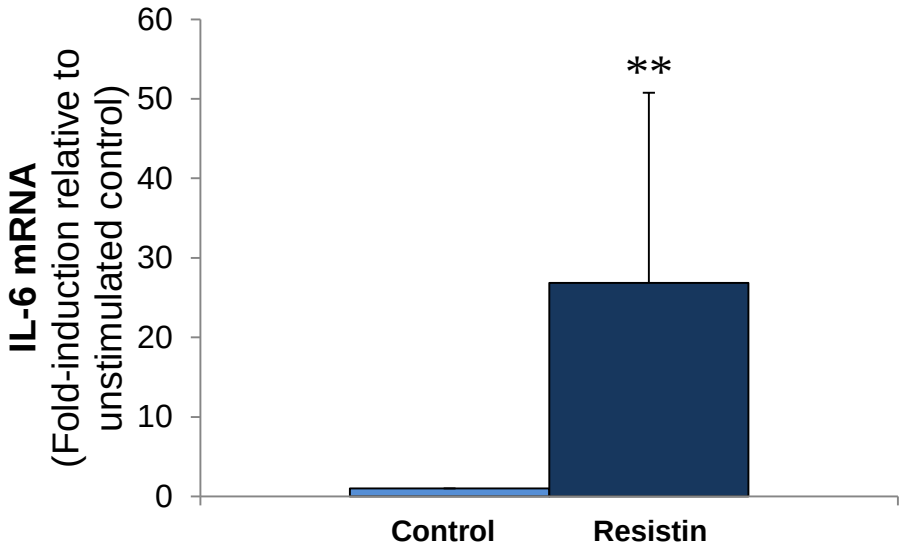


Figure 6.11 The effect of resistin on IL-6 mRNA expression in THP-1 monocytes

THP-1 monocytes (4 x 10^6) were stimulated with resistin (500 ng/ml) for 2 h. The IL-6 mRNA expression was quantified by Real-Time PCR. The data are expressed as mean fold-induction of 3 independent cell culture experiments (each comprising duplicate cultures i.e. n=6 in total). The mRNA expression was normalized to polymerase II RNA. Statistical analysis was performed on δCt values using Mann-Whitney test. ** P<0.01 compared with controls.

6.3 Discussion

A large and growing body of literature designate resistin as a significant local and systemic modulator of inflammation (Lehrke *et al.*, 2004; Bokarewa *et al.*, 2005; Nagaev *et al.*, 2006). Resistin induces the synthesis of the pro-inflammatory cytokines TNF, IL-1, IL-6 and IL-12 by various cell types through an NF-κB dependent pathway (Bokarewa *et al.*, 2005; Silswal *et al.*, 2005; Lee *et al.*, 2009). In particular, monocytes have recently been reported as resistin sensitive cells, responding to resistin stimulation with upregulation of multiple cytokines and chemokines (Lee *et al.*, 2009), indicating that resistin sensitive cells may be broadly distributed throughout the body and that resistin can act to evoke inflammatory responses from primary monocytes. Substantial evidence testifies to the importance of monocytes as circulating cells functional in pathogen recognition, since these cells are precursors of tissue macrophages and dendritic cells which play a pivotal role in the initial sensing of bacteria (Gordon and Taylor, 2005; Randolph *et al.*, 2008). Indeed, monocytes are important component of innate immunity in periodontal disease. Inflammatory signals instigate monocytes to migrate to sites of infection in the tissues and differentiate into macrophages which can successfully combat invading microbes. The phagocytosis of bacteria by macrophages gives rise to cytokine release and antigen presentation which ultimately incite a more efficient adaptive immunity (Teng, 2006; Liu *et al.*, 2010). Generally, individuals with diabetes are more susceptible to periodontal inflammation than non-diabetic (Bascones-Martinez *et al.*, 2011). Interestingly, higher levels of serum resistin are observed in diabetic patients (Tokuyama *et al.*, 2007; Chen *et al.*, 2009; Lau and Muniandy, 2011) as well as in periodontitis patients (Furugen *et al.*, 2008; Saito *et al.*, 2008) when compared with healthy controls. Thus we hypothesized that human resistin, as an inflammatory mediator, might be a causal factor of periodontitis particularly in diabetic

patients, and here we report that human resistin can stimulate the synthesis of various cytokines and chemokines which may potentially aggravate inflammation in periodontium and modulate the clinical course of periodontal disease.

The present study demonstrated for the first time that resistin enhances the expression of 28 cytokine and chemokine genes in human monocytes. In addition, new data are presented which reveal a significant upregulation in the gene expression and protein secretion of MIP-1α in response to resistin. Furthermore, resistin significantly enhanced CXCL10 expression in THP-1 monocytes. Moreover, resistin significantly upregulated TNF-α on both gene and protein levels. Besides, resistin enhanced IL-12 gene expression but not the protein secretion in THP-1 monocytes. Finally, this study also demonstrated that resistin significantly upregulated the gene expression of IL-1β and IL-6 in THP-1 monocytes.

In the current study we first carried out a preliminary experiment to determine the optimal concentration of resistin for inflammatory induction. The results from this study showed that incubation of THP-1 monocytes with increasing concentration of resistin appears to stimulate the production of TNF-α, in particular, at a concentration of 500 ng/ml. However, other studies demonstrated that resistin requires a higher concentration (30 µg/ml) in order to induce the secretion of TNF-α in U937 macrophages (Silswal *et al.*, 2005), and (10 µg/ml) in primary monocytes (Lee *et al.*, 2009). The observed difference in results could be attributed to the differential sensitivity to resistin in different cell types. Furthermore, the upregulation of TNF-α release was observed at all time points (1, 6, 24 and 48 h stimulation) compared with un-stimulated controls.

To the best of our knowledge, this is the first study to investigate the effect of resistin on MIP-1α/CCL3 production in human monocytes. The most interesting and novel finding in the current study was that resistin upregulates both the gene expression and protein secretion of MIP-1α/CCL3 in THP-1 monocytes, as revealed by ELISA data, Real-Time RT-PCR and

TLDA analysis. It is encouraging to compare this figure with that found by Zhang et al (2010) who reported that MIP-1α gene is one of the genes that was upregulated by human chondrocytes in response to resistin treatment (Zhang et al., 2010). This finding also corroborates the findings of Lee et al (2009), who demonstrated that resistin induced the release of MIP-1α from human primary monocytes (Lee et al., 2009). There are several lines of evidence that implicate MIP-1α in the pathogenesis of periodontal disease, and therefore our data provides some support for the conceptual premise that resistin is possibly contribute to the inflammatory reaction in periodontium. Previous research of chemokine expression in gingival biopsies identified that MIP-1α and its receptors were more prevalent in aggressive periodontitis and associated with higher INF-γ and lower IL-10 expression (Garlet et al., 2003). MIP-1α expression is also enhanced in epithelial cells and polymorphonuclear leukocytes by IL-1β, *P.gingivalis* and *A.actinomycetemcomitans* LPS (Ryu et al., 2007). Recent findings indicated that MIP-1α was expressed and produced by both human periodontal ligament and gingival fibroblasts challenged with lipopolysaccharide (LPS) from P.gingivalis (Morandini et al., 2010). Furthermore, *P.gingivalis* enhanced the production of MIP-1α by DCs, monocytes, and THP-1 cells (Cohen et al., 2004). MIP-1α was recognized to be the most abundantly expressed chemokine in periodontitis tissues, with its expression localized in the connective tissue subjacent to the pocket epithelium of inflamed gingival tissues (Gemmell et al., 2001; Kabashima et al., 2002). It has also been demonstrated that MIP-1α positive cells increase in number with increasing severity of periodontal disease (Kabashima et al., 2002). Intriguingly, a multiplex analysis of GCF cytokine levels in patients with periodontitis undergoing initial periodontal therapy revealed that the levels of MIP-α at diseased sites reduced post-treatment (Thunell et al., 2010). MIP-1α has a wide range of proinflammatory activities, for example Fahey et al. (1992) demonstrated that recombinant MIP-1α stimulates the secretion of proinflammatory cytokines TNF-α, IL-1β, and IL-6 by

peritoneal macrophages and act as an autocrine modulator of its cells of origin (Fahey *et al.*, 1992). Additionally, MIP-1α orchestrates acute and chronic inflammatory host responses at the site of injury or infection mainly by recruiting a wide range of proinflammatory cells such as macrophages, neutrophils and lymphocytes (Schall *et al.*, 1993; Maurer and von Stebut, 2004; Silva *et al.*, 2007; Hsieh *et al.*, 2008), and hence may play a role in the recruitment of inflammatory leukocytes into the inflamed periodontal tissues. Interestingly, MIP-1α can induce the chemotaxis and differentiation of osteoclast precursors into osteoclasts in periodontium, which could contribute to bone resorption and periodontal disease severity (Silva *et al.*, 2007). Considering this finding it can be expected that this high level increase in MIP-1α gene expression and protein secretion in response to resistin stimulation will have significant impact on the pathogenesis of periodontal disease.

Another novel finding revealed in the current study, and shown by the results from Real-Time RT-PCR and TLDAs analysis, was the significant upregulation in CXCL10/IP-10 gene expression by THP-1 monocytes in response to resistin treatment. In reviewing the literature, no data was found on the influence of resistin on CXCL10 regulation. However, the importance of CXCL10 stems from the fact that this chemokine is intensely expressed in diseased periodontal tissues (Kabashima et al., 2002; Garlet et al., 2003). Previous studies of chemokines expression in gingival biopsies revealed that IP-10 and its receptors are more prevalent in aggressive periodontitis and correlates with higher levels of IFN-γ in these tissues (Garlet et al., 2003). Indeed, CXCL10 has been associated with the development of Th1 cell responses; as it attracts activated Th1 cell into inflammatory sites through interaction with its receptor (CXCR3) (Kabashima et al., 2002; Silva et al., 2007). Th1 cells synthesize and secrete IFN-γ which promotes the activation of macrophages to enhance their phagocytosis and secretion of cytokines (O'Garra, 1989). Because IFN-γ-producing Th1 lymphocytes are classically implicated in the activation of macrophages (Burger and Dayer,

2002; Ma et al., 2003), their chemotaxis could contribute to disease progression. It can therefore be assumed that the resistin-induced upregulation of CXCL10 gene could contribute to enhanced severity of periodontal disease.

A further intriguing observation in the current study (revealed by ELISA data, Real-Time RT-PCR and TLDAs analysis) is that resistin significantly enhanced TNF-α mRNA expression and protein secretion in THP-1 monocytes. This finding supports previous research which reported a significant upregulation of TNF-α gene expression and/or protein release upon resistin stimulation in human PBMC, human primary monocytes, adipocytes, and in human chondrocytes (Bokarewa et al., 2005; Fu et al., 2006; Kusminski et al., 2007; Lee et al., 2009; Zhang et al., 2010). In addition, it was found that resistin induced the secretion of TNF-α in human and mouse macrophages, and that the combination of resistin with LPS stimulation had an additive influence on TNF-α secretion by these cells (Silswal et al., 2005). In contrast, the production of TNF-α was significantly decreased by resistin treatment of LTA- induced DCs, however, resistin alone had no effect on TNF-α production by mature DCs (Son et al., 2008).

Interestingly, our data from both TLDAs and real Time RT-PCR analysis showed that resistin significantly upregulated IL-1β gene expression in THP-1 monocytes. The data are in agreement and extending previous reports demonstrating that IL-1β mRNA expression and/or protein secretion was significantly enhanced by resistin stimulation in human PBMC (Bokarewa et al., 2005), human primary monocytes (Lee et al., 2009), and human chondrocytes (Zhang et al., 2010). These data clearly support the potent proinflammatory regulatory functions of resistin.

The effect of resistin on IL-6 expression was assessed at mRNA level by both Real-Time PCR and TLDAs analysis. Intriguingly, stimulation of THP-1 monocytes with resistin in vitro led to significant upregulation in IL-6 mRNA levels. This finding is broadly consistent

with earlier studies which demonstrated an enhancement of IL-6 gene expression and/or protein secretion in response to resistin stimulation by PBMCs (Bokarewa et al., 2005), human primary monocytes (Lee et al., 2009), human chondrocytes (Zhang et al., 2010), and in human adipocytes (Fu et al., 2006; Nagaev et al., 2006; Kusminski et al., 2007). In contrast, Son et al (2010) reported that resistin supressed the expression of IL-6 in human monocyte-derived dendritic cells (Son et al., 2010). Also, resistin has been reported to supress IL-6 production in DCs stimulated with LTA which support an immunomodulatory effect of resistin on DCs to suppress cell-mediated immunity (Son et al., 2008).

In the present study, Real-Time RT-PCR and TLDAs analysis showed that IL-12 gene expression was upregulated in THP-1 monocytes upon resistin stimulation, however, this upregulation failed to reach statistical significance. In contrast, Son et al (2010) reported that resistin supressed IL-12 p40 expression in human monocyte-derived dendritic cells (Son et al., 2010). The current study also demonstrated that resistin had no effect on IL-12 release in THP-1 monocytes as shown by the results from ELISA. Although, these results differ from an earlier observation which showed an enhancement in the secretion of IL-12 by macrophages in response to resistin (Silswal et al., 2005), they are consistent with other research which demonstrated that resistin alone had no direct effect on IL-12 p40 production in DCs, however it significantly attenuates the production of this cytokine in LTA- induced DCs (Son et al., 2008).

To provide an overall view of the primary response of THP-1 monocytes to resistin, we carried out a large scale screening procedure where 96 different genes related to inflammatory cascade were analysed at once using Taqman Low Density Arrays (TLDAs). The present study is the first study to show that the gene expression profiling reveals a profound upregulation in a large set of inflammatory genes in THP-1 monocytes by exposure to resistin treatment, these genes including IL-1α, IL-1β, IL-6, TNF-α, IL-12β, IL-13, IL-15,

NFκB-2, ICAM-1, IL-8, CXCL10, CXCL11, CCL2, CCL3, CCL19, CCR4, CD40, CD80, CSF-1, CSF-3, CYP7A1, EDN1, LRP-2, LTA, PTGS-2, SMAD-7, TBX21, and VEGF.

In inflamed periodontal tissues, cytokines and chemokines are produced by various cell types such as monocytes, macrophages, neutrophils, keratinocytes, lymphocytes, epithelial cells and fibroblasts, and they are thought to be key regulators of inflammatory process (Seymour and Gemmell, 2001; Preshaw and Taylor, 2011). Cytokines enhanced the production of inflammatory mediators in the periodontium (e.g. prostaglandins, cytokines, chemokines and MMPs) driving tissue destruction (Preshaw and Taylor, 2011). Manifold feedback loops generate; for instance, cytokines stimulate the release of prostaglandins and elevated prostaglandin concentrations result in enhanced cytokine secretion (Noguchi *et al.*, 2007). Therefore, the production of chemokines and cytokines under the influence of resistin could exacerbate the inflammation in the periodontium. Cytokines and chemokines that are highly upregulated by resistin in inflammation have not previously been shown to be regulated by resistin in human monocytes. However, it is encouraging to compare the preliminary data obtained from TLDAs in the current study with that found by Zhang *et al* (2010), who demonstrated that resistin enhanced the expression of multiple cytokines and chemokines in human articular chondrocytes including TNF-α, IL-6, IL-1β, IL-1α, IL-8, CCL2, CCL3, CCL3L1, CCL4, CCL5, CCL8, CXCL1, CXCL2, CXCL3, CXCL6, MMP1, and MMP13 (Zhang *et al.*, 2010b). Our results also confirm those of previous reports showing the proinflammatory effect of resistin and its ability to activate immune responses and to induce the local recruitment of immune cells in inflammatory lesion (Bokarewa *et al.*, 2005; Silswal *et al.*, 2005; Nagaev *et al.*, 2006).

Chemokines have been shown to play an important role in a variety of chronic inflammatory disease, including periodontitis and rheumatoid arthritis (Silva *et al.*, 2007; Szekanecz *et al.*, 2010). Chemokines are large family of small (7-15 kDa), structurally related proteins that

induce leukocytes chemotaxis *in vitro*. These proteins are classified into four families depending on structural properties and primary amino acid sequence, it includes CXC, CC, C, CX3C (Zlotnik and Yoshie, 2000; Charo and Ransohoff, 2006). In the present study several chemokines/receptors were upregulated in response to resistin stimulation. These include CXCL8, CXCL10, CXCL11, CCL2, CCL3, CCL19, and the chemokine receptor CCR4. Although the junctional epithelium of healthy periodontium shows a slight expression of CXCL8/IL-8, inflamed periodontal tissues reveal an elevated expression of this chemokine (Silva *et al.*, 2007). Moreover, a reduction in the GCF levels of IL-8 has been recorded after periodontal treatment for chronic periodontitis patients (Zhu and Liu, 2010), which suggest a close association between IL-8 and the development and severity of periodontitis. The up-regulation of IL-8 gene in response to resistin stimulation, revealed in the current study, is also accord with other earlier observations which showed that resistin stimulation enhanced the mRNA expression and/or protein secretion of IL-8 in human monocytes (Lee *et al.*, 2009), adipocytes (Nagaev *et al.*, 2006), and in human chondrocytes (Zhang *et al.*, 2010b).

In reviewing the literature, no data was found on the effect of resistin on the expression of CXCL10, CXCL11, CCL19, and the chemokine receptor CCR4. In effect, CXCL11 production was enhanced both in (TNF-α)-treated human gingival fibroblasts (HGFs) stimulated with MDP (the NOD2 agonist) (Hosokawa *et al.*, 2010a) and in (IFN-γ)-treated HGFs stimulated with TNFSF14 (tumor necrosis factor superfamily 14) (Hosokawa *et al.*, 2010b).

The enhanced expression of CCL2 found in the present study also consistent with those of Zhang et al (2010) who reported that CCL2 expression was upregulated in human chondrocytes in response to resistin stimulation (Zhang *et al.*, 2010b). Moreover, recent study has shown that resistin induced the secretion of CCL2 in mouse cartilage (Lee *et al.*, 2009). Interestingly, Scheres et al (2011) demonstrated that stimulating periodontal ligament

fibroblasts and gingival fibroblasts with viable *P.gingivalis* lead to the induction of CCL2 (Scheres *et al.*, 2011) suggesting a potential role in periodontal disease.

As previously mentioned, no data was reported in reviewing the literature about the effect of resistin on CCL19 (also named MIP-3β) regulation. It has been shown that the expression and secretion of CCL19 (a chemotactic factor for macrophages, T cells and dendritic cells) can be enhanced by stimulating human neutrophil with either LPS, TNF-α, Gram positive or Gram negative bacteria (Scapini *et al.*, 2001; Akahoshi *et al.*, 2003). Therefore, the ability of resistin to enhance CCL19 expression shown in the current study, while preliminary, suggests that resistin may contribute indirectly to the orchestrated recruitment of innate and adaptive immune cells to the inflamed periodontal lesion.

In the present study, CCR4 gene expression was enhanced in response to resistin stimulation. Previous studies have reported that CCR4, which binds CCL17 and CCL22, is expressed on dendritic cells, T cells, and monocytes/macrophages, and particularly on the T cells and dendritic cell infiltrating the lung of asthmatics in response to allergen challenge (Panina-Bordignon *et al.*, 2001; Pilette *et al.*, 2004).

In the present study, resistin upregulated the expression of colony stimulating factors; CSF-1 and CSF-3. CSF-1 (macrophage-colony stimulating factor) and CSF-3 (granulocytes-colony stimulating factor) are hematopoietic growth factors that are involved in the proliferation, differentiation, function and survival of mononuclear phagocytes (Stanley *et al.*, 1997; Chitu and Stanley, 2006; He *et al.*, 2008). In effect, CSF-1 plays vital roles in innate immunity, cancer and inflammatory diseases such as rheumatoid arthritis and obesity (Chitu and Stanley, 2006). In mice, CSF-1 production enhanced in response to TNF-α, IFN-γ (Satriano *et al.*, 1993) and LPS stimulation (Roth *et al.*, 1997). Notably, it has been demonstrated that CSF-1 gene was significantly associated with aggressive periodontitis (Rabello *et al.*, 2006).

On the other hand, CSF-3 provokes the production of TNF-α in vivo (Xu *et al.*, 2000) and is thought to play a critical role in driving joint inflammation (Lawlor *et al.*, 2004).

The results from this study showed that resistin enhanced the expression of co-stimulatory molecules CD40 and CD80 in THP-1 monocytes, suggesting a possible "indirect" role of resistin in the regulation of effective adaptive immune response. Our results concur with those of Fang et al (2011) who demonstrated that a dramatic increase in the expression levels of CD40 was found in human umbilical vein endothelial cells (HUVECs) treated with resistin (Fang *et al.*, 2011). In 2003, Verma *et al* demonstrated that incubation of endothelial cells with human recombinant resistin resulted in enhanced CD40 ligand-induced MCP-1 production with a concomitant reductions in TRAF-3 (an inhibitor of CD40 ligand signalling) expression, however, it did not alter CD40 receptor expression in these cells (Verma *et al.*, 2003). Recent evidence revealed that CD80 expression was upregulated by IFN-γ and GM-CSF on both monocytes and T helper cells (Yokozeki *et al.*, 1998; Liu *et al.*, 1999).

In addition, new data are presented in the current study which reveals that resistin is capable of upregulating a variety of genes possessing pleiotropic activities in THP-1 monocytes, these include CYP7A1, EDN1, LRP-2, LTA, PTGS-2, SMAD-7, TBX21 and VEGF. Each of these genes encodes a protein that possess a critical function in regulating diverse cellular processes. For instance, CYP7A1 gene encoding a microsomal enzyme that is tightly regulated to the pathway through which cholesterol is converted into bile acids, and thereby control bile acid synthesis and maintain lipid homeostasis (Song and Chiang, 2006). Therefore, the upregulation of this gene by resistin stimulation may suggest a possible contribution of resistin in lipid haemostasis. Whereas EDN1 Gene encoding a peptide hormone (endothelin-1) with extremely potent biologically discrepant actions, and is apparently implicated in cardiovascular, neural, pulmonary, reproductive, and renal physiology as well as in immune functions and inflammation (Stow *et al.*, 2011). On the

other hand, LRP-2 gene encoding a membrane glycoprotein (also known as Megalin) that belongs to the low-density lipoprotein receptor (LDLR) family and it represents an endocytic receptor expressed on the apical surface of several epithelial cells and internalizes a variety of molecules. Once internalized, these molecules are directed to the lysosomal degradation pathway or transported by transcytosis from one side of the cell to the opposite membrane. LRP-2 implicated in processes that are crucial during development and adult life and are impaired in several pathologic conditions that compromise the kidney and the central nervous system (Cabezas *et al.*, 2011; Marzolo and Farfan, 2011). SMAD7 gene encodes an inhibitory protein that blocks transforming growth factor-beta (TGF-β) signalling via multiple mechanisms in the cytoplasm and in the nucleus and therefore antagonizes various cellular processes regulated by TGF-β such as cell proliferation, differentiation, apoptosis, adhesion and migration. Consequently, an alteration in SMAD7 expression is often associated with several human diseases such as cancer, tissue fibrosis and inflammatory diseases (Yan and Chen, 2011). TBX21 gene encoding a transcription factor responsible for the differentiation of naïve Th cells into a Th1 lineage (Li *et al.*, 2012), which may indicate a potential role of resistin in promoting Th progenitor cells differentiation. VEGF gene encodes a Vascular endothelial growth factor which is a known to increase vascular permeability and vasodilatation (Ferrara, 2004). In addition, there is evidence that VEGF is implicated in the pathogenesis of cancer, arteriosclerosis, obesity, and diabetes mellitus-related complications such as diabetic retinopathy (Bates *et al.*, 2002; Ferrara, 2004; Silha *et al.*, 2005).

Lymphotoxin alpha (LTA) (formerly known as TNF-β) have been identified as a member of the tumor necrosis factor cytokine superfamily, and is implicated as important regulator and developmental factor for the immune system and inflammation (Williams-Abbott *et al.*, 1997; Calmon-Hamaty *et al.*, 2011). The results of this study demonstrated that THP-1

monocytes respond to exogenous resistin by upregulating the expression of LTA gene, which add a further evidence to confirm the proinflammatory features of resistin. In this study, resistin was found to cause a raise in the expression of IL-13 gene, which is well known to posses an anti-inflammatory properties *in vivo* (de Vries, 1998). It is well established that IL-13 is known to promote inflammation associated with allergic disorders and contribute in asthma pathology, and inhibit the production of proinflammatory mediators (Hershey, 2003). Interestingly, IL-13 suppressed CXCL10 production in (IFN-γ or TNF-α)-stimulated human gingival fibroblasts (Hosokawa *et al.*, 2009).

It is interesting to note that the stimulation of THP-1 monocyte with resistin in this study resulted in the upregulation of IL-15 gene expression. A large body of evidence documented the importance of IL-15 in activating the proliferation and cytotoxicity of NK cell, stimulating the production of cytokines and chemokines by these cells and regulating the interaction between NK cell and macrophage. In addition, IL-15 has the capacity to induce the production of proinflammatory cytokine in macrophages and phagocytosis in neutrophils and therefore contributing in innate immune responses and maintaining neutrophil-mediated inflammatory processes. Accordingly, IL-15 is a pivotal cytokine with pleiotropic activity that drive immunoregulatory cross-talk between natural and specific immune cells and bridges the innate and adaptive immune systems (Fehniger and Caligiuri, 2001). It is therefore likely that resistin participate in the orchestration of the innate immune response as well as influences the developing adaptive response.

In this study resistin was also found to enhance the gene expression of PTGS-2 (prostaglandin-endoperoxide synthase 2) that encodes an enzyme known as cyclooxygenase-2 (COX-2), which plays a key role in regulating the production of prostaglandins. The prostaglandins produced by COX-2 are involved in inflammation and pain response in

diverse tissues in the body, and therefore, COX-2 is an inflammation-induced enzyme that remains undetectable in most tissues under normal conditions and is highly expressed at the sites of inflammation (Grosser *et al.*, 2006; Mitchell and Warner, 2006). The findings of the current study are consistent with those of Zhang *et al* (2010a) who found a significant upregulation in COX-2 gene expression in mouse macrophages upon exposure to resistin treatment (Zhang *et al.*, 2010a). It is therefore likely that the upregulation of COX-2 by resistin stimulation might amplify and enhance inflammatory reaction.

Previously published research have shown that the expression of intercellular adhesion molecule-1 (ICAM) in endothelial cells was enhanced in response to resistin stimulation (Kawanami *et al.*, 2004; Hsu *et al.*, 2011). Likewise, the current results indicate that resistin upregulates the gene expression of ICAM in THP-1 monocytes. Interestingly, ICAM-1 plays a role in inflammatory processes and in the T-cell mediated immunity (van de Stolpe and van der Saag, 1996), therefore supporting the notion of resistin implication in inflammation.

Emanating from studies of cytokines in chronic inflammatory diseases is the concept that the IL-1 family cytokines may be of fundamental importance in the pathogenesis of periodontal disease and rheumatoid arthritis (Graves and Cochran, 2003; Barksby *et al.*, 2007; Dinarello, 2007). Interestingly, two principal members of IL-1 cytokine family (IL-1α and IL-1β) were upregulated by resistin stimulation in the current study. Research has found that both IL-1α and IL-1β were upregulated in response to stimulation with whole periodontal bacteria as well as by *P.gingivalis* and *E. coli* LPS (Lindemann *et al.*, 1988; Sandros *et al.*, 2000; Kusumoto *et al.*, 2004; Barksby *et al.*, 2009). In the present study, the upregulated expression of IL-1α gene in response to resistin treatment was consistent with those of Zhang *et al* (2010) who found that resistin enhanced IL-1α gene expression in human chondrocytes (Zhang *et al.*, 2010b). This finding is also accords with a previous observation, which

showed that resistin-treated human primary monocytes enhanced the release of IL-1α protein (Lee *et al.*, 2009).

An abundance of evidence has emerged suggested that the proinflammatory influences of resistin stems from the activation of several intracellular signalling pathways. Interestingly, resistin activates NFκB, resulting in the translocation of both p65 and p50 subunits from the cytoplasm to the nucleus (Bokarewa *et al.*, 2005); this occurs through resistin-mediated phosphorylation of the inhibitory protein IκBα and the p65 subunit of NFκB (Hu *et al.*, 2007b). Also, resistin phosphorylate both MAPKs; such as Erk or p38; and Akt as a downstream substrate of PI3K in several cell lines (Calabro *et al.*, 2004; Kushiyama *et al.*, 2005; Bertolani *et al.*, 2006; Mu *et al.*, 2006). Moreover, resistin enhances the cytosolic calcium concentration through both the influx of calcium from the extracellular environment and the PLC activation resulting in the release of calcium from intracellular pools such as endoplasmic reticulum (Bertolani *et al.*, 2006).

Another interesting observation in the current study is that resistin upregulated the gene expression of NF-κB in THP-1 monocytes. The present findings seem to be consistent with other research which reported a significant upregulation of NF-κB subunits p65 at both the mRNA and the protein levels in mouse macrophages in response to resistin treatment (Zhang et al., 2009). Besides, resistin enhanced the DNA-protein complex of NF-κB subunits p50 and p65 in the nuclear extracts derived from U937 macrophages and PBMCs treated with human resistin (Bokarewa et al., 2005; Silswal et al., 2005). Moreover, the nuclear level of p65 (NF-κB subunit) in human endothelial cells was significantly increased upon exposure to resistin stimulation (Manduteanu et al., 2009). Likewise, NF-κB protein expression in adipocytes was increased in response to resistin treatment (Kusminski et al., 2007). Hence, it could conceivably be hypothesised that exogenous resistin stimulates the expression of a key

component of the innate immune pathway represented by NF-κB and its localization into the nucleus, which strongly affirm the proinflammatory regulatory properties of resistin.

In conclusion, the present study supports that resistin functions as a pivotal inflammatogenic cytokine which exacerbates and triggers inflammatory response through the upregulation of a wide range of cytokines and chemokines. The proinflammatory influence of resistin on the synthesis of MIP-1α, IP-10, TNF-α, IL-1β, and IL-6 may play a role on cross-susceptibility between periodontal disease and diabetes.

Chapter 7 General discussion

The link between diabetes and periodontal disease has been widely discussed in literature. Resistin is one of the important cytokines that may contribute to the inflammatory process (Bokarewa *et al.*, 2005; Nagaev *et al.*, 2006). The serum levels of resistin have been reported to be increased in diabetes and were significantly correlated to increased risk of type 2 diabetes (Chen *et al.*, 2009; Lau and Muniandy, 2011). Emerging research is highlighting the importance of cytokines as diagnostic markers and rational targets for therapy of immune-mediated diseases, including periodontal disease (Preshaw, 2008a; Giannobile *et al.*, 2009).

The present study demonstrated for the first time that salivary resistin is significantly elevated in periodontitis subjects as compared to gingivitis and periodontally healthy subjects in both T2DM and non-diabetic group. However, there were no significant differences in salivary resistin levels between T2DM and non-diabetic groups irrespective of periodontal status. Thence, it is reasonable to hypothesise that elevated salivary resistin is a mirror of the local inflammation in periodontitis, and that this, and not the diabetic status, has a profound impact on the salivary resistin levels. The relationship between increasing levels of resistin in saliva and the presence of periodontal disease is further supported by significant positive correlations that were demonstrated for the first time in the current study between salivary resistin levels and the clinical periodontal parameters (%BOP, mean PD, mean LOA and PISA). This also reflects the potential importance of salivary resistin as a diagnostic marker for periodontal disease. The current study demonstrated for the first time that saliva samples from both T2DM and non-diabetic subjects showed significant reductions in

resistin levels at 3, 6 and 12 months after non-surgical periodontal management. It seems possible that these reductions in salivary resistin levels mirror improvements in periodontal inflammation following non-surgical periodontal management.

The current study has been unable to demonstrate a significant difference in GCF resistin levels between different periodontal categories within T2DM and non-diabetic groups, and even between T2DM and non-diabetic subjects irrespective of periodontal status. However, the relationship between resistin levels in GCF and the presence of periodontal disease is evidenced by significant positive correlations that were demonstrated for the first time in the current study between the clinical periodontal parameters (%BOP, mean PD and PISA) and resistin levels in GCF. This study provided the first direct evidence that GCF resistin levels were significantly and positively correlated with resistin levels in saliva. Taken collectively, it is now possible to state that resistin in saliva and GCF is affiliated with the level of local inflammation in periodontal disease. This permitted a broad and comprehensive portrayal of the utility of salivary resistin as indicator of inflammatory status in the periodontal tissues.

In accord with prior research (Chen *et al.*, 2009; Lau and Muniandy, 2011); the current study reported a significant increase in serum levels of resistin in T2DM subjects compared to non-diabetic controls. The relation between increasing levels of resistin in serum and the presence of T2DM is further supported by significant positive correlations that were demonstrated in the current study between serum resistin levels and HbA1c, BMI, and hsCRP. This combination of findings provides some support for the conceptual premise that resistin may be useful as biomarkers to reflect the increased risk of T2DM.

Although, serum resistin levels showed no significant difference between various periodontal category within T2DM and non-diabetic groups in the current study, significant positive correlations were demonstrated between serum resistin levels and the clinical periodontal parameters (%BOP, mean PD and PISA), which suggest a possible relationship between increasing serum resistin levels and the presence of periodontal disease. It is possible, therefore, to hypothesise that elevated levels of serum resistin related to increased risk of T2DM which was partially attributable to the inflammatory process.

It is becoming apparent that periodontal disease is driven by various oral bacteria which have different forms of LPS, and it is the host inflammatory response to uncontrolled bacterial challenge that primarily mediates tissue damage (Barksby *et al.*, 2009; Hajishengallis, 2009). In the present study, LPS from both *P.gingivalis* and *E.coli* significantly enhanced resistin expression and secretion in human monocytes and macrophages. Along with the principal role of LPS in the pathogenesis of periodontal disease (Jain and Darveau, 2010), LPS was also reported to induce insulin resistance in human (Agwunobi *et al.*, 2000). Furthermore, an association was found between elevated serum LPS levels and T2DM (Creely *et al.*, 2007). Additionally, the role of human resistin in the development of insulin resistance has been well established (Li *et al.*, 2009a; Park *et al.*, 2011). Taking into consideration the upregulation of resistin expression and secretion by human monocytes and macrophages in response to LPS stimulation in the current study, it can be suggested that the insulin resistance provoked by LPS might at least be partially mediated by resistin.

The current study demonstrated a direct role for resistin in upregulating the expression and secretion of a wide range of proinflammatory cytokines, chemokines, and other

inflammatory mediators in THP-1 monocytes. It is possible; therefore, that resistin stimulation results in a hyper-inflammatory state in the local periodontal tissues. Cytokines, chemokines and inflammatory mediators secreted by monocytes in response to resistin induction drive the tissue destruction that results in the clinical manifestation of periodontitis.

Evidence for a key role of pro-inflammatory cytokines such as TNF-α and IL-6 as being involved in the development of insulin resistance have been well established (Tilg and Moschen, 2008a). It can therefore be assumed that the elevated circulating levels of cytokines stemming from continuous and/or excessive production of these mediators in periodontal tissues as a result of resistin stimulation may contribute to the proinflammatory milieu which is proposed to play a role in the development of insulin resistance. This observation not only provides a new link how resistin might affect insulin resistance but also again demonstrates the possible role of the resistin in the relationship between periodontal disease and diabetes.

This study represents a first attempt to study the effect of resistin in the modulation of T2DM and periodontal disease. Our samples comprised mostly Caucasians from the UK population. The covariates in term of ages, ethnicity, gender and smoking status were homogenous and were matched with the case-control groups. Clinical measurements were taken using standardized protocols and resistin was measured using assay with good precision. Nevertheless, this study had limitations. The results of the analysis of resistin in serum were based on single cross-sectional measurements. Further longitudinal analyses are, therefore, required to investigate further potential associations between circulating resistin and periodontal disease, and to provide novel insights into the pattern of response with regard to changes in serum resistin levels after non-surgical periodontal management. The differences in the

recruitment pools used for subjects with T2DM and non-diabetic subjects represent another weakness in the current study, since the subjects with T2DM were recruited from medical databases of T2DM patients held in both primary and secondary care settings whereas the non-diabetic subjects were recruited from patients referred from general dental practice or from patients seen on student treatment clinics within the Newcastle Dental Hospital. Suffice to say, diabetes was studied in diabetes cohort, and periodontal condition was appraised in subjects came to Dental Hospital seeking for dental treatment, therefore, to which extent those two cohorts are valid to be studied as a representative for community is difficult to be determined.

In the current study, subjects with T2DM and non-diabetic subjects were matched based on their periodontal diagnosis; however, the extent and severity of periodontal disease were not considered in this process. Therefore, it would be interesting to stratify periodontal case selection basing on extent and severity of disease in future studies in order to ensure more robust matching of groups with regards to periodontal status. The groups examined in the current study were relatively small; hence it is likely that further investigation with a larger number of subjects would increase the power of analysis and therefore permitted a better portrayal of the resistin levels and its modulation in periodontal disease and T2DM. Although matched for known confounding factors, residual confounders which are unmatched cannot be excluded. Consequently, it is recommended that case-controlled study matching other confounding factors such as BMI would provide more robust results in future research. Another limitation in the current study is the technique used to sample saliva; it involved an oral rinse using 10ml of saline which consequently diluted the saliva sample. This dilution might have resulted in an underestimation of the actual salivary levels of the resistin tested. Particularly because the actual volume of whole

saliva in each sample is not known; therefore, it is not possible to clarify the levels of dilution each sample underwent, and all this limits the diagnostic value of the introduced method. Thus, further research is needed; implementing another technical procedure in saliva sampling, possibly by accumulating saliva in the mouth and expectorating into collection tubes and thereby obtaining autonomous production and secretion of saliva (un-stimulated whole saliva sample). Further, other biomarkers associated with the development of periodontal diseases and diabetes mellitus not examined here might still be present in serum, saliva and GCF at levels that could be used to discriminate between health and disease; and also to correlate with the disease pathogenesis. Therefore, it would be interesting to study the interaction effect of multiple adipokines and cytokines in the modulation of T2DM and periodontal disease. This could be achieved in prospective research by quantifying a number of inflammatory mediators using a multiplex immunoassay. By measuring the levels of different mediators in body fluids, the unidentifiable biomarkers can be explored and a novel index might be established to provide a better indicator of periodontal disease and T2DM.

The current findings add substantially to our understanding of the proinflammatory cytokines and chemokines that are highly upregulated by resistin in inflammation, and which have not previously been shown to be regulated by resistin in human monocytes. The fact that resistin activates monocytes for proinflammatory mediators production points to the importance of the monocytes/macrophage signal cascades in obesity, diabetes and periodontal disease. Additionally, the present study suggests that resistin modulates inflammation in periodontitis and may be a new marker of periodontitis. Further investigation of the actions of resistin on periodontal tissues, including gingival epithelial cells/fibroblasts, periodontal ligament fibroblasts,

osteoblasts/osteoclasts, endothelial cells and immune cells, is necessary to elucidate the role of resistin in periodontal diseases. The data in the current study clearly indicate that resistin also plays a pivotal role in amplifying inflammation–resistin–inflammation cascade thereby adding a new dimension to the physiological function of this important protein. However, this project was limited in several ways. First, the project was unable to analyse the events that follow the interaction of resistin with responsive cells and the intracellular signalling pathways of resistin was not evaluated. Therefore, further experimental investigations are needed to explore the potential intracellular signalling pathways for resistin-induced inflammation. The capability of monocyte in responding to resistin stimulation with cytokine release shown in the present study clearly indicates that monocytes express the, so far, unidentified receptor for resistin. Consequently, further investigation and experimentation into the process of identification of resistin receptor(s) is strongly recommended. In effect, it would be interesting to assess the detailed molecular pathways whereby resistin interacts with cells and specific molecules, such as receptors, proteins, transcription factors and target genes, as well as individual genomic variability within these mediators. Detailed characterization of the signal transduction pathways involved in the proinflammatory cytokine induction by resistin represent an exciting field for future study and the translation of basic science discoveries to clinical application and therefore would be of great interest in the generation of pharmacological modulators in the treatment of inflammatory disorders such as periodontal disease.

Inflammation in periodontitis is induced by a spectrum of cytokines which function in a complex networks; therefore, the identification of principal regulatory mediators in this complex may provide a logical target for therapeutic modalities. Several studies

suggest that IL-1β and TNF-α could be a potential targets for therapeutic intervention in periodontal inflammation (Graves and Cochran, 2003; Liu *et al.*, 2010). Blocking of IL-1β and TNF-α in animal model of periodontitis has shown promising returns (Zhang *et al.*, 2004). For instance, exogenous IL-1β and TNF-α antagonists caused a significant improvement in periodontal healing as shown by a reduction in inflammatory cell infiltration, alveolar bone loss and connective tissue breakdown (Delima *et al.*, 2001; Zhang *et al.*, 2004). Nonetheless, research into the use of anti-cytokines in the therapeutic modalities of periodontitis is still at a very early stage, and its impact on periodontal disease has not yet investigated in humans.

The immunopathological role of resistin has been well documented, but anti-resistin therapies (which may one day prove to be a powerful anti-inflammatory treatment for a spectrum of disorders) have not been studied yet. Resistin represents an amplification signal for components of the innate and adaptive immune responses. Therefore, addressing the clinical implication and pathologic mechanism of resistin in periodontal disease progression are warranted. The current study has found that generally resistin in saliva was linked to the intensity of periodontal inflammation, whereas serum resistin levels were associated with diabetes mellitus. Although further studies may be necessary to confirm the efficacy of measuring salivary resistin levels in the prediction, diagnosis and management of periodontal disease, our study certainly highlights the potential for the salivary resistin to move one step closer to becoming an established biomarker of inflammatory activity in periodontal disease. Hence, periodontal disease progression might play a significant role in predicting high risk individuals for one of the most common diseases of the present era (i.e. diabetes mellitus). Further, periodontal therapy might decrease the risk of severity of this

systemic disease because it reduces the inflammatory burden, not only in systemically compromised but also in otherwise healthy individuals.

Consequently, information about host responses and modulation factors (such as resistin) in diabetes, periodontitis and diabetes-associated periodontitis may be used for therapeutic purposes. As our comprehension of these diseases deepens, the focus is shifting from diagnosis and treatment to prevention and health promotion. Numerous cases of diabetes may stay un-diagnosed, and the profiteer screening for diabetes in the dental clinic (which depends on self-reported data and clinical periodontal parameters) might be efficient in identifying some of these cases. Active and adjunctive therapy to improve insulin sensitivity and glycaemic control, such as preventing the recurrence of periodontal disease and tooth mortality in patients with diabetes, should be considered substantial components of treatment. As proofs of the close liaison between inflammatory periodontal diseases and diabetes mellitus continue to accumulate; physicians, dentists and oral health professionals should interact to a greater extent, to improve general health care and glycaemic control and to prevent complications among subjects with diabetes.

In conclusion, the findings of the present study contribute to the knowledge of the role of resistin as a potential mediator linking T2DM and periodontal disease. The up-regulation of serum resistin levels may be directly relevant to periodontal destruction in diabetic subjects. The oral inflammatory burden, as assessed by elevated levels of inflammatory mediator resistin in saliva, is related to the intensity of local inflammation (periodontal disease), therefore, the current study demonstrate resistin as a novel local biomarker for periodontal disease. Our results indicate that resistin is important member of the cytokine family with potent immune-regulatory functions; which may play a pivotal role in the exacerbation of periodontal inflammatory

response and the development of insulin resistance. Resistin may therefore be crucial in the regulatory control of inflammatory responses in periodontal disease and may also contribute to the cross-susceptibility between type 2 diabetes and periodontal disease.

Chapter 8 References

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