DOCTOR OF PHILOSOPHY

The Production And Distribution Of Factors Relating To The Regulation Of Wound Healing In Oral Health And Disease

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Thesis submitted for the degree of Doctor of Philosophy

in Dentistry.

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Dedication

This thesis is dedicated to my wife, Fleur Davey, and to my two children, Alexander and Laura Davey. Without their love, support and encouragement, I would not have had the strength to complete my thesis. I also dedicate this thesis to my loving parents, Lyndon and Vivienne Davey, who inspired me to further my education and to broaden my view of the world.

Quotation

"I was taught that the way of progress was neither swift nor easy" – Marie Curie.

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Special thanks must also be given to my wife and children for their on-going love and support during my time as a PhD student.

Finally, I would also like to acknowledge the financial support provided by the British Society of Periodontology (BSP) and the Tattersall Trust.

Declaration

I declare that I am the author of this thesis and that I have consulted all the references cited. The work, of which this thesis is a record, has not been previously accepted for a higher degree. This work has been carried out in the Cell and Molecular Biology laboratories in the School of Dentistry (University of Dundee), under the supervision of Prof. Mark Hector and Dr. Sarah J Jones.

Signature

Date 11/05/21

Kevin John Davey

Certificate

I hereby certify that Kevin John Davey has fulfilled the condition of Ordinance 39 of the University of Dundee and is qualified to submit this thesis for the degree of doctor of philosophy.

Prof. Mark Hector

Dean School of Dentistry

Signed Date 11/05/21

Dr. Sarah J. Jones

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Signed Date 11/05/21

All of the School of Dentistry, University of Dundee, UK.

Abbreviations

Aa	*Aggregatibacter actinomycetemcomitans*
ACJ	Amelocemental Junction
ADH	Antidiuretic Hormone (Vasopressin)
ADP	Adenosine-5'-Diphosphate
AF	Access flap (surgery)
AGE	Advanced Glycation End-products
AgP	Aggressive Periodontitis
a-MVD	average-Microvascular Density
ANG	Angiogenin
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
Ang-4	Angiopoietin-4
ANOVA	Analysis Of Variance
ALK	Activin receptor-Like Kinase
APC	Antigen Presenting Cells
ATP	Adenosine-5'-Triphosphate
BMI	Body Mass Index
BOP	Bleeding On Probing
BSA	Bovine Serum Albumin
BSP	British Society of Periodontology
CD105	Endoglin
CL	Chemiluminescence

CRP	C-Reactive Protein
CVD	Cardiovascular Disease
DAB	3,3’ Diaminobenzidine
DKA	Diabetic Ketoacidosis
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELAM-1	Endothelial Adhesion Molecule-1
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron Microscopy
EMBL	European Molecular Biology Laboratory
FAK	Focal Adhesion Kinase
FGF	Fibroblast Growth Factors (FGF)
FGF-2	Fibroblast Growth Factor-2 (basic-FGF)
FGFR-	Fibroblast Growth Factor Receptor (1-4)
Fn	*Fusobacterium nucleatum*
Fn1	Fibronectin encoding gene
GCF	Gingival Crevicular Fluid
GH	Growth Hormone
GMP	General Medical Practitioner
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor

GTR	Guided Tissue Regeneration
HbA1c	Glycated/Glycosylated Haemoglobin
H and E	Haematoxylin and Eosin
HGF	Hepatocyte Growth Factor
HIF	Hypoxia-Inducible Factor
HLA	Human Leukocyte Antigen
h-MVD	highest-Microvascular Density
h-PC	high-Point Counting
HRE	Hypoxia Response Element (VEGF gene)
HRP	Horseradish Peroxidase
IBM	International Business Machines
ICAM-1	Intercellular Adhesion Molecule-1
IFN-γ	Interferon-gamma
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein (1-6)
IgG	Immunoglobulin G
IL-1	Interleukin-1 (α -alpha or β -beta)
IL-2	Interleukin-2
IL-6	Interleukin-6
IQR	Interquartile Range
kb	Kilobase
kDa	Kilodalton
KGF	Keratinocyte Growth Factor

KJD	Kevin John Davey
LED	Light-Emitting Diode
LOA	Loss of Attachment
LOD	Limit of Detection
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
LTB_4	Leukotriene B_4
MAC	Membrane Attack Complex (C5b6789)
MAPK	Mitogen-activated Protein Kinase pathway
MCP-1	Monocyte Chemotactic Protein-1
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase/s
MPO	Myeloperoxidase
mRNA	Messenger Ribonucleic Acid
MRI	Magnetic Resonance Imaging
MSF	Migration Stimulating Factor
MSFI	Migration Stimulating Factor Inhibitor (NGAL)
MVC	Microvessel Count
MVD	Microvascular Density
MVV	Microvascular Volume
NAD	Nicotinamide Adenine Dinucleotide
NGAL	Neutrophil Gelatinase-Associated Lipocalin (Lipocalin-2/MSFI)
NGF	Nerve Growth Factor

NGS	Normal Goat Serum
NO	Nitric Oxide
NOM	Normal Oral Mucosa (Peri-tumour)
NRP-1	Neuropilin-1
NSAID	Non-Steroidal Anti-Inflammatory Drugs
NSB	Non-Specific Binding
OSCC	Oral Squamous Cell Carcinoma/s
OD	Optical Density (ELISA)
PAF	Platelet Activating Factor
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline, Tween 20
PBS-T-PI	Phosphate Buffered Saline, Tween 20, Protease Inhibitor
PCR	Polymerase Chain Reaction
PD	Pocket Depth
PD-ECGF	Platelet-Derived Endothelial Cell Growth Factor
PDGF	Platelet-Derived Growth Factor
PDL	Periodontal Ligament
PET	Positron Emission Tomography
Pg	*Porphyromonas gingivalis*
PG	Periapical Granuloma
PGE_2	Prostaglandin E_2
Pi	*Prevotella intermedia*
PIGF	Placenta Growth Factor

PKC	Protein Kinase C pathway
PMN	Polymorphonuclear Neutrophils
PTN	Pleiotrophin
RAGE	Receptor for Advanced Glycation End-products
RANK	Receptor Activator of Nuclear factor-kappa beta
RANKL	Receptor Activator of Nuclear factor-kappa beta Ligand
rhMSF	recombinant human Migration Stimulating Factor
rRNA	ribosomal Ribonucleic Acid
ROI	Reactive Oxygen Intermediates
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SD	Standard Deviation
SE	Standard Error
SF	Serum-Free (medium)
SIRT1	Silent Information Regulator 2 Homologue 1
α-SMA	α-Smooth Muscle Actin
SPECT	Single-Photon Emission Computed Tomography
SPSS	Statistical Package for the Social Sciences
SS	Stimulated Saliva
TACE	Tumour Necrosis factor Converting Enzyme
TβR1-3	Transforming Growth Factor-Beta Receptors 1 to 3
TCR	T-Cell Receptor
Td	*Treponema denticola*

TβR	TGF-β receptor
Tf	*Tannerella forsythia*
TGF-α	Transforming Growth Factor-alpha
TGF-β	Transforming Growth Factor-beta
TIMP	Tissue Inhibitors of Metalloproteinases
TMB	Tetramethylbenzidine
TNF-α	Tumour Necrosis Factor-alpha
TSP-	Thrombospondin (1-2)
TXA_2	Thromboxane A_2
US	Unstimulated Saliva
USA	United States of America
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor (A, B, C and D)
VEGFR-	Vascular Endothelial Growth Factor Receptor (1 to 3).
VHD	VEGF Homology Domain
VPF	Vascular Permeability Factor (VEGF)
v/v	Volume per volume
vWF	von Willebrand Factor (Factor VIII related antigen)
w/v	Weight by volume

Abstract

Background: Wound healing of the oral soft tissues, compared with skin and other epidermal tissues, is associated with enhanced healing capacity and reduced scarring. Angiogenesis is dependent on many angiogenic factors including angiopoietin-1 (Ang-1), migration stimulating factor (MSF) and vascular endothelial growth factor (VEGF), and the inhibitor endostatin. Angiogenesis is an important feature in both wound healing and periodontitis; both known to be compromised by factors including smoking and poorly controlled diabetes.

Aims: To investigate whether:

i) serum and salivary concentrations of Ang-1, MSF, VEGF and endostatin differ significantly between periodontal health and severe periodontitis, smoking and diabetes.

ii) the quantification of vascularity in periapical granulomas (PG) and healthy periodontal ligament (PDL), using different endothelial markers, has value as an index of angiogenesis.

Methods: Following research ethics approval, 102 adult subjects were recruited and divided into three study groups: systemically healthy (n=53), smokers (n=20) and subjects with diabetes (n=29). Each group was sub-divided into periodontally healthy or those with severe periodontitis. Serum, saliva (whole mouth, unstimulated and stimulated) and gingival crevicular fluid samples were collected. Ang-1, MSF, VEGF and endostatin concentrations were determined using enzyme-linked immunosorbent assays (ELISA). Paraffin-embedded sections of PG and PDL were stained with vWF and CD105, and standard microscopic methods used to quantify vascularity.

Results: Salivary concentrations of Ang-1 and endostatin are reported for the first time. Salivary concentrations of VEGF were significantly raised in diabetes ($p<0.05$) and serum endostatin concentrations were significantly reduced in smokers ($p<0.001$). No significant differences were found in Ang-1 or MSF concentrations between the study groups, in either serum or saliva. No significant differences were found in staining or measures of vascularity between PG-PDL, whilst clear evidence of angiogenesis was found in oral squamous cell carcinoma (OSCC) control samples.

Conclusions: Smoking and diabetes were found to significantly alter levels of endostatin and VEGF respectively. No evidence in angiogenic activity was found in the PGs and CD105 was not found to be a specific angiogenic marker.

Chapter 1

Introduction and Literature Review

1.1 Introduction

In this chapter, the key background concepts related to this thesis have been discussed in order to set the scene and to justify the research undertaken. Firstly, an outline of wound healing, in general, has been provided and highlights the important role angiogenesis has in this process. This is followed by a discussion of the processes involved in angiogenesis and a review of the literature of the angiogenic factors and inhibitors related to this thesis. Angiogenesis is then discussed in the context of the oral soft tissues, which are associated with enhanced wound healing and reduced scarring, in comparison to skin. The various theories, which have been proposed to account for the enhanced oral soft tissue healing, including the angiogenic factor content in saliva, is then explored. Finally, the potential impact of periodontal disease on angiogenic factors levels is discussed, including the potential negative effect of smoking and diabetes, which are both significant risk factors for poor wound healing and increased risk of periodontal disease.

1.2 Wound healing – A general overview

1.2.1 Introduction

The key biological process studied in this thesis is angiogenesis and its vital role in oral wound healing, namely the re-establishment of a functional vascular network. Angiogenesis was defined by Folkman and Shing (1992) as "the formation of new blood vessels from the pre-existing vascular network". An overview of the important stages of cutaneous wound healing is described below, as evidence suggests oral wound healing follows a similar pattern (DiPietro and Schrementi, 2018). The following section is designed to place angiogenesis into the wider context of wound healing.

Wound healing is a fundamental process of life and involves a complex array of dynamic spatial events necessary to maintain the integrity of the organism (Gurtner et al., 2008). This process is known to involve specific time critical interactions between various soluble mediators, blood cells, parenchymal cells and the extracellular matrix (ECM). Wound healing is categorised in terms of healing time, either acute or chronic (Dreifke et al., 2015). Acute wounds, for example non-infected surgical wounds, tend to heal quickly within 3 to 6 weeks, while chronic wounds take at least 3 months. Unfortunately, due to the inherent

complexity there are several points where this process can be disturbed leading to abnormal chronic healing. Risk factors for chronic wound healing include: advanced age, poor nutrition (vitamin A and C deficiency), smoking, systemic disease (e.g. poorly controlled diabetes), side effects of various drugs (steroids, chemotherapy and immunosuppressants) and wound infection (Guo and Dipietro, 2010, Sorensen, 2012a, Levine, 2017).

Although there are a large variety of wounds from simple scratches, to surgical incisions, burns and gross trauma, the fundamental processes of healing are common to all (Bielefeld et al., 2013, DiPietro and Schrementi, 2018). Classically, clinicians categorise wounds, depending on whether the wound edges are closely apposed, as being primary intention, or apart, as being secondary intention (Johnstone and Farley, 2005). Biologically wounds heal by undergoing four major overlapping phases: haemostasis phase, an inflammatory phase, a proliferation/granulation phase and a remodelling/maturation phase (Singer and Clark, 1999, Gosain and DiPietro, 2004), though there are some variations in the terminology used in the literature. The length of time particular wounds take to transit through each phase varies depending upon the location, the nature of the wound (such as the extent of tissue loss or infection), and the natural variation in healing potential between individuals (Sciubba et al., 1978). Infected chronic wounds may move backwards and forwards through these phases, associated with prolonged inflammatory and proliferative phases, with little functional tissue repair (Guerra et al., 2018).

Ideally, following injury the damaged tissue would be restored to its original structure (regeneration), but this is often not achieved leading to scar tissue formation (repair). In contrast, wound healing in foetal tissue is rapid with minimal scarring (Whitby and Ferguson, 1991, Larson et al., 2010, Branford and Rolfe, 2018). Furthermore, oral soft tissue wound healing shares many of the characteristics of foetal healing, with more rapid healing and minimal scarring compared with other adult tissues (DiPietro and Schrementi, 2018). Understanding the processes involved in foetal and oral soft tissue wound healing is seen as key to enhancing wound care and reducing debilitating scars. Proposed mechanisms for enhanced foetal wound healing include: significant differences in the ECM such as higher levels of hyaluronic acid, splice variants of fibronectin (including Migration Stimulating

Factor (MSF)) and the presence of the more reticular pattern of the type III collagen; different concentrations and temporal release of various cytokines and growth/angiogenic factors; reduced inflammation (duration and extent) and different characteristics of foetal inflammatory cells compared to adults (Schor et al., 1988a, Branford and Rolfe, 2018, Pratsinis et al., 2019). However, the exact nature of the processes underlying the enhanced wound healing in foetal tissue is extremely complex and is poorly understood, with many contradictory findings in the literature. Studies suggest, however, that foetal scar-free healing is in some way intrinsic to the tissue as human foetal skin heals without scaring when transplanted subcutaneously into an adult athymic mouse (Lorenz et al., 1992). Conversely, when adult sheep skin was transplanted onto a lamb foetus and subsequently incised, the wound healed with scar formation (Longaker et al., 1994). Currently, many aspects of adult wound healing are also poorly understood and it is likely to be many years before controlled tissue regeneration is possible.

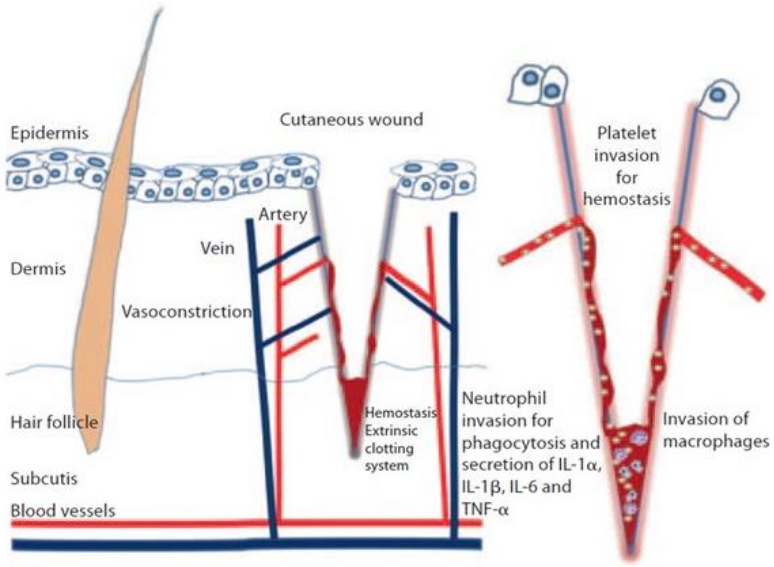
The overview of the four phases of wound healing below describes the general events following an incisive injury of an epithelial-lined tissue such as the skin or the oral mucosa, but also directly relates to periodontal wound healing. Some of the processes described below are highly complex and are not fully understood, so the general processes have been summarised for clarity (Singer and Clark, 1999, Hakkinen et al., 2000, Diegelmann and Evans, 2004, Velnar et al., 2009, Schultz et al., 2011, Gonzalez et al., 2016, Morand et al., 2017, DiPietro and Schrementi, 2018, Guerra et al., 2018, Uluer et al., 2018, desJardins-Park et al., 2019).

1.2.2 Haemostasis phase

Damage to the microvasculature leads to haemorrhage which is initially controlled by platelet aggregation and the formation of the primary platelet plug. This process is aided by rapid vasoconstriction, within five to ten minutes of the injury, mediated by the release of epinephrine (adrenaline), norepinephrine (noradrenaline), serotonin (5HT) and prostaglandins. Platelets initially adhere to the exposed sub-endothelial collagen by the interaction of the platelet membrane glycoprotein Ib/IX and the endothelial cell derived protein von Willebrand factor (vWF). Platelet adhesion causes platelet activation and

degranulation with the release of various growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), and vasoactive molecules such as serotonin and histamine. Other factors released include proteases and two major platelet activation factors, adenosine-5'-phosphate (ADP) and the prostaglandin, thromboxane A_2 (TXA_2). Platelet activation causes conformational changes leading to the activation of the platelet surface glycoprotein IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) receptor, which has affinity for adhesive molecules with the RGD peptide sequence (arginine (R) – glycine (G) – aspartate (D)) such as fibrinogen, vWF, fibronectin and vitronectin. This receptor has a high affinity for fibrinogen and so promotes platelet aggregation creating a primary platelet plug. The key processes in the haemostasis phase are summarised in Figure 1.1.

Figure 1.1 A summary of the key processes in the haemostasis and early inflammatory phases of cutaneous wound healing (diagram from Reinke and Sorg (2012)).



The primary platelet plug requires to be further stabilised by fibrin produced as the result of activation of the intrinsic and extrinsic coagulation pathways. The intrinsic pathway is activated when Hageman factor (factor XII) is exposed to extravascular surfaces, while the extrinsic pathway is initiated by the activation of tissue factor, which is found in extravascular cells, in the presence of factor VII and VIIa. Both pathways merge at factor X, which when activated is bound to the surface of platelets, which enables the clotting process to remain

localised to the site of injury. Factor X then converts prothrombin into thrombin, which in turn converts soluble fibrinogen into insoluble fibrin. Factor XIII is required to promote the cross linking and stabilisation of the fibrin, which in conjunction with the platelet plug forms the final blood clot. The resultant fibrin scaffold has been shown to be important in the later migration of inflammatory cells and without it wound healing is impeded (Greiling and Clark, 1997). Like many processes in the body there are coagulation inhibitors, such as antithrombin III, protein C and protein S, which prevent excessive clot formation.

1.2.3 Inflammatory phase

Vasoconstriction associated with the haemostasis phase is followed by a prolonged period of vasodilation, increased blood flow and vascular permeability mediated by the release of various factors such as bradykinin and histamine. Bradykinin is formed by the activation of the kinin pathway by Hageman factor. Histamine is released by the degranulation of the platelets and mast cells, leading to the production of the vasoactive prostaglandins E_1 and E_2. Complement fragments C3a, C4a and C5a are important factors in mast cell degranulation. Epidermal damage results in keratinocyte activation leading to enhanced expression of keratins K6, K16 and 17, which are important in re-epithelialisation (Patel et al., 2006).

Hageman factor from the blood coagulation cascade also activates the classical complement cascade, which initiates the host inflammatory reaction. The production of complement C5a and C567, as well as the large quantities of TGF-β released during platelet degranulation, mediates the chemotaxis of polymorphonuclear neutrophils (PMN), which are the predominant inflammatory cell in the first 24 to 48 hours, into the wound site. This process is aided by the endothelial cells expressing surface adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), endothelial adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1), which enable the PMN to leave the bloodstream by binding to the endothelial cells (leucocyte adhesion cascade). The PMN progress between the endothelial cells and through the basement membrane (diapedesis) and follow the chemotactic gradient into the wound site aided by the cytokine induced (TGF-β, interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α)) release of elastase and collagenases. PMN are short-lived phagocytic cells which ingest bacteria and foreign material and destroy them

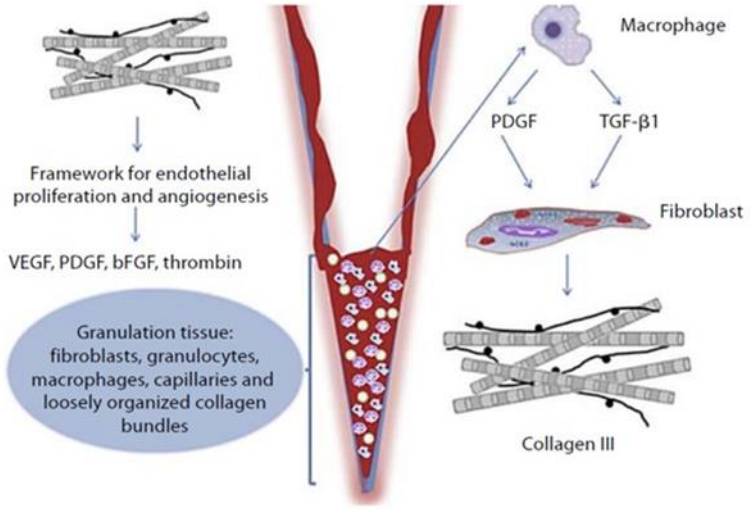
internally using various hydrolytic enzymes, such as acid hydrolases and lysozyme, lactoferrin and by the production of reactive oxygen intermediates (ROI) (Respiratory burst). Complement fragment C3b causes opsonisation, aiding PMN phagocytosis and complement also leads directly to bacterial cell lysis by the membrane attack complex (MAC) (C5b6789). When the PMN die these toxic mediators are released into the surrounding tissue leading to extensive tissue damage. PMN also enhance the inflammatory response by producing and releasing TNF-α and IL-1.

After 48 to 72 hours, monocytes are also attracted to the wound site by chemotactic factors, such as TGF-β, collagen and fibronectin fragments, and as they leave the blood stream they differentiate into macrophages. Macrophages have been shown to be a fundamental component in the early phases of wound healing and their absence causes impaired healing (Leibovich and Ross, 1975). Macrophages are long-lived phagocytes which are important in the removal of bacteria, foreign and devitalised material, but they also play a major co-ordination role both in the inflammatory response and in wound healing. Macrophages secrete various cytokines and growth factors, such as IL-1, interleukin-6 (IL-6), TNF-α, fibroblast growth factors (FGF), insulin-like growth factor (IGF), epidermal growth factor (EGF), PDGF and TGF-β, all of which are involved in the recruitment of cells important for the proliferative phase (Koh and DiPietro, 2011). After about three days post injury T-lymphocytes migrate into the wound, in response to the local release of IL-1, and are thought to have a significant role in the regulation of collagen and ECM production (Efron et al., 1990).

1.2.4 Proliferative phase

Aspects of this phase are usually apparent between two to twenty one days post injury and result in the replacement of the platelet-fibrin clot with new vascular connective tissue. This phase consists of three overlapping principal processes: re-epithelialisation, angiogenesis and granulation tissue formation. The key processes in the proliferative phase are summarised in Figure 1.2.

Figure 1.2 A summary of the key processes in the proliferative phase of cutaneous wound healing (diagram from Reinke and Sorg, 2012).



1.2.4.1 Re-epithelialisation

Re-epithelialisation involves the formation of a new epithelium over the denuded area of a wound and tends to be completed within days of an injury in order to protect the underlying wound site from microbial invasion. The time taken to achieve this depends on the width of the denuded area, the extent of any underlying tissue damage, infection and the environment (moist conditions promote epithelialisation) (Jones, 2005). At the wound edge structural changes occur which detach the epidermal cells from each other and from the underlying basement membrane, and intracellular actin microfilaments are formed. These changes allow the immature epidermal cells to migrate across the wound surface in an amoeboid-like action separating the non-viable superficial tissue (eschar) from the underlying wound. This process is aided by the epidermal cells secreting collagenases and plasminogen activator, the latter of which stimulates the production of plasmin and so helps to breakdown the clot. At the wound edges, basal layer keratinocytes proliferate in response to the local release of growth factors such as EGF and keratinocyte growth factor (KGF) (Barrandon and Green, 1987, Werner et al., 1994). As the sheets of epidermal cells converge in the centre of the wound, mitosis and migration are inhibited by contact inhibition. The epidermal cells then form new desmosomal linkages between each other and hemi-desmosomal linkages to the underlying basement membrane. In the skin and the oral mucosa, these cells differentiate into a stratified

squamous epithelium. Evidence also suggests that the underlying ECM components, such as fibronectin, influence epidermal cell proliferation and migration (Li et al., 2003).

1.2.4.2 Granulation tissue formation and angiogenesis

Underlying the newly reformed epithelium is the developing granulation tissue, which is mainly composed of macrophages, fibroblasts and newly formed blood vessels embedded in a loose matrix of ECM components including fibrin, fibronectin and collagen. Granulation tissue has a high metabolic demand and, in order to maintain this, new blood vessels are formed through angiogenesis. Angiogenesis is thought to be initiated by the secretion of various angiogenic factors such as VEGF, fibroblast growth factor-2 (FGF-2), nitric oxide (NO) and TGF-β and by local environmental factors such as hypoxia, low pH and high lactate levels (Nissen et al., 1998, Tonnesen et al., 2000, Bhushan et al., 2002, Greaves et al., 2013).

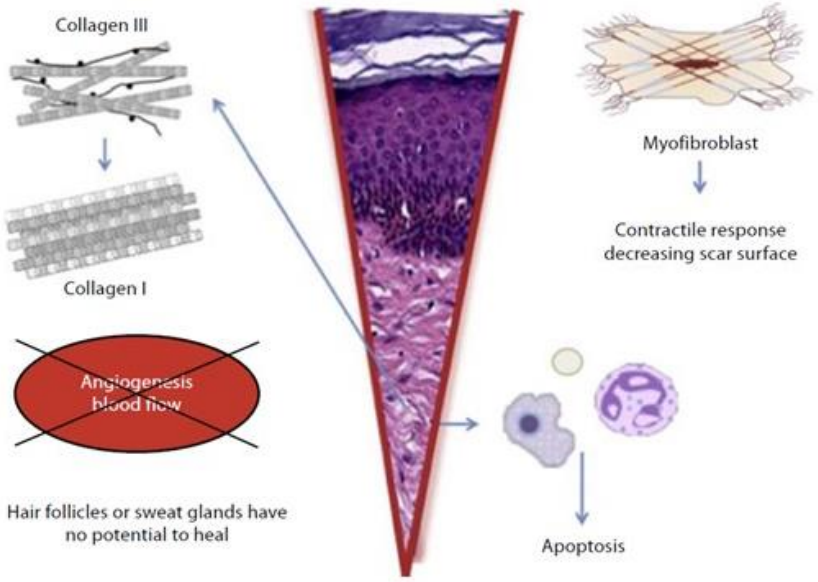
The key cell in the formation and maturation of the granulation tissue is the fibroblast, either derived from tissue fibroblasts or from undifferentiated mesenchymal cells, which migrate into the wound site from the surrounding tissue. Several cytokines and growth factors are thought to promote fibroblast proliferation and migration such as PDGF, FGF-2, TGF-β and MSF, as well as C5a, fibronectin and its fragments (Schor et al., 1993, Greiling and Clark, 1997, Marcopoulou et al., 2003). Fibroblast migration is determined by the concentration gradient of the chemotactic factors and is guided by the alignment of the fibrils in the ECM. These processes are aided by the binding of the fibroblast surface integrin receptors with ECM components such as collagen, fibronectin, vitronectin and fibrin. Fibroblast migration and the breakdown of the ECM components of the provisional matrix are achieved by the extracellular secretion of matrix metalloproteinases (MMP). These proteases are particularly important in removing the denatured collagen in the provisional matrix in order for the fibroblasts to re-establish an organised and strengthened ECM. Fibroblasts also secrete tissue inhibitors of metalloproteinases (TIMP) to prevent the uncontrolled degradation of the ECM by the MMPs. Fibroblasts produce a multitude of ECM components including Type III collagen, glycosaminoglycans, hyaluronic acid, chondroitin sulphate, dermatin sulphate, heparin sulphate and elastin. During this phase fibroblasts have the ability to differentiate into contractile myofibroblasts, which possess high levels of α-smooth muscle actin, and are

thought to be important in reducing the size of wounds. Factors such as increased stiffness of the ECM and the presence of TGFβ are thought to be important in the formation of myofibroblasts (Tomasek et al., 2002, Hinz, 2015). Although wound contraction is maximal over the first 5 to 15 days post injury, it continues over a prolonged period and into the maturation phase.

1.2.5 Remodelling (maturation) phase

This prolonged phase starts from about three weeks post injury and continues up to two years, with fibroblasts being the principal cell involved during this process (Figure 1.3). Collagen continues to be remodelled resulting in greater organisation, increasing wound strength and possibly some further wound contraction. Elements of the immature provisional matrix, such as Type III collagen, fibronectin, hyaluronic acid and glycosaminoglycans are progressively replaced by Type I collagen and proteoglycans (Yates et al., 2011). A reduction in cellular activity and metabolic load leads to reduced vascular density (vascular pruning) with loss of surplus capillaries and the maturation and formation of more large vessels.

Figure 1.3 A summary of the key processes in the remodelling (maturation) phase of cutaneous wound healing (diagram from Reinke and Sorg, 2012).



Healing rarely results in complete regeneration of the tissue and often leads to scar tissue formation (repair). Keloids and hypertrophic scars are both associated with excessive collagen deposition and lack differentiated structures such as hair follicles, sebaceous glands and sweat glands. Several studies have reported that scar tissue formation may be related to the expression of different isoforms of TGF-β (Shah et al., 1999, Gorvy et al., 2005). For example, Shah et al. (1995) reported TGF-β1 and TGF-β2 expression was associated with scarring, while TGF-β3 expression was associated with reduced cutaneous scarring. Wound healing is a complex multi-stage process involving the temporal interaction between various cell types, cytokines, growth factors and components of the ECM, with angiogenesis being a fundamental element.

1.3 Angiogenesis

1.3.1 Introduction

Angiogenesis is a complex and highly co-ordinated process involving the dynamic interaction between endothelial cells, inflammatory cells, adhesion molecules, ECM, various cytokines and growth factors resulting in the formation of blood vessels (Carmeliet and Jain, 2011a). Angiogenesis was first described by the British surgeon Dr John Hunter in 1787 whilst studying blood vessel growth in reindeer antlers, although its significance was not appreciated until the work of Folkman (1971), who hypothesised that tumour growth was dependent on new blood vessel growth. Folkman later defined angiogenesis as the, "formation of new blood vessels from the pre-existing vascular network" (Folkman and Shing, 1992).

Angiogenesis differs from vasculogenesis, which is the de novo formation of primitive vascular networks of blood vessels by angioblasts (endothelial precursor cells), during early embryonic development (Risau, 1997). These initial blood vessels are subsequently remodelled and expanded by angiogenesis in order to meet the functional demands of embryonic growth. There is limited evidence that vasculogenesis occurs in adults and may be involved in collateral vessel growth in ischaemic tissue and in the growth of some tumours (Asahara et al., 1997, Drake, 2003).

Angiogenesis is vital in childhood physiological growth and development, but is maintained at a relatively low level in healthy adults, apart from higher activity in the female reproductive cycle and in wound healing (Saravanan et al., 2020). Evidence also suggests that angiogenesis may be involved in neural development and learning (Zhang et al., 2003, Greenberg and Jin, 2005, Fujioka et al., 2019). In health, angiogenesis is tightly controlled by the dynamic balance between angiogenic factors (Table 1.1) and inhibitors (Table 1.2), often referred to as the angiogenic switch (Hanahan and Folkman, 1996, Iruela-Arispe and Dvorak, 1997). Most of these factors are still relatively poorly understood, especially in terms of the interaction between themselves and with the ECM. The major angiogenic factors and inhibitors are briefly described below, with particular emphasis on those related to this thesis (Chapter 1.5.3 and 1.5.4). These factors will be further discussed in relation to their roles in oral health and periodontitis later in this chapter (Chapter 1.6.6).

Table 1.1 Known angiogenic factors.

- Angiogenin.
- Angiopoietin-1.
- Fibroblast Growth Factors (FGF-1 & FGF-2).
- Epidermal Growth Factor (EGF).
- Granulocyte Colony Stimulating Factor (G-CSF).
- Hepatocyte Growth Factor (HGF) / Scatter Factor.
- Insulin-like Growth Factor I & II
- Integrins $\alpha_v\beta_3$, $a_v\beta_5$
- Interleukin 8 (IL-8).
- Matrix Metalloproteinases (MMP)
- Migration Stimulating Factor (MSF)
- Nerve Growth Factor (NGF)
- Placental Growth Factor
- Platelet-Derived Endothelial Cell Growth Factor (PD-ECGF)
- Platelet-Derived Growth Factor (PDGF)
- Pleiotrophin (PTN)
- Progranulin
- Proliferin
- Transforming Growth Factors (TGF-α and TGF-β).
- Tumour Necrosis Factor alpha (TNF-α).
- Vascular Endothelial Growth Factor (VEGF).

Table 1.2 Known angiogenic inhibitors.

- Angiopoietin-2
- Angiostatin.
- Arresten
- CD59 complement fragment
- Endorepellin
- Endostatin.
- Fibronectin fragments
- Interferons (IFN-α, IFN-β , IFN- γ)
- Interleukin 12 (IL-12).
- Hepatocyte Growth Factor fragment NK1
- Metalloproteinase inhibitors (TIMP)
- Migration Stimulating Factor Inhibitor (MSFI)/ Neutrophil Gelatinase-Associated Lipocalin (NGAL)
- Platelet Factor-4
- Thrombospondin-1
- Thrombostatin
- Tissue Inhibitor of Metalloproteinases (TIMP) 1/2/3
- Transforming Growth Factor beta (TGF β)
- Troponin-1
- Tumstatin
- Vasostatin.

Dysfunctional regulation of angiogenesis has been implicated in several disease processes, especially in neoplasia (Polverini, 1995, Carmeliet and Jain, 2011b). Excessive angiogenesis has been implicated in the pathogenesis of several conditions including neoplasia, diabetic retinopathy, arthritis and asthma, whilst insufficient angiogenesis is associated with atherosclerosis, strokes, diabetic neuropathy and pre-eclampsia (Carmeliet, 2003, Carmeliet and Jain, 2011b, Okonkwo and DiPietro, 2017, Balogh et al., 2019). Although the exact mechanisms involved in angiogenesis have not been fully determined, it is likely that they will differ depending on the circumstances. For instance, the micro-environment in a rapidly developing embryo, where the angiogenic activity is high, will differ from that of a quiescent adult vascular bed. These will differ again from that of an aggressive metastatic tumour where loss of normal angiogenic control is considered to be a key feature.

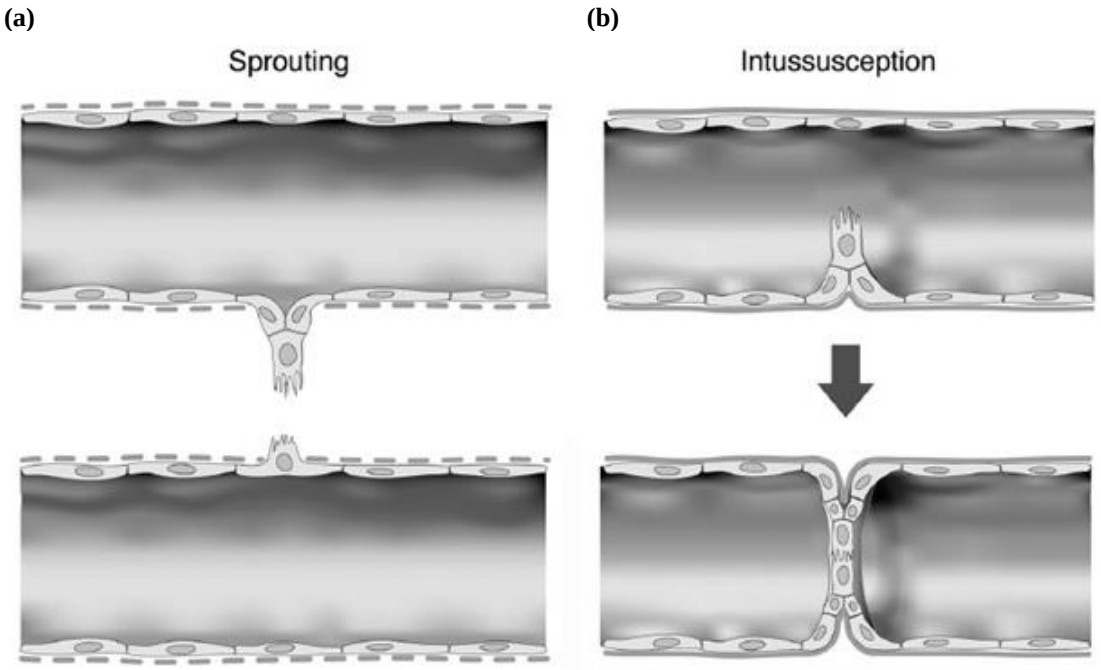
1.3.2 Types of angiogenesis

There are thought to be two distinct mechanisms by which of angiogenesis occurs, sprouting and intussusceptive angiogenesis.

1.3.2.1 Sprouting angiogenesis

Sprouting angiogenesis is a well described complex process involving the ordered array of multiple growth/angiogenic factors, cytokines, ECM components and alterations in gene expression (Carmeliet, 2003, Karamysheva, 2008, Velnar et al., 2009, Carmeliet and Jain, 2011a, Guerra et al., 2018). The process is initiated following the release of angiogenic factors, often as a result of hypoxia, such as VEGF, FGFs and Angiopoietin-2 (Ang-2). This leads to detachment of pericytes and the release of matrix metalloproteinases, leading to breakdown of the basement membrane and loosening of the endothelial cell attachments. Subsequent vasodilation and increased vascular permeability permits plasma proteins to enter the surrounding ECM. Endothelial cells then migrate into the ECM as a result of integrin signalling and the release of further angiogenic factors, such as VEGF and FGFs, from the ECM (Figure 1.4(a)). The endothelial cells migrate towards the angiogenic stimulus under the guidance of a single lead endothelial cell, called the tip cell. The adjacent endothelial cells, called stalk cells, proliferate to form solid sprouts which subsequently develop a lumen. The new vessel is formed when the endothelial cells attach to an adjacent vessel, which is aided by myeloid bridge cells, and is stabilised by the release of angiogenic factors such as PDGF-β, Ang-1 and TGF-β. The vessel basement membrane is re-established by the release of various proteases inhibitors, such as tissue inhibitors of matrix metalloproteinases (TIMP) and plasminogen activator inhibitor. Subsequently, vascular density is further increased by new vessels connecting with adjacent vessels to form three dimensional loops and arcades (anastomosis). Thus, angiogenesis typically results in excessive vascular density, which leads to vascular pruning to remove under perfused vessels (Bluff et al., 2006). The differentiation of the vessels into arteries and veins is genetically determined and requires the recruitment of vascular smooth muscle cells and pericytes. It has been proposed that distinct subsets of angioblasts result in either the formation of arteries or veins (Dor et al., 2003).

Figure 1.4 Comparison of the initial processes involved in (a) sprouting and (b) intussusceptive angiogenesis. The dotted line represents the breakdown of the basement membrane in sprouting angiogenesis (diagram from Mentzer and Konerding, 2014).



1.3.2.2 Intussusceptive angiogenesis

Intussusceptive or splitting/non-sprouting angiogenesis involves the splitting of vessels into two by the extension of the capillary wall and was first observed in postnatal rat lung development (Caduff et al., 1986). This is a complex and still poorly understood process which appears to have several functions including the duplication of vessels, removal of redundant vessel and modification of the angle between bifurcated vessels (Mentzer and Konerding, 2014). Essentially this form of angiogenesis involves the formation of an intraluminal bridge or pillar by the endothelial cells on opposite sides of a vessel protruding into the lumen, until contact is made (Figure 1.4(b)) (Burri et al., 2004, De Spiegelaere et al., 2012, Mentzer and Konerding, 2014). This is followed by reorganisation of the endothelial cell junctions and interstitial tissue formation within the intraluminal pillar. A core is then formed between the two new vessels into which pericytes and myofibroblasts invade, with the latter cells producing the ECM, allowing the two vessels to bifurcate and continue to grow in size. The key difference between the two types of angiogenesis is that intussusceptive angiogenesis involves a large increase in the number of capillaries, with

relatively little increase in the number of endothelial cells, and may be an efficient mechanism of expanding vascular networks following initial sprouting angiogenesis (Mentzer and Konerding, 2014). Unlike sprouting angiogenesis, relatively little is known about the regulation of intussusceptive angiogenesis and the role of angiogenic factors, such as VEGF, although hypoxia has been shown to initiate it mice (Taylor et al., 2010). This form of angiogenesis is thought to be important in embryonic development and its involvement in the growth of some human tumours, such as gliomas, may explain their resistance to anti-angiogenic therapy (Burri and Djonov, 2002, Nico et al., 2010, Saravanan et al., 2020).

1.3.3 Angiogenesis in neoplasia

Extensive research has been published into the role of aberrant angiogenesis in the development, growth and metastasis of neoplastic lesions. It has been shown that for tumours to grow beyond 1-2mm, the maximum distance which sufficient nutrients and oxygen can diffuse, angiogenesis is required (Folkman, 1990). Therefore, hypoxia is thought to be an important pathway for neoplastic growth through activation of a number of complex pathways including the hypoxia-inducible factors (HIF) leading to the production of VEGF (Chen et al., 2009). Furthermore, angiogenesis is also fundamental for the ability of many tumours to metastasise and grow at secondary sites (Folkman, 1995). Tumour angiogenic vessels tend to be more immature and disorganised in structure with more permeable and convoluted, often with disrupted blood flow (Carmeliet and Jain, 2011b). Evidence suggests that the blood vessel luminal surfaces are composed of a mosaic of endothelial cells and tumour cells, which may account for the metastatic potential of some tumours (Chang et al., 2000). New vessel growth is thought to occur not only from pre-existing vessels, but also from the recruitment of circulating endothelial precursor cells (Lyden et al., 2001). Many neoplastic lesions show dysfunctional cellular signalling, leading to a change in the angiogenic switch in favour of uncontrolled angiogenesis (Carmeliet and Jain, 2000). Thus, various anti-angiogenic drugs designed to restrict tumour vascular growth are now available, such as Avastin (bevacizumab) which is a monoclonal antibody against VEGF (Vasudev and Reynolds, 2014). However, the current effectiveness of anti-angiogenic therapy varies between types of tumours and further development is required.

1.4 Oral wound healing

1.4.1 Introduction

Many aspects of oral soft tissue wound healing are identical to cutaneous wound healing, although oral soft tissues display enhanced healing with minimal scarring, despite the high microbial load and wet environment (Sciubba et al., 1978, Schor et al., 1996, Szpaderska et al., 2003, Wong et al., 2009). Hence, the oral soft tissues are described as being a privileged site for wound healing (Schor et al., 1996). Several mechanisms have been proposed to account for this enhanced healing ability which fall into two broad categories: (i) factors intrinsic to the oral soft tissues and (ii) factors present in saliva. Current evidence indicates that all of these mechanisms, discussed in further detail below, are likely to contribute to the enhanced oral mucosal healing, although the exact mechanisms are poorly understood.

1.4.2 Intrinsic tissue factors

Several studies have described the oral mucosa as having intrinsic characteristics in common with foetal tissue, which may explain its enhanced healing capacity compared with dermal tissue (Schor et al., 1996). These characteristics include rapid re-epithelisation, reduced inflammatory infiltration and angiogenic response, the presence of foetal-like fibroblasts and minimal scarring (Szpaderska et al., 2003, Szpaderska et al., 2005, Mak et al., 2009, Turabelidze et al., 2014, DiPietro and Schrementi, 2018). Underpinning the various intrinsic mechanisms are active stem cell populations in the oral epithelial and connective tissues (Izumi et al., 2007, Zhang et al., 2012). As previously discussed (Chapter 1.2), animal studies have shown that transplanted adult skin tissue maintains its original characteristics (i.e. heals with scaring) in a foetal recipient site, suggesting that a tissue's healing characteristics are intrinsic to the tissue and not due to the environment (Longaker et al., 1994). Likewise, skin grafts placed on the oral mucosa can form keloid scars, suggesting that the enhanced oral mucosal healing is in some way related to the intrinsic nature of the tissue (Reilly et al., 1980).

1.4.2.1 Re-epithelisation

Another unique feature of oral mucosal wound healing is rapid re-epithelisation by keratinocytes, which have significantly higher proliferation rates and migration compared to skin keratinocytes (Szpaderska et al., 2003, Schrementi et al., 2008, Turabelidze et al., 2014). For example, Schrementi et al. (2008) reported that 1mm excisional wounds in mice resulted in 100% re-epithelisation in the oral mucosa compared with less than 25% re-epithelisation in the dermal wounds over a 24 hour period. There is also some evidence that oral mucosal keratinocytes may be less differentiated, compared with dermal keratinocytes, effectively allowing more rapid proliferation (Iglesias-Bartolome et al., 2018).

1.4.2.2 Inflammatory reaction

Several studies have indicated that oral mucosal healing is associated with lower levels of inflammatory infiltrate, which leads to lower levels of pro-inflammatory cytokines and growth/angiogenic factors (Szpaderska et al., 2003, Szpaderska et al., 2005, Mak et al., 2009, Chen et al., 2010, DiPietro, 2016). Szpaderska et al. (2003) reported significantly less inflammatory cell infiltration (neutrophils, macrophages and T-lymphocytes), lower levels of the pro-inflammatory cytokine IL-6 and significantly more rapid wound closure in oral excisional wounds in mice compared to dermal wounds. A similar study, also in mice, compared the healing of punch biopsies of the hard palate with similar wounds on the scalp (Nooh and Graves, 2003). They found that if there was significant connective tissue loss, oral healing was delayed, compared to the equivalent sized dermal wounds and these oral wounds had a persistently and significantly higher inflammatory infiltration. The group hypothesised that the delayed healing associated with large oral wounds was due to the significantly higher bacterial load leading to a persistent and elevated infiltratory response.

In conjunction with a reduced inflammatory infiltrate in oral mucosal healing, several studies have reported significantly reduced levels of TGF-β1, a growth factor associated by myofibroblast differentiation and increased scar tissue formation (Lin et al., 1995, Shah et al., 1995, Szpaderska et al., 2003, Schrementi et al., 2008). Furthermore, Schrementi et al. (2008) found a significant increase in the TGF-β3 to TGF-β1 ratio in the first 24 hours in mouse oral wounds, a finding consistent with a study in rats, where high ratios of TGF-β3 to

TGF-β1 were associated with reduced scarring in dermal tissue wounds (Shah et al., 1995). Schrementi et al. (2008) proposed that high TGF-β3 to TGF-β1 ratios partly accounted for the oral mucosa being a privileged site for healing.

1.4.2.3 Angiogenic response

A seemingly paradoxical finding is that oral wound healing is associated with reduced angiogenic activity compared to skin, probably as the result of the reduced inflammatory response (Szpaderska et al., 2003, Szpaderska et al., 2005). VEGF levels were also found to be reduced in oral tissue wounds compared with skin (Szpaderska et al., 2005). From these findings Szpaderska et al. (2005) hypothesised that wound healing in skin resulted in an excessive angiogenic response, beyond the physiological needs of the tissue. The implication being that oral wound healing is associated with sufficient levels of angiogenesis to form enough viable vessels to meet the physiological requirements of the healing tissue (DiPietro, 2016). Furthermore, evidence from a skin wound healing study in mice demonstrated that partial interference of the angiogenic response, using an anti-VEGF antibody, resulted in a reduced vascular response and reduced scarring (Wilgus et al., 2008). This suggests that the angiogenic response may also play a role in scar formation.

1.4.2.4 Fibroblast heterogeneity

Fibroblast heterogeneity has been shown both within sites and between different tissues in the body, including the oral mucosa and periodontal tissues, with fibroblast sub-populations showing different characteristics (Harper and Grove, 1979, Hassell and Stanek, 1983, Schor and Schor, 1987, Smith et al., 2019). Several studies have shown that oral mucosal and gingival fibroblasts display many of the characteristics of foetal fibroblasts, in terms of enhanced migration and the production of various growth factors and cytokines, such as MSF (Schor et al., 1988a, Grey et al., 1989, Schor et al., 1996). For example, Irwin et al. (1994) studied the migratory characteristics of gingival fibroblasts using 3D collagen gel cultures and found that gingival fibroblasts harvested from the gingival papilla showed enhanced migratory phenotypes similar to that of foetal fibroblasts. This enhanced migratory ability was associated with the production of MSF, a cytokine which had previously been shown to be produced by foetal fibroblasts and not by normal adult tissue fibroblasts (Schor et al.,

1988a). Fibroblasts harvested from the reticular levels of the gingival tissue however, showed more adult characteristics both in terms of their migratory characteristics and in their lack of MSF production.

Interestingly, several tissue-culture studies have shown human oral fibroblasts cell lines have an enhanced ability to contract collagen gels, implying increased wound contraction (Stephens et al., 1996, Irwin et al., 1998, Shannon et al., 2006). However, a similar tissue culture study reported oral mucosal fibroblasts had lower ability to contract compared with dermal fibroblasts (Lee and Eun, 1999). Furthermore, Mak et al. (2009) reported lower numbers of myofibroblasts and significantly less wound contraction in oral mucosal wounds in pigs compared with dermal wounds. This dichotomy in findings is probably related to the difference in cellular behaviour associated with different oral fibroblast populations and the difficulty of reproducing the complex conditions associated with wound healing in the laboratory.

1.5 Saliva and oral wound healing

1.5.1 Introduction

Saliva is a complex oral fluid known to contain approximately 2000 proteins, many of which have functions beneficial to wound healing such as various growth factors, cytokines, antibacterial agents and mucins (Dawes et al., 2015, Proctor, 2018). Saliva has been known for centuries to promote healing and both the ancient Greeks and Egyptians used dog and snake saliva to enhance wound healing (Angeletti et al., 1992). It is a common observation that animals lick their wounds and it has been proposed that this is a mechanism for delivering healing factors found in saliva to the wound (Hutson et al., 1979). Evidence from a dermal wound healing study in calves found that wounds treated with saliva healed faster and had a shorten inflammatory phase compared with wounds treated with saline alone (Varshney et al., 1997). Furthermore, a recent tissue culture study reported that sterilised human saliva enhanced migration of both dermal and oral mucosal keratinocytes and fibroblasts, leading to the suggestion that saliva could be used therapeutically to enhance healing (Rodrigues Neves et al., 2019). However, wound debridement through wound licking may also account for some of the beneficial effects on wound healing.

Several studies by Bodner et al reported reduced cutaneous and oral wound healing capacity, including extraction site healing, following the removal of the major salivary glands (sialadenectomy) in rodents (Bodner, 1991, Bodner et al., 1991a, Bodner et al., 1991b, Dayan et al., 1992, Bodner et al., 1993). More specifically, submandibular and sublingual saliva appeared to be important in the enhancement of cutaneous wound healing in these animal models (Bodner, 1991). The reduction in oral wound healing in desalivated rodents appeared to be more pronounced in wounds with connective tissue damage (Bodner and Dayan, 1995). Re-epithelialisation was unaffected but the granulation tissue formation and wound contraction were significantly impaired (Niall et al., 1982, Bodner et al., 1992). In fact, in two studies on palatal wound healing it was noted that there were more intense inflammatory reactions in the desalivated rats, this being more pronounced in larger wounds (Bodner et al., 1992, Bodner et al., 1993). The implication of this finding is that the delayed healing was probably due to the more intense inflammatory reaction induced by the resultant xerostomia. It is likely, however, that the resultant reduced healing may be due to other causes in addition to the reduced salivary growth/angiogenic factors reported in these studies.

1.5.2 Angiogenic factors in saliva

The concentrations of relatively few angiogenic factors have been examined in human saliva and currently those examined include EGF, FGF-2, Nerve Growth Factor (NGF), TGF-α, TGF-β, VEGF and Insulin-like Growth Factor I and II (Cohen, 1962, Costigan et al., 1988, Glantz et al., 1989, Amano et al., 1991, Humphreys-Beher et al., 1994, van Setten, 1995, Taichman et al., 1998). Furthermore, there have been relatively few publications which have reported the relationship between the angiogenic factor content of saliva in relation to oral health and disease, in particular periodontal disease. Taichman et al. (1998) postulated that oral angiogenic factors, such as VEGF in saliva, could account for the enhanced oral healing and could be important in the maintenance of the oral cavity and the upper gastro-intestinal tract. Currently, there have been few publications which have reported angiogenic inhibitor content, such as endostatin, in human saliva and so the balance between angiogenic factors and inhibitors in saliva is poorly understood. It is therefore relevant in this thesis to examine the possible role of angiogenic factors and inhibitors in human saliva in relation to wound healing and periodontal disease.

When studying angiogenic factors in saliva it is important to take account of several factors that may influence the findings. For example, there is evidence that the salivary levels of some angiogenic factors, such as epidermal growth factor (EGF), are affected by circadian rhythm in a similar way to many hormones, such as cortisol (Ferguson et al., 1973, Dawes, 1975, Ino et al., 1993). Therefore, in the present study saliva collection was taken between 9:30am and 11am in order to take this issue into account (Chapter 2.1.6). Other possible factors which may affect the salivary angiogenic factor concentration include age, how the saliva is sampled (unstimulated or stimulated saliva), type of saliva (whole saliva or whether it is from specific salivary glands), smoking, medications, medical conditions, periodontal health and the time since the last meal (Humphrey and Williamson, 2001).

Several angiogenic factors, such as EGF and VEGF, have been shown to be produced by the acinar cells within the salivary glands (Taichman et al., 1998, Cossu et al., 2000, Lantini et al., 2001). However, the origin of several salivary related angiogenic factors, such as angiopoietin-1 (Ang-1) and endostatin, have still to be determined and some may enter saliva from the blood in a similar fashion to insulin (Vallejo et al., 1984). Many of these angiogenic factors have also been detected in gingival crevicular fluid (GCF), especially in periodontal disease, and so may contribute to the salivary levels of angiogenic factors.

In the following sections, the structure and function of the main angiogenic factors and inhibitors is outlined and the current literature base is discussed in relation to each factor in saliva.

1.5.3 Angiogenic factors in oral wound healing and saliva

1.5.3.1 Angiogenin (ANG)

Angiogenin (ANG), also referred to as ribonuclease 5, is a 14kDa, non-glycosylated angiogenic polypeptide with weak ribonuclease activity and is a member of the ribonuclease superfamily (Adams and Subramanian, 1999, Sheng and Xu, 2016). On binding with its receptors, it is endocytosed, transported to the nucleus where it accumulates in the nucleolus, where ultimately it stimulates ribosomal ribonucleic acid (rRNA) transcription (Xu et al.,

2002). ANG is a potent angiogenic factor, shown to induce endothelial cell proliferation, migration and differentiation (Hu et al., 1994, Hu et al., 1997, Soncin, 1992), and is thought to act in conjunction with other angiogenic factors such as FGF-1, FGF-2, EGF, and VEGF (Kishimoto et al., 2005). ANG angiogenic effects can be inhibited by the aminoglycoside antibiotic Neomycin, which blocks ANG nuclear translocation (Hu, 1998).

To date there have been no studies which have reported actual ANG concentrations in saliva. A recent study by Roca et al. (2019) studied the effect of nutritional supplements on salivary immunity in non-elite marathon runners and reported a small but significant reduction in salivary ANG levels, relative to total salivary protein concentrations, 48 hours after a marathon. However, salivary concentrations of ANG were not reported in the paper.

1.5.3.2 Angiopoietin-1 (Ang-1)

The Angiopoietin family consists of three human forms, named Angiopoietin-1, 2 and 4 (Ang-1, Ang-2 and Ang-4), Ang-3 being found in mice (Davis et al., 1996, Maisonpierre et al., 1997, Valenzuela et al., 1999). These factors all have two characteristic domains, a N-terminal coiled-coiled domain and a C-terminal fibrinogen-like domain which binds to the receptor tyrosine kinase Tie2 (Davis et al., 1996). Ang-1 has the highest affinity for the Tie2 receptors and is thought to be responsible for the majority of the angiogenesis mediated by Tie2 activation (Suri et al., 1996), with Ang-2 acting as a competitive inhibitor for Ang-1 (Maisonpierre et al., 1997, Gale et al., 2002). There are at least three splice variants of Ang-1, but only one has been found to activate Tie2, while the others may act as suppressors of Ang-1 activity (Huang et al., 2000).

Ang-1 has been shown to be an angiogenic factor through promotion of endothelial cell migration, sprouting, lumen formation, stabilisation and in the recruitment of support cells for both the endothelial cells and peri-endothelial cells (Koblizek et al., 1998, Witzenbichler et al., 1998, Kwak et al., 1999, Papapetropoulos et al., 1999, Teichert-Kuliszewska et al., 2001). Thus, Ang-1 is thought to be important in angiogenesis and in the stabilisation and long-term maintenance of the adult microvascular network, but it requires the presence of other angiogenic factors, such as VEGF, to initiate angiogenesis (Wong et al., 1997, Yancopoulos et al., 2000, Brindle et al., 2006). Due to many of the actions of Ang-1

involving the later stages of angiogenesis, and its involvement in vessel maintenance, some consider it to be an angiogenic inhibitor. Evidence also suggests that Ang-1 has a role in lymphatic vessel formation, PMN adhesion and migration, blood vessel permeability and neuronal development (Thurston et al., 2000, Gale et al., 2002, Valable et al., 2003, Lemieux et al., 2005).

Currently, there have been no publications which have reported Ang-1 concentrations in saliva, although two studies have examined Ang-1 levels in GCF following periodontal surgery (Rakmanee et al., 2010, Rakmanee et al., 2019). Hence, salivary concentrations of Ang-1 have been studied in this thesis and has been discussed in more detail Chapter 3.3.

1.5.3.3 Epidermal Growth Factor (EGF)

Epidermal Growth Factor (EGF) is the principal member of the EGF family of growth factors, which includes another angiogenic factor, Transforming Growth Factor-alpha (TGF-α), both of these factors bind to the tyrosine kinase Epidermal Growth Factor Receptor (EGFR) (Zeng and Harris, 2014). EGF was initially found to be a potent mitogen for fibroblasts and stimulated the proliferation and differentiation of epithelial and epidermal cells (Carpenter and Cohen, 1976). EGF is a 53 amino acid globular protein formed by proteolytic cleavage of a large precursor transmembrane protein, which contains at least seven EGF-like sequences (Lu et al., 2001). EGF is stored in platelets and released during degranulation and has been detected in various human bodily fluids such as in serum, urine, gastric fluids, breast milk and saliva (Cohen and Carpenter, 1975, Gregory et al., 1979, Carpenter, 1980, MacNeil et al., 1988, Aybay et al., 2006). EGF has been shown to induce epithelial development, promote angiogenesis and wound healing *in vivo* (Schreiber et al., 1986, Schultz et al., 1991).

EGF was originally isolated from male mouse submandibular glands (Cohen, 1962). As a consequence of this early discovery it is the most widely studied salivary angiogenic factor with 862 studies reported on Medline (June 2020). Early studies using desalivated rodent models suggested that salivary EGF is important both in cutaneous wound healing (animal wound licking behaviour) and oral wound healing, as well as playing a role in gastro-intestinal maintenance (Hutson et al., 1979, Niall et al., 1982, Olsen et al., 1984, Konturek et

al., 1988, Noguchi et al., 1991). These conclusions were based on desalivation impairing wound healing, which was restored by the topical application of EGF or the application of saliva containing EGF, but not by EGF-free saliva. The removal of the major salivary glands in these studies impaired wound healing, but did not totally prevent it suggesting that the salivary growth/angiogenic factors are not totally responsible for wound healing. In mice there is a gender difference in the levels of EGF, with male mice having over ten times the levels of EGF in their saliva compared with the female (Noguchi et al., 1991). No such gender difference has been found in humans (Thesleff et al., 1988).

Human EGF salivary secretion has been shown to be affected by diurnal variation in relation to meal times (McGurk et al., 1990), a factor taken into account in the thesis protocol, which was not apparent in either the serum or urinary EGF levels (Ino et al., 1993). Interestingly, no correlation was found between the EGF levels in saliva, serum and urine. The EGF concentration in human unstimulated whole saliva was about 1000pg ml^{-1}, although significant differences were found in the EGF concentrations of saliva derived from different salivary glands, with the parotid gland saliva containing approximately 2500pg ml^{-1} (Thesleff et al., 1988, Ino et al., 1993, Oxford et al., 2000). Whole saliva concentrations of EGF were found to be significantly less in young children compared with adults, although high levels of this factor were present in both groups (Ino et al., 1993). Electron-microscope studies have detected EGF in both acinar and ductal cells, in human submandibular and parotid glands. In both cases, clearly stained secretory granules were found in the acinar cells suggesting that EGF is produced in the glands and is subsequently released into the saliva by granular exocytosis (Cossu et al., 2000, Lantini et al., 2001).

Several studies have compared salivary EGF in periodontal health and disease. Moosavijazi et al. (2014) reported significantly higher concentrations of EGF in unstimulated whole saliva in periodontal healthy subjects compared with subjects with gingivitis and severe periodontitis. However, this was a small study with only 37 subjects across the three study groups and the exclusion criteria did not rule out smokers. In an earlier study by Hormia et al. (1993), significantly higher salivary EGF levels were initially found in subjects with Juvenile Periodontitis (probably equating to Stage 3/4 Grade C Periodontitis in the 2017

World Workshop classification). However, following more detailed investigation of a few subjects, salivary EGF concentration was found to be increased in comparison to healthy controls, but not significantly so. Significantly higher EGF levels were found in stimulated saliva from patients with Juvenile Periodontitis when the EGF levels were expressed against total salivary protein levels. With regards to wound healing following periodontal and oral surgery, salivary levels of EGF were found to be significantly raised within the first 48 hours post-surgery (Oxford et al., 1998, Oxford et al., 1999). Interestingly, some studies have reported significantly reduced salivary EGF concentrations in smokers (Jones et al., 1992, Wang et al., 1992) and in patients with diabetes (Oxford et al., 2000).

1.5.3.4 Fibroblast Growth Factors (FGF)

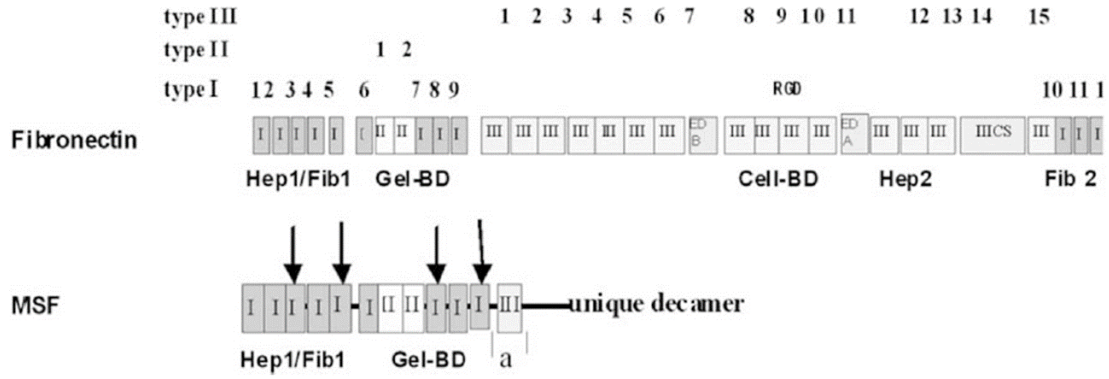
Currently there are 23 members of this family of structurally related heparin-binding signalling proteins, named FGF1-23 (Itoh and Ornitz, 2004). The name was derived from the mitogenic effect of pituitary extracts on mouse fibroblasts and the active factors were identified and named acidic FGF (FGF-1) and basic FGF (FGF-2). The majority of these factors bind and activate a family of four high-affinity cell surface tyrosine kinase receptors (FGFR1-4) leading to a wide range of cellular events involved in embryonic and postnatal development, many of which involve angiogenesis (Johnson and Williams, 1993). FGF-1 and FGF-2 are the mostly widely studied members of the family and have been shown to be involved in several stages of angiogenesis, including endothelial cell proliferation, migration, protease production, integrin receptor expression and vessel maturation. FGF-2 is thought to be a major angiogenic factor and plays an important role in wound healing by promoting fibroblast proliferation and granulation tissue formation (Pierce et al., 1992). Evidence suggests FGF-2 and VEGF interact during angiogenesis. Tillie et al. (2001) found that inhibition of VEGFR2 inhibited both VEGF and FGF-2 induced angiogenesis *in vivo* and *in vitro*. These two factors were also found to have a synergistic effect on blood vessel density in xenografts of a human tumour cell line studied in a murine model. However, these factors were shown to have differing effects on the blood vessel maturation and function, suggesting they have different roles in angiogenesis (Giavazzi et al., 2003).

FGF-1 has been detected in healthy and diseased human salivary gland tissue using immunohistochemistry (Myoken et al., 1996, Kusafuka et al., 2001), but has not been reported in saliva. This may either be due to FGF-1 not being present in human saliva or that the levels are below the detectable range of the assays. FGF-2 has been detected in salivary tissue and saliva (van Setten, 1995, Kongara et al., 2001). A large study by Westermark et al. (2002) examined the FGF-2 levels in unstimulated whole saliva in 182 healthy non-smokers and found that there were significantly higher concentrations of FGF-2 in young individuals compared to the middle-aged, who in turn had significantly higher levels than the elderly. An earlier study by the same group found significantly higher levels of FGF-2 in middle-aged smokers, although the numbers used in this study was very small, only six smokers, so this finding should be viewed with caution (Ishizaki et al., 2000). Neither of these studies found any gender or diurnal differences in the levels of salivary FGF-2. Raised salivary FGF-2 has also been found in patients with oral lichen planus and in oral squamous cell carcinoma (OSCC) (Magnusson et al., 2004, Vucicevic Boras et al., 2005, Gorugantula et al., 2012). Studies in animal models suggested that salivary FGF-2 can accelerate salivary gland and mucosal wound healing in desalivated rats and rabbits, although its effect on normal control animals was minimal (Kagami et al., 2000, Fujisawa et al., 2003). Szabo et al.(1994) found that oral FGF-2 enhanced healing of experimentally induced duodenal ulcers in rats, which was associated with significantly increased vascularity of the ulcer beds. It is possible that salivary FGF-2 may play a role in the maintenance of the entire upper gastro-intestinal tract.

1.5.3.5 Migration Stimulating Factor (MSF)

Migration Stimulating Factor (MSF) is a 70kDa soluble protein encoded by the *Fn1* gene on chromosome 2 (Grey et al., 1989, Schor et al., 1993, Schor and Schor, 2001, Schor et al., 2003). This protein is a truncated isoform of fibronectin (MSF; accession number AJ535086) corresponding to the amino-terminus of fibronectin with a unique 10 amino acid carboxyl-terminus (Figure 1.5).

Figure 1.5 Structural homology between fibronectin and MSF (diagram from Schor and Schor, 2010).



MSF has a range of biological activities including the induction of cell migration and hyaluronan synthesis, and is thought to be important in foetal development, wound healing and carcinogenesis (Schor et al., 1988a, Picardo et al., 1991, Schor and Schor, 2001, Schor et al., 2003, Houard et al., 2005, Perrier et al., 2012). MSF has shown to be expressed in various foetal and tumour cells, in particular by fibroblasts, endothelial and epithelial cells, but is not normally expressed in healthy adult cells (Schor et al., 1988a, Schor et al., 2003, Schor and Schor, 2010). One exception is in wound healing, where MSF is thought to play an important role through the induction of angiogenesis and its motogenic effect on various cell types, including fibroblasts, endothelial cells and pericytes (Ellis et al., 2010, Schor and Schor, 2010). *In vivo* evidence for the possible role of MSF in physiological wound healing was shown by Picardo et al. (1992) who reported that MSF activity was detected in wound fluid in over 90% of cases undergoing surgery for non-malignant conditions. However, MSF activity in serum was only detected in a small minority of cases pre-operatively, and in no cases post-operatively, implying MSF was produced locally at the wound site, rather than systemically. More recently, Jones et al. (2007) demonstrated the presence of a MSF inhibitor (MSFI) in keratinocyte conditioned medium and identified this to be neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2.

Recombinant human MSF (rhMSF) has been shown to induce sprouting angiogenesis in embryonic chick yolk sac membrane assays and pre-treatment with rhMSF leads to profound new blood vessel formation within avascular collagen implants placed subcutaneously in rats

(Dr AM Schor: personal communication and discussed in Schor and Schor (2010)). Conversely, inhibition of MSF activity by use of an anti-MSF specific monoclonal antibody leads to the premature death of sprouting-phenotype endothelial cells, but leaves cobblestone resting cells intact, suggesting that MSF may act as a survival factor for sprouting endothelial cells (Schor et al., 2003).

With regards to the periodontium, Irwin et al. (1994) demonstrated that sub-populations of oral gingival fibroblasts displayed more foetal-like phenotypes and were able to secrete high levels of MSF, which may account in some way for the enhanced wound healing characteristics of gingival tissue. To date there has only been one publication which has examined MSF expression in oral disease. In this immunohistochemistry study, Aljorani et al. (2011) examined MSF expression in benign (n=7) and malignant salivary tumours (n=27) in relation to adjacent histologically normal salivary tissue (n=16), which was used as a control. MSF expression was found to significantly increase in a "stepwise fashion" from the normal, benign and malignant tumours (p=0.04-0.0001), with MSF expression being found in both epithelial and connective tissue cells (fibroblasts, endothelial and inflammatory cells) particularly in the malignant tumours. Unexpectedly, there was some MSF expression found in the control samples, however, as these samples were adjacent to the tumours (i.e. not true healthy control tissue) it could not be ruled out that a cancerisation/field change effect had occurred.

Currently, no studies have examined MSF levels in serum, saliva or GCF in either periodontal health or in severe periodontitis. Furthermore, it is not known whether smoking or diabetes significantly affects MSF levels, although MSF gene expression has been reported to be upregulated in a bronchioloalveolar carcinoma cell line following exposure to benzopyrene, a constituent of cigarette smoke (Yoshino et al., 2007). Therefore, MSF has been studied in this thesis and has been discussed in more detail Chapter 3.4.

1.5.3.6 Nerve Growth Factor (NGF)

Nerve Growth Factor (NGF), also called neurotrophin, is a 26kDa peptide derived from the proNGF precursor (Coelho et al., 2019). NGF was originally isolated from mouse

submandibular glands (Cohen, 1960) and has mainly been associated with neuronal survival, growth and differentiation (Rocco et al., 2018). It is only more recently that its angiogenic activity has been demonstrated, with one study finding that NGF induced dose-dependent angiogenesis in quail chorioallantoic membrane assays (Lazarovici et al., 2006).

NGF importance in oral wound healing was suggested by the accelerated wound healing rate, through significantly reduced wound contraction times, when topical NGF was applied to wounds in sialoadenectomised mice (Li et al., 1980). Although there have been a large number of studies which have examined NGF concentrations in human saliva, there has been few large studies. Nam et al. (2007) measured salivary NGF in 127 healthy non-smoking individuals ranging from the ages of 20 to 81 years. High mean concentrations ($\pm$SE) of NGF were recorded in unstimulated whole saliva (901.4$\pm$75.6pg ml^{-1}), stimulated parotid saliva (885.9$\pm$79.9pg ml^{-1}) and stimulated submandibular/sublingual saliva (1066.1$\pm$88.1pg ml^{-1}). Interestingly, NGF was found to be significantly higher in women and its concentration in stimulated submandibular/sublingual saliva reduced significantly with age. Unfortunately, this study did not measure the NGF concentration in stimulated whole saliva, although a different small study (10 control subjects) examined salivary NFG concentration in cases of gingival overgrowth and found a median concentration of NGF in stimulated whole saliva of 9644pg ml^{-1} (Ruhl et al., 2004).

To date there have been no studies which have examined salivary levels of NGF in periodontal health and disease, or in smokers and non-smokers. Likewise, there has only been one study which examined NGF concentrations in unstimulated whole saliva in humans with Type 2 diabetes and reported no statistical difference between diabetics and healthy controls (Tvarijonaviciute et al., 2017). One criticism of this study is that they did not exclude smokers, which may have influenced the results, although they did exclude subjects with periodontal disease or recent periodontal treatment.

Evidence now suggests that the early studies on mice do not equate to human saliva, where the pro-NGF precursors, which have angiogenic activity, are the main form of NGF in human saliva (Naesse et al., 2013). In the review article by Schenck et al. (2017), they suggested

that this issue has led to an over-estimation of the active NGF levels in human saliva reported in studies which have used ELISAs. A further complication, is the likelihood that endogenous factors in human saliva interfere with many NGF ELISA-kits, which have not been optimised for saliva studies (Engen et al., 2017).

1.5.3.7 Platelet Derived Growth Factor (PDGF)

PDGF has a dimeric structure made up of two structurally similar subunits, PDGF A and B chains, linked together by a disulphide bond. Thus, PDGF exists in either homodimer isoforms, PDGF-AA or PDGF-BB, or in a heterodimer form, PDGF-AB. PDGF is produced by a wide range of cells including endothelial cells, vascular smooth muscle cells, fibroblasts, macrophages/monocytes and is released from the α granules on platelet activation (Heldin and Westermark, 1999). PDGF binds to two types of structurally related cell surface tyrosine kinase receptors termed α receptors and β receptors. The α receptor has high affinity for both PDGF A and B chains while the β receptor only has high affinity for the B chains (Claesson-Welsh et al., 1989). PDGF is an important mitogen for endothelial cells, fibroblasts and vascular smooth muscle cells as well as being an angiogenic factor and so is an important factor in wound healing (Sato et al., 1993, Battegay et al., 1994).

Several studies have detected PDGF during the early stages of human oral wound healing, following which the PDGF levels reduce (Green et al., 1997, Morelli et al., 2011, Pirebas et al., 2018). A few studies have examined PDGF concentration in human saliva in relation to periodontal therapy which suggested salivary PDGF is not significantly different between periodontal health and periodontitis. For example, Lee et al. (2018) reported PDGF concentrations in unstimulated whole saliva to be 2.7pg ml^{-1} (IQR 0.8-7.0pg ml^{-1}) in periodontally healthy controls compared with 3.8pg ml^{-1} (IQR 0.8-6.2pg ml^{-1}) in patients with severe periodontitis. Similar findings were reported in patients with rapidly progressive periodontitis compared with periodontally healthy controls (probably equating to Stage 3/4 Grade C Periodontitis in the 2017 World Workshop classification) (Pietruska et al., 2000). Kaval et al. (2014), however, in a small study (n=15 per group) reported reduced concentrations of PDGF in unstimulated whole saliva in smokers (3.87 ± 5.36pg ml^{-1}) compared with non-smokers (7.96 ± 17.33pg ml^{-1}), although the result was not significant.

1.5.3.8 Transforming Growth Factor-α (TGF-α)

Transforming Growth Factor-α has close structural homology to EGF and shares the same receptor (EGFR or erbB1). TGF-α is synthesised as a 160 amino acid transmembrane precursor molecule, which is cleaved by TNF-α converting enzyme (TACE) (Peschon et al., 1998) to produce the active 50 amino acid TGF-α molecule (Schreiber et al., 1986). TGF-α contains an EGF-like motif which has a complex 3D structure which is important for its high affinity receptor binding. TGF-α has been detected, generally at low concentrations, in a wide variety of normal adult and foetal tissues, as well as in some tumours (Singh and Coffey, 2014). Evidence suggests that it is an important factor in epithelial cell growth, wound healing, angiogenesis and possibly in neuronal development (Junier, 2000, Singh and Coffey, 2014).

Transforming growth factor-α has been detected in significant concentrations in both unstimulated and stimulated whole human saliva, and the concentration in unstimulated saliva was found to significantly reduce with age (Humphreys-Beher et al., 1994). This study also provided evidence that TGF-α is produced locally within the parotid and submandibular glands in rats and mice. Immunohistochemical techniques localised TGF-α to the granular ductal cells and RT-PCR showed the presence of TGF-α mRNA. A similar study in human major and minor salivary glands also found significant TGF-α expression in ductal cells and in intra-ductal secretions strongly suggesting local production and secretion into the saliva (Ogbureke et al., 1995). To date there have been no studies which have examined TGF-α concentration in human saliva in relation to periodontal health and disease.

1.5.3.9 Transforming Growth Factor-β (TGF-β)

Transforming Growth Factor-β is a ubiquitous multifunctional cytokine known to be important in the regulation of cellular proliferation, differentiation, migration and in angiogenesis (Pepper, 1997). There are three principal isoforms of TGF-β expressed in mammals (TGF-β1-3) with TGF-β1 and TGFβ-3 being the most abundant isoforms in adults and in foetal tissue respectively (Millan et al., 1991). TGF-β is secreted into the ECM in a latent form, which is thought to be activated by enzymatic cleavage. TGF-β binds to and activates three high affinity specific cell surface receptors. Type I TGF-β receptor (TβR1)

or Activin receptor-like kinase (ALK 1 to 7) and Type II TGF-β receptor (TβRII 1-5) are both serine/threonine kinase receptors. Type III TGF-β receptor (TβRIII) is a transmembrane receptor called betaglycan, although in endothelial cells endoglin (CD105) can act as an accessory receptor in the presence of a Type II TGF-β receptor (Bertolino et al., 2005). TGF-β is an important stimulant for fibroblast proliferation and ECM production and is known to play an important role in wound healing. The levels of TGF-β1 are thought to be an important difference between scar free foetal wound healing, where its levels are low, and scars associated with adult skin wound healing, where its levels are high. Lin et al. (1995) found that the addition of exogenous TGF-β1 to foetal wounds resulted in scar formation. Likewise, Shah et al. (1995) found that the addition of TGF-β1 neutralising antibodies reduced adult skin scar formation in mice.

In angiogenesis TGF-β has been shown to be important in the regulation of endothelial cell proliferation, migration, survival and differentiation as well as in the behaviour of the vascular smooth muscle cells. Although inhibition of TGF-β with a neutralising antibody has been shown to strongly inhibit angiogenesis, the mechanisms underlying this are unknown (Tuxhorn et al., 2002). Several *in vitro* studies have suggested that TGF-β appears to have bifunctional effects both on the behaviour of endothelial cells and on fibroblasts depending on the context, such as the origin of the cells, the tissue culture conditions and on the matrix used (Pepper et al., 1993, Pepper, 1997, Ellis and Schor, 1998). TGF-β is known to be important in the regulation of vascular homeostasis and it has been proposed that the balance between two distinct TGF-β type 1 receptors, endothelial-restricted ALK-1 and the widely expressed ALK-5 receptors, may induce opposite effects on endothelial cell behaviour and angiogenesis seen *in vitro* (Bertolino et al., 2005). Overall, the *in vivo* evidence strongly suggests that TGF-β is a potent angiogenic factor because mutation of endoglin and ALK-1 leads to the vascular disorder, hereditary haemorrhagic telangiectasia (Fernandez et al., 2006). Furthermore, over-expression of endoglin is associated with increased angiogenesis in some forms of human neoplasia (Burrows et al., 1995).

Immunohistochemistry studies have detected all three TGF-β isoforms in normal human salivary gland tissue (Brennan and Fox, 2000, Kusafuka et al., 2001), although one study

failed to detect the TGF-β1 isoform (Kusafuka et al., 2001). Evidence suggests TGF-β is produced within salivary tissue and is subsequently secreted into the saliva (Amano et al., 1991, Brennan and Fox, 2000, Kusafuka et al., 2001). Two studies reported significantly different TGF-β concentrations in the healthy control groups (unstimulated saliva). One study found a mean (±SD) TGF-β1 concentration of 24.96±1.67pg ml^{-1} in 22 women (mean age 57 years) (Yousefzadeh et al., 2006), while the other found a mean (±SD) TGF-β1 concentration of 2.36±0.52ng ml^{-1} in 20 age and sex matched subjects (mean age 41 years) (Rezaie et al., 2006). This difference may be age-related although no studies have examined the effect of age on TGF-β1 concentration in unstimulated whole saliva. One study has examined the effect of age on TGF-β1 concentration in stimulated saliva and found that the concentration increased with age, this being associated with a reduction in stimulated saliva flow with increasing age (Streckfus et al., 2002). Currently, there have been few studies which have examined TGF-β concentrations in human saliva in periodontal health and disease, however, Khalaf et al. (2014) reported significantly raised TGF-β concentrations in serum, GCF and in unstimulated whole saliva in subjects with periodontitis compared with healthy controls.

1.5.3.10 Vascular Endothelial Growth Factor (VEGF)

VEGF is a family of growth factors, VEGF-A to VEGF-D inclusive and placenta growth factor (PIGF), each having the characteristic VEGF homology domain (VHD) which contains receptor binding sites and a conserved cysteine-knot motif (McDonald and Hendrickson, 1993, Roy et al., 2006). These factors bind, to a greater or lesser degree, to three structurally similar cell surface tyrosine kinase receptors called VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4). Furthermore, neuropilin receptors (NRP-1 and NRP-2) can also act as co-receptors to the VEGFRs (Guerra et al., 2018). The VEGFRs consist of an extracellular portion made up of seven immunoglobulin-like domains, a transmembrane region and an intracellular portion containing a split tyrosine-kinase domain. VEGF binding to these receptors causes dimerisation of the receptor and activation by transphosphorylation leading to stimulation of a cellular response (Ferrara et al., 2003). VEGF-C and D have been shown to be important in the regulation of lymphatic angiogenesis by binding to VEGFR-3 (Enholm et al., 2001). The most important member of the VEGF family in terms of angiogenesis is VEGF-A, or more commonly known as VEGF.

VEGF is a potent key initiating factor in vasculogenesis and angiogenesis as well as causing increased vascular permeability through the induction of post capillary endothelial cell fenestration, hence it is also known as Vascular Permeability Factor (VPF) (Dvorak et al., 1995, Roberts and Palade, 1995, Gale and Yancopoulos, 1999). VEGF primarily affects the proliferation and migration of endothelial cells but has also been shown to have similar effects on monocytes/macrophages and on vascular smooth muscle (Clauss et al., 1990, Gerber et al., 2002, Bhardwaj et al., 2005). The VEGF gene has been located to 6p21.3 and alternative mRNA splicing results in six isoforms, named by the number of amino acids they contain ($VEGF_{121/145/165/183/189/206}$) (Vincenti et al., 1996). The size of the isoforms affects their biological activities with the smallest isoform $VEGF_{121}$ being freely soluble and the larger isoforms $VEGF_{189}$ and $VEGF_{206}$ being completely bound to the ECM and cell surfaces (Houck et al., 1992). The latter two isoforms of VEGF become biologically active once they have been released from the ECM by the action of heparinase or plasmin. Thus, it has been proposed that the ECM acts as a reservoir of growth factors which are only activated during its degradation such as in wound healing and angiogenesis (Park et al., 1993). $VEGF_{165}$ is the dominant isoform and occurs in ECM as bound and soluble forms.

VEGF exerts its biological effects through binding to the VEGFR-1 and VEGFR-2 receptors. VEGFR-2 binding has been shown to cause the majority of VEGF's cellular responses, while the exact action of binding to VEGFR-1 is still largely unknown (Carmeliet and Jain, 2011a). VEGFR-1 may modulate the VEGFR-2 signal expression or act as a decoy receptor reducing the amount of VEGF available to bind to VEGFR-2 (Apte et al., 2019). Although VEGF mRNA transcription is induced by various growth factors and cytokines, such as PDGF, EGF, TNF-α, TGF-β and IL-1, tissue hypoxia is thought to be a major initial stimulus for angiogenesis. Hypoxia induces the production of HIF which upregulates VEGF gene transcription (Elson et al., 2000).

The importance of VEGF in wound healing has been shown by various tissue culture, immunohistochemistry, *in-situ* hybridisation and polymerase chain reaction (PCR) studies which have all shown high levels of VEGF expression in endothelial cells, keratinocytes and macrophages at various stages in wound healing (Brown et al., 1992, Frank et al., 1995,

Nissen et al., 1998, Lauer et al., 2000). In the porcine wound model, the introduction of VEGF neutralising antibodies caused significant impairment of wound angiogenesis and reduced granulation tissue formation (Howdieshell et al., 2001). Furthermore, disruption of either the VEGF receptors or the VEGF gene itself resulted in death of murine embryos at about nine days suggesting this factor is also crucial in embryonic development (Fong et al., 1995, Shalaby et al., 1995, Carmeliet et al., 1996, Ferrara et al., 1996).

VEGF has been detected in unstimulated and stimulated whole saliva in the majority of human subjects tested, although there appears to be a large range in the mean levels found. Pammer et al. (1998) found the mean unstimulated saliva VEGF concentration in 24 subjects to be 1400pg ml^{-1} (SD$\pm$770pg ml^{-1}) compared to Booth et al. (1998) who found the mean to be around 2500pg ml^{-1} (the actual mean and standard deviation was not available in the paper) in 12 subjects. In both papers it was unclear if any of the healthy subjects were either smokers or previous smokers, and there was no indication of the time of day the saliva was collected in the Booth et al. (1998) paper, both of which could influence the findings. The Brozovic et al. (2002) study may give a more reliable mean unstimulated saliva VEGF concentration, 1652pg ml^{-1}(SD$\pm$567pg ml^{-1}), as they clearly stated that their control group (n=27) of healthy individuals were systemically and orally healthy, non-smokers and diurnal variation was taken into account. Taichman et al. (1998) is the only study to examine VEGF concentrations in stimulated whole saliva and from the individual major salivary glands. Mean VEGF concentrations were 693pg ml^{-1} (SD$\pm$543pg ml^{-1}) for stimulated whole saliva, 424pg ml^{-1} ($\pm$470pg ml^{-1}) for parotid saliva and 131pg ml^{-1} ($\pm$100pg ml^{-1}) for submandibular-sublingual saliva. Apart from the submandibular-sublingual saliva, the saliva VEGF concentrations were significantly higher than that found in the subject's serum (168$\pm$58pg ml^{-1}). Once again, certain confounding factors were not taken into account in this study regarding smoking, time of day of saliva collection, small number of subjects relative to the age range and the possible effects of various medical conditions and their associated medication. To date there is no evidence that salivary VEGF concentration is significantly affected by age. This has been supported by an immunohistochemistry study which showed no statistical difference in VEGF expression in minor salivary glands with age (de Oliveira et al., 2002). Some of the deficiencies in the above studies will be addressed in this thesis (Chapter 3.5).

Evidence suggests that VEGF is produced by the parotid, submandibular and minor salivary glands as VEGF and VEGF mRNA have been localised in serous acinar cells (Pammer et al., 1998, Taichman et al., 1998). Taichman et al. (1998) speculated that as there was little evidence of angiogenesis occurring within healthy salivary glands, the production of VEGF may play a role in the maintenance of the salivary gland tissue itself. However, VEGF was also expressed in the ductal cells and in the infiltrating inflammatory cells in chronically inflamed salivary tissue, which possibly accounts for the increased salivary tissue vascularity found in some chronic salivary gland conditions such as sialadentitis (Pammer et al., 1998).

1.5.4 Angiogenic inhibitors in oral wound healing and saliva

1.5.4.1 Angiostatin

Angiostatin is an endogenous angiogenic inhibitor derived by the proteolytic cleavage, probably by MMP-2/3/7/9 and 12, of the amino-terminal of plasminogen which contains the first four Kringle domains (Cao et al., 1996, Cornelius et al., 1998). The resultant 38kDa fragments have been shown to have anti-angiogenic properties by the inhibition of endothelial cell migration, tube formation and proliferation (O'Reilly et al., 1994, Claesson-Welsh et al., 1998). Kringle 1-3 fragment appears to be mainly responsible for the inhibition of endothelial cell proliferation, while fragments containing Kringle 4 inhibit endothelial cell migration (Ji et al., 1998). The exact mechanism of angiostatin's anti-angiogenic action is poorly understood, but several mechanisms have been proposed. Angiostatin is known to bind to both mitochondrial and endothelial cell surface ATP synthase, the latter causing inhibition of the proton pump, which leads to a reduction in intracellular pH and apoptosis (Moser et al., 1999). Another proposed mechanism is that angiostatin, like plasmin, binds to $\alpha_v\beta_3$ integrin due to it containing an RGD sequence, which leads to inhibition of plasmin-induced endothelial cell migration (Tarui et al., 2001). Finally, angiostatin binds to cells which contain angiomotin, which enables angiostatin to be internalised into the cell. This leads to induction of Focal Adhesion Kinase (FAK), which is involved in cell motility, adhesion-dependent cell survival and proton transport. This is thought to inhibit endothelial cell migration and induce apoptosis (Plopper et al., 1995). To date there have been no

publications which have reported angiostatin concentrations in human saliva or GCF levels in periodontal health and disease.

1.5.4.2 Endostatin

Endostatin is a matrix-derived 20kDa carboxy-terminal fragment of Collagen XVIII C terminal domain released by proteolytic cleavage and was initially found to inhibit growth of primary tumours and metastasis in several types of tumour in mice (Boehm et al., 1997, O'Reilly et al., 1997). Endostatin has been shown to inhibit endothelial cell proliferation, migration and survival along with promoting new vessel stabilisation leading to inhibition of angiogenesis (O'Reilly et al., 1997, Yamaguchi et al., 1999, Dixelius et al., 2000, Ergun et al., 2001, Skovseth et al., 2005, Zhuo et al., 2011). Several *in vitro* studies have shown that endostatin interferes with the action of several angiogenic factors, such as HIFα-1, which may account for its anti-angiogenic properties (Abdollahi et al., 2004). Hanai et al. (2002) reported that endostatin inhibited the Wnt signalling pathway leading to suppressed VEGF and FGF-2 induced endothelial cell migration. Other studies have suggested that endostatin interferes with FGF-2 signal transduction and cell-matrix adhesion, notably by the interaction with integrin $\alpha_v\beta_1$ on the cell surface of endothelial cells, leading to reduced endothelial cell motility (Dixelius et al., 2002, Wickstrom et al., 2002). Although caution is needed with the interpretation of these studies due to the form of endostatin used, as with many factors, endostatin's behaviour appears to vary depending on whether it is in the soluble or immobilised forms. Rehn et al. (2001) suggested that only soluble endostatin inhibits endothelial cell migration again by interacting with integrins. Another possible mechanism is by direct interaction of endostatin with VEGF-R2 receptor kinases leading to reduced VEGF-mediated signalling (Kim et al., 2002, Schmidt et al., 2005). Endostatin is also thought to induce apoptosis in endothelial cells by down-regulating a variety of genes in growing endothelial cells, including genes for the anti-apoptotic proteins Bcl-2 and Bcl-XL (Dhanabal et al., 1999). Endostatin may inhibit endothelial migration and tumour invasion by inhibiting the activation and activity of MMP-2, 9 and 13 (Kim et al., 2000, Nyberg et al., 2003).

Currently, there have been no publications which have reported the endostatin concentrations in human saliva or GCF either in oral health or disease. Furthermore, endostatin expression has not been reported in human gingival or periodontal tissues, either in periodontal health or periodontal disease. As far as can be ascertained, there have been no publications which have examined the balance between endostatin and pro-angiogenic factors, such as Ang-1, MSF or VEGF in oral health or in periodontal disease. This will be addressed in Chapter 3.6.

1.5.4.3 Thrombospondin (TSP)

The thrombospondin family consists of five members, numbered TSP-1 to 5 respectively, and contains two subgroups. Only TSP-1 and TSP-2, which structurally consist of three identical subunits (homotrimers), have been shown to have anti-angiogenic effects (Armstrong and Bornstein, 2003). TSP-1 has been shown to have multiple biological functions including anti-angiogenic effects, induction of apoptosis, activation of TGF-β, immune regulation effects and protease activation (Nyberg et al., 2005). TSP-1 has multiple receptors including CD36, CD47 and integrins. TSP-1 is thought to inhibit angiogenesis through the interaction with the scavenger receptor CD36 expressed on endothelial cell surfaces, which leads to inhibition of endothelial cell proliferation and migration, and by directly suppressing FGF-2 mediated angiogenesis (Dawson et al., 1997, Armstrong and Bornstein, 2003). Furthermore, the binding of TSP-1 and its fragments to CD36 is thought to lead to the activation of Fas and caspases leading to endothelial cell apoptosis (Jimenez et al., 2000). Like TSP-1, TSP-2 anti-angiogenic activity appears to be mediated through the activation of CD36, but unlike TSP-1, it lacks the ability to activate TGF-β (Simantov et al., 2005). Noh et al. (2003) also showed TSP-2 inhibited VEGF induced endothelial cell migration, tube formation and increased endothelial cell apoptosis *in vivo*.

In a small study by Crombie et al. (1998), the mean TSP-1 concentration in whole unstimulated saliva was found to be 4.1µg ml^{-1} (range 1.1-12.8µg ml^{-1}). Likewise, TSP-2 concentrations have been reported in unstimulated saliva in a larger study, where it was found to be significantly raised in OSCC (n=49, mean 12.90ng ml^{-1}, (SD)$\pm$32.81ng ml^{-1}) compared with healthy controls (n=47, mean 0.68ng ml^{-1}, (SD)$\pm$0.73ng ml^{-1}) (Hsu et al., 2014).

Currently, there have not been any studies which have examined either TSP-1 or TSP-2 in oral fluids (saliva or GCF) in periodontal health or disease. Likewise, no studies have examined the effect of smoking or diabetes on TSP-1 or TSP-2 levels in oral fluids.

1.6 Periodontal disease

1.6.1 Introduction

Periodontal disease is a group of multifactorial inflammatory diseases affecting the supporting structures of the dentition involving the complex interaction between bacterial plaque biofilms, the host inflammatory/immune response, genetic/epigenetic factors and various modifying risk factors (Offenbacher et al., 2009, Meyle and Chapple, 2015). The two most prevalent periodontal diseases are gingivitis, which causes no permanent damage to the periodontal support, and periodontitis, which results in bone loss and loss of attachment (LOA) (clinical attachment loss). At the time of planning this thesis, the 1999 International Workshop for the Classification of Periodontal Diseases and Conditions was the standard classification system used in periodontal research (Armitage, 1999). Hence, the clinical study selection criteria used in this thesis are based on the 1999 Periodontal Classification (Chapter 2.1.2 and 2.1.3). Subsequently, at the time of writing this thesis the World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions (2017) had been published (Table 1.3) (Caton et al., 2018), therefore it has been necessary to refer to both classifications in this thesis. The 2017 World Workshop Periodontal Classification was developed following significant advances in periodontal research into the relationship between plaque biofilms and the host response during the transition from periodontal health (symbiosis) to gingivitis and periodontitis (dysbiosis) (Meyle and Chapple, 2015, Caton et al., 2018, Rosier et al., 2018).

Table 1.3 World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions (2017) had been published (Caton et al., 2018).

i) Periodontal health, gingival diseases and conditions (Chapple et al., 2018):	
	1. Periodontal health and gingival health (Lang and Bartold, 2018).
	2. Gingivitis - dental biofilm-induced (Murakami et al., 2018, Trombelli et al., 2018).
	3. Gingival diseases – non-dental biofilm-induced.
ii) Periodontitis (Papapanou et al., 2018a):	
	1. Necrotising periodontal diseases.
	2. Periodontitis as a manifestation of systemic diseases.
	3. Periodontitis (Tonetti et al., 2018).
iii) Other conditions affecting the periodontium* (Jepsen et al., 2018).	
iv) Peri-implant diseases and conditions* (Berglundh et al., 2018).	

* These categories are sub-divided but are not outlined as they are not relevant to this thesis.

One of the key changes in the 2017 Periodontal diseases classification was the definition of clinical periodontal health, which had not previously been formally defined, as being cases with no LOA, pockets depths 3mm or less (≤4mm, if post treatment) with less than 10% sites with bleeding on probing (BOP) (Chapple et al., 2018). This differs slightly from the definition for periodontal health used in this thesis study protocol (Palmer and Floyd, 1995) (Chapter 2.1.2), which used BOP of 15% or less. This means that a small number of subjects in the periodontally healthy (sub-group A) categories in this thesis would now be defined according to the 2017 Periodontal classification as having localised gingivitis (i.e. subjects with BOP scores from 10% to 15%), although it is unlikely that this issue would have had a significant impact on the findings of the present study.

1.6.1.1 Gingivitis

Clinical features of gingivitis include the classical signs of inflammation (redness and oedema but generally no pain), loss of mucosal stippling, increased flow of gingival crevicular fluid (GCF), BOP and increased pocket depths but no LOA or bone loss (Loe et

al., 1965, Loe and Holm-Pedersen, 1965, Muhlemann and Son, 1971, Mariotti, 1999, Trombelli et al., 2018). Evidence from the classic experimental gingivitis studies demonstrated that gingivitis is a plaque-induced inflammatory disease (Loe et al., 1965), which does not inevitably progress to periodontitis in every individual or at every site in a susceptible individual (Lang et al., 1973, Baelum et al., 1988). Epidemiological studies have shown that gingivitis has a high prevalence globally across all age groups, although there are large variations in the prevalence data due to the inconsistencies in the epidemiological criteria used (Hugoson and Jordan, 1982, Stamm, 1986, Brown et al., 1989, White et al., 2012, Trombelli et al., 2018). Furthermore, like periodontitis, the prevalence of gingivitis is increased by a number of risk/modifying factors such as poor restorative margins, which promote plaque accumulation, and hormonal changes associated with puberty, pregnancy and the early forms of the oral contraceptive pill (progesterone and oestrogen) (Sutcliffe, 1972, Mombelli et al., 1989, Nakagawa et al., 1994, Mariotti and Mawhinney, 2013, Niederman, 2013).

1.6.1.2 Periodontitis

Periodontitis shares several of the clinical features of gingivitis but also causes varying degrees of permanent damage to the periodontal support (bone loss and LOA) resulting in gingival recession, increased tooth mobility, furcation involvement, migration (drifting) and in severe cases, tooth loss (Papapanou et al., 2018a, Tonetti et al., 2018). The resultant damage caused by periodontitis can lead to significant aesthetic and functional issues, leading to impaired quality of life and expensive dental care (Ferreira et al., 2017).

One of the most significant changes in 2017 Periodontal Classifications is the incorporation of aggressive periodontitis (AgP) (1999 Classification) (Armitage, 1999) into the periodontitis category (Caton et al., 2018). Although, the clinical features of localised AgP are probably the best defined disease characteristics in periodontology, there is no clear diagnostic criteria to justify retaining AgP as a separate disease entity (Papapanou et al., 2018a, Tonetti et al., 2018). Therefore, periodontitis is considered to cover a wide spectrum of patients whose susceptibility will vary greatly from highly susceptible patients, who develop rapidly destructive periodontitis at a young age (formerly classified as AgP), to those

who develop mild periodontitis by old age. This change to the classification does not impact upon the selection criteria for this thesis, as the subjects with very high susceptibility to periodontitis, which equated to AgP or now Stage 3/4 Grade C in the 2017 classification, were excluded from the study (Chapter 2.1.3).

Data from epidemiological studies show the high prevalence of periodontitis with approximately 50% of adults over 30 years old in the United States having periodontitis and approximately 10-15% globally affected by severe periodontitis (Eke et al., 2012, Kassebaum et al., 2014). Furthermore, the key longitudinal epidemiological study by Loe et al. (1986) on Sri Lankan tea workers showed approximately 10% were at risk of rapid disease progression, 80% from moderate disease progression and 10% were resistant to periodontitis, which did not progress beyond gingivitis. This study also showed that in this cohort, who had limited access to dental care, plaque and calculus alone could not account for individual susceptibility for periodontitis and other risk factors, such as genetics, must also be involved. As with the gingivitis prevalence studies, there are large variations in the prevalence data reported for periodontitis due to the variation in the disease criteria used.

Studies have shown that gingivitis does not inevitably progress to periodontitis in all individuals or affect all sites equally in susceptible subjects, suggesting that innate susceptibility varies greatly between individuals (Baelum et al., 1986, Loe et al., 1986). Currently, there are no reliable means of predicting which sites, if any, will progress in any individual (Haffajee et al., 1983, Lang et al., 1986, Claffey et al., 1990, Kaldahl et al., 1990, Lang et al., 1990, Grbic et al., 1991, Haffajee et al., 1991). Clinically BOP from the base of the pocket is commonly used to assess disease activity, but this has been shown to be a poor indicator of future disease progression. Lang et al.(1986) showed that sites with persistent BOP on four consecutive occasions had a 30% risk of progression, although no attempt was made to account for the masking effect of smoking on BOP. However, a similar study found that 98% of sites with persistent lack of BOP showed evidence of long-term periodontal stability making lack of BOP a more reliable indicator (Lang et al., 1990, Lang et al., 2009). Although the possession of risk factors, such as smoking, have been shown to increase the likelihood of periodontal disease progression, the best predictor for future disease progression is the previous disease experience (Machtei et al., 1997, Machtei et al., 1999).

Evidence from early epidemiological studies, which examined population levels of LOA in relation to age, suggested that the progression of periodontitis was an inevitable continuous process (linear model) unless treated (Loe et al., 1978). This theory did take into account that some subjects were more susceptible and had more rapid disease progression. Later longitudinal studies, which examined the behaviour of individual untreated periodontal sites, suggested that periodontitis is a dynamic condition involving progression, remission and periods of inactivity (Goodson et al., 1982). This was termed the Random Burst Theory by Socransky et al. (1984) where episodic bursts of periodontal destruction occurred at random sites throughout the mouth, followed by long periods of quiescence, while other sites remained stable (Socransky et al., 1984). An alternative model, the Asynchronous Multiple Burst Model, proposed clustered bursts of periodontal disease progression over a relatively short period of time followed by periods of quiescence. However, these studies were conducted using manual periodontal probes which required high thresholds (2.5-3mm) in order to detect changes in pocket depths/LOA. The later use of significantly more sensitive electronic pressure-sensitive periodontal probes suggested that the majority of progressive sites underwent a more gradual continuous LOA, with a small percentage of sites undergoing a more burst-like pattern (Jeffcoat and Reddy, 1991). Current thinking is that periodontitis progresses in a more gradual continuous pattern with the potential for some sites to undergo more rapid burst-like progression, particularly in subjects with higher susceptibility.

1.6.2 Aetiology and pathogenesis of periodontitis

Although the histological changes that take place between periodontal health, gingivitis and periodontitis described by Page and Schroeder (1976), updated by Kinane et al. (2008) and summarised by Preshaw (2019), are still relevant there has been a significant shift in philosophy in recent years regarding the relationship between the periodontal plaque biofilm and the host response (inflammatory and immunological). Evidence from experimental gingivitis studies have demonstrated that clinically healthy human gingival connective tissues contain low levels of inflammatory infiltrate, which is referred to as the physiological immune surveillance, even when extremely high levels of oral hygiene are maintained long-term (Brecx et al., 1987a, Brecx et al., 1987b, Lang and Bartold, 2018). Therefore, it is now

recognised that in clinical periodontal health a state of symbiosis is maintained between the plaque biofilm (low pathogenicity) in the gingival sulcus and the low levels of inflammatory infiltrate in the underlying gingival connective tissue. However, the balance in the symbiotic relationship is disrupted if sufficient quantities of undisturbed supra-gingival plaque accumulates for sufficient time at the gingival margin. Depending on the patient's susceptibility, which is largely dictated by genetic factors, this will lead to gingival inflammation (gingivitis), as demonstrated by the classical experimental gingivitis studies (Loe et al., 1965, Theilade et al., 1966, Loe et al., 1967, Jensen et al., 1968, Loe and Schiott, 1970). This evidence was used to support the non-specific plaque hypothesis, which essentially proposed that the quantity of plaque, and the associated virulence factors, overwhelms the host response leading to gingival inflammation (Theilade, 1986).

Although the non-specific plaque hypothesis could account for the development of gingivitis, it does not explain why some subjects never progress from gingivitis to periodontitis even in the presence of long-term poor oral hygiene (Baelum et al., 1986, Loe et al., 1986). Subsequently, evidence from checkerboard DNA-DNA hybridisation studies demonstrated close associations between microbial complexes of specific periodontal pathogenic bacteria (orange and red complexes) in periodontitis (Socransky et al., 1994, Socransky et al., 1998). This led to the development of the specific plaque hypothesis where bacteria, such as the red complex bacteria *Porphyromonas gingivalis (Pg)*, *Tannerella forsythia (Tf)* and *Treponema denticola (Td)*, were found to be associated with the development and progression of periodontitis (Loesche, 1976, Loesche, 1979, Socransky et al., 1994, Socransky et al., 1998). However, this theory could not adequately account for all the characteristics associated with periodontitis, for instance the presence of low levels of the supposedly pathogenic periodontal bacteria in healthy sites and the inability to cause periodontitis by inoculating sites with periodontal pathogens, such as *Aggregatibacter actinomycetemcomitans* (*Aa*) (Christersson et al., 1985, Bartold and Van Dyke, 2013).

Essentially, the non-specific and specific plaque hypotheses suggested that the development of periodontitis is primarily driven by changes in the ecology of the bacterial plaque biofilm, from the predominately Gram positive aerobes in supragingival plaque in health, to a relatively small number of pathogenic bacterial species in the subgingival plaque biofilms in

periodontitis. The development of gingivitis and periodontitis is now thought to be primarily driven by the host response to the presence of plaque bacteria, and if other modulating factors are present (e.g. genetic/epigenetic, behavioural and/or environmental risk factors), the host response leads not only to the majority of the periodontal tissue destruction associated with periodontitis, but also to creating the environment to allow the growth of the pathogenic anaerobic bacteria (Grossi et al., 1994, Marsh, 2003, Bartold and Van Dyke, 2013). This equates to the ecological plaque hypothesis proposed by Marsh (1994), which describes the synergistic balance between host response and the plaque biofilm in periodontal health (symbiosis) and the breakdown of this relationship (dysbiosis) during the development of gingivitis through to periodontitis. For example, the host response to plaque not only leads to inflammatory swelling and the development of periodontal pockets, which creates a sheltered environment supporting the ecological shift to anaerobic bacterial species, but in susceptible patients, to the release of excessive pro-inflammatory mediators which further drives the host response (Marsh, 2003, Curtis et al., 2020). Furthermore, tissue damage from the release of various host factors, such as matrix metalloproteinases, oxygen radicals and various cytokines, leads to the release of products which act as nutrients for the growth of particular pathogenic species. For example, the release of haem favours the growth of *Pg* (McKee et al., 1986).

There are, however, many unresolved questions, such as what are the underlying mechanisms which trigger the progression from gingivitis to periodontitis in some patients/individual sites, but not in others? Further research is on-going to resolve these issues, but it is likely to involve complex changes in the dynamic relationship between the host response and the plaque biofilm leading to changes in the inflammatory drive. These changes are likely to involve genetic and epigenetic factors, as well as behavioural, environmental and nutritional factors (Kornman et al., 1997, Michalowicz et al., 2000, Genco and Borgnakke, 2013, Meyle and Chapple, 2015). There is increasing evidence that epigenetic factors can influence the expression of a wide range of factors including pro-inflammatory mediators and angiogenic factors. For example, Offenbacher et al. (2009) showed significant changes in gene expression between periodontal health and gingival inflammation using a human experimental gingivitis model. They reported that development of gingivitis resulted in raised levels of expression of a wide range of genes for pro-inflammatory mediators, such as IL-1α,

IL-1β and IL-8R1, as well as angiogenic factors, such as Angiopoietin-like 1, which is resolved following the return to gingival health. Interestingly, EGF gene expression is reduced during the development of gingivitis and increased on the return to gingival health. However, this study examined only a small number of the likely genes affected in periodontal inflammation and it is likely to be many years before there is a detailed understanding of the complex interactions between all the genes involved. What is important, in terms of the management of gingivitis and periodontitis, is that the host-biofilm relationship is bi-directional allowing conventional periodontal therapy (i.e. risk factor management, debridement, etc) to restore a healthy symbiotic relationship in most cases (Curtis et al., 2020).

1.6.2.1 Vascular changes in periodontal inflammation

Multiple studies in both animal models and humans have shown that plaque-induced gingival inflammation results in vasodilation, activation of inactive capillary beds and angiogenesis, with increasing vascularity in the gingival connective tissue from gingival health to periodontitis (Page and Schroeder, 1976, Hock and Kim, 1987, Zoellner and Hunter, 1991, Bergstrom, 1992, Bonakdar et al., 1997, Johnson et al., 1999a, Chapple et al., 2000, Vladau et al., 2016). However, there have been few studies in humans which have investigated changes in angiogenic factor expression in relation to changes in gingival vascularity in plaque-induced inflammation. Johnson et al. (1999a) reported significantly raised vascular density and tissue concentrations of VEGF and IL-6 in severe periodontitis compared with periodontal health, although the highest VEGF concentrations was reported in gingival tissue from moderate periodontitis (pocket depths 4mm-6mm with BOP). The group speculated that the reduced VEGF concentration in severe periodontitis (pocket depths greater than 6mm with BOP) was probably due to the higher chance of enzymatic degradation or being bound to heparin in deeper pockets. In an immunohistochemistry study using gingival biopsies harvested following extractions, Chapple et al. (2000) reported higher FGF-2 and VEGF expression in periodontally health (n=12) compared with severe periodontitis (n=10). Consistent with previous studies, vascular density in the gingival connective tissue sub-adjacent to the pocket epithelium was significantly higher in severe periodontitis compared

with the gingivally healthy samples. There were no significant differences in the FGF-2 staining profile, which was mainly associated with the basement membranes of vessels, between the periodontally healthy and severe periodontitis samples. VEGF staining was found to be associated with vessels, inflammatory cells and keratinocytes, although reduced staining intensity was found in the pocket epithelium. In a larger study, Vladau et al. (2016) reported significantly increased vascularity associated with increased expression VEGF and VEGF receptor-2 (VEGFR2) in endothelial and epithelial cells between periodontal health and severe periodontitis. However, further research is required to ascertain the role of the various angiogenic factors and inhibitors in the progression of periodontitis.

1.6.3 Risk factors for periodontitis

A further complication to the understanding of the development and progression of periodontitis is the presence of risk or modifying factors. Although bacterial plaque is the principal aetiological factor for periodontal disease, it is clear that the disease trajectory can be greatly influenced by modifying risk factors (Salvi et al., 1997b, Genco and Borgnakke, 2013). A risk or modifying factor can be defined as an aspect of personal behaviour or lifestyle, an environmental exposure, or an inborn or inherited characteristic, which on the basis of epidemiological evidence is known to be associated with a health-related condition (Last, 1988). Although a risk factor may increase the probability of an individual acquiring a disease, it does not necessarily imply that there is a direct cause and effect relationship.

Systemic risk (modifying) factors for periodontal disease include genetic factors (Beaty et al., 1993, van der Velden et al., 1993, Hart and Kornman, 1997, Kornman et al., 1997, McDevitt et al., 2000, Michalowicz et al., 2000, Loos and Van Dyke, 2020), haematological disorders (Seymour and Heasman, 1992), behavioural risk factors such as smoking (Haber, 1994, Haffajee and Socransky, 2001) and alcohol consumption (Tezal et al., 2001, Pitiphat et al., 2003, Tezal et al., 2004, Park et al., 2014), environmental risk factors such as drug influenced gingival enlargement (Seymour and Heasman, 1992), metabolic risk factors such as diabetes (Glavind et al., 1968, Cianciola et al., 1982, Shlossman et al., 1990, Emrich et al., 1991, Oliver and Tervonen, 1994, Genco and Borgnakke, 2013) and life-style risk factors such as stress (Moss et al., 1996, Croucher et al., 1997, Genco et al., 1999, Aleksejuniene et

al., 2002, Vettore et al., 2003, Rai et al., 2011, Warren et al., 2014). The major modifiable systemic risk factors for periodontitis, smoking and diabetes which are directly relevant to this thesis, will be discussed in more detail in Chapters 1.7 and 1.8 respectively.

Local risk (predisposing) factors are essentially plaque retentive factors such as calculus, overhanging restorations, furcation involvements, root grooves and mal-aligned teeth (Leknes et al., 1994, Chapple et al., 2015, Chapple et al., 2018). Ultimately, the management of modifiable risk factors (e.g. through oral hygiene instruction, smoking cessation and the removal of plaque retentive factors) is fundamental for successful management of periodontal disease and maintaining long-term stability.

1.6.4 Association between periodontitis and systemic disease

Since Mattila et al. (1989) proposed a link between poor dental health and acute myocardial infarction there been great interest into whether there are associations between periodontal disease and various systemic diseases such as cardiovascular disease (CVD) (Genco et al., 2002, Janket et al., 2003, Bahekar et al., 2007, Carrizales-Sepulveda et al., 2018), Type II diabetes (Khader et al., 2006, Nascimento et al., 2018), kidney disease (Deschamps-Lenhardt et al., 2019, Kapellas et al., 2019, Zhao et al., 2020), pre-term pregnancy and low birth weight (Offenbacher et al., 1996, Corbella et al., 2016, Manrique-Corredor et al., 2019), rheumatoid arthritis (Kaur et al., 2013, Fuggle et al., 2016) and dementia/Alzheimer's disease (Leira et al., 2017, Nadim et al., 2020). However, these conditions have complex multifactorial aetiologies with evidence of both genetic and environment input, often sharing the same risk factors as periodontal disease such as smoking, stress, poor diet and low socio-economic status. Therefore, evidence may show an association between various systemic conditions and periodontitis, but there is generally insufficient evidence of a direct cause and effect relationship between them (Williams et al., 2008, Cullinan and Seymour, 2013).

The key issue arising from these studies is that it provides evidence of the intimate connection between the periodontal tissues and the systemic circulation. Studies have shown that during periodontal inflammation the surface area of the exposed ulcerated periodontal pockets open to the systemic circulation is extensive, perhaps as high as $300cm^2$ in a subject with

generalised 6mm-7mm pocketing (Waite and Bradley, 1965, Offenbacher, 1996, Hujoel et al., 2001, Park et al., 2017). Immediately adjacent to the ulcerated periodontal pocket walls is the underlying periodontal connective tissue, which is effectively a large highly vascular chronically inflamed soft tissue wound covered in plaque biofilm. Therefore, it has been proposed that periodontal pathogenic bacteria from the periodontal pocket biofilm and/or inflammatory mediators from the chronically inflamed periodontal connective tissue can readily enter the systemic bloodstream leading to systemic effects. For example, evidence from a human autopsy study of carotid endarterectomy specimens reported 44% of samples were positive to one or more putative periodontal pathogens (*Aa*, *Prevotella intermedia* (*Pi*), *Pg* and *Tf*) (Haraszthy et al., 2000). There is increasing evidence to support the theory that periodontal inflammation contributes to systemic inflammation, potentially exacerbating pre-existing chronic systemic conditions (e.g. cardiovascular disease, diabetes, etc.) (D'Aiuto et al., 2013, Pink et al., 2015). For example, several studies have associated periodontitis with increased systemic levels of factors including C-reactive protein (CRP), IL-1β, IL-2, TNFα and IFN-γ compared to periodontal health (Gorska et al., 2003, Paraskevas et al., 2008, Noack et al., 2001, Roca-Millan et al., 2018). Furthermore, meta-analyses of the literature have also demonstrated that periodontal therapy results in significant reductions in systemic CRP levels (Paraskevas et al., 2008, Freitas et al., 2012, Roca-Millan et al., 2018). However, there are currently insufficient long-term, large scale intervention studies, with the exception of poorly controlled diabetes, which provide any evidence that periodontal therapy reduces the severity or slows the progression of these systemic conditions (Beck et al., 2019).

1.6.5 Gingival Crevicular Fluid (GCF)

Gingival Crevicular Fluid (GCF) is generally considered to be a transudate of serum in gingival health and an inflammatory exudate in periodontal inflammation (Uitto, 2003, Wassall and Preshaw, 2016). GCF is derived from the post-capillary venules of the dentogingival plexus sub-adjacent to the junctional epithelium, but also contains various factors from the periodontal tissues including neutrophils, pro-inflammatory mediators and angiogenic factors (Attstrom, 1971, Lamster, 1992, Armitage, 2004). GCF flows between the keratinocytes of the junctional epithelium into the gingival sulcus/periodontal pocket and

finally enters the oral cavity to become a minor constituent of saliva (Booth et al., 1998). Similar to saliva, GCF flow is subject to circadian rhythm and is generally higher during the day than at night (Bissada et al., 1967). Experimental gingivitis studies demonstrated that plaque-induced inflammation causes vasodilation and increased vascular permeability within the gingival tissues leading to increased GCF flow (Garnick et al., 1979, Armitage, 1995). In gingival health, especially in smokers, it can be extremely difficult to accurately record the GCF flow rate due to the very low volumes involved, particularly if below 0.1µl (Persson et al., 1999, Griffiths, 2003). GCF collection is considered to be a relatively non-invasive procedure allowing factors to be sampled from the gingival tissues without the need for biopsy (Griffiths, 2003, Armitage, 2004, Wassall and Preshaw, 2016). However, it is extremely time consuming and technique-sensitive in terms of its collection, processing and storage (Curtis et al., 1988, Wassall and Preshaw, 2016). Various methods are available to collect GCF from the gingival crevice/pocket, including washing, micropipettes and by the use of filter papers, such as the PerioPapers used in the present study (Skapski and Lehner, 1976, Griffiths et al., 1988, Salonen and Paunio, 1991, Griffiths, 2003). The use of filter papers has the advantage over the other techniques as it allows immediate measurement of the volume of GCF using a calibrated Periotron (Preshaw et al., 1996, Ciantar and Caruana, 1998, Chapple et al., 1999, Wassall and Preshaw, 2016). Details of the protocols used in this thesis to collect and quantify the volumes of GCF are outlined in Chapter 2.1.8 and in Appendix 4.

1.6.6 Angiogenic factor levels in periodontitis

1.6.6.1 Angiogenin (ANG)

ANG was first detected in GCF by Sakai et al. (2006) as part of a GCF cytokine array study examining differences in various cytokine and growth factor levels in periodontal health and disease. Significantly higher signal intensity for ANG was found in periodontitis (53%) compared with the periodontal health (11%), suggesting ANG levels are significantly raised in periodontal disease. Two other studies have examined ANG in GCF in samples taken prior to and in the weeks following augmented periodontal surgical therapy versus conventional surgical grafting techniques. In both studies ANG expression peaked during the early stages

of wound healing, the first few days post-surgery, and then rapidly diminished (Morelli et al., 2011, Pirebas et al., 2018).

1.6.6.2 Angiopoietin-1 (Ang-1)

Currently there is little data on the role of Ang-1 in gingival health and disease. In a small immunohistochemistry study, which examined Ang-1 expression in human gingival biopsy tissue, Ang-1 expression was reported to be higher in periodontally diseased tissue compared with gingival health, although not significantly so (Yuan et al., 2000b). However, Ang-1 expression was only reported in a small proportion of the study samples and there was no information stated regarding the severity of the periodontal disease. A much larger study, by Lester et al. (2009), examined the concentrations of a range of inflammatory and angiogenic factors, including Ang-1, in gingival tissue harvested following extractions. Prior to sampling, the gingival health of the tissue was stratified into periodontal health and varying degrees of periodontal disease (mild, moderate and severe) using pocket depths and whether BOP was present. Gingival tissue Ang-1 concentrations were found to be inversely correlated to periodontal health status (i.e. significantly lower Ang-1 concentrations in severe periodontal disease compared with gingival health). The opposite findings were reported for other factors such as IL-1β, IL-6, TNFα, VEGF and endothelin-1. Currently, it is not known whether the reduced gingival tissue levels of Ang-1 in severe periodontitis correlates with levels of Ang-1 found in GCF or in saliva, hence salivary levels of Ang-1 will be investigated in this thesis. There have been two studies which have examined Ang-1 levels in GCF following periodontal surgery and these studies have been discussed in more detail in Chapter 3.3.1.2 (Rakmanee et al., 2010, Rakmanee et al., 2019).

1.6.6.3 Epidermal Growth Factor (EGF)

Like salivary levels of EGF, there are contradictory findings reported in the literature regarding EGF concentrations in GCF between periodontal health and disease. Chang et al. (1996) reported significantly lower GCF concentrations of EGF in deep periodontal pockets ($\geq$5mm) compared with healthy sites. EGF receptor expression and binding capacity was also found to be higher in the deep periodontal pockets, suggesting that the lower GCF

concentrations of EGF in diseased sites was due to increased receptor binding. In a significantly larger study, Mogi et al. (1999) also reported lower EGF concentrations in severe periodontitis compared with healthy controls, although the results were not significant. However, in a more recent antibody array analysis study, GCF levels of EGF were significantly higher in severe periodontitis compared with periodontal health (Sakai et al., 2006). Whether the difference in these findings is technique-based or due to other factors, is currently unknown.

1.6.6.4 Fibroblast Growth Factor-2 (FGF-2)

Currently, there have been few studies which have reported FGF-2 in GCF in periodontal health and disease, although there have been several studies which have reported levels following periodontal therapy, conventional periodontal surgery and regenerative techniques (Rakmanee et al., 2010, Zekeridou et al., 2017, Pirebas et al., 2018). However, Zekeridou et al. (2017) reported significantly lower GCF concentrations of FGF-2 in subjects with severe periodontitis, in both shallow and deep periodontal pockets, compared with periodontally healthy subjects. Interestingly, Ozdemir et al. (2016) reported no significant difference in GCF levels of FGF-2 between periodontal health and gingivitis in both smokers and non-smokers. Taken together, the findings of these two studies suggest that the role of FGF-2 changes between periodontal health-gingivitis and the development of periodontitis.

1.6.6.5 Platelet Derived Growth Factor (PDGF)

In terms of PDGF levels in periodontal disease, Pinheiro et al. (2003) reported significantly higher PDGF expression in human gingival biopsy tissue from periodontitis sites compared with periodontally healthy sites. Furthermore, PDGF expression was most prominent in the periodontal pocket epithelial cells and the underlying pocket wall connective tissue cells. Interestingly, the findings of this study do not correspond to the PDGF levels reported in GCF. For example, Zhu et al. (2015) reported no significant difference in serum or GCF PDGF concentrations in subjects with cardiovascular disease and periodontitis compared with healthy controls groups. Likewise, no significant difference was found in GCF concentrations of PDGF between healthy controls and subjects with severe periodontitis

(generalised aggressive periodontitis) (Romano et al., 2018). However, GCF levels of PDGF were found to be significant lower in periodontally healthy smokers compared with non-smokers controls (p=0.014) (Eren et al., 2015).

Several studies have detected high levels of PDGF during the early stages of human oral wound healing, following which the PDGF levels reduce (Green et al., 1997, Morelli et al., 2011, Pirebas et al., 2018, Rakmanee et al., 2019). Furthermore, the therapeutic addition of PDGF and IGF-1 has been shown to result in significantly improved periodontal regeneration around teeth and implants in surgical studies in dogs, suggesting PDGF may have a potential use in human periodontal regenerative surgery (Lynch et al., 1991, Stefani et al., 2000, Li et al., 2017).

1.6.6.6 Vascular Endothelial Growth Factor (VEGF)

VEGF levels in GCF have been widely reported to be significantly raised in subjects with periodontitis compared with healthy controls (Booth et al., 1998, Lee et al., 2003, Guneri et al., 2004, Prapulla et al., 2007, Pradeep et al., 2011, Padma et al., 2014, Sakallioglu et al., 2015, Zhu et al., 2015, Pannicker and Mehta, 2016, Turer et al., 2017, Romano et al., 2018, Afacan et al., 2019, Tayman et al., 2019), with levels of which being significantly reduced following periodontal therapy (Prapulla et al., 2007, Pradeep et al., 2011, Padma et al., 2014, Pannicker and Mehta, 2016, Turer et al., 2017, Romano et al., 2018). These studies have been discussed in more detail in Chapter 3.5.1.3.

1.6.6.7 Angiostatin

To date there have been no publications which have reported angiostatin concentrations, or other angiogenic inhibitors, in human saliva or GCF levels in periodontal health and disease. Two studies have reported elevated angiostatin levels in GCF immediately following periodontal surgery (Morelli et al., 2011, Pirebas et al., 2018). However, significantly reduced angiostatin expression has been reported in an immunohistochemistry study in gingival samples from periodontitis cases compared with periodontally health subjects (Yuan et al., 2000a).

1.7 Smoking and periodontal disease

1.7.1 Introduction

It has been estimated that smoking accounts directly for at least 30% of deaths in the developed world (Vineis et al., 2004). Although globally, the prevalence of smoking has reduced (men 41% in 1980, 31% in 2012; women 10% in 1980, 6% in 2012), the numbers of smokers is increasing due to population growth (Ng et al., 2014). Tobacco smoke is considered to be a multiple organ site carcinogen strongly linked with various cancers, especially lung cancer, as well cancers of the oesophagus, larynx, mouth, kidneys, pancreas and cervix (Warnakulasuriya et al., 2010, Preshaw et al., 2019). Smoking is also a major dose-dependent risk factor for oral cancer, especially if combined with alcohol consumption (Figuero Ruiz et al., 2004). Furthermore, smoking is a major risk factor for several chronic conditions associated with high mortality rates such as chronic respiratory diseases and various forms of cardiovascular disease (Wald and Hackshaw, 1996, Doll et al., 2004, Vineis et al., 2004). Smoking is also a major risk factor in periodontitis and is associated with significantly reduced treatments outcomes following non-surgical and surgical periodontal therapy, as well as increased risk of implant failure (Genco and Borgnakke, 2013, Nociti et al., 2015, Buduneli and Scott, 2018, Leite et al., 2018b).

Although nicotine is the most researched constituent, it is estimated that tobacco smoke contains over 4000 substances, of which at least 50 are known carcinogens or procarcinogens (Arbes et al., 2001, Roemer et al., 2004, Ho et al., 2007). Procarcinogens require metabolic activation in order to exert their toxic effects and include polycyclic aromatic hydrocarbons, nitrosamines and aromatic amines. Other toxic substances include gaseous toxins such as carbon monoxide, hydrogen cyanide, ammonia and acrolein as well as oxygen radicals and radioactive elements such as Polonium 210. Tobacco smoke also has a particulate phase containing a variety of tar compounds, including nicotine. Evidence suggests that smoking results in widespread molecular changes which significantly compromise host response and affect all stages of wound healing, including angiogenesis (Jones and Triplett, 1992, Towler, 2000, Palmer et al., 2005, Guo and Dipietro, 2010, Sorensen, 2012a, Sorensen, 2012b, McDaniel and Browning, 2014).

Nicotine in tobacco smoke is highly lipid soluble and rapidly enters the blood stream via the lungs and results in activation of the nicotine receptors in the mesostriatal dopaminergic neurones in the brain leading to dopamine release resulting in a sensation of pleasure (Pontieri et al., 1996). Due to the relatively short half-life of nicotine, approximately 1-2 hours, smokers tend to smoke regularly leading to chronic exposure to the toxic effects of tobacco smoke (Pilotti, 1980). The most common method for assessing long-term cumulative smoking exposure, or dose, in periodontal research is through smoking histories and self-reporting questionnaires. This information allows the calculation of Pack Years which is the number of packets of cigarettes smoked per day (1 packet = 20 cigarettes) multiplied by the number of years smoked or for loose tobacco 1g is the equivalent to one cigarette (Grossi et al., 1994). The conversion for cigar smoking is one small cigar is the equivalent of three cigarettes and a large cigar is the equivalent of five cigarettes (Jensen et al., 1998). However, self-reporting tends to under-estimate the true cumulative dose, due to subjects either consciously or subconsciously denying the true extent of their habit (Scott et al., 2001, Rebagliato, 2002). Various biochemical methods are available to assess current smoking status including carbon monoxide levels in exhaled air and blood (carboxyhaemoglobin) or systemic measurement of cotinine, nicotine and thiocyanate in plasma, saliva or urine (Marrone et al., 2011).

1.7.2 Association between smoking and periodontitis

Typical clinical features associated with smoking include staining, increased calculus deposits, deeper pocket depths, increased LOA and reduced evidence of gingival inflammation, visually and in terms of BOP, in comparison to the levels of plaque present (Bergstrom and Preber, 1986, Bergstrom, 1990, Grossi et al., 1994, Bergstrom et al., 2000, Dietrich et al., 2004). Radiographically, smoking is associated with increased bone loss and reduced bone density compared to non-smokers (Bergstrom and Floderus-Myrhed, 1983, Rosa et al., 2008). Smoking is also associated with reduced baseline GCF flow compared with non-smokers (Persson et al., 1999, Apatzidou et al., 2005, Rosa et al., 2008).

There is strong evidence that cigarette smoking is a major modifiable risk factor for periodontitis (Bergstrom, 1989, Grossi et al., 1994, Grossi et al., 1995, Gelskey et al., 1998,

Tomar and Asma, 2000, Calsina et al., 2002, Susin et al., 2004, Genco and Borgnakke, 2013, Leite et al., 2018a, Helal et al., 2019). Once confounding variables, such as oral hygiene, have been statistically accounted for, these studies reported odds ratios for the effect of smoking on periodontitis between two and eight depending on the study criteria used (Bergstrom, 2003). Evidence also suggests there is a dose-dependent related relationship between smoking and periodontitis (Grossi et al., 1994, Grossi et al., 1995, Tomar and Asma, 2000, Han et al., 2012). For example, Grossi et al. (1994) reported LOA was directly and positively related to the number of pack years smoked, with relative risk ranging from 2.05 for light smokers to 4.75 for heavy smokers. Smokers also have a higher risk of periodontal disease progression. Data from longitudinal studies, varying from one to ten years, demonstrate that smokers are significantly at more risk of periodontal disease progression compared with non-smokers (Machtei et al., 1997, Bergstrom et al., 2000, Paulander et al., 2004).

Smoking has also been shown to significantly compromise treatment outcomes of non-surgical periodontal treatment, compared with non-smokers, in terms of pocket depth reduction (Ah et al., 1994, Preber et al., 1995, Kaldahl et al., 1996, Grossi et al., 1997b, Haffajee et al., 1997, Machtei et al., 1998, Jin et al., 2000, Darby et al., 2005, Heasman et al., 2006), gain in clinical attachment (Grossi et al., 1997b, Haffajee et al., 1997, Machtei et al., 1997, Jin et al., 2000) and gain in bone levels (Machtei et al., 1997, Machtei et al., 1998). The majority of studies have shown clinical improvements in the periodontal parameters in smokers suggesting that smoking does not completely nullify the benefits of non-surgical periodontal treatment. Evidence also demonstrates the benefits of stopping smoking, with previous smokers having periodontal treatment outcomes between those of smokers and never smokers, with the positive effects increasing with time following cessation (Bolin et al., 1993, Krall et al., 1997, Bergstrom et al., 2000, Tomar and Asma, 2000, Preshaw et al., 2005, Heasman et al., 2006, Do et al., 2008, Chambrone et al., 2013, Leite et al., 2018a, SSY et al., 2019). Interestingly, the suppression of BOP and GCF production have been shown to return to normal levels within weeks of stopping smoking (Morozumi et al., 2004). However, more longitudinal smoking cessation studies are required to fully ascertain the potential benefits on periodontal health.

Likewise, smoking has also been shown to significantly reduce treatment outcomes of various periodontal surgical procedures, including regenerative techniques (Preber and Bergstrom, 1990, Ah et al., 1994, Tonetti et al., 1995, Kaldahl et al., 1996, Bostrom et al., 1998, Scabbia et al., 2001, Tonetti et al., 2002, Trombelli et al., 2003, Stavropoulos et al., 2004, Slotte et al., 2007, Kotsakis et al., 2015). Furthermore, smoking is associated with reduced implant treatment outcomes including increased risk of marginal bone loss and implant failure (Klokkevold and Han, 2007, Chrcanovic et al., 2015, Alfadda, 2018).

1.7.3 Effect of smoking on the periodontium

It is often suggested that the increased association between smoking and periodontal disease is due to higher levels of plaque found in smokers (Preber et al., 1980), however, studies which have taken plaque levels into account have not reported a significant association between them (Bergstrom, 1989). Evidence suggests that smoking has widespread negative effects on periodontal health through changes in the relationship between host response to the plaque biofilm, the gingival vasculature and tissue cell function. Many of these changes, especially with regard to the effects on the host tissue response and angiogenesis, are still poorly understood.

1.7.3.1 Effect of smoking on the host response

There is now widespread evidence that smoking compromises the host response, both in terms of the inflammatory and immunological response, in favour of increased tissue damage, although the exact mechanisms for this are varied and still poorly understood (Mooney et al., 2001). Smoking has been shown to have multiple negative effects on neutrophil function including reduced chemotaxis and phagocytosis, defective respiratory burst leading to reduced bactericidal effects and increased release of damaging oxidative products (Seow et al., 1994, Ryder et al., 1998, Darby et al., 2005, Xu et al., 2008). Although smoking is associated with increased levels of neutrophils in the peripheral blood there is reduced neutrophil migration into the periodontal tissues in smokers (Pauletto et al., 2000, Sorensen et al., 2004). This is thought to be due to changes in the actin component of the neutrophil cytoskeleton, resulting in reduced deformability, and to changes in the expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin

(Drost et al., 1992, Palmer et al., 2002). Vascular expression of both ICAM-1 and E-selectin have been found to be significantly higher in the periodontally inflamed sites in both smokers and non-smokers compared with healthy sites (Rezavandi et al., 2002). Furthermore, there was significantly higher number of vessels which expressed ICAM-1 in non-inflamed areas in non-smokers than smokers. This group hypothesised that the inflammatory response in smokers with periodontitis was associated with reduced expression of endothelial ICAM-1 resulting in reduced neutrophil trafficking. The same group also reported a dose-dependent increase in circulating levels of soluble ICAM-1 in the peripheral blood of smokers, which reduced to the level of non-smokers a year after stopping smoking (Palmer et al., 2002). Furthermore, nicotine has been shown *in vitro* to promote neutrophil degranulation potentially increasing the release of damaging enzymes such as elastase (Seow et al., 1994).

Potentially, many of the negatives effects of smoking on neutrophil function also apply to macrophage function in the periodontal tissues (Pabst et al., 1995). Nicotine has also been shown to significantly increase the secretion of prostaglandin-E_2 (PGE_2), an important mediator in periodontal bone loss, by *Pg* lipopolysaccharide (LPS) stimulated peripheral blood monocytes (Payne et al., 1996). Nicotine also causes up-regulation of genes involved in PGE_2 production in a human macrophage-like cell line (Koshi et al., 2007). A similar study reported that when peripheral blood monocytes from healthy non-smokers were exposed to tobacco smoke it resulted in up-regulation of several genes for factors known to be important in the pathogenesis of periodontitis including IL-1α, cyclooxygenase-2, NADH dehydrogenase, cathepsin L and plasminogen activator (Ryder et al., 2004).

1.7.3.2 Effect of smoking on periodontal pocket flora

Smoking per se does not appear to cause significantly increased supragingival plaque accumulation (Bergstrom and Preber, 1986), but the literature is split on whether smoking is associated with significant changes in the subgingival microflora, in particular on the presence of periodontal pathogens. Zambon et al. (1996) used indirect immunofluorescence microscopy to study the subgingival microflora in 628 non-smokers and 798 previous/current smokers. The prevalence of *Aa*, *Pg* and *Tf* was reported to be increased in current smokers, with the relative risk of *Tf* infection being 2.3 times that of previous or non-smokers. Studies

using other laboratory techniques have reported smokers to higher prevalence of *Td* using PCR techniques (Umeda et al., 1998), while higher prevalence of *Fn* and *Pi* were reported using culturing techniques (van Winkelhoff et al., 2001). One major study, which used checkerboard DNA-DNA hybridisation, showed not only increased prevalence of *Fn*, *Pi*, *Tf*, *Pg* and *Td* in smokers but this was particularly the case in shallow pockets (<4mm) (Haffajee and Socransky, 2001). This finding suggested that smoking promoted the development of pathogenic subgingival microflora in shallow pockets, which may strongly influence future disease progression.

However, many studies using similar techniques have failed to report any significant difference in the prevalence of periodontal pathogens in smokers (Preber et al., 1992, Darby et al., 2000, Van der Velden et al., 2003, Apatzidou et al., 2005, Natto et al., 2005). The cause of this discrepancy in the literature is likely to be due to differences in the sampling and bacterial identification methodology. Current evidence suggests that the altered host response in smokers results in changes in the subgingival micro-environment (dysbiosis) which favour the growth of a more pathogenic microflora (Haffajee et al., 1997, Darby et al., 2005, Guglielmetti et al., 2014, Feres et al., 2015, Moon et al., 2015).

1.7.3.3 Effect of smoking on gingival blood flow and vascularity

Smoking has been shown to have a strong dose-dependent suppressive effect on gingival inflammation (Dietrich et al., 2004), clinically shown by reduced BOP, although the exact cause of this effect is currently a controversial subject. For many years this was thought to be due to sympathetic-induced vasoconstrictive effects of nicotine, leading to reduced gingival blood flow (Clarke et al., 1981). This resulted in reduced pocket oxygen tension, compromised nutrient and inflammatory cell supply, and reduced removal of metabolic waste products (Hanioka et al., 2000). However, laser doppler flowmetry studies have contradicted this view (Baab and Oberg, 1987, Meekin et al., 2000, Mavropoulos et al., 2003, Morozumi et al., 2004). Baab and Oberg (1987) reported increased relative gingival blood flow, compared with resting levels, immediately following smoking in 12 young smokers. Evidence of a simultaneous minor reduction in forearm skin blood flow suggested that smoking caused peripheral vasoconstriction, but not in the gingival tissues. A similar finding

was reported by Mavropoulos et al. (2003), who hypothesised that the vasoconstrictive effect of nicotine was partially compensated for by the increased heart rate and blood pressure resulting in increased gingival blood flow. Another study was unable to demonstrate any significant difference in relative gingival blood flow between non-smokers, light and heavy smokers, although light smokers were found to have significantly increased forehead skin blood flow following smoking (Meekin et al., 2000). This group hypothesised that heavy smoking resulted in vascular tolerance in terms of gingival blood flow. However, gingival blood flow and GCF volume were found to significantly increase five days following smoking cessation in periodontally healthy male smokers (Morozumi et al., 2004). This suggested that gingival blood flow is in some way suppressed in smoking and smoking cessation results in rapid improvement in vascular supply. Interestingly, Mavropoulos et al. (2007) reported that there was no significant difference in resting gingival blood flow between smokers and non-smoker, however, smoking may result in reduced gingival blood flow in patients with periodontitis. Further studies are required to clarify the gingival blood flow characteristics in smokers, especially in periodontitis.

Evidence from immunohistochemistry and stereophotography studies which examined vascularity in gingival tissue, have reported that there were no significant differences in vascular densities in the gingival tissues between non-smokers and smokers (Persson and Bergstrom, 1998, Mirbod et al., 2001, Sonmez et al., 2003). Several studies, however, have reported significant differences in the structure of the gingival vessels between smokers and non-smokers. In a small study carried out by Mirbod et al. (2001), the gingival vasculature of smokers consisted of a higher percentage of small vessels and a lower percentage of large vessels compared with the non-smokers. This finding was consistent with a clinical study which used video capillaroscopy to study the morphology of the gingival vessels in smokers and non-smokers (Scardina and Messina, 2005). The gingival vasculature associated with smokers consisted of significantly higher numbers of smaller calibre capillaries and the papillary vessels were significantly more tortuous compared to those seen in non-smokers. Although the development of periodontitis is known to be associated with increased vascularity and vessel size, the gingival tissue is not uniformly affected (Zoellner and Hunter, 1991). When vascularity was compared between healthy and sites of periodontal

inflammation there were clear differences between smokers and non-smokers (Rezavandi et al., 2002). In non-smokers there was a significant increase in the numbers of vessels (capillaries and post-capillary venules) in the inflamed gingival tissue compared with the non-inflamed tissue. No such difference was found in the equivalent tissues in smokers. Furthermore, the number of vessels was significantly higher in the inflamed tissue in the non-smokers compared with the smokers. Therefore, the inflammatory reaction to plaque in smokers appears to result in a reduced vascular response/suppressed angiogenesis, which may in turn account for the reduced BOP in smokers (Scott and Singer, 2004, Buduneli and Scott, 2018). Currently, there have been few studies which have compared angiogenic factors levels in periodontal health and periodontitis in smokers.

1.7.3.4 Effect of smoking on gingival and periodontal fibroblasts

The importance of gingival fibroblasts in oral wound healing has been discussed in Chapter 1.4.2.4. Tissue culture studies have suggested that nicotine impairs gingival fibroblast migration, proliferation, attachment and impairs myofibroblast differentiation (Tipton and Dabbous, 1995, Tanur et al., 2000, Fang and Svoboda, 2005a, Fang and Svoboda, 2005b). Furthermore, nicotine also impaired gingival fibroblast ECM production by significantly reducing fibronectin and type I collagen synthesis, while also significantly increasing collagenase production (Tipton and Dabbous, 1995). Similar negative effects been reported as a result of exposure to volatile components of tobacco smoke, such as acrolein and acetaldehyde, on gingival fibroblast attachment and proliferation (Cattaneo et al., 2000, Poggi et al., 2002).

Similar findings have been reported in periodontal ligament (PDL) fibroblasts with nicotine causing dose-dependent inhibition of fibroblast proliferation, chemotaxis, attachment and protein synthesis (Giannopoulou et al., 1999, Chang et al., 2002). Nicotine has been reported to be present on the root surfaces in smokers with periodontitis and its levels can be reduced by non-surgical periodontal treatment (Cuff et al., 1989). In light of these findings, Gamal and Bayomy (2002) studied the effect of smoking on PDL fibroblast attachment to extracted teeth root surfaces which had undergone non-surgical periodontal treatment one week prior to extraction. PDL fibroblast attachment to the root surfaces was significantly reduced in

smokers compared to non-smokers, but no significant difference in attachment was found between light and heavy smokers.

Overall, these studies suggest that smoking impairs many of the important functions of both gingival and periodontal fibroblasts in tissue maintenance and in wound healing, however, a recent tissue culture study reported that smoking has a greater negative impact on periodontal fibroblast function than gingival fibroblasts (Lallier et al., 2017). However, caution needs to be applied in the interpretation of these tissue culture studies, especially with regard to the culture conditions and doses of the components of smoke.

1.8 Diabetes mellitus (DM) and periodontal disease

1.8.1 Introduction

Diabetes mellitus (DM) is a heterogeneous group of metabolic disorders which result in chronic hyperglycaemia leading to significant morbidity and mortality (Chapple et al., 2013, American Diabetes, 2018). The two main types of diabetes are Type 1 DM and Type 2 DM, however gestational diabetes may occur during pregnancy (American Diabetes, 2018, WHO, 2019). Type 1 DM is caused by autoimmune destruction of the insulin producing beta cells in the pancreatic Islets of Langerhans leading to chronic insufficient insulin production (Eisenbarth, 1986). Type 1 DM has a strong genetic basis, but usually requires the presence of an environment factor, such as a viral infection, to initiate the disease (Skamagas et al., 2008, Tuomi et al., 2014). In Type 2 DM insulin production is maintained, although often reduced, and there is peripheral resistance or reduced sensitivity to circulating insulin (a qualitative reduction). Type 2 DM accounts for approximately 90% of cases and the prevalence is increasing globally, especially in Western societies where it has been linked with increasing obesity and lack of exercise, although genetic factors are also involved (Ford et al., 1997, Tuomi et al., 2014). This has resulted in Type 2 DM becoming more common in younger age groups and many of these subjects may go undiagnosed for many years, increasing the risk of complications from the condition (Tuomi et al., 2014, Cai et al., 2020). The higher prevalence of Type 2 DM in the UK population accounts for why the majority of diabetic subjects studied in this thesis were from this category. There is also an increasing prevalence of pre-diabetes globally, which has a significant risk of progressing to full

diabetes (Tabak et al., 2012). The prevalence of diabetes in the UK in 2019 was 3.8 million people, up from 1.4 million in 1996, with an estimated additional one million people with undiagnosed diabetes (Diabetes, 2019). Globally it has been estimated that diabetes is now the seventh most common cause of death (WHO, 2018).

Although the aetiology of Type 1 and Type 2 diabetes are different, the consequences of the resultant chronic hyperglycaemia are similar with varying degrees of dysfunction of the carbohydrate, lipid and protein metabolism causing widespread cellular and molecular dysfunction (Tuomi et al., 2014, Graves et al., 2020). This causes significant disturbance of immune response, angiogenesis and wound healing leading to microvascular and macrovascular systemic pathology (Soory, 2002, Fowler, 2011). Classically DM is described as having five major complications which are atherosclerosis, retinopathy, nephropathy, neuropathy and impaired wound healing. Later, Loe (1993) described periodontal disease as being the sixth complication of diabetes. Macrovascular changes result in potentially life threatening complications associated with large vessel disease (atherosclerosis), which is often exacerbated by other environmental factors such as smoking and poor diet, leading to ischaemic heart disease, strokes and peripheral vascular disease (Fowler, 2011, Cai et al., 2020). Microvascular changes particular affect the small vessels in the retina, renal glomerulus and the nerve sheath leading to significant ocular, kidney and neuronal problems respectively (Fowler, 2011). Impaired wound healing in diabetes is thought to play a significant role in many of the pathological conditions and surgical complications associated with diabetes (Tuomi et al., 2014).

1.8.2 Association between diabetes mellitus and periodontal disease

The association between DM and periodontal disease was first reported in a cross-sectional study where hospital patients with poorly controlled diabetes were associated with higher levels of periodontal destruction (Sandler and Stahl, 1960). Several cross-sectional studies have demonstrated that gingivitis (gingival inflammation) is significantly more prevalent in children and young adults with Type 1 DM, and is associated with poor glycaemic control (Gislen et al., 1980, Novaes Junior et al., 1991, de Pommereau et al., 1992, Pinson et al., 1995, Karjalainen and Knuuttila, 1996).

Epidemiological studies have found significantly higher prevalence of periodontitis in adults in both Type 1 DM (Hugoson et al., 1989, Thorstensson and Hugoson, 1993, Moore et al., 1999) and Type 2 DM (Nelson et al., 1990, Emrich et al., 1991, Grossi et al., 1994, Taylor et al., 1998a, Taylor et al., 1998b) compared with healthy non-diabetic subjects. Many of these studies involved Pima Indian populations, who have a very high prevalence of Type 2 DM (Knowler et al., 1990). Nelson et al. (1990) examined 2273 adult Pima Indians and reported the prevalence of severe periodontitis, quantified by tooth and alveolar bone loss, in Type 2 DM to be 60% compared to 36% in the non-diabetic controls. In both Type 1 and Type 2 DM the prevalence, severity and risk of progression of periodontitis is strongly associated with the poor long-term glycaemic control (Gusberti et al., 1983, Ervasti et al., 1985, Seppala et al., 1993, Tervonen and Oliver, 1993, Seppala and Ainamo, 1994, Karjalainen and Knuuttila, 1996, Novaes Junior et al., 1996, Taylor et al., 1998b, Tsai et al., 2002, Lim et al., 2007, Demmer et al., 2012). Furthermore, evidence suggests that there is a dose-dependent relationship between glycaemic control and the severity and risk of progression of periodontitis, including in the periodontal maintenance phase (i.e. higher glycated haemoglobin scores are associated with a higher risk of periodontal destruction) (Seppala et al., 1993, Tervonen and Oliver, 1993, Tervonen and Karjalainen, 1997, Costa et al., 2013). Furthermore, there is increasing evidence that being overweight or obese can increase the risk of periodontitis and the likelihood of reduced treatment outcomes following periodontal therapy (Keller et al., 2015). Conversely, patients with well controlled diabetes (glycated haemoglobin scores $\leq$7%; 53 mmol mol^{-1}) have a similar risk of periodontitis as non-diabetic subjects (Tervonen and Knuuttila, 1986, Tervonen and Oliver, 1993).

Likewise, studies have reported that the outcome of both non-surgical and surgical periodontal therapy in subjects with well controlled diabetes, both Type 1 and Type 2 DM, is similar to healthy controls (Bay et al., 1974, Tervonen et al., 1991, Westfelt et al., 1996, Christgau et al., 1998). However, the response to periodontal therapy was significantly impaired in subjects with poor glycaemic control (Seppala et al., 1993, Tervonen and Karjalainen, 1997).

There is growing evidence that periodontitis may exacerbate various systemic chronic conditions, probably by increasing systemic inflammation, with the strongest evidence being the relationship between periodontitis and diabetes (Genco et al., 2020). There is strong evidence that there is a bi-directional relationship between diabetes and periodontitis, where poorly controlled diabetes increases the risk of periodontal disease (see above) but successful periodontal therapy, especially involving severe periodontitis, has a positive effect on glycaemic control in both Type 1 and 2 DM (Grossi et al., 1996, Grossi et al., 1997a, Grossi and Genco, 1998, Stewart et al., 2001, Rodrigues et al., 2003, Kiran et al., 2005, Promsudthi et al., 2005). Although meta-analysis studies reported that periodontal therapy results in a relatively small reduction in glycated haemoglobin, by approximately 0.4%, it is likely to be sufficient to result in clinical benefits with regards to reducing the complications of diabetes (Janket et al., 2005, Simpson et al., 2010, Sgolastra et al., 2013, Casanova et al., 2014). Furthermore, evidence from a meta-analysis of the literature suggested that periodontal therapy in subjects with Type 2 DM resulted in significant reductions in some markers of systemic inflammation, such as CRP and TNFα (Artese et al., 2015).

1.8.3 Effect of DM on the periodontium

The mechanisms by which poorly controlled diabetes impacts on periodontal health are complex and are not fully understood, but are thought to involve several mechanisms which negatively affect the host response to the plaque biofilm and collagen metabolism as well as reducing wound healing potential, partly due to reduced angiogenesis (Graves et al., 2020, Polak et al., 2020). Although there is good evidence that there is a greater inflammatory response to periodontal plaque bacteria in diabetes, at least in rodents, there is contradictory evidence regarding whether diabetes affects the composition of the plaque biofilm (Graves et al., 2005, Pacios et al., 2012). Current consensus is that diabetes does not significantly alter bacterial composition of the plaque biofilm (Chapple et al., 2013, Taylor et al., 2013), however, a more recent study in mice found diabetes reduced bacterial diversity in favour of more pathogenic species, which in turn promotes dysbiosis (Xiao et al., 2017). Furthermore, as briefly discussed in Chapter 1.6.4, there is evidence that systemic spill over of periodontal pathogens into the systemic circulation (bacteraemia) may contribute to increased systemic

inflammation leading to raised hyperglycaemia and acerbation of diabetes (Genco et al., 2020).

1.8.3.1 Advanced Glycation End (AGE) products

Prolonged hyperglycaemia promotes glycation of lipids, proteins and nucleic acids with greater glycation being associated with poorer levels of control (Brownlee, 1994, Monnier et al., 1996, Graves et al., 2006, Cho et al., 2007, Singh et al., 2014, Shaikh-Kader et al., 2019). The non-enzymatic glycation of proteins results in stable AGE products, which alters their structural and functional properties, leading to changes in cellular and matrix interactions. Excessive accumulation of AGE products is closely linked to both the macro- and microvascular complications of DM (Fowler, 2011). AGE-modification impairs collagen turnover through cross-linkage of tissue collagen, reduced collagen production and increased MMP production by fibroblasts. Macrovascular complications result from AGE-modified collagen accumulating in large vessel walls leading to vessel wall thickening and narrowing of the vessel lumen (Monnier et al., 1996). Microvascular changes occur by the accumulation of AGE-modified collagen in the basement membranes of small blood vessels increasing membrane thickness and altering molecular transport across the membrane. With regards to periodontitis, significantly higher levels of AGE products has been reported in periodontal tissues in both Type 1 and Type 2 DM compared with healthy controls (Zizzi et al., 2013b). Furthermore, this group found a significant positive correlation between AGE expression and the length of time since diagnosis with DM, but not with other factors such as glycated haemoglobin levels.

AGE products also affects cellular function through binding to specific cell surface receptors (RAGE) on various cell types, such as endothelial cells, fibroblasts, vascular smooth muscle cells, neurones, monocytes and macrophages (Brett et al., 1993). RAGE activation results in increased cellular oxidative stress and activation of the transcription factor nuclear factor-kB, which stimulates periodontal bone loss, and causes apoptosis of periodontal ligament fibroblasts (Schmidt et al., 1996, Vlassara and Bucala, 1996, Li et al., 2014). In addition to high levels of RAGE, hyperglycaemia has also been shown to cause periodontal ligament fibroblast apoptosis via the caspase-3 activation (Liu et al., 2013). Hyperglycaemia increases RAGE expression leading to increased vascular permeability and increased expression of

Vascular Cell Adhesion Molecule-1 (VCAM-1), which is potentially involved in monocyte related vascular complications of diabetes (Esposito et al., 1989, Schmidt et al., 1995). Lalla et al. (2000) showed the importance of RAGE in *Pg* induced alveolar bone loss in diabetic mice. However blockading RAGE using soluble RAGE resulted in a dose-dependent reduction of alveolar bone loss, reduced levels of pro-inflammatory mediators, such as TNFα and IL-6, and reduced MMP levels. A similar study reported blocking RAGE resulted in enhanced wound closure and healing times associated with a reduced inflammatory response in diabetic mice (Goova et al., 2001). A recent tissue culture study showed that AGE resulted in raised expression of RAGE, IL-6, ICAM-1 and reactive oxygen species in human gingival fibroblasts, all of which could potentially promote periodontal damage (Nonaka et al., 2018).

1.8.3.2 Effect of DM on the inflammatory response and periodontal bone loss

Diabetes has been shown to lead to a dysfunctional host response to the plaque biofilm. Hyperglycaemia causes impaired neutrophil adherence, chemotaxis and phagocytosis, as well as increased production of various cytokines (IL-1, IL-6 and TNFα) and the release of damaging MMPs and reactive oxygen species (Manouchehr-Pour et al., 1981, McMullen et al., 1981, Marhoffer et al., 1993, Gursoy et al., 2008, Omori et al., 2008, Roberts et al., 2015). Macrophages tend to display a hyper-responsive phenotype resulting in increased secretion of pro-inflammatory mediators, such as IL-1, TNF-α and PGE_2, and MMPs leading to the pronounced chronic inflammatory response associated with DM (Salvi et al., 1997a, Salvi et al., 1997c, Salvi et al., 1998, Mirza and Koh, 2011). Furthermore, macrophages in diabetic wounds have been shown to have reduced production of important angiogenic factors, such as VEGF and Insulin-like Growth Factor-1 (IGF-1) (Mirza and Koh, 2011). This phenotypic change is thought to be mediated through cell surface AGE-RAGE interaction (Schmidt et al., 1996). Consequently, these changes lead to an exaggerated inflammatory response to the plaque biofilm resulting in excessive periodontal tissue damage.

Hyperglycaemia has also been shown to cause increased periodontal bone loss through changes in the receptor activator of nuclear factor kappa-β ligand (RANKL) to osteoprotegerin ratio in favour of RANKL (Santos et al., 2010). Clearly, poorly controlled

diabetes has widespread profound negative effects on the periodontium, leading to increased risk of periodontal disease progression.

1.8.3.3 Effect of DM on angiogenesis

With particular regards to this thesis, it has been shown that diabetes affects all stages of wound healing, in particular the angiogenic element of the proliferative phase, leading to increased risk of poor dermal and oral wound healing (Abiko and Selimovic, 2010, Guo and Dipietro, 2010, Okonkwo and DiPietro, 2017). Furthermore, many of the processes described above also have a negative impact on diabetic wound healing. Evidence suggests that diabetic wound healing is associated with down-regulated angiogenic activity, although in certain conditions, such as diabetic retinopathy, angiogenic activity is increased leading to pathology (Simo-Servat et al., 2019). As a result of down regulated angiogenesis, diabetic wounds are less vascular and show evidence of reduced branching and total vessel length, and delayed vessel maturation (Urao et al., 2016, Okonkwo et al., 2020). It is likely that the reduced angiogenic activity in diabetes is the result of several complex mechanisms, many of which are still poorly understood. Several studies have shown that diabetic wound healing is associated with significant reductions in several angiogenic factors, such as VEGF, PDGF and IGFs (Frank et al., 1995, Beer et al., 1997, Brown et al., 1997, Okonkwo et al., 2020). Furthermore, the normal balance between angiogenic factors and inhibitors appears to be altered in diabetic wounds. For example, the ratio of Ang-1 to Ang-2 in diabetic wounds was found to be reduced, in addition to reduced VEGF levels, which may partly account for the prolonged delay in vascular maturity (Kampfer et al., 2001, Isidori et al., 2016). Currently, the relationship between angiogenic factors and inhibitors in diabetic wound healing is poorly understood and further research is required.

Other factors shown to be negatively affected in diabetic wound healing, which have an impact on angiogenesis, include dysfunctional endothelial cell and pericyte function, and reduced recruitment of endothelial progenitor cells (Tepper et al., 2002, Piconi et al., 2006, Kolluru et al., 2012, Bodnar et al., 2016). A recent study by Okonkwo et al. (2020), reported pericyte coverage of newly formed vessels was significantly reduced leading to increased vascular permeability and delayed vessel maturation.

Currently, there is little data on angiogenic factor levels in saliva and GCF in periodontal health and disease in subjects with diabetes. This will be discussed further with regards to Ang-1, MSF, VEGF and endostatin in the relevant chapters in this thesis.

1.9 Aims of the thesis

The aims of this thesis were:

1) To investigate whether serum and salivary concentrations of the angiogenic factors Ang-1, MSF and VEGF, and the inhibitor endostatin, differ significantly between periodontal health and severe periodontitis.
2) To investigate whether serum and salivary concentrations of Ang-1, MSF, VEGF and endostatin are significantly altered in smokers and in patients with diabetes, compared with matched healthy controls.
3) To investigate whether the quantification of vascularity in periapical granulomas and healthy periodontal ligament, using different endothelial markers, has value as an index of angiogenesis.

There were two broad elements to this thesis, the ELISA and immunohistochemistry studies (Chapters 3 and 4 respectively). One of the initial aims was to examine MSF expression in healthy PDL tissue and correlate it to MSF levels in GCF and saliva, which is the reason why MSF was the only angiogenic factor examined in the immunohistochemistry study. Another link between the two elements of the thesis was to establish whether a periodontal-related model of angiogenesis could be developed similar to the OSCC-NOM model commonly used in the study of angiogenesis in the development of tumours (Chapter 4). A periodontal-related model of angiogenesis would provide actual evidence of angiogenic activity in relation to tissue expression of angiogenic factors, which could be correlated to the levels of the same factors in oral fluids and serum. This could provide important information with regards to the role angiogenesis in the development of periodontal disease.

1.10 Null hypotheses of the thesis

The null hypotheses for this thesis were:

1. Severe periodontitis does not result in significant changes in serum and salivary concentrations of Ang-1, MSF, VEGF and endostatin compared with periodontal health.
2. Smoking and diabetes does not result in significant changes in serum and salivary concentrations of Ang-1, MSF, VEGF and endostatin compared with systemically healthy controls.
3. Indices of vascularity and MSF expression do not provide evidence of angiogenesis in periapical granulomas.

Chapter 2

Material and Methods

2.1 Clinical techniques

2.1.1 Patient selection protocol

Ethical approval for this study was obtained from The Tayside Committee on Medical Research Ethics (129/02). Subjects were recruited and selected into one of three study groups (Table 2.1). Groups 1 and 2 both comprised of systemically fit and healthy patients who were not taking any medication. A full list of patient exclusion criteria is given in section 2.1.3. These exclusions were necessary to rule out confounding effects of various systemic diseases and medications. In addition, Group 1 subjects had never smoked, while Group 2 subjects were current long-term smokers (smoked ≥10 years). Pack Years was calculated to estimate the individual cumulative exposure to tobacco smoke (Prignot, 1987) and smokers were further stratified into either light smokers (<20 pack years) or heavy smokers (≥20 pack years) (Elter et al., 2003). Subjects with a previous history of smoking were excluded to make the differentiation between Groups 1 and 2 as clear as possible.

Table 2.1 Classification of the clinical samples according to the clinical characteristics of the donors.

Study Group	Sub-groups	Study Group Criteria
1. Systemically healthy non-smokers	**1a.** Periodontally healthy **1b.** Periodontitis	• Never smoked
2. Systemically healthy smokers	**2a.** Periodontally healthy **2b.** Periodontitis	• Current long-term smokers (smoked ≥10 years) Cumulative smoking exposure calculated in Pack Years
3. Subjects with Diabetes	**3a.** Periodontally healthy **3b.** Periodontitis	• Smokers and non-smokers Cumulative smoking exposure calculated in Pack Years • Diabetes Types 1 and 2 included Diabetic control quantified by most recent glycated haemoglobin (HbA1c) result.

Group 3 comprised a wide range of subjects with Diabetes mellitus (Type 1 and Type 2). Many patients with diabetes take multiple medications, not only to help to stabilise their blood glucose levels, but also to prevent/treat the side effects of the condition. This was true for many subjects in this study group. Long-term glycaemic control was assessed using the subject's most recent glycated haemoglobin (HbA1c) score, retrieved from either their General Medical Practitioner (GMP) or the diabetes clinic at Ninewells Hospital, Dundee.

2.1.2 Clinical sub-groups

Each of the three study groups were further divided into two sub-groups, (a) and (b). Sub-group (a) subjects were periodontally healthy and Sub-group (b) subjects had severe Periodontitis. These sub-groups were defined as follows:

- Sub-group (a): Periodontally healthy subjects with no loss of attachment (LOA), periodontal probing depths ≤3mm and minimal full mouth bleeding on probing scores (BOP) (<15%) (Palmer and Floyd, 1995).
- Sub-group (b): Subjects with evidence of severe periodontitis (Papapanou et al., 2018b) with significant LOA (≥5mm), ≥5mm periodontal probing depths (true pocketing) and generalised BOP (>40% full mouth bleeding scores) (Palmer and Floyd, 1995).

2.1.3 Exclusion criteria

The following were applied:

- Subjects needed to have at least two bilateral upper posterior teeth to allow standardised Gingival Crevicular Fluid (GCF) sampling.
- Periodontal treatment within the last six months were excluded to ensure the periodontal microenvironment was undisturbed prior to sampling.
- History of previous or irregular smoking were excluded to ensure clear differentiation between Group 1 (never smokers) and Group 2 (current long-term smokers).
- Subjects diagnosed with Periodontitis Stage 3 or 4 Grade C (Papapanou et al., 2018b), where there was evidence of rapidly progressing disease were excluded due to the potential of confounding factors.
- Pregnant subjects were excluded to ensure that the associated periodontal effects did not affect the results. Women of child-bearing age were specifically asked if they were likely to be pregnant, if so, they were also excluded. This was a requirement of the Ethics Committee approval.
- Subjects who were currently taking or had recently taken medications that could affect the periodontal health were excluded. These included:
    - Non-Steroidal Anti-Inflammatory Drugs (NSAID)

- Steroids
- Antibiotics within the previous three months

- Subjects with complex medical histories where recording of clinical data and/or taking of clinical samples would require extra pre-cautions to be taken for patient safety reasons were excluded. These included:
  - Patients with a known infectious disease (Hepatitis B/C, HIV)
  - Immuno-compromised patients

2.1.4 Power calculation

The initial aim was to only measure Migration Stimulating Factor (MSF) levels in the clinical samples. As there was limited data at the time on MSF levels in serum, saliva and GCF, it was not possible to carry out a power calculation to estimate the number of subjects required. It was initially estimated, after consulting a statistician, that a total of 100-120 subjects, equally spread between the study groups, would be required to ensure significant differences could be detected with a high degree of statistical confidence. Subsequently, a post-hoc power sample size calculation was carried out using G*Power software (Faul et al., 2009), which estimated that 64 subjects would be required to achieve an adequate level of power at the 80% level (effect size f=0.40; p=0.05; df=2; 6 groups). Greater than 64 samples (serum or saliva) were assayed for each of the angiogenic factors examined in this thesis.

2.1.5 Clinical sampling

Subjects were recruited from the Periodontology Clinic at Dundee Dental Hospital and School. A brief verbal explanation of the study was given and followed up with a Patient Information Sheet giving details about the study (Appendix 1). Ethics Committee requirements ensured that subjects were given a 24-hour period to reconsider their choice to take part in the study. Subjects were booked in for a single one hour morning appointment, prior to any periodontal treatment appointments, in order to collect the clinical samples and clinical data for this cross-sectional study. All sampling, recording of clinical data and laboratory work was carried out by one investigator (KJD). Prior to sampling, consent forms and detailed medical, dental, family and social history forms were completed (Appendix 1).

In this thesis, three pro-angiogenic factors (Angiopoietin-1 (Ang-1), Migration Stimulating Factor (MSF) and Vascular Endothelial Growth Factor (VEGF)) and one anti-angiogenic factor (Endostatin) were investigated. Unstimulated and stimulated whole saliva samples were collected in order to ascertain whether there were any differences in angiogenic factor content. Gingival Crevicular Fluid (GCF) was sampled from standard sites as detailed below (Section 2.1.8). Blood samples were taken in order to harvest serum. This permitted the comparison of the concentration of factors in the oral fluid samples (saliva and GCF) with the systemic circulation (serum). Standard clinical periodontal measurements (Section 2.1.9) were recorded using a 20g constant pressure electronic Florida probe system (Florida Probe Corporation) and the data stored on a laptop computer (Samsung Corporation).

2.1.6 Saliva collection, laboratory processing and storage

The protocols used were adapted from previous publications (Chapple et al., 1997, Booth et al., 1998, Taichman et al., 1998). Unstimulated saliva was collected first using a protocol described by Booth et al. (1998). Subjects were advised not to eat, chew gum, brush their teeth or smoke for two hours prior to sampling. All saliva sampling was carried out between 9:30-11:00am in order to overcome the diurnal variation in saliva production (Chapple et al., 1997). Prior to sampling, subjects rinsed their mouths with sterile water to remove any food debris or previously pooled saliva, and then sat upright quietly for two to three minutes. Subjects were then left alone to drool saliva for five minutes into a sterile Universal container (Sterilin). The Universal containers were then placed into an ice bucket prior to the samples being centrifuged at 5000g at 4^{o}C for five minutes to remove bacterial and cellular debris. The saliva was carefully aliquoted into non-stick 0.5ml Eppendorf tubes in order not to disturb the pellet of debris. Non-stick Eppendorf tubes were used to reduce the risk of proteins binding to the sides of the container during storage. The volume of the saliva was also measured during aliquoting. The aliquots were labelled using an anonymous subject reference number (section 2.1.10) and stored at -80^{o}C prior to assaying.

An almost identical protocol was followed for the stimulated saliva sampling except that a sterile marble was used to stimulate saliva flow (Chapple et al., 1997). The subjects were instructed not to bite, swallow or inhale the marble and if the patients felt that the marble was

going to cause a problem, they were instructed to immediately spit the marble into the collecting vessel.

2.1.7 Venepuncture, serum collection and storage

Approximately 10ml of venous blood was collected from the antecubital fossa using a standard venepuncture technique. Blood was collected using the BD Vacutainer system (Becton, Dickinson and Company) into untreated (red) 10ml Vacutainer tubes. Samples were allowed to stand for at least 30 minutes at 4^{o}C to clot and then sterile plastic spatulas were used to rim the internal circumference of the Vacutainer tubes. This procedure ensured good layering of the serum during centrifugation. Samples were centrifuged at 10000g for five minutes at 4^{o}C. The resultant serum was carefully pipetted into sterile Falcon tubes, using wide-bore Pasteur pipettes. This ensured that the serum samples were homogenous prior to aliquoting into 0.5ml non-stick Eppendorf tubes. These were then stored at -80^{o}C prior to assaying.

2.1.8 Clinical Gingival Crevicular Fluid (GCF) collection, processing and storage

The protocol used was adapted from Chen et al. (1998). GCF flow rates vary in different areas of the mouth, with lower GCF flow rates in anterior sites than posterior sites, so consistency in sampling sites was important (Smith et al., 1992, Ozkavaf et al., 2000). Mesial buccal sites on the upper pre-molars and first molars were used for GCF collection, due to easier access, and the mesial buccal sites on upper second molars were only used when the first upper molars were missing. GCF samples were collected from both upper posterior sextants. In the periodontally healthy study sub-groups, four sites were sampled on each side which were not immediately adjacent to a diseased site: i.e. not in the same embrasure. In the periodontally diseased sub-groups, GCF was sampled from at least four periodontally diseased sites and from four periodontally healthy sites on each side. Probing of pockets immediately prior to GCF sampling was avoided, as this greatly changes the nature of the GCF samples, thus the sampling sites were pre-selected at the most recent periodontal appointments.

The GCF sample sites were isolated with cotton wool rolls and carefully air dried using the 3-in-1 handpiece. Air was not blown directly into the periodontal pockets to avoid disturbing the GCF. Buccal and palatal sides of the tooth were carefully dried to prevent pooling of saliva in the embrasure area. Any supragingival plaque was carefully removed with a straight probe without entering the gingival sulcus (Chapple et al., 1997). Sterile PerioPaper collection strips (Pro Flow) was placed into the selected periodontal pockets using sterile College tweezers, with the white end of the strip going into the pocket until gentle resistance was detected. The collection strips were left for 30 seconds prior to being removed and immediately read on a pre-calibrated Periotron 8000 (Oraflow Inc) (Chapple et al., 1999). This machine allowed the minute volumes of GCF collected on the PerioPaper collection strips to be calculated from a standardised calibration curve (Appendix 4). To ease handling of the PerioPaper collection strips a PerioPaper Holder (Oraflow Inc) was used. The Periotron reading was recorded and the collection strips were placed into 0.5ml Eppendorf tubes containing 30µl of sterile Phosphate Buffered Saline (PBS), making sure that the white filter paper end of the collection strips were immersed in the fluid. Two collection strips from site-matched GCF sample sites were pooled into each Eppendorf, which was then placed into an ice bucket prior to elution and storage. The orange section of the collection strips only were handled so that the collected GCF sample was not contaminated. During sampling if there was any bleeding from the pocket or a likelihood of salivary contamination the collection strips were discarded.

The GCF was eluted from the collection strips using an adapted soak and centrifugal method described by Booth et al. (1998). The GCF collection strips were incubated in 0.5 ml Eppendorf tubes at 4^{o}C for 60 minutes to encourage proteins to elute from the PerioPaper collection strips into the PBS. A hole was then made in the base of the Eppendorf tubes with a gauge-25 needle, making sure that the lids of the Eppendorf tubes were open otherwise the eluate would be lost. Fresh needles were used for each sample to prevent cross contamination. The Eppendorf tubes were then placed into 1.5 ml non-stick Eppendorf tubes and centrifuged at 10000g for five minutes at 4^{o}C. A further 30µl of sterile PBS was pipetted onto the tops of the collection strips and incubated for a further five minutes at 4^{o}C prior to repeating the centrifuge step. A further 30µl of sterile PBS was added, as above, and the

centrifuge step repeated. The resultant supernatant, in the 1.5ml Eppendorf tubes, was aliquoted into two 40µl aliquots (in 0.5 ml non-stick Eppendorf tubes) and stored at -80^{o}C prior to assaying.

2.1.9 Periodontal clinical data

Periodontal clinical data was recorded using the Florida probe system (Florida Probe Corporation) in conjunction with a standard laptop computer (Samsung Corporation). The Florida probe is a constant pressure electronic probing system which records pocket depths more accurately than standard manual probes. This is due to the system using a constant probing force and it records measurements electronically rather than being judged by eye. A 20g Florida Pocket Probe Handpiece was used in this study. A six point full mouth double periodontal pocket chart was recorded for each subject which included the following parameters:

- Periodontal probing depths
- Gingival margin levels
- Dichotomous plaque scores
- Dichotomous bleeding scores
- Mobility (Miller's index)
- Furcation involvement (Hamp et al., 1975)

One major problem with the Florida probe FP32 software (Florida Probe Corporation) was that it assumed that the gingival margin was at or apical to the Amelocemental Junction (ACJ). In circumstances when the gingival margin level was coronal to the ACJ, the software recorded the gingival margin at the ACJ, which resulted in the LOA being over-estimated. The charts were stored on the laptop computer, but the raw data could also be converted into Excel files (Microsoft) using a data converter programme supplied with the Florida probe system. This allowed the data to be analysed using other software.

2.1.10 Clinical study documentation

As a requirement of the Ethics Committee approval each subject was assigned an anonymous reference number. All of the study documentation (Appendix 1) was kept in sealed A4 envelopes with only the subject's reference number on the outside. All documentation was

securely stored in a locked cabinet and only the principal researcher (KJD) had access to this information. The clinical information from the Florida probe was password protected on the laptop (Data Protection Act registered).

2.1.11 Travelling expenses

Full travelling expenses and a small inconvenience fee of £5 was paid to each subject as requested by the Local Ethics Committee. This was funded by a grant from the British Society of Periodontology (BSP).

2.2 Enzyme-Linked Immunosorbent Assay (ELISA)

The clinical samples were analysed for various angiogenic and anti-angiogenic factors using ELISAs. The Migration Stimulating Factor (MSF) ELISA was developed in the laboratory in collaboration with Dr Katerina Kankova, while commercial ELISA kits were used for all of the other factors. These ELISA kits required optimisation prior to assaying the clinical samples.

2.2.1 General DuoSet ELISA Development System protocol

Commercial ELISA kits (DuoSet ELISA Development System, R & D Systems) were used to assay the clinical samples for Angiopoietin-1 (Ang-1), Vascular Endothelial Growth Factor (VEGF) and Endostatin. These basic ELISA kits supplied the specific antibodies, recombinant factors as standards and Streptavidin-Horseradish Peroxidase (Section 2.53), with each kit sharing the same general protocol (supplied by the manufacturer). Many of these kits had not been previously calibrated for use on some of the types of clinical samples used in this thesis and so some further optimisation was required (Section 2.2.3).

These indirect sandwich ELISA kits required a two-stage protocol with an initial ELISA plate preparation stage, an overnight incubation and then the assaying stage. Due to these assays requiring multiple steps great care was taken to prevent experimental errors. Potential sources of experimental errors included inaccurate pipetting, inadequate washing stages, differential plate temperatures and dehydration during incubation stages (Crowther, 2001). These ELISA kits required stationary incubation, which resulted in long incubation times at

a constant room temperature (air conditioned to 19^{o}C). During incubation the plates were kept away from direct sunlight and machinery to ensure even heating. However, the air conditioning in the laboratory was turned off during washing and pipetting stages to prevent drying of the plates.

In all of the assays, colourless BD Falcon Microtest 96-well ELISA Plates (BD Biosciences) were used. For each assay the component reagents were aliquoted and frozen according to the manufacturer's instructions. The mouse anti-human capture antibodies were diluted to the working concentration (Table 2.2) in sterile PBS and 100µl was used to coat each well in the ELISA plate. After pipetting it was important to gently tap the plates to ensure thorough mixing and to eradicate any air bubbles. This was necessary as air bubbles can greatly reduce the coverage of the walls of the wells by the assay reagents and reduce the effectiveness of washing stages, thus leading to differences between the duplicate wells. A reverse pipetting technique was used to reduce the production of air bubbles during pipetting (Crowther, 2001). To prevent dehydration, the ELISA plates were sealed with Parafilm (Alcan Packaging) and placed flat into a sealed humidified container (lined with paper towels dampened with distilled water). The plates were then incubated overnight at room temperature (air conditioned to 19^{o}C for 15 hours).

The next morning the ELISA plate seals were removed and the wells checked for signs of dehydration. If such a change was detected the wells were noted. The capture antibody was then forcibly expelled into a sink and the plate inverted and tapped sharply onto clean paper towels to remove as much fluid as possible. Using a multi-channel micropipette (Gilson) each well was washed with 400µl of wash buffer (0.05% v/v Tween 20 in PBS). Then the fluid was again forcibly expelled into a sink and the plate tapped dry onto clean paper towels. The washing process was repeated a further two times. This washing procedure was important to remove all of the unbound antibody to ensure good assay performance.

Table 2.2 Summary of the ELISA protocol antibodies (capture, secondary and detection) and the recombinant human factors.

Factor	Capture Antibody	Detection Antibody	Recombinant Standard Factor
Ang-1	Mouse anti-human Ang-1 monoclonal antibody (R & D Systems) diluted to 4µg ml^{-1} in sterile PBS. No cross-reactivity or interference with recombinant human:- • Ang-2 • Ang-4	Biotinylated goat anti-human Ang-1 antibody (R & D Systems) diluted to 200ng ml^{-1} in 1% w/v sterile BSA.	Human Ang-1 (R & D Systems) diluted in 1% w/v sterile BSA to give a working range (serial dilution) of 10000 to 156pg ml^{-1}.
MSF	Mouse anti-human Pep Q5.1 monoclonal antibody (Moravin Biotech) (Schor et al., 2003) diluted to 10µg ml^{-1} in coating buffer (Thermo Fischer Scientific Inc). No cross-reactivity or interference with recombinant human:- • Fibronectin	Rabbit anti-human Rp VSI polyclonal secondary antibody (Moravin Biotech) diluted to 10µg ml^{-1} in 1% w/v sterile BSA. Goat anti-rabbit HRP detection polyclonal antibody (Thermo Fisher Scientific Inc.) diluted 1:1000 in 1% w/v sterile BSA.	Bacterial rhMSF-aa (Dundee) diluted in 1% w/v sterile BSA to give a working range (serial dilution) of 200 to 3.125ng ml^{-1}.
VEGF	Mouse anti-human VEGF monoclonal antibody (R & D Systems) diluted to 1µg ml^{-1} in sterile PBS. Specific for human $VEGF_{121}$ and $_{165}$. No cross-reactivity or interference with recombinant human:- • PIGF • VEGF-C • VEGF-D	Biotinylated goat anti-human VEGF polyclonal antibody (R & D Systems) diluted to 50ng ml^{-1} in 1% w/v sterile BSA.	Human VEGF (R & D Systems) diluted in 1% w/v sterile BSA to give a working range (serial dilution) of 2000 to 31.25pg ml^{-1}.
Endostatin	Mouse anti-human Endostatin monoclonal antibody (R & D Systems) diluted to 4µg ml^{-1} in sterile PBS. No cross-reactivity or interference with recombinant human:- • Ang-2 • Ephrin-A5/Fc Chimera • Tie-2/Fc Chimera	Biotinylated goat anti-human Endostatin polyclonal antibody (R & D Systems) diluted to 100ng ml^{-1} in 1% w/v sterile BSA.	Human Endostatin (R & D Systems) diluted in 1% w/v sterile BSA to give a working range (serial dilution) of 4000 to 62.5pg ml^{-1}.

Prevention of non-specific binding (NSB) of other proteins to the wells in the plates was achieved by blocking them by the addition of 300µl of ELISA reagent diluent (1% w/v BSA in PBS) to each well and incubating for one hour at room temperature. The plates were then sealed to prevent dehydration in the same way used during the overnight incubation. The plates were then washed using the same protocol as described above. Next the plates were incubated with either 100µl per well of the diluted clinical samples or the recombinant factor standards dilution (Section 2.2.3 and Table 2.2). For each assay, clinical samples were selected to cover all the study groups and storage times. All of the dilutions were made up in the ELISA reagent diluent, including the standards, and each sample was assayed in duplicate. Recombinant factors were supplied with the kits and diluted to the recommended high standard concentration (Table 2.2). These were then diluted to give a series of seven factor-of-two standard dilutions, using a passing down the line method, with the most concentrated being the recommended high standard concentration supplied by the manufacturer. This standard dilution series allowed a standard curve to be drawn for each factor in order to allow the concentrations of the factors in each clinical sample to be calculated. It was important that a standard curve was done for every plate to compensate for the differences in the readings due to the changes in the conditions for each assay run. When pipetting the serial dilutions onto the plate, the most dilute serial concentration was pipetted first, and after expelling all of the remaining solution in the tip, the same pipette tip was used for the next dilution in the series. This process was repeated up the standard factor serial dilutions. This resulted in efficient plating and a good standard curve. Two blank wells, which only contained ELISA reagent diluent, were used to record the background noise of the assay.

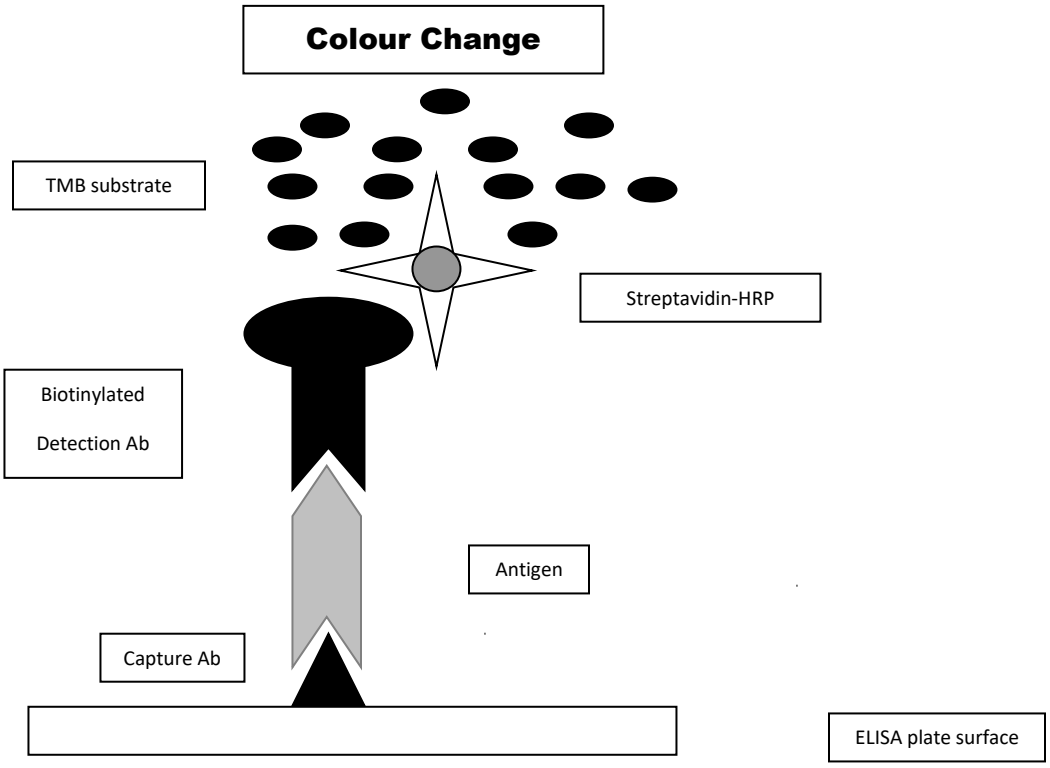
Forty duplicate clinical samples could be assayed on each plate. All dilutions were made up in 1.5ml Eppendorf tubes and stored in an ice bucket immediately before use. The plates were sealed and incubated, as before, for two hours at room temperature and then the plates were washed as before. Throughout the assay any unused wells were filled with reagent diluent in order to prevent “edge effects” which may affect the readings in the adjacent test wells (Crowther, 2001).

The factor specific anti-human biotinylated detection antibody was diluted with ELISA reagent diluent to the working concentration recommended by the manufacturer (Table 2.2) and 100µl was added to each well. The plates were then sealed and incubated for two hours at room temperature and then washed as before. Streptavidin-Horseradish Peroxidase was diluted 1 in 200 in ELISA reagent diluent (actual concentration not supplied) and 100µl was added to each well. This was sealed and incubated for 20 minutes at room temperature avoiding direct sunlight. The plates were then washed as before.

Then 100µl of undiluted Tetramethylbenzidine (TMB) Substrate-Chromogen (DAKO Corporation) ELISA Substrate Solution was added to each well and incubated for 20 minutes at room temperature, again avoiding direct sunlight. Wells containing any bound factor resulted in a blue colour change during this incubation. After 20 minutes 50µl of Stop Solution (2M H_2SO_4) was added to each well and the wells containing any bound factor changed to a yellow colour. The Optical Density (OD) of each well was immediately read using a microplate reader (MRX ELISA Reader, Dynex Technologies) set to 450nm. This machine was wavelength corrected to 630nm. The average of the duplicate OD readings for each standard and clinical sample were calculated and the average zero standard OD (blank wells) was subtracted from these to give the actual OD readings. This could be calculated automatically by the Dynex Revelation 3.2 software (Dynex Technologies) supplied with the microplate reader. Any sample duplicate readings with a greater than 5% variation were discarded and the sample re-assayed.

A summary of this ELISA technique is shown in Figure 2.1. In order to detect any bound factor in the wells the ELISA kits rely on the strong affinity reaction between Avidin and Biotin. The detection antibody is biotinylated and strongly binds to the Avidin component of the Streptavidin-Horseradish Peroxidase. The Horseradish Peroxidase (HRP) component then converts the TMB substrate solution into a coloured substrate allowing detection by the ELISA plate reader.

Figure 2.1 A diagram summarising the direct-ELISA protocol.

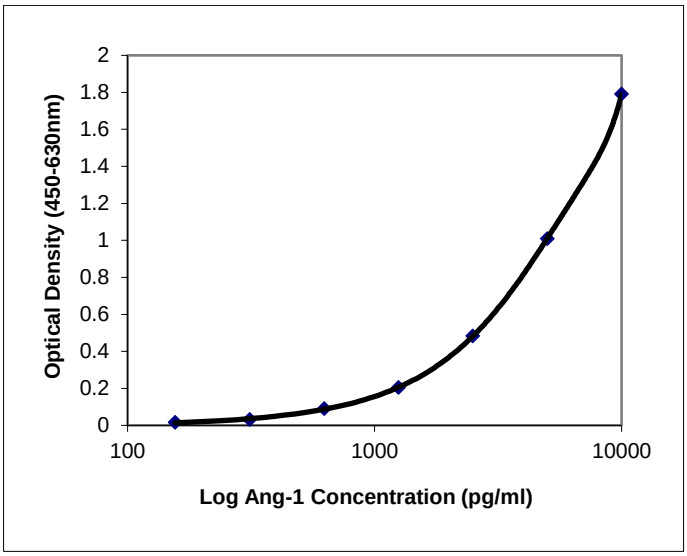


In order to calculate the concentration of the factors in the clinical samples, standard curves were produced using Excel (Microsoft Corporation) from the averaged and corrected optical density readings generated from the two-fold serial dilutions of recombinant human standard factors (Figure 2.2). Corrected optical density readings refers to the duplicate blank wells, containing only reagent diluent (100µl), used to record the background noise of the assays. The averaged optical density readings from these blank wells (background noise) were subtracted from both the optical density readings from the recombinant standards wells and the sample wells to give the final readings. The mean optical density reading for each standard concentration (y-axis) was plotted against the log of the standard concentration (x-axis) using a four-parameter logistic curve-fit (4^{th} polynomial), as recommended by the manufacturer (R & D Systems). The concentration of the factors could then be calculated from the graph using the mathematical formula generated from the graph. The resultant trend lines showed a very high level of precision fit to the data points (R^2=1.0), with the working ranges for each assay largely being within the straight-line aspect of the standard curves. As

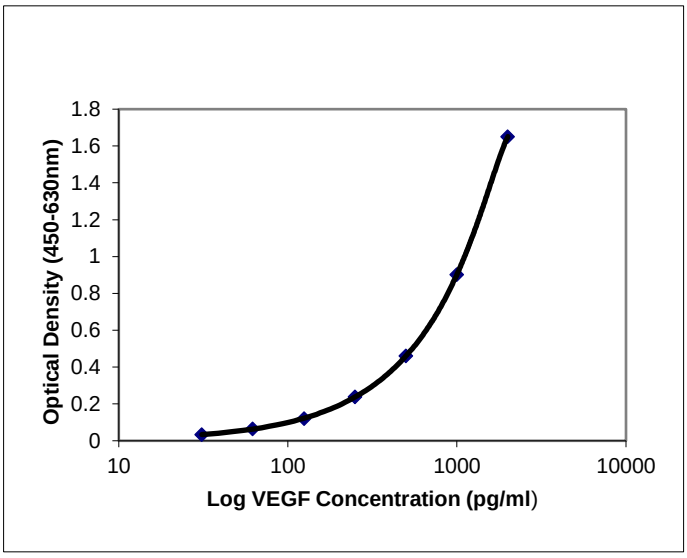
the clinical samples were diluted in the ELISA reagent diluent these results were multiplied by this dilution factor to give the actual factor concentration in the clinical samples.

Figure 2.2 Calibration curves for the ELISA kits: (a) Ang-1, (b) VEGF and (c) Endostain. Optical density versus the log of the concentration of recombinant human VEGF (pg ml^{-1}).

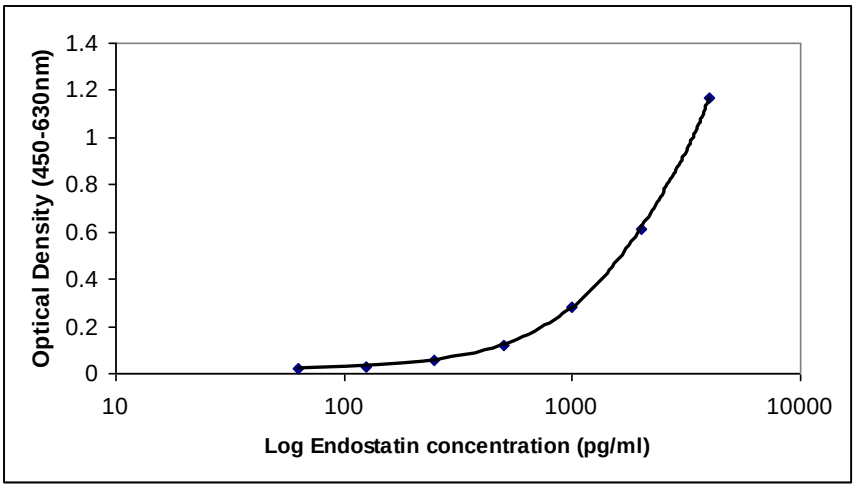
(a)



(b)



(c)



Standard curve plotted to the 4th polynomial as recommended by the manufacturer (R&D Systems), trend line fit R^2=1.

Confidence in the protocol was demonstrated by the very low optical density readings for the blank wells (background noise) which suggested there was no significant non-specific binding and no additional blocking was required. It was noted that the background noise for the MSF ELISA increased in later assays, probably as a result of the degradation of the MSF capture antibody (Chapter 3.4).

Intra-assay variation was examined in two ways, the percentage co-efficient of variation between duplicate wells (Table 2.3) and, for some factors, having two or more sets of the same sample in different parts of the plate (cross plate variation). The percentage co-efficient of variation between duplicate wells were found to be low in both the serial dilutions of the recombinant factors and the clinical samples (range 1.6% to 4.5%), which suggested consistent mixing of the samples and accurate pipetting. Cross plate variation was examined for the Endostatin ELISA where six serum samples were assayed in different parts of the same plate (in duplicates), resulting in an overall mean co-efficient of variation of 5.4% across all the samples. Cross plate variation was also examined in some of the MSF ELISAs, where one of the samples was plated in different parts of the plates with co-efficient of variation ranging from 3.8% to 9.1%. These findings are consistent with acceptable levels of intra-assay variation of 10% or below. In order to ensure consistency in the resultant

concentrations generated between plates reference serial dilutions of the recombinant factors were used (Table 2.2). Although pooled clinical samples were not routinely used between plates, inter-assay variation was examined by assessing the co-efficient of variation of the highest concentration of recombinant factor (Ang-1 19.9%; MSF 4.9%; VEGF 12.9%; Endostatin 14.3%). In all of the ELISA kits the highest recombinant factor optical density reading was found to increase near to the use by date of the kits, leading to increased variation. For the MSF ELISA, some clinical samples were repeated between plates with co-efficient of variation ranging from 9.1% to 11.6%. These findings are generally consistent with acceptable levels of inter-assay variation of 15% or below.

Table 2.3 Co-efficient of variation for each factor ELISA kit between: (i) duplicate wells for the serial dilution of the recombinant factors and (ii) multiple serum, unstimulated and stimulated saliva samples.

Factor	**Co-efficient of Variation (%±SD)**			
	Recombinant Standards	**Serum**	**Unstimulated Saliva**	**Stimulated Saliva**
Ang-1	2.6%	1.6%	2.3%	2.2%
Endostatin	2.9%	2.2%	-	-
MSF	1.8%	2.1%	-	-
VEGF	3.8%	1.9%	3.1%	4.5%

The limit of detection (LOD) of the assays was defined as the analyte concentration which was equivalent to the mean optical density reading of the dilution medium plus three standard deviations (Steiner et al., 2000, Crowther, 2001, Hosseini et al., 2018). Using data from five independent plates the maximum sensitivity of each assay was calculated (Table 2.4). For each ELISA the LOD of the assay was found to be above the lowest point in the working range quoted by the manufacturer, although this may be accounted for by the different definitions used to calculate LOD.

Table 2.4 Comparison of the ELISA kits limit of detection (manufacturer and calculated).

Factor	Sensitivity (LOD) (pg ml^{-1})	
	Manufacturer	**Calculated**
Ang-1	156pg ml^{-1}	370pg ml^{-1}
Endostatin	62.5pg ml^{-1}	148pg ml^{-1}
MSF	-	2ng ml^{-1}*
VEGF	31.25pg ml^{-1}	81pg ml^{-1}

*ELISA developed in-house; Personal communication Dr Katerina Kankova.

2.2.2 MSF ELISA protocol

The MSF indirect sandwich ELISA was developed and optimised for use with serum samples by Dr Katerina Kankova and the protocol was similar to the general ELISA protocol outlined in Section 2.2.1. As this was a non-commercial ELISA, more extensive optimisation was required prior to assaying the clinical samples (Chapter 3.4.2). In this assay colourless Costar 3590 96-well ELISA plates (Corning Incorporated) were used. The plate washing procedure consisted of consecutive washes, 200µl per well, of PBS, twice with PBST (0.05% v/v) and finally with PBS. The plates were blocked with 1% w/v sterile BSA buffer under constant orbital shaking for 1 hour, and the subsequent antibodies (Table 2.2) were all diluted in 1% w/v sterile BSA and incubated (stationary) at room temperature (air conditioned to 19^{o}C) for 90 minutes. Refer to Section 2.2.3 for details regarding the optimisation for the clinical samples. The TMB (DAKO) step consisted of the addition of 50µl per well and the plate was placed onto the orbital shaker for 10 minutes prior to the addition of the stop solution. The OD was read using the Orion II Microplate Luminometer (Berthold Detection Systems) set to 450nm and 570 nm (for reference).

2.2.3 Optimisation of the ELISAs with the clinical samples

An initial optimisation procedure was carried out to assess the performance of each ELISA kit in relation to both the manufacturer's performance data and to ensure optimal performance with the clinical samples collected. Optimisation was carried out on a broad range of samples from each study group in order to account for possible variations in factor levels due to individual variation, age effects and storage time. Serial dilutions of serum and stimulated saliva samples were assessed to find the sample dilutions which consistently resulted in OD

readings (averaged and corrected) within the straight-line aspect of the standard curves for each factor (Table 2.5). Dilutions less than 1:10 were not examined due to the high risk of non-specific binding with the serum samples (Personal communication: Dr Richard Kay) and the saliva samples being too viscous to accurately pipette and to homogenise. Stimulated saliva was used for the optimisation of the various ELISAs due to the small volumes of unstimulated saliva harvested from most subjects.

Table 2.5 Optimised clinical sample dilutions (serum and stimulated saliva) for the ELISA kits.

Factor	Ang-1	MSF	VEGF	Endostatin
Serum	1:10 dilution in 1% sterile BSA	1:100 dilution in sterile PBS-T-PI	1:10 dilution in 1% sterile BSA	1:20 dilution in 1% sterile BSA
Stimulated Saliva	1:10 dilution in 1% sterile BSA	1:10 dilution in sterile PBS-T-PI	1:10 dilution in 1% sterile BSA	1:10 dilution in 1% sterile BSA

Key:
BSA – Bovine Serum Albumin
PBS-T-PI – Phosphate Buffered Saline, 0.5% v/v Tween 20, Protease Inhibitor (Roche)

2.3 Immunohistochemistry – Periapical granuloma study

2.3.1 Specimens

A total of 44 formalin-fixed paraffin-embedded sections were obtained from the archives of the Oral Pathology laboratory, University of Dundee and from the University of Münster, Germany. These samples were collected under the Ethics regulations current at the time. The specimens included 13 chronic periapical granulomas (PG), obtained after extraction or periradicular surgery, and 13 normal periodontal ligaments (PDL) obtained from extracted teeth. In addition, four PDL specimens from adjacent non-endodontically involved teeth were also examined. Histologically, the chronic PG specimens consisted of granulomatous tissue containing varying degrees of inflammatory cell infiltrate (lymphocytes, plasma cells and macrophages), fibroblasts, vessels and in some specimens epithelial cells. Fourteen oral squamous cell carcinoma (OSCC) specimens, a tissue known to have high angiogenic activity, were used as a positive control. Six of the OSCC sections showed histologically normal peri-tumour oral mucosa (NOM) adjacent to the tumours. This normal tissue was used as a control for the OSCC. Consecutive sections from the middle third of each specimen were alternately stained with CD105, MSF and vWF antibodies. At least two sections from

each specimen were stained with each antibody. A limited number (up to six) of random sections from other areas of the specimens were also examined for a small number (n=10) of specimens. No significant histological variation was observed between the replicate sections. All laboratory work was carried out by the principal researcher (KJD) unless otherwise stated.

2.3.2 General immunohistochemistry staining protocol

Specimens were sectioned at 5µm using a Microtome (Microm HM320, GmbH) and mounted on silane-coated slides (Superfrost microscope slides, Shandon). The sections were placed into a metal slide rack with duplicate slides placed apart in order to check for differences due to the processing procedure. Slides were deparaffinised by placing twice in 100% xylene for five minutes and then rehydrating for two minutes in 100% and then 95% ethanol followed by distilled water. Endogenous peroxidase activity was blocked by immersion of the slides into 3% v/v hydrogen peroxide solution in PBS (40ml 30% hydrogen peroxide + 360ml PBS) for 30 minutes followed by washing them twice in PBS for five minutes, with all of these stages being carried out on a rocking platform. The slides were carefully dried using paper tissues, without touching the actual tissue section, and never allowing the tissue sections to dry out. Individual tissue sections were then ringed with a Dako pen (DakoCytomation) to restrict the loss of reagents from the tissue sections during incubation. Antigen retrieval was enhanced by use of one of three types of pre-treatment detailed below (see section 2.3.3). Slides were then washed twice for five minutes in PBS and following this, the Dako pen ring was examined and any defects in continuity were repaired as required.

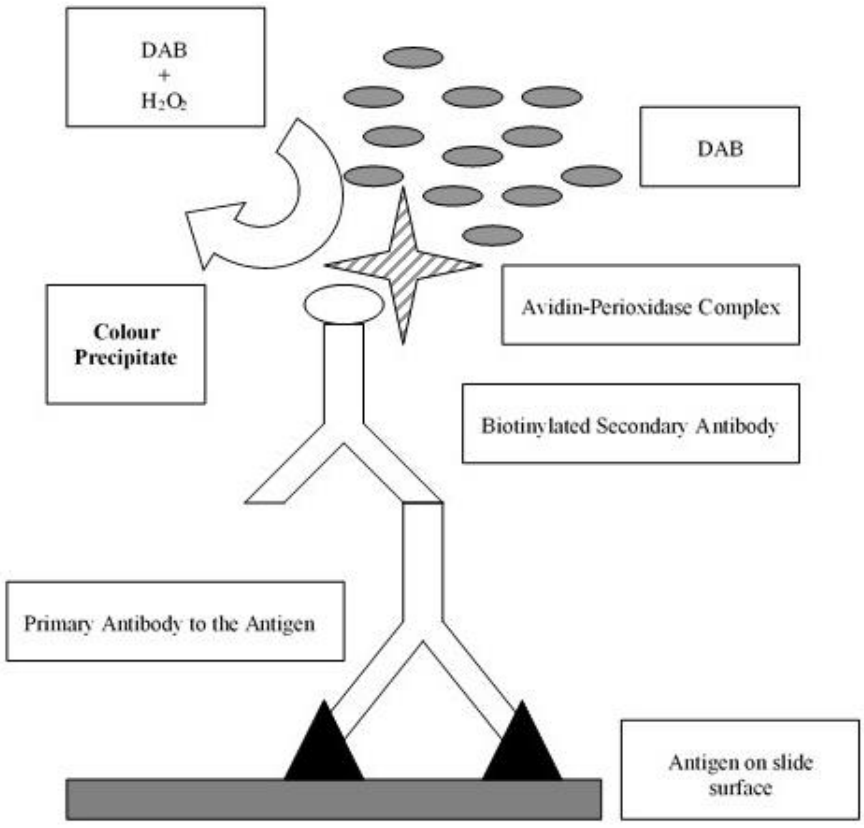
Non-specific binding was blocked using 100% v/v normal goat serum (NGS) (Diagnostics Scotland) for 20 minutes in a humidified container at room temperature. In all of the incubation steps in this protocol the actual volume of reagents used depended on the size of the tissue section (30µl for small sections, 100µl for standard sections and 250µl for large sections). All of the incubations were carried out in sealed humidified plastic containers lined with damp paper (distilled water) at room temperature, unless otherwise stated. This was followed by two further five-minute washes in PBS. A further blocking stage was

carried out using an Avidin-Biotin Blocking kit (Vector Labs Ltd). This involved two 15 minute incubations, the first in Avidin D (bottle A) and the second in Biotin (bottle B), and between these two incubations the sections were washed with PBS from a wash bottle and then gently dried with tissue paper. The Biotin was tipped off, the sections dried with tissue paper and the sections were then incubated with the primary antibody (CD105, MSF or vWF) overnight at 4^{o}C in sealed humidified containers (16 hours). For the negative control sections, mouse IgG (Dako) was used at a dilution equal to the primary antibody protein levels in the test sections. Due to the large number of sections used, staining was carried out on different occasions, so a positive standardised control slide was used to compare the staining intensity between staining runs. This quality control measure allowed staining to be compared between staining runs. Throughout the protocols all antibody dilutions were made up and placed in ice immediately, prior to use.

The next morning the primary antibody was washed off using PBS from a wash bottle followed by two five-minute washes in 0.05% v/v Tween 20 and a five-minute wash in PBS. The sections were dried with paper tissues as before. A further blocking stage was carried out by incubating with 100% NGS for 10 minutes. The NGS was tipped off and tissue paper was used to remove the majority of the serum. Then the slides were incubated for 40 minutes with the biotinylated secondary antibody (Vector Labs Ltd) and during this incubation the Avidin-Biotin peroxidase ABC complex (Vector Labs Ltd) was prepared. This stage was necessary as this complex must be made up at least 30 minutes prior to use at room temperature. The secondary antibody was washed off the slides and a further Tween 20 and PBS wash sequence was repeated as above and the slides dried with paper tissues. The slides were incubated for 30 minutes with the ABC complex followed by the Tween 20 and PBS wash sequence. The slides were not dried after this but were immediately placed into 0.04% v/v DAB solution (3,3' diaminobenzidine, Sigma) in PBS for 5 minutes on a rocking platform. This process allowed the visualisation of the antibody complex. A small quantity of the ABC complex was retained and 200µl of DAB solution was added to it in order to check that the DAB solution was working. The slides were then washed in running tap water for two minutes and then counterstained by being placed into Mayer's haematoxylin (TBS) for 30 seconds. The slides were again washed for two minutes in running tap water and then

placed in Blueing agent (Thermo Shandon) for one minute followed by another two-minute wash in running tap water. The sections were then dehydrated by placing in 96% v/v and then 100% ethanol for one minute followed by a further two minutes in 100% ethanol and finally five minutes in Xylene. The slides were remounted using DPX microscopy mountant (BDH Laboratory Supplies), glass cover slips were applied and the slides were dried at 37°C for 3-4 hours. Figure 2.3 shows a summary of the immunohistochemistry protocol described above.

Figure 2.3 Summary diagram of the immunohistochemistry protocol.



2.3.3 Pre-treatments

Three types of pre-treatments were used to enhance antigen retrieval, either by heat treatment (Autoclave and Microwave pre-treatments) in fresh citrate buffer (2.1g citric acid dissolved in 1L distilled water and adjusted to pH6 with 1M NaOH) or by the enzyme Protease XXIV (Sigma-Aldrich).

The Autoclave pre-treatment required the slides to be placed into a metal rack, with the rest of the rack being filled with blank slides to ensure even heating. This rack was placed into a plastic container of citrate buffer solution, ensuring that the slides were completely immersed. A large plastic beaker was placed upside down in the autoclave and the plastic container containing the slides placed on top of it. The slides were autoclaved for 11 minutes at 126^0C (Prestige Medical Series 2100 Clinical Autoclave) with the whole process taking about 45 minutes. Once cool enough to handle, the sections were washed twice in distilled water for five minutes on a rocking platform.

The Microwave pre-treatment required the slides to be placed into a plastic rack, with the rest of the rack being filled with blank slides to ensure even heating. This rack was placed into a plastic container of citrate buffer solution, ensuring that the slides were completely immersed, and the plastic container covered in cling film. The slides were then microwaved (Tecnolec Microwave T250T 750W) for five minutes and then the citrate buffer was topped up before another five-minute microwave treatment. The cling film was removed and the buffer was allowed to cool for ten minutes before transferring the slides into a metal rack, which was placed in distilled water. The sections were washed twice in distilled water for five minutes on a rocking platform.

The Protease XXIV enzymatic pre-treatment was carried out by adding 100µl 0.01% w/v Protease XXIV (Sigma) and incubating for 20 minutes at 37ºC in a humidified sealed box lined with damp paper (distilled water). The Protease XXIV was then carefully washed off with PBS from a wash bottle, while being careful not to wash directly onto the tissue.

2.3.4 MSF immunohistochemistry staining protocol

The protocol used for the MSF expression was based on the general protocol above (Chapter 4.3.4 details the optimisation process adopted). The final MSF staining protocol used the MSF 7.1 Ascites Fluid (AF) mouse anti-human monoclonal primary antibody (Moravin Biotech) diluted 1:1200 in PBS. The secondary antibody was a goat anti-mouse biotinylated secondary antibody (Vector Labs) diluted in a 1 in 5 dilution of NGS in PBS to give a working concentration of 9µg ml^{-1}. No pre-treatment was used.

2.3.5 vWF immunohistochemistry staining protocol

A similar protocol was used for vWF except that 0.01% w/v Protease XXIV (Sigma) pre-treatment was used. No Avidin-Biotin blocking stage was done and the primary monoclonal antibody was a vWF IgG rabbit anti-human antibody (stock 1.5 mg ml^{-1}) diluted 1:3000 (DAKO) in PBS. The negative control was a rabbit IgG (DAKO) diluted 1 in 10500 to give the equivalent antibody proteins levels as the test samples. The secondary antibody was a biotinylated goat anti-rabbit antibody (Vector Labs) diluted in a 1 in 5 dilution of NGS in PBS to give a working concentration 9µg ml^{-1}.

2.3.6 CD105 (Endoglin) immunohistochemistry staining protocol

The protocol for the CD105 also used an autoclave pre-treatment step. The primary antibody was mouse anti-human CD105 monoclonal antibody (Novocasta) diluted 1 in 75 in PBS. The secondary antibody was a biotinylated goat anti-mouse IgG (Vector Labs) diluted in a 1 in 5 dilution of NGS in PBS to give a working concentration 9µg ml^{-1}.

2.3.7 Quantification of antibody staining

The intensity of the antibody staining was graded using pre-determined in-house calibration slides as either no staining (-), weak staining (+), moderate (++) or strong MSF (+++) staining using a standard binocular light microscope (Olympus BH2 Binocular Phase Contrast Microscope, Olympus America Inc). The slides were assessed at low magnification (x100), to get a general overall staining profile, and at high magnification (x200-x400) to assess the staining of the various cell types. The slides were graded by two to four independent observers and the final grades were obtained by consensus.

2.3.8 Quantification of vascularity

Modified standard light microscopy stereological methods were used to measure the Microvascular volume (MVV), average-Microvascular Density (MVD) and highest-Microvascular Density (h-MVD) for the vWF and CD105 stained sections using an eyepiece graticule (Mertz 100 point) (Chandrachud et al., 1997, Schor et al., 1998b). Initial training for MVV and MVD assessment was carried out using three standardised vWF stained human papilloma training slides (h-MVD was introduced later after the introduction of oral squamous cell carcinoma positive control sections). Instead of the standard 100 point counts

at x200 magnification, 25 point counts were carried out at x400 magnification (Davey et al., 2008). This was done to allow meaningful counts from the very thin periodontal ligament sections (Chapter 4.3.2.1). Fifteen random fields were counted for each section (375 points) for both the normal periodontal ligament (PDL) sections and the periapical granuloma (PG) sections. The final methodology was as follows:

(i) Microvascular volume (MVV): Stained vessel walls which coincided with the graticule points were counted in 15 random fields per section (375 points) and the results expressed as percentage, taking the mean (± standard deviation (SD)) for the 15 fields.

(ii) Average-Microvascular Density (MVD): Using the same grid, magnification and number of random fields, all stained vessels that fell within the area of the grid were counted. Vessels that touched the margins of the grid were only counted if they touched the upper or right-hand margins (unbiased counting frame). The results were converted to mean vessels per millimetre square (mm^{-2}) (± SD) for the 15 fields.

(iii) Highest-Microvascular Density (h-MVD): The area of highest microvascular density was located by scanning the section at x100 magnification. Three separate fields were counted in the same way as average-MVD, and the mean value was taken as the h-MVD and expressed as the number of vessels per mm^2 (±SD). No vascular hot spots were found in the PG and PDL sections, so h-MVD was calculated as the mean of the highest three MVD field values found per section.

Following initial training, the study sections were quantified by two to four independent observers and any sections with an inter-observer variation above 15% were recounted and agreed by consensus. Intra-observer variation was assessed by one observer (KJD) recounting all the sections on two separate occasions.

2.3.9 Assessment of inflammatory infiltration

One slide from each tissue block was stained with Haematoxylin (TBS) and Eosin (TBS) (H and E) using a standard protocol (Appendix 5). The extent of the inflammatory infiltration of each section was independently assessed by two examiners using four pre-determined

calibration H and E stained slides. The slides were scored either as Grade 0 (no inflammatory infiltrate), Grade 1 (mild degree of inflammatory infiltrate), Grade 2 (moderate degree of inflammatory infiltrate) or Grade 3 (Severe inflammatory infiltration). Precautions to minimise inter and intra-observer variation was taken (Chapter 4.4.1). The final scores were agreed by consensus.

2.4 Statistical analysis

All data was analysed at the time of the study using non-parametric tests with SPSS version 15 (SPSS Inc.). At the time of completion of the thesis the data was reanalysed using SPSS version 22 (IBM). Data for individual groups were pooled and means, standard deviations (SD), medians and interquartile ranges were determined. Two-way analysis of variance (ANOVA) and Bonferroni correction were used to compare the angiogenic factor levels in the multiple groups in the clinical samples. Diagnostic statistical analysis of the data was carried using Levene's, Standardized Residual and Cook's Distance tests. When appropriate a Bootstrapping methodology was carried out to ensure accuracy of the 95% confidence intervals. Comparisons between any two groups were carried out using Mann-Whitney U-test and correlations using Spearman rank correlation. The Chi-square test was used to compare frequencies. Intra- and inter-observer variation were analysed using Wilcoxon paired sample test. Results were considered to be significant at the 95% level of confidence ($p<0.05$).

Concentrations of angiogenic factors levels in clinical samples quoted in the literature (e.g. in serum, plasma, GCF and saliva), are by convention, stated in pg ml^{-1}. This convention has been used in thesis, unless otherwise stated.

2.5 Hardware, software and reagents

2.5.1 General hardware

Florida Probe System: Including 20g Florida Pocket Probe Handpiece. Florida Probe Corporation, Gainesville FL 32606, USA.

Microm HM320 Microtome: GmbH, Heidelberg 36900, Germany.

Microwave T250T 750W Oven: Tecnolec, Malaysia.

MRX ELISA Reader: Dynex Technologies, Chantilly, Virginia 20151-1621 USA.

Olympus BH2 Binocular Phase Contrast Microscope: Olympus America Inc, Center Valley, Pennsylvania 18034-0610 USA.

Orion II Microplate Luminometer: Berthold Detection Systems, Pforzheim D-75173, Germany.

Periotron 8000: Oraflow Inc, Plainview, New York, USA. Calibrated every three months with a standardised serum according to the manufacturer's recommendations.

Prestige Medical Series 2100 Clinical Autoclave: Prestige Medical Limited, Blackburn, Lancashire, UK.

Samsung V20 Laptop computer: Samsung Corporation, Suwon City, Kyungki-Do, Korea.

2.5.2 Software packages

Access XP: Database package, Microsoft Corporation, Redmond, WA 98052-6399, USA.

Dynex Revelation 3.2: ELISA plate reader software, Dynex Technologies, Chantilly, Virginia 20151-1621 USA.

Excel versions 97-2003 and 2013: Microsoft Corporation, Redmond, WA 98052-6399, USA.

G*Power: Statistical package (power calculations), version 3.1.9.7, Heinrich Heine University, Düsseldorf, Germany.
https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower.html

FP32: Periodontal Charting Programme, Florida Probe Corporation, Gainesville, FL 32606, USA.

Simplicity 4.02: Orion II Luminometer plate reader software, Berthold Detection Systems, Pforzheim D-75173, Germany.

SPSS version 15: Statistical package for the Social Sciences (SPSS 15), SPSS Inc., Chicago, IL, USA.

SPSS version 25: IBM SPSS statistics for Windows, version 25.0. IBM Corp., Armonk, New York, USA.

2.5.3 Antibodies and recombinant human protein standards

CD105 Mouse Anti-Human Monoclonal Antibody: Novocasta, Newcastle upon Tyne, UK. 1.5mg ml^{-1} stock diluted 1:5 NGS:PBS to give a working concentration of 9µg ml^{-1}.

DuoSet ELISA Development System kits: R & D Systems Inc., Minneapolis, MN 554 13, USA. See Table 2.2.

Goat Anti-Mouse Biotinylated Secondary Antibody: Vector Labs Ltd, Burlingame, USA. 1.5mg ml^{-1} stock diluted 1:5 NGS:PBS to give a working concentration of 9µg ml^{-1}.

Goat Anti-Rabbit Biotinylated Secondary Antibody: Vector Labs, Burlingame, USA. 1.5mg ml^{-1} stock diluted 1:5 NGS:PBS to give a working concentration of 9µg ml^{-1}.

Goat Anti-Rabbit IgG HRP Conjugate: Thermo Fisher Scientific Inc, PO Box 117, Rockford, IL 61105, USA. 0.8mg ml^{-1} stock diluted 1:1000 in dried milk buffer or 1% w/v BSA.

Mouse IgG Negative Control: Dako, Glostrup, Denmark. Diluted in PBS to give a protein equivalence to the primary antibody used.

MSF 7.1AF: Mouse anti-human monoclonal antibody, Moravin Biotech, Brno, Czech Republic. Diluted 1:1200 in PBS.

MSF Pep Q 5.1: Mouse anti-human monoclonal antibody raised against the IGD region of MSF. Batch 5 group1. Moravin Biotech, Brno, Czech Republic. 2.25mg ml^{-1} stock diluted to a working concentration of 10µg ml^{-1} in either 1% w/v dried milk or 1% w/v BSA buffer.

MSF RpVSI: Rabbit anti-human polyclonal antibody raised against the MSF unique C-terminal 10 amino acid sequence (VSIPPRNLGY). Moravin Biotech, Brno, Czech Republic. 4.92mg ml^{-1} stock diluted to a working concentration of 16.7µl ml^{-1} in either 1% w/v dried milk or 1% w/v BSA buffer.

Rabbit IgG Negative Control: DAKO, Glostrup, Denmark.

Recombinant MSF: Bacterial rMSF-aa Batch 7. University of Dundee. 30µg ml^{-1} diluted in blocking buffer to give a working range of 200 to 3.125ng ml^{-1}.

vWF IgG Rabbit Anti-Human Monoclonal Antibody: DAKO, Glostrup, Denmark.

2.5.4 Standard buffers and reagents

Avidin-Biotin Blocking kit: Vector Labs Ltd, Peterborough, UK.

ABC Complex: Vector Labs Ltd, Peterborough, UK. 12µl of each reagent per ml PBS. [36µl reagent A + 36µl reagent B + 3ml PBS]. Must be made up at least 30 minutes prior to use (room temperature).

Blueing Agent: Thermo Shandon, Runcorn, Cheshire, UK.

Bovine Serum Albumin (BSA): Sigma-Aldrich Co, PO Box 14508, St Louis MO 63178, USA.

Citrate Buffer: 9ml 0.1M Citric acid + 41ml 0.1M Sodium citrate + 10 drops of Conc. Sodium hydroxide + 450ml distilled water + check pH at pH6.

DAB Solution (3,3' diaminobenzidine): Sigma, Gillingham, UK. 0.04% in PBS. 400ml PBS + 400µl 30% H_2O_2 + 2 x 800µl 10% DAB aliquots.

DPX Microscopy Mountant: BDH Laboratory Supplies, Poole, Dorset, UK.

Dried Milk Buffer: 1% w/v in PBST buffer. Marvel dried skimmed milk, Premier International Foods, Spalding, Lincolnshire, UK.

ELISA Reagent Diluent: 1% w/v BSA in PBS, pH 7.2-7.4, 0.2µm filtered 1% w/v BSA in PBS, pH 7.2-7.4, 0.2µm filtered.

- 2g BSA in 200ml of distilled water with 1 PBS tablet.

ELISA Stop Solution: 2M H_2SO_4. 5.46 ml Sulphuric acid (18.3 Molar) in 94.54ml distilled water.

ELISA Streptavidin-HRP (R&D Systems Part 890803): 1ml of Streptavidin conjugated to horseradish-peroxidase. Must not be frozen. Diluted 1 in 200 in ELISA reagent diluent.

ELISA Substrate Solution: TMB (Tetramethylbenzidine) + Substrate-Chromogen. DAKO Corporation, Carpinteria CA, USA.

Eosin: TBS, Skelmersdale, UK.

Ethanol: BDH Laboratory Supplies, Poole, Dorset, UK.

Hydrogen Peroxide 30%: Sigma-Aldrich Co., St Louis Mo 63103, USA.

Mayer's haematoxylin: TBS, Skelmersdale, UK.

MSF Coating Buffer: BupH 0.2M Carbonate-Bicarbonate buffer pH 9.4. Dissolve 1 pouch in 500ml distilled water. Filtered. Thermo Fisher Scientific Inc, PO Box 117, Rockford IL 61105, USA.

Normal Goat Serum: Diagnostics Scotland, Law Hospital, Carluke, UK.

PBS: 137 mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5 mM $KH2P0_4$, pH 7.2-7.4. Sigma-Aldrich Co, PO Box 14508, St Louis MO 63178, USA.

Protease XXIV: Sigma-Aldrich, Gillingham, UK. Diluted in PBS 1% stock to give 0.01% working concentration.

Protease Inhibitor Cocktail Tablets: EDTA-free. Dilute in 10ml filtered PBS or PBST. Roche Diagnostics GmbH, Mannheim +49 621 75 90, Germany.

Tween 20 Washing Buffer (PBST): Sigma-Aldrich Co, PO Box 14508, St Louis MO 63178, USA. Diluted to 0.05% in PBS, pH 7.2-7.4.

- 2 litres PBS + 1ml Tween 20.

Xylene: BDH Laboratory Supplies, Poole, Dorset, UK.

2.5.5 General laboratory plasticware/consumables

BD Falcon Microtest 96-well ELISA Plate: colourless enhanced surface. BD Biosciences, 2 Oak Park, Bedford MA 01730, USA.

BD Vacutainer System: Becton Dickinson and Company, Franklin Lakes, NJ 07417, USA.

Costar 3590 96-well ELISA Plate: colourless high binding polystyrene. Corning Incorporated, Corning NY 14831, USA.

Dako Pen: DAKO Corporation, Carpinteria CA, USA.

Eppendorf 0.5ml and 1.5ml Non-stick Microtubes: Alpha Laboratories Ltd, Eastleigh, Hampshire, UK.

Falcon 15ml Polypropylene Conical Tubes: Becton Dickinson and Company, Franklin Lakes, NJ 07417, USA.

Filter 0.45µm Membranes: Millipore Corporation, Bedford MA 01730, USA.

Parafilm: Alcan Packaging, Neenah WI 54956, USA.

PerioPaper GCF Collection Strips: Pro Flow, Amityville, New York 11701, USA. LOT 6178.

Slide Cover Glass Slips (22x22mm and 22x32mm): VWR International, Lutterworth, Leicestershire, UK.

Sterilin Bijou 7ml Containers: Barloworld Scientific Ltd, Stone, Staffordshire, UK.

Sterilin Universal Containers: Barloworld Scientific Ltd, Stone, Staffordshire, UK.

Superfrost microscope slides: Shandon, Cheshire, UK.

Chapter 3

Angiogenic Factor Levels in Oral Health and Periodontal Disease

3.1 Aims of the study

The aims of this study were:

1. To determine concentrations of Ang-1, MSF, VEGF and endostatin in human serum and saliva (unstimulated and stimulated saliva).
2. To determine MSF concentrations in GCF.
3. To investigate whether Ang-1, MSF, VEGF and endostatin concentrations in serum and saliva, including MSF concentrations in GCF, are significantly different between periodontal health and severe periodontitis.
4. To investigate whether Ang-1, MSF, VEGF and endostatin concentrations in serum and saliva, including MSF concentrations in GCF, are significantly altered in smokers and in patients with diabetes, compared with samples from matched healthy controls.
5. To explore the relationship between endostatin and the pro-angiogenic factors Ang-1, MSF and VEGF.

3.2 Methods

Details of the materials and methods used in this study are outlined in Chapter 2.2.

3.3 Angiopoietin-1 (Ang-1): Levels in Oral Health and Periodontal Disease

3.3.1 Background

3.3.1.1 Ang-1 levels in serum and plasma

Serum and plasma concentrations of the pro-angiogenic factor Ang-1 have been reported in a variety of human pathological conditions, in comparison to healthy control subjects (Chapter 1.5.3.2). Significantly raised serum and plasma Ang-1 concentrations, in comparison with healthy control subjects, have been reported in breast cancer (Caine et al., 2003), proliferative sickle retinopathy (Mohan et al., 2005) and in pregnancy (Nadar et al., 2005) (Table 3.1). However, some studies have reported no significant differences in Ang-1 levels, for example in prostate cancer (Caine et al., 2003) and sickle cell disease (Duits et al., 2006), while significantly reduced concentrations has been reported in thyroid cancer (Niedzwiecki et al., 2006).

Table 3.1 shows a representative selection of publications describing serum Ang-1 concentrations in healthy control subjects and provides a baseline level to compare with the subjects examined in this thesis. Currently, there have been no publications which have compared serum Ang-1 concentrations in periodontal health with periodontitis, or whether smoking or diabetes have any additional effects on serum Ang-1 levels.

3.3.1.2 Ang-1 levels in saliva and GCF

Currently no publications have reported Ang-1 levels in saliva or GCF either in periodontal health or severe periodontitis, although two studies have examined Ang-1 levels in GCF following periodontal surgery (Rakmanee et al., 2010, Rakmanee et al., 2019). Rakmanee et al. (2010) studied GCF levels of Ang-1 (total protein levels) at periodontally healthy sites (n=15) and compared this with periodontitis sites seven days post periodontal surgery (n=15). Significantly higher GCF Ang-1 levels were found at surgical sites compared with healthy control sites in the same subjects ($p<0.05$). In a more recent study, Rakmanee et al. (2019) carried out a 12 month longitudinal study in patients with aggressive periodontitis (1999 Periodontal classification (Lang et al., 1999)) who underwent either guided tissue regeneration (GTR) or conventional access flap (AF) surgery (n=18 in both groups). In both types of surgery, maximum GCF levels of Ang-1 were found 7 days post-surgery, after which the levels returned to baseline levels. GCF levels of Ang-1 (total protein per site) was significantly higher in the GTR group 30 days post-surgery compared with the healthy controls. However, at 6 months there were no significant differences in total GCF Ang-1 levels between the GTR or AF sites. Neither study examined GCF Ang-1 concentrations in periodontal health compared with conventional periodontitis.

Table 3.1 A representative selection of publications for Ang-1 concentrations in human serum and plasma.

SERUM	PLASMA
Duits et al. (2006) • Healthy controls (n=35) mean=39109pg ml^{-1} • Sickle cell disease (n=23) mean=29136pg ml^{-1} • No significant difference (p=0.13)	Caine et al. (2003) • Healthy female controls (n=12) mean 4ng ml^{-1} • Breast cancer (n=30) mean=15.5ng ml^{-1} • Significantly higher Ang-1 concentration in breast cancer> control (p=0.0005) • Prostate cancer (n=30) mean 8ng ml^{-1}; healthy male controls (n=12) mean 8ng ml^{-1} • No significant difference between prostate cancer and healthy controls (p=0.072) • Significantly higher concentration in male healthy controls versus female (p=0.0073)
Niedzwiecki et al. (2006) • Healthy controls (n=27) mean=51108±3084pg ml^{-1} (SE) • Thyroid cancers patients (n=52) mean=29307±2305pg ml^{-1} (SE) • Significantly lower Ang-1 concentrations in thyroid cancer patients than controls (p<0.003)	Nadar et al. (2005) • Healthy non-pregnant controls (n=30) mean=3ng ml^{-1} • Healthy pregnant (n=64) mean=8ng ml^{-1} • Pregnancy hypertension (n=37) mean=15ng ml^{-1} • Pre-eclampsia (n=35) mean=23ng ml^{-1} • Significantly increased Ang-1 concentrations of all pregnancy groups compared with healthy non-pregnant controls (p<0.001)
Iribarren et al. (2011) • Healthy controls (n=345) median=32.5ng ml^{-1} (IQR=12.7) • Acute myocardial infarction (n=347) median =33.1ng ml^{-1} (IQR 13.6) • No significant difference in serum Ang-1 concentration in acute myocardial infarction group compared with healthy controls (p=0.52)	Mohan et al. (2005) • Healthy controls (n=23) mean=0.5ng ml^{-1} (range 0.5-2.5ng ml^{-1}) • Proliferative sickle retinopathy (n=24) mean=2.2ng ml^{-1} (range 1.0-11.4ng ml^{-1}) • Significantly increased Ang-1 concentrations in proliferative sickle retinopathy compared with healthy controls (p=0.0004)
Guveli et al. (2016) • Healthy controls (n=20) median=41.65pg ml^{-1} (min=9.8; max=86.9pg ml^{-1}) • Nasopharynx cancer (n=40) median=33.35pg ml^{-1} (min=11.8; max=128.3pg ml^{-1}) • Larynx cancer (n=20) median=42pg ml^{-1} (min=18.1; max=109.8pg ml^{-1}) • Significantly raised Ang-1 concentration in advanced nasopharyngeal cancers compared with healthy controls and early stages of tumours	X
Data from manufacturer • Healthy controls (n=46) mean=37122pg ml^{-1} (range 14272-65570pg ml^{-1})	x

3.3.2 Results

3.3.2.1 Ang-1 concentration in serum

Ang-1 was detected in the majority of serum samples and the results are summarised in Table 3.2 and are shown in Figure 3.1. Descriptors of the study groups and sub-groups are shown in Chapter 2.1.1 and 2.1.2 respectively. Ang-1 was quantified in 96 serum samples with mean concentrations for the study sub-groups ranging from 32910 to 46560pg ml^{-1}, although there were large variations in the levels detected. No significant differences were found between either the study groups (Two-way ANOVA test $F(2,90)=1.229$, $p=0.297$) or between periodontal health and severe periodontitis sub-groups (Two-way ANOVA test

F(1,90)=0.885, p=0.349), and there was no significant interaction found between periodontal health status and the study groups (Table 3.3). A non-significant reduction was found in the smoking group (Group 2) in periodontal health compared with the healthy control (Group 1) and diabetes groups (Group 3) (Figure 3.2). Bonferroni post hoc tests confirmed that there were no significant differences between the study sub-groups (Table 3.4). Diagnostic statistical analysis revealed that there was an outlier in the data which had a significant influence on the overall statistical outcome (Studentized residual highest=3.01, lowest= -1.77; highest Cook's distance=0.14; sample KJD58 was deemed to be an outlier). Sensitivity analysis, where data from the outlier was removed from the dataset, confirmed there were no significant differences in the serum Ang-1 levels between the study groups (Two-way ANOVA test F(2,89)=2.261, p=0.110; Table 3.3 and Figure 3.3). Bonferroni post hoc tests revealed, however, significantly higher serum Ang-1 concentrations in the systemically healthy non-smokers (study group 1) compared with the diabetes group (study group 3) (study group 1>3 p=0.034) (Table 3.4). No significant difference was found between the systemically healthy and smoking groups.

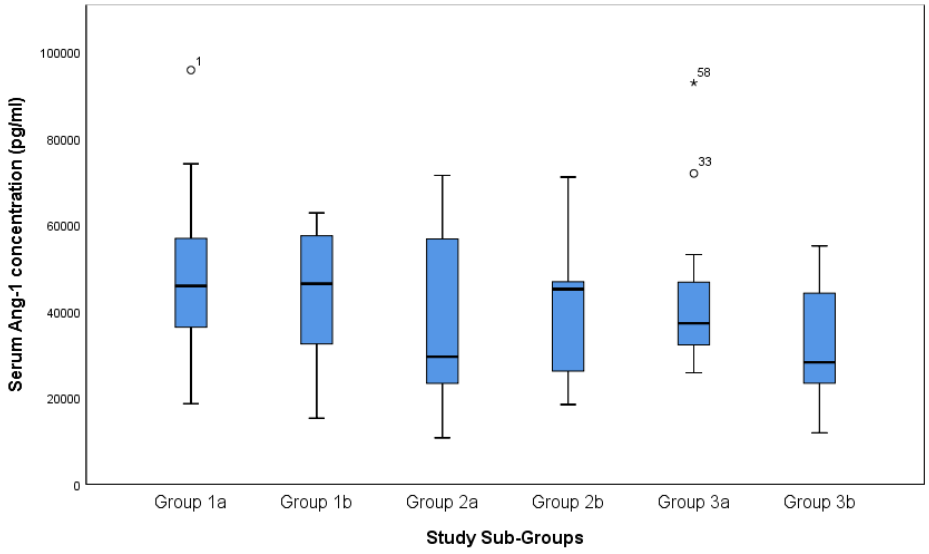
Table 3.2 Descriptive statistics for Ang-1 serum concentrations (pg ml^{-1}) for the study groups and sub-groups. Statistical data in brackets are the results following removal of extreme outliers (>3xSD) from the data set.

Group	Number Valid Cases*	Mean ± SD (pg ml^{-1})	Median (pg ml^{-1})	Interquartile Range	Range
1a	37	46560±15739	45916	20975	77238
1b	12	43646±15469	46415	27177	47539
Study Group 1	49	45847±15563	45916	22440	80582
2a	11	39555±22338	29506	41041	60743
2b	9	42526±17900	45156	34660	52670
Study Group 2	20	40892±19996	43890	34336	60743
3a	12 (11)	43871±19827 (39411±13035)	37275 (35280)	18642 (9956)	67102 (46117)
3b	15	32910±13982	28225	25183	43247
Study Group 3	27 (26)	37781±17389 (35660±13718)	34150 (34132)	22931 (17129)	81013 (60028)

*Excluding either no sample available or factor not detected.

Figure 3.1 Serum Ang-1 concentrations (pg ml^{-1}) for (a) study sub-groups and (b) study groups. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

(a)



(b)

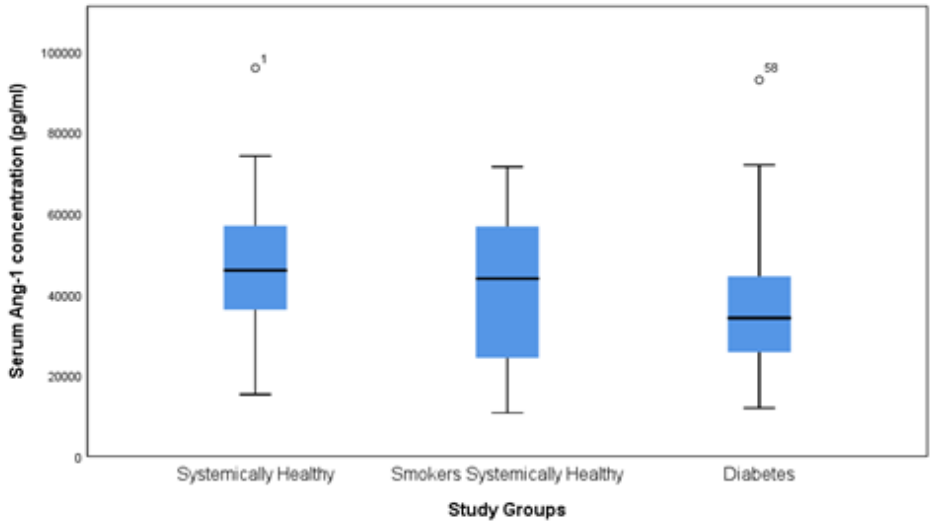


Table 3.3 Statistical comparison of serum Ang-1 concentrations of the study groups and periodontal health-severe periodontitis using Two-way ANOVA test. Data in brackets are the results following removal of outlier KJD58 from the data set.

Group	Degrees of Freedom (df)	F	Significance (p-value)
Periodontal Health – Severe Periodontitis	1	0.885 (0.334)	0.349 (0.565)
Study Groups (1, 2 and 3)	2	1.229 (2.261)	0.297 (0.110)
Periodontal Health – Severe Periodontitis Versus Study Groups (interaction)	2	0.987 (0.475)	0.377 (0.624)

Table 3.4 Statistical comparison between study sub-groups for serum Ang-1 (Bonferroni test p-values). Data in brackets are the results following removal of outlier KJD58 from the data set.

Study Groups		Mean Difference	Significance (p-value)	95% Confidence Interval	
				Lower Bound	Upper Bound
Group 1	Group 2	4954.71 (4954.71)	0.829 (0.762)	-6082.33 (-5574.89)	15991.76 (15484.32)
	Group 3	8065.42 (10186.37)	0.154 (0.034*)	-1903.96 (558.13)	18034.80 (19814.60)
Group 2	Group 1	-4954.71 (-4954.71)	0.829 (0.762)	-15991.76 (-15484.32)	6082.33 (5574.89)
	Group 3	3110.70 (5231.65)	1.000 (0.847)	-9160.67 (-6570.95)	15382.08 (17034.26)
Group 3	Group 1	-8065.42 (-10186.37)	0.154 (0.034*)	-18034.80 (-19814.60)	1903.96 (-558.13)
	Group 2	-3110.70 (-5231.65)	1.000 (0.847)	-15382.08 (-17034.26)	9160.67 (6570.95)

* - Significant difference <0.05

Figure 3.2 Profile plot of periodontal health and severe periodontitis against estimated marginal mean serum Ang-1 concentrations (pg ml^{-1}) for the three study groups.

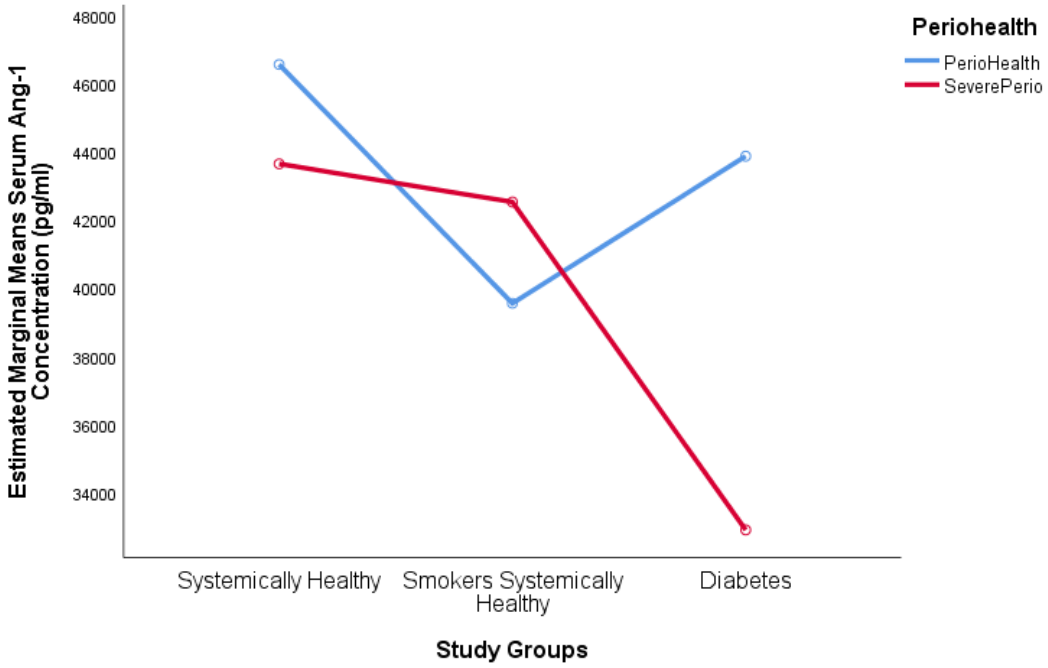
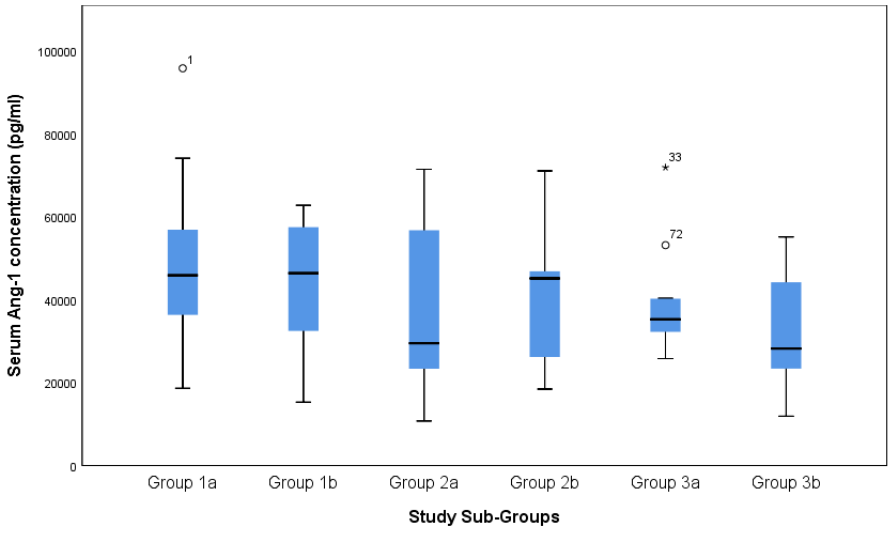
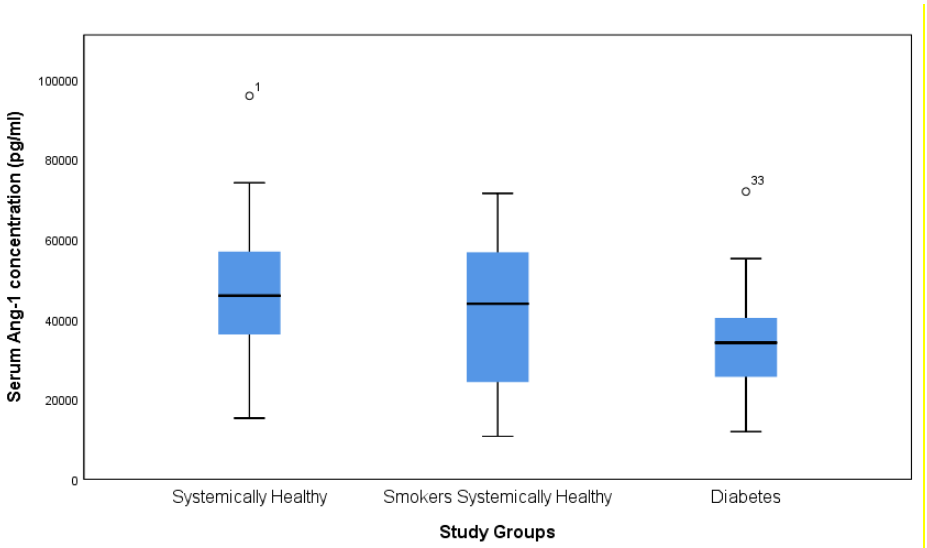


Figure 3.3 Serum Ang-1 concentrations (pg ml^{-1}) with the extreme outlier (>3xSD – KJD58) removed for (a) study sub-groups and (b) study groups. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

(a)

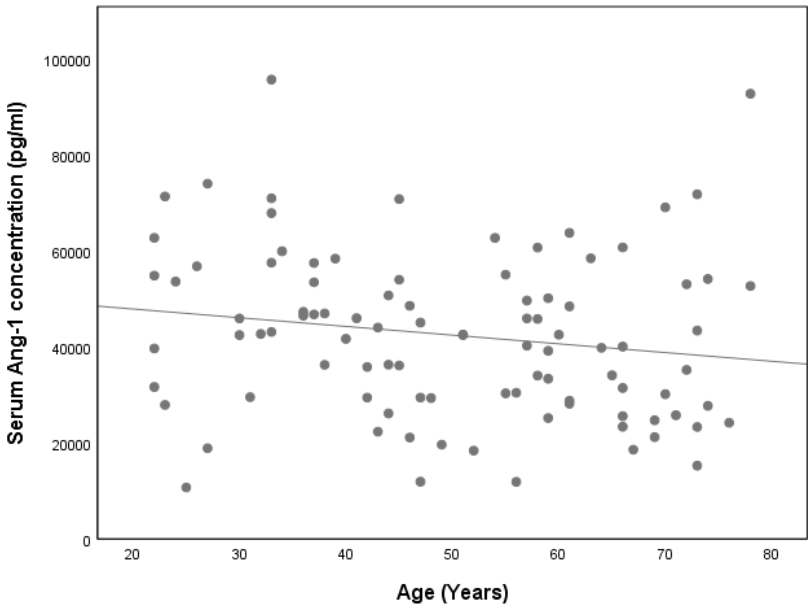


(b)



No significant correlations were found between serum Ang-1 concentration with smoking dose (Spearman rho -0.143, p=0.170) or glycaemic control (Spearman rho -0.219, p=0.271), however, a significant weak negative correlation was found with age (Spearman rho -0.211, p=0.039; without outliers Spearman rho=-0.248, p=0.015) (Figure 3.4). No significant difference was found regarding gender and serum Ang-1 concentration across the whole sample (Mann Whitney p=0.352).

Figure 3.4 Scatterplot of the correlation between serum Ang-1 concentration (pg ml^{-1}) with age (Years). Line of best fit shown*.



*Line of best fit R^2=0.029 (Spearman rho -0.211, p=0.039; without outliers Spearman rho=-0.248, p=0.015).

3.3.2.2 Ang-1 concentration in saliva

3.3.2.2.1 Ang-1 concentration in stimulated saliva

Ang-1 was detected in the majority of stimulated saliva samples and the results are summarised in Table 3.5 and shown in Figure 3.5. Ang-1 was quantified in 84 stimulated saliva samples with mean concentrations for the study sub-groups ranging from 26879 to 47399pg ml^{-1}, although there were large variations in the levels found. No significant differences were found between either the study groups (Two-way ANOVA test $F(2,78)=0.694$, $p=0.503$) or between periodontally healthy and severe periodontitis sub-groups (Two-way ANOVA test $F(1,78)=0.866$, $p=0.355$), and there was no significant interaction found between periodontal health status and the study groups (Table 3.6). A non-significant reduction was found in the smoking group (Group 2) in periodontal health compared with the healthy control (Group 1) and the diabetes (Group 3) groups (Figure 3.6). Bonferroni post hoc tests confirmed that there were no significant differences between the study sub-groups (Table 3.7). Diagnostic statistical analysis revealed that there were outliers in the data which had a significant influence on the overall statistical outcome (Studentized residual highest=2.97, lowest=-1.54; highest Cook's distance=0.10; samples KJD20 and KJD25 were deemed to be outliers). Sensitivity analysis, where the outliers (KJD20 and KJD25) were removed from the dataset, confirmed no significant differences between the study groups (Two-way ANOVA test $F(2,78)=0.443$, $p=0.644$) or between periodontally healthy and severe periodontitis sub-groups (Two-way ANOVA test $F(1,78)=1.412$, $p=0.238$) (Table 3.6 and Figure 3.7). There was no significant interaction found between periodontal health status and the study groups. Bonferroni post hoc tests confirmed that there were no significant differences in Ang-1 concentration in stimulated saliva between the study groups (Table 3.7).

No significant correlations were found between Ang-1 concentration in stimulated saliva with smoking dose (Spearman rho 0.078, $p=0.483$), glycaemic control (Spearman rho 0.038, $p=0.852$) or age (Spearman rho 0.038, $p=0.852$). No significant difference was found regarding gender and stimulated saliva Ang-1 concentration across the whole sample (Mann Whitney $p=0.324$).

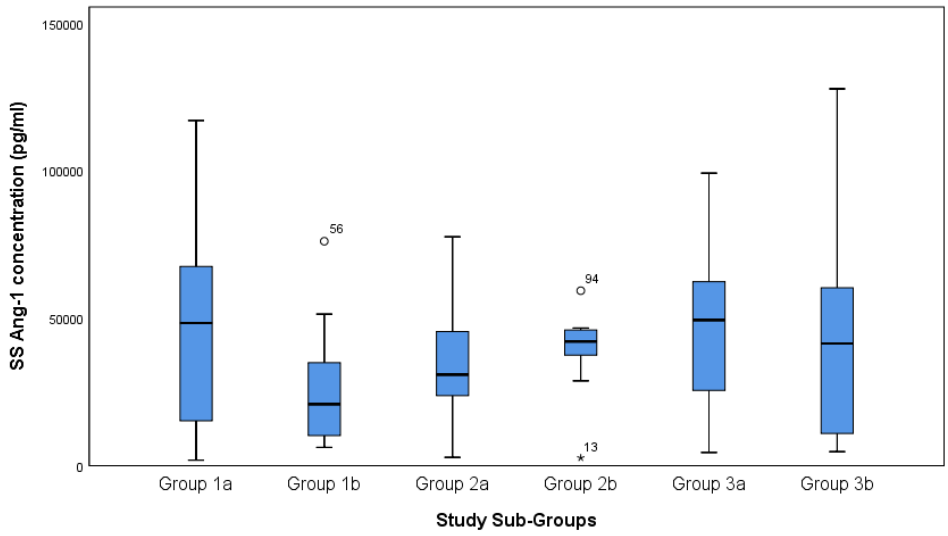
Table 3.5 Descriptive statistics for Ang-1 stimulated (SS) and unstimulated saliva (US) concentrations (pg ml^{-1}) for the study groups and sub-groups. Statistical data in brackets are the results following removal of extreme outliers (>3xSD) from the data set.

	Group	Number Valid Cases*	Mean ± SD (pg ml^{-1})	Median (pg ml^{-1})	Interquartile Range	Range
SS	**1a**	26 (25)	46808±34569 (43998±32108)	48406 (44071)	55037 (56558)	115174 (108528)
	1b	13	26879±20329	20889	26713	69880
	Study Group 1	39 (38)	40165±31737 (38141±29504)	34946 (34730)	53875 (52167)	115174 (108528)
	2a	9	32729±22404	30874	29937	74772
	2b	9	38767±15809	42140	13234	56654
	Study Group 2	18	35748±19065	38968	21920	74936
	3a	12	47399±29226	49379	45819	94765
	3b	15 (14)	42036±36133 (35911±28285)	41438 (34827)	57855 (46537)	123008 (82903)
	Study Group 3	27 (26)	44420+32738 (41213±28740)	46537 (44943)	54766 (50230)	123312 (94765)
US	**1a**	26	45338±35630	37034	53278	114472
	1b	14	30644±27499	20299	39308	85744
	Study Group 1	40	40195±33410	30055	51867	114472
	2a	8	29017±25739	31381	40844	73572
	2b	9	36288±16725	36121	18787	58757
	Study Group 2	17	32866±21064	36121	29354	73572
	3a	13	50159±33383	43479	51663	101087
	3b	15	36904±27736	33915	49582	95148
	Study Group 3	28	43058±30651	41783	53900	101087

*Excluding either no sample available or factor not detected.

Figure 3.5 Stimulated saliva Ang-1 concentrations (pg ml^{-1}) for (a) study sub-groups and (b) study groups. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

(a)



(b)

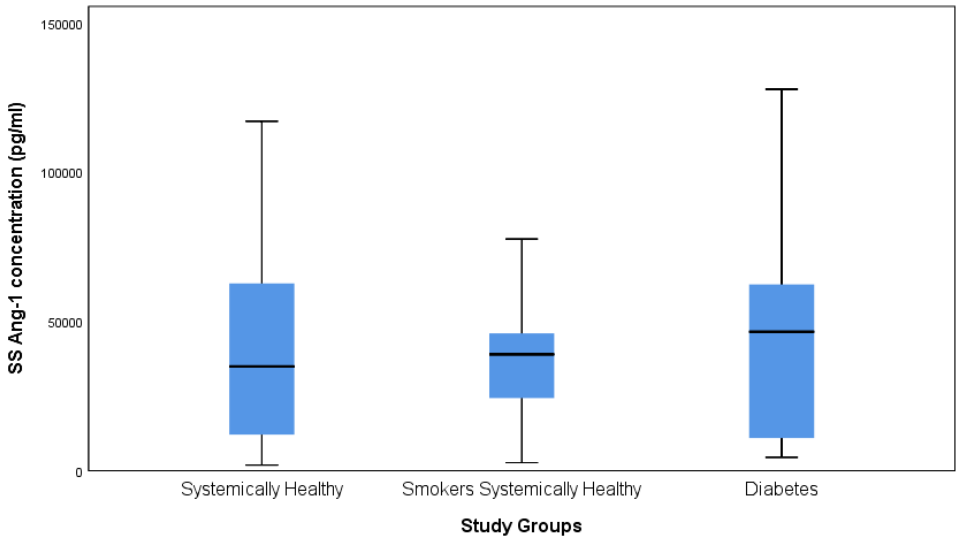


Table 3.6 Statistical comparison of stimulated saliva Ang-1 concentrations of the study groups and periodontal health-severe periodontitis using Two-way ANOVA test. Data in brackets are the results following removal of outliers KJD020 and KJD025 from the data set.

Group	Degrees of Freedom (df)	F	Significance (p-value)
Periodontal Health – Severe Periodontitis	1	0.866 (1.412)	0.355 (0.238)
Study Groups (1, 2 and 3)	2	0.694 (0.443)	0.503 (0.644)
Periodontal Health – Severe Periodontitis Versus Study Groups (interaction)	2	1.218 (1.099)	0.301 (0.339)

Table 3.7 Statistical comparison between study sub-groups for stimulated saliva Ang-1 (Bonferroni test p-values). Data in brackets are the results following removal of outliers KJD020 and KJD025 from the data set.

Study Groups		Mean Difference	Significance (p-value)	95% Confidence Interval	
				Lower Bound	Upper Bound
Group 1	Group 2	4416.94 (2393.37)	1.000 (1.000)	-16247.38 (-16538.19)	25081.27 (21324.92)
	Group 3	-4254.70 (-3071.96)	0.154 (1.000)	-22410.28 (-19911.56)	13900.88 (13767.64)
Group 2	Group 1	-4416.94 (-2393.37)	1.000 (1.000)	-25081.27 (-21324.92)	16247.38 (16538.19)
	Group 3	-8671.65 (-5465.33)	1.000 (1.000)	-30738.50 (-25752.60)	13395.21 (14821.95)
Group 3	Group 1	4254.70 (3071.96)	1.000 (1.000)	-13900.88 (-13767.64)	22410.28 (19911.56)
	Group 2	8671.65 (5465.33)	1.000 (1.000)	-13395.21 (-14821.95)	30738.50 (25752.60)

Figure 3.6 Profile plot of periodontal health and severe periodontitis against estimated marginal mean stimulated saliva Ang-1 concentrations (pg ml^{-1}) for the three study groups.

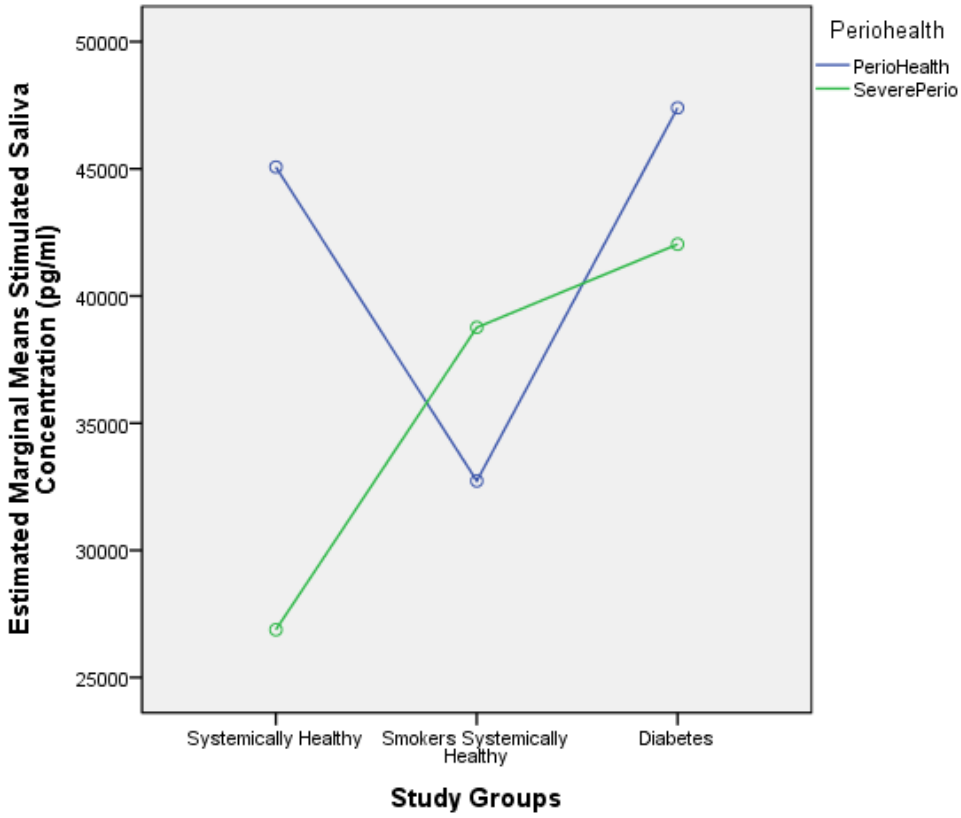
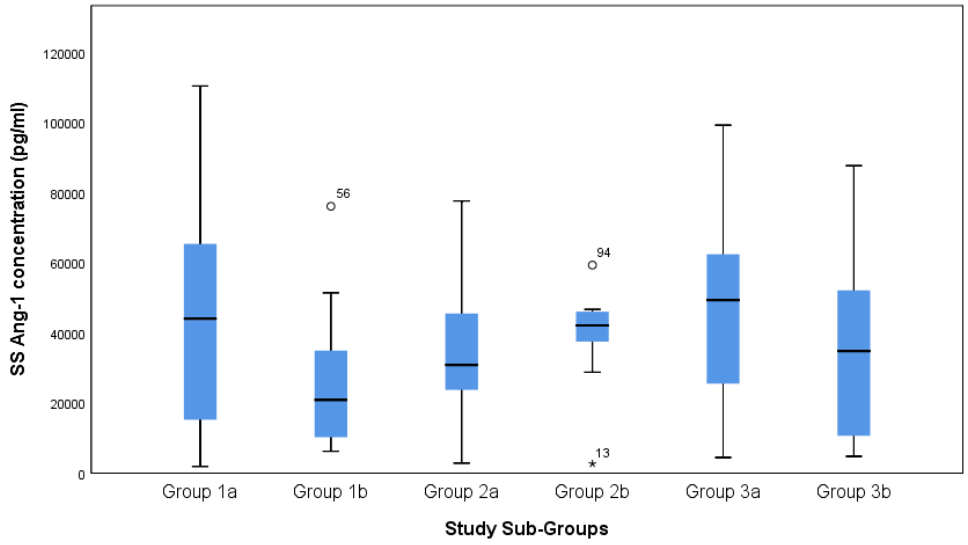
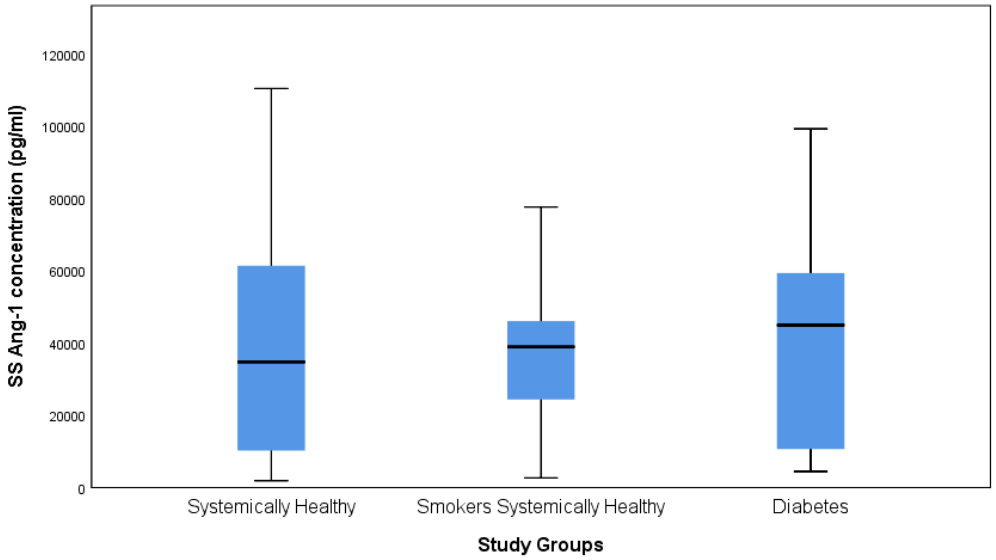


Figure 3.7 Stimulated saliva Ang-1 concentrations (pg ml^{-1}) with the extreme outlier (>3xSD – KJD58) removed for (a) study sub-groups and (b) study groups. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

(a)



(b)

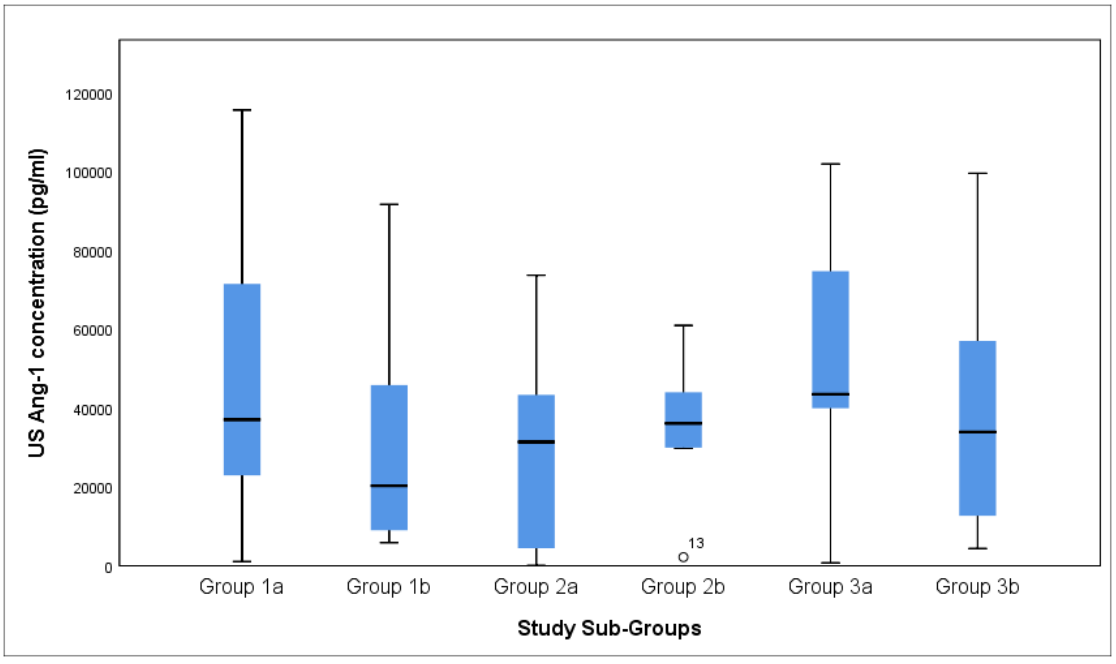


3.3.2.2.2 Ang-1 concentration in unstimulated saliva

Ang-1 was detected in the majority of unstimulated saliva samples and the results are summarised in Table 3.5 and shown in Figure 3.8. Ang-1 was quantified in 85 unstimulated saliva samples with mean concentrations for the study sub-groups ranging from 29017 to 50159pg ml^{-1}, although there were large variations in the levels found. No significant

differences were found between either the study groups (Two-way ANOVA test F(2,82)=0.701, p=0.499) or between periodontally healthy and severe periodontitis sub-groups (Two-way ANOVA test F(1,82)=0.950, p=0.333), and there was no significant interaction found between periodontal health status and the study groups (Table 3.8). A non-significant reduction was found in the smoking group (Group 2) in periodontal health compared with the healthy control (Group 1) and diabetes (Group 3) groups (Figure 3.9). Bonferroni post hoc tests confirmed that there were no significant differences between the study sub-groups (Table 3.9). Diagnostic statistical analysis revealed that there were no significant outliers in the data and the influence of any outliers on the overall statistical outcome was very low (Studentized residual highest=2.44, lowest=-1.68; highest Cook's distance=0.06). Therefore, no further diagnostic statistics was necessary.

Figure 3.8 Unstimulated saliva Ang-1 concentrations (pg ml^{-1}) for (a) study sub-groups and (b) study groups. The box represents the interquartile range, the horizontal line the median and the whiskers the range.
(a)



(b)

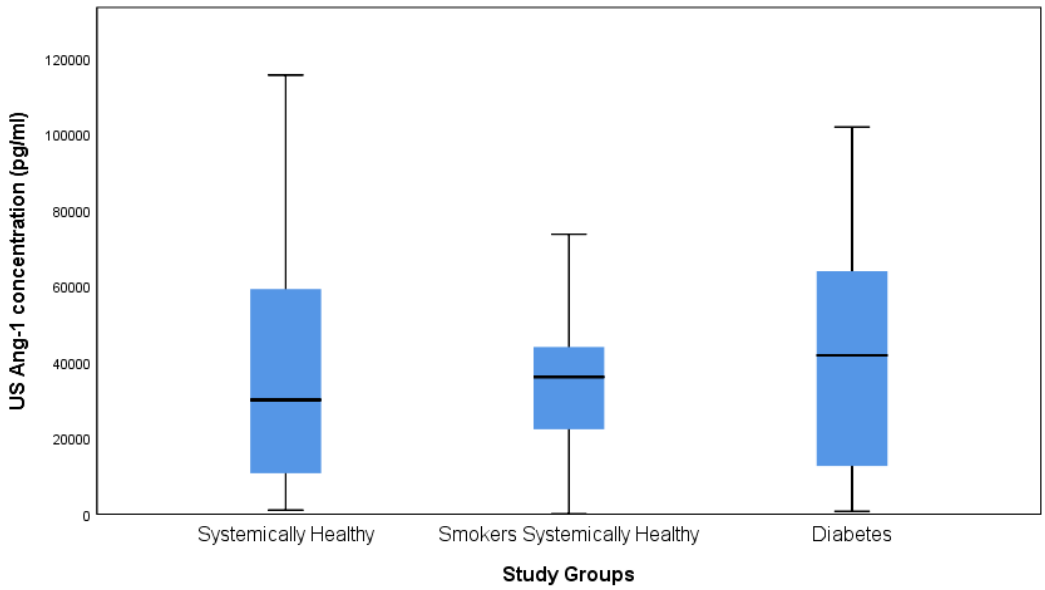


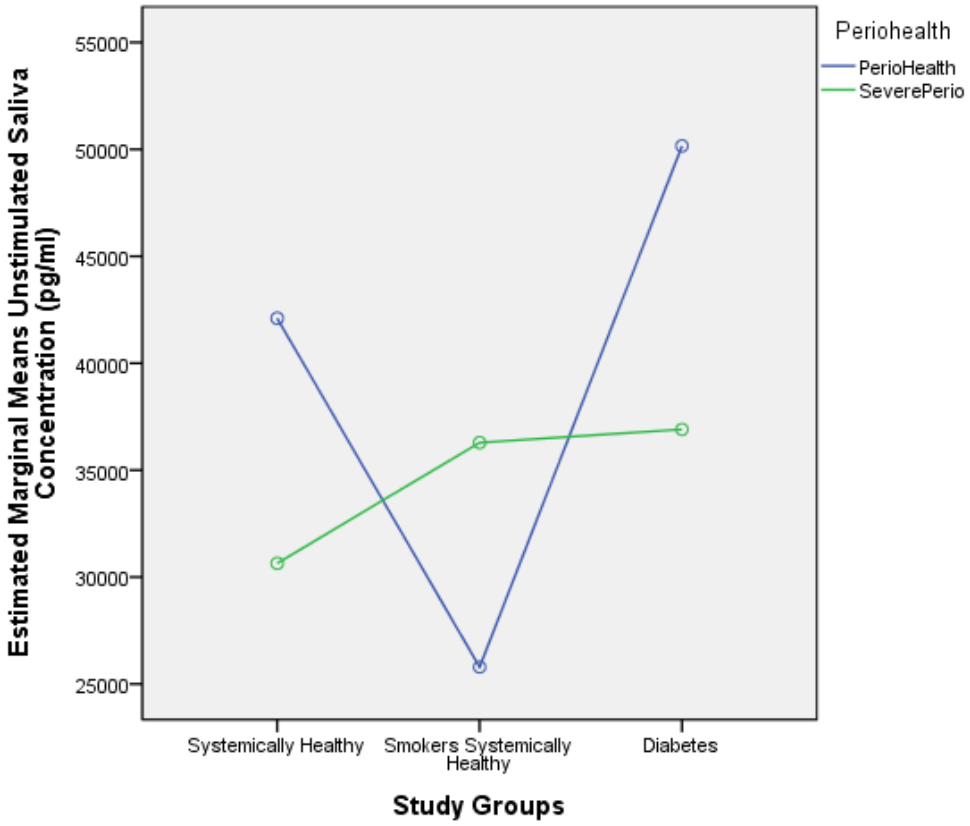
Table 3.8 Statistical comparison of unstimulated saliva Ang-1 concentrations of the study groups and periodontal health-severe periodontitis using Two-way ANOVA test.

Group	Degrees of Freedom (df)	F	Significance (p-value)
Periodontal Health – Severe Periodontitis	1	0.950	0.333
Study Groups (1, 2 and 3)	2	0.701	0.499
Periodontal Health – Severe Periodontitis Versus Study Groups (interaction)	2	0.835	0.438

Table 3.9 Statistical comparison between study sub-groups for unstimulated saliva Ang-1 (Bonferroni test p-values).

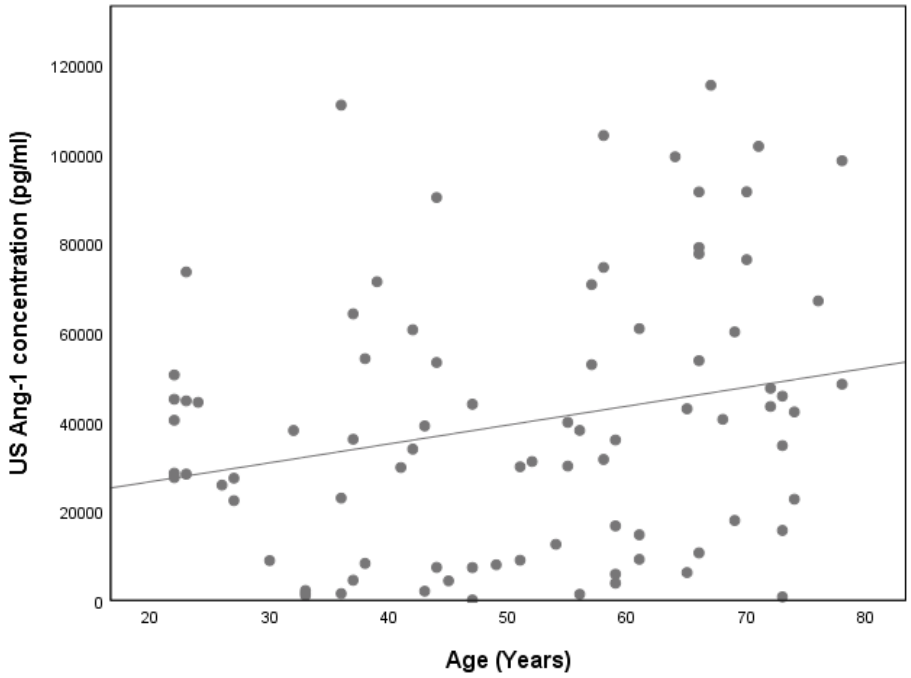
Study Groups		Mean Difference	Significance (p-value)	95% Confidence Interval	
				Lower Bound	Upper Bound
Group 1	Group 2	7328.86	1.000	-14137.36	28795.09
	Group 3	-2863.22	1.000	-21132.36	15405.92
Group 2	Group 1	-7328.86	1.000	-28795.09	14137.36
	Group 3	-10192.09	0.832	-32988.97	12604.79
Group 3	Group 1	2863.22	1.000	-15405.92	21132.36
	Group 2	10192.09	0.832	-12604.79	32988.97

Figure 3.9 Profile plot of periodontal health and severe periodontitis against estimated marginal mean unstimulated saliva Ang-1 concentrations (pg ml^{-1}) for the three study groups.



No significant correlations were found between Ang-1 concentration in unstimulated saliva with smoking dose (Spearman rho 0.006, p=0.956) or glycaemic control (Spearman rho -0.048, p=0.810). A significant weak positive correlation was found between Ang-1 concentration in unstimulated saliva and age (Spearman rho 0.237, p=0.029) (Figure 3.10). No significant difference was found regarding gender and unstimulated saliva Ang-1 concentration across the whole sample (Mann Whitney p=0.475).

Figure 3.10 Scatterplot of the correlation between unstimulated saliva Ang-1 concentration (pg ml^{-1}) with Age (Years). Line of best fit shown*.

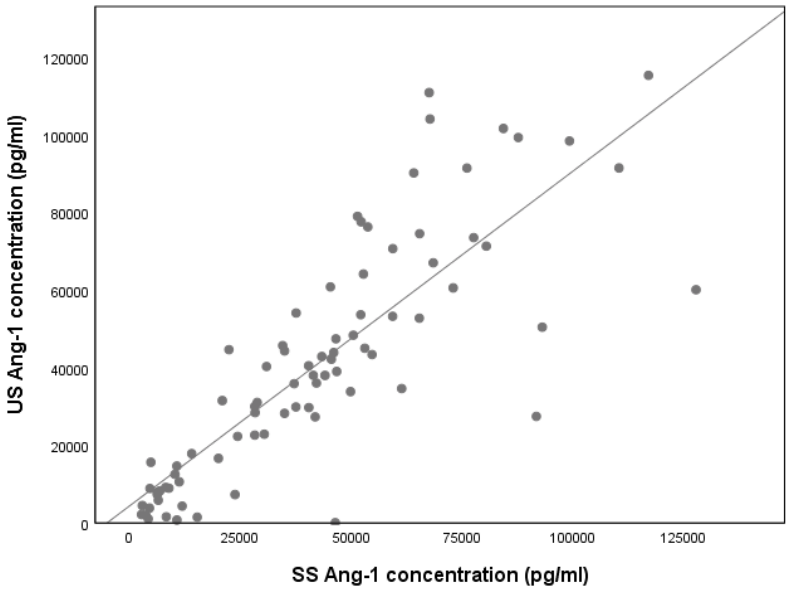


*Line of best fit R^2=0.056 (Spearman rho 0.237, p=0.029).

3.3.2.3 Relationship between Ang-1 concentrations in serum and saliva

No significant correlations were found between serum Ang-1 concentrations with either the levels found in the unstimulated (Spearman rho=-0.68, p=0.554) or stimulated saliva (Spearman rho=-0.059, p=0.607). A highly significant very strong positive correlation was found between paired unstimulated and stimulated saliva Ang-1 concentrations (Spearman rho=0.862, p<0.001; without outliers Spearman rho=0.858, p<0.001) (Figure 3.11).

Figure 3.11 Scatterplot of the correlation between the Ang-1 concentration (pg ml^{-1}) in unstimulated and stimulated saliva. Line of best fit shown*.



*Line of best fit R^2=0.689 (Spearman rho 0.862, p<0.001; without outliers Spearman rho=0.858, p<0.001).

3.3.3 Discussion

In this study, the concentration of the pro-angiogenic factor Ang-1 was measured in serum, unstimulated and stimulated saliva samples from three groups of subjects: (i) systemically healthy, (ii) systemically healthy smokers and in (iii) subjects with diabetes. Each clinical group was divided into two sub-groups: (a) periodontally healthy and (b) subjects with severe periodontitis. Confidence in the assay methodology was demonstrated by the good assay standard curves, low assay background noise, acceptable levels of intra- and inter-assay variation. Ang-1 was detected in the majority of samples assayed and the mean serum concentrations, standard deviations and ranges were found to be within levels previously reported in the literature, (Tables 3.1). High levels of Ang-1 were observed in serum, and for the first time in both unstimulated and stimulated saliva, however, no significant differences were observed between any of the sub-groups or study groups. No significant differences were found in Ang-1 concentrations in either serum or saliva (unstimulated and stimulated saliva) between the periodontally healthy or periodontitis sub-groups. However, once a statistical outlier was removed from the data, serum Ang-1 concentrations were found to be significantly higher in the systemically healthy non-smokers (Group 1) compared with

the diabetes group (Group 3). Significant correlations were found between Ang-1 concentrations in unstimulated and stimulated saliva and between Ang-1 concentrations in unstimulated saliva and age. Furthermore, a significant negative correlation was found between serum Ang-1 concentration and age.

As far as can be ascertained this is the first study to examine serum Ang-l concentrations in periodontal health and disease. Serum Ang-1 concentrations have previously been studied in several human conditions, in comparison with healthy control subjects. Currently there is great interest regarding the relationship between periodontal disease and systemic health with increasing evidence that periodontal inflammation may contribute to systemic inflammation (D'Aiuto et al., 2013, Pink et al., 2015). For example, several studies have associated periodontitis with increased systemic levels of factors including C-reactive protein (CRP), IL-1β, IL-2, TNFα and IFN-γ (Noack et al., 2001, Gorska et al., 2003, Paraskevas et al., 2008). Therefore, systemic spill over from periodontal inflammation is thought to acerbate various chronic systemic diseases, which are also known to have altered angiogenic-mediated responses, such as diabetes mellitus (Khader et al., 2006, Nascimento et al., 2018), cardiovascular disease (Genco et al., 2002, Janket et al., 2003, Bahekar et al., 2007, Carrizales-Sepulveda et al., 2018) and rheumatoid arthritis (Kaur et al., 2013, Fuggle et al., 2016). However, there was no evidence indicated in the present study that severe periodontitis significantly altered serum or salivary Ang-1 concentrations.

An explanation for this finding may come from an ELISA study by Lester et al. (2009) which reported the concentrations of a range of inflammatory and angiogenic factors, including Ang-1, in gingival biopsies harvested from extracted teeth. Prior to sampling, the health of the tissue was stratified into periodontal health and varying degrees of periodontal disease (mild, moderate and severe) using pocket depths and whether bleeding on probing was present. Ang-1 concentrations were found to be inversely correlated to periodontal health status (i.e. significantly lower Ang-1 concentrations in severe periodontal disease compared with gingival health). The opposite findings were reported for other factors such as IL-1β, IL-6, TNFα, VEGF and endothelin-1. From this finding the group hypothesised that reduced Ang-1 levels, in periodontal disease, promoted inflammation through reduced inhibition of

VEGF and endothelin. However, a major criticism of this study was that smoking was not taken into account, which may have influenced the findings. Although no significant differences were found between the study groups in serum or salivary Ang-1 concentrations in the present study, non-significant reductions in Ang-1 concentrations were found in periodontally healthy smokers in serum and saliva (stimulated and unstimulated saliva). However, these were non-significant results and require further investigation.

An interesting finding in the present study was the significant, albeit weak, negative correlation between age and serum Ang-1 concentration suggesting that serum Ang-1 concentration reduces with increasing age. Similar findings have been previously reported with other angiogenic factors, for example, mean serum VEGF concentrations were found to be significantly higher in children compared with adults (Okamoto et al., 2008). Relatively few studies have examined the effect of age on serum or plasma Ang-1 concentrations, however, the consensus suggests that Ang-1 concentration is not significantly influenced by age (Lim et al., 2005, Lukasz et al., 2008, Meng et al., 2009). Although, one study did report a significant positive correlation with age (Bennett et al., 2013). A possible reason for this discrepancy could be due to the relatively low number of subjects examined in many of these studies, which may have reduced the statistical power of the data. A note of caution needs to be taken with the finding of the significant correlation in the present study due to the relatively weak strength of the correlation, suggesting this may have been a statistical anomaly and further research is required to verify this finding.

As far as can be ascertained, this is the first study to have investigated the concentration of Ang-1 in saliva. Both stimulated and unstimulated saliva were found to contain high concentrations of Ang-1 suggesting that Ang-1, along with other pro-angiogenic factors in saliva, may be important in oral wound healing and maintenance of the oral soft tissues. It had been anticipated that there would be significant reductions in salivary levels of Ang-1 in the smoking and diabetes study groups. However, no significant differences were found in the present study between these groups and the healthy controls. Another unexpected finding was the significant, albeit weak, positive correlation between unstimulated salivary Ang-1 concentrations and age (i.e. the mean Ang-1 concentration in unstimulated saliva increased

with age). This finding differs from other pro-angiogenic factors recorded in saliva, such as Fibroblast Growth Factor-2 (FGF-2) and Nerve Growth Factor (NGF), which have been found to be significantly reduced in older subjects (Westermark et al., 2002). However, a similar reduction in factor concentration with age was reported in a small study which examined VEGF concentrations in unstimulated saliva (Upile et al., 2009). Unfortunately, at the time of writing there have been no publications indicating whether Ang-1 is produced in salivary glands, and if it is, whether its production is significantly reduced in older subjects. A possible explanation for the increased Ang-1 concentration in unstimulated saliva could be due to the reduced unstimulated salivary flow rates associated with old age (Affoo et al., 2015). Stimulated saliva flow rates have been shown not to be significantly affected in older people, which may explain why no significant correlation was found in the present study between the Ang-1 concentration in stimulated saliva with age. Future studies could examine flow rate, as well as Ang-1 concentration and total protein concentration, in order to ascertain whether age-related changes in flow rate account for the findings in this study. Furthermore, immunohistochemistry and *in-situ* hybridisation studies could determine whether and where Ang-1 is produced in salivary gland tissue.

Smoking is known to have a negative effect of oral and periodontal health, and significantly reduces oral wound healing potential (Chapter 1.7). Several theories have been proposed to account for this, such as the toxic effects of the chemical constituents of smoke on tissue cells, reduced inflammatory reaction and its influence on the levels of the angiogenic factor levels (Chapter 1.7.3). Currently, few studies have reported the effect of smoking on Ang-1 levels, especially between healthy smokers and non-smoking controls. Bennett et al. (2013) reported no statistical difference in serum Ang-1 concentrations between healthy smokers and healthy non-smokers. Ang-1 concentrations in sputum have been reported in asthma studies with contradictory results. Kanazawa et al. (2009) found no significant difference in sputum Ang-1 levels between healthy smokers and non-smokers, while Petta et al. (2015) reported significantly higher levels in smokers. In the present study, there was no evidence that smoking significantly influenced the concentration of Ang-1 found either in serum or in saliva, both in terms of smoking per se or the number of pack years (dose). This suggested that the negative influence of smoking on oral/periodontal health was not directly attributable

to changes in levels of Ang-1 in either serum or saliva. However, non-significant decreases in serum and salivary Ang-1 concentrations were found in periodontally healthy smokers compared with the healthy control and diabetes study groups. Although this finding was non-significant, and should be viewed with caution, it may be in part due to the reduced gingival inflammatory reaction and/or changes in vasculature in smokers (Pauletto et al., 2000, Mirbod et al., 2001, Rezavandi et al., 2002, Scardina and Messina, 2005). Further studies are required to ascertain whether smoking significantly affects the expression of Ang-1 in periodontal tissue and levels in GCF. Also, an immunohistochemistry study could examine Ang-1 expression in relation to vascularity in gingival tissue, in periodontal health and periodontitis, including samples from smokers and non-smokers.

Altered angiogenesis is widely thought to be one of many mechanisms by which chronic hyperglycaemia in diabetes results in poor wound healing (Chapter 1.8) (Goodson and Hung, 1977, Bohlen and Niggl, 1979, Rasul et al., 2012). In the present study, no significant differences were found initially in the serum and salivary Ang-1 concentrations between the diabetes and healthy controls groups. This finding was consistent with previous studies which have reported no significant differences in serum and plasma concentrations of Ang-1 between subjects with Type 2 diabetes and healthy controls (Lim et al., 2004, Lim et al., 2005, Gui et al., 2013, Zeng et al., 2013, Chen et al., 2015). Interestingly in the present study, once an extreme outlier was removed from the dataset, serum Ang-1 concentrations were found to be significantly lower in the diabetes group compared with the healthy controls. Diabetes is associated with raised systemic levels of other angiogenic factors, such as VEGF and Ang-2 (Lim et al., 2005, Chen et al., 2015), suggesting that diabetes is associated with changes to the balance of angiogenic factors leading to dysfunctional angiogenesis and immature vascular development. Therefore, the finding of significantly reduced serum Ang-1 concentrations in the diabetes group would further exacerbate the difference in the Ang-1 levels compared with Ang-2 and VEGF.

Ang-2 is thought to act as a competitive inhibitor for Ang-1, through binding to the Tie2 receptor, and is important in vessel disruption required early in angiogenesis (Chapter 1.5.3.2) (Maisonpierre et al., 1997, Gale et al., 2002, Bogdanovic et al., 2006). Several

studies have reported increased ratios of Ang-1 to Ang-2 in diabetes compared with healthy controls both in plasma (Lim et al., 2004, Lim et al., 2005, Yeboah et al., 2016) and in serum (Chen et al., 2015, Li et al., 2015). Furthermore, studies in diabetic mouse wound healing models have shown at tissue level prolonged increased Ang-1/Ang-2 ratios, suppressed levels of VEGF and reduced endothelial cell counts following cutaneous injury in comparison with non-diabetic controls (Kampfer et al., 2001). Similar studies have also shown that therapeutic use of Ang-1 results in enhanced wound healing associated with faster re-epithelialisation, increased levels of angiogenesis and blood flow (Cho et al., 2006, Balaji et al., 2015). This raises the possibility of using Ang-1 to promote wound healing is patients with diabetes, although there are currently concerns regarding side effects and the potential to promote tumour progression (Koh, 2013).

Potential limitations in the present study include the narrow range of glycaemic control, with the majority of the subjects with diabetes being relatively well controlled with glycated haemoglobin scores around the target of 7%. Therefore, this study really assessed the effects of relatively well controlled diabetes, rather than poorly controlled diabetes, which may have had different outcomes. Furthermore, by the nature of Type 2 diabetes, the age range of this group tended to be older (i.e. middle aged and above) than the other study groups. Further research is required to clarify the relationship between Ang-1 levels in serum and saliva with a broader range of glycaemic control.

Currently, there have been surprisingly few publications which have examined the significance of Ang-1 in oral health and pathology, especially its potential role in periodontal healthy and disease. Further studies could examine Ang-1 levels, in comparison with Ang-2 and VEGF, in GCF and saliva in periodontal health and disease. These studies could also examine the potential effect of smoking and diabetes to the levels and ratios between these factors. Immunohistochemistry and *in-situ* hybridisation studies could determine whether the salivary glands are the primary source of Ang-1 in saliva.

3.4 Migration Stimulating Factor (MSF)

3.4.1 Background

3.4.1.1 MSF levels in serum and plasma

As discussed in Chapter 1.5.3.5 and Chapter 3.4.1, MSF is a 70kDa soluble protein encoded by the *Fn1* gene on chromosome 2 (Grey et al., 1989, Schor et al., 1993). This protein is a truncated isoform of fibronectin (MSF; accession number AJ535086) corresponding to the amino-terminus of fibronectin with a unique 10 amino acid carboxyl-terminus. MSF has a range of biological activities including the induction of cell migration and angiogenesis, and is thought to play important roles in foetal development, wound healing and carcinogenesis (Schor et al., 1988a, Picardo et al., 1991, Schor and Schor, 2001, Schor et al., 2003, Houard et al., 2005, Aljorani et al., 2011, Perrier et al., 2012).

Currently, there have been no studies which have directly examined MSF concentrations in either human serum or plasma, however, tissue culture studies have reported detectable levels of MSF activity in human serum. Picardo et al. (1991) reported a high proportion of serum samples from patients with breast cancer, pre- and post-surgery, had MSF activity compared with a small proportion of systemically healthy gender and age-matched controls (Picardo et al., 1991). Interestingly, this study reported the minimum level of MSF activity corresponded to a serum MSF concentration of 0.5ng ml^{-1}. A similar study in patients undergoing surgery for non-malignant conditions reported MSF activity in a high proportion of wound fluid samples (17 out of 18) (Picardo et al., 1992). Pre- and post-operative serum samples were collected from five subjects with MSF positive activity in their wound fluids, with only 1 out of 5 serum samples showing MSF activity pre-operatively and none post-operatively. Furthermore, high levels of MSF activity were found in wound fluid and in pre- and post-operative serum samples taken from a sample number of patients undergoing surgery for a range of different malignant tumours. The implication taken from these findings was that MSF found in wound fluid was derived locally from fibroblasts involved in wound healing rather than systemically, and high serum MSF levels were associated with neoplasia.

To date there have been no publications which have examined the effect of smoking and diabetes, which are major risk factors for periodontitis and poor wound healing (Chapter 1.7 and 1.8 respectively), on the MSF levels in serum and oral fluids. However, there has been one study which reported increased MSF gene expression in a bronchioloalveolar carcinoma cell line following exposure to benzopyrene, a constituent of cigarette smoke (Yoshino et al., 2007).

3.4.1.2 MSF levels in saliva and GCF

Currently, there have been no publications which have reported MSF concentrations in human saliva or gingival crevicular fluid in periodontal health and periodontitis. Furthermore, there have been few publications relating to MSF and oral tissues. As discussed in Chapter 1.4.2.4, Irwin et al. (1994) reported that there were different sub-populations of gingival fibroblasts, with those derived from the gingival papillary tips displaying more foetal-like characteristics, including the secretion of high levels of MSF. Whilst, the sub-population of gingival fibroblasts derived from the deeper reticular layers were larger and displayed more adult cell characteristics, including no MSF production. The authors of the study hypothesised that the foetal-like production of MSF by some populations of gingival fibroblasts may contribute to the enhanced and foetal-like nature of wound healing in the oral cavity. Whether MSF derived from the gingival tissues has any influence on GCF and salivary levels of MSF will be investigated in this thesis.

To date there has only been one publication which has examined MSF expression in oral disease. In this immunohistochemistry study, Aljorani et al. (2011) examined MSF expression in benign (n=7) and malignant salivary tumours (n=27) in relation to adjacent histologically normal salivary tissue (n=16), which was used as a control. MSF expression increased significantly from normal salivary tissue, benign and malignant tumours ($p=0.04$-0.0001). MSF expression was found in both epithelial and connective tissue cells (fibroblasts, endothelial and inflammatory cells), especially in malignant tumours. Low level MSF expression was found in some of the control samples, however, as these samples were adjacent to the tumours (i.e. not true healthy control tissue) it is possible that field change/cancerisation had occurred. Furthermore, unpublished data has indicated MSF

expression is also upregulated in oral squamous cell carcinomas and is associated with reduced survival rates (Dr G Ohe: personal communication and quoted in (Schor and Schor, 2010)).

3.4.2 MSF ELISA optimisation

The MSF ELISA was developed and partially optimised for use with serum by Dr Katerina Kankova. As this was a non-commercial ELISA kit more extensive optimisation was required prior to assaying the clinical samples. Details of the ELISA reagents are outlined in Chapter 2.2 and the optimised MSF ELISA protocol is described in Chapter 2.2.2.

3.4.2.1 MSF ELISA optimisation: serum and stimulated saliva

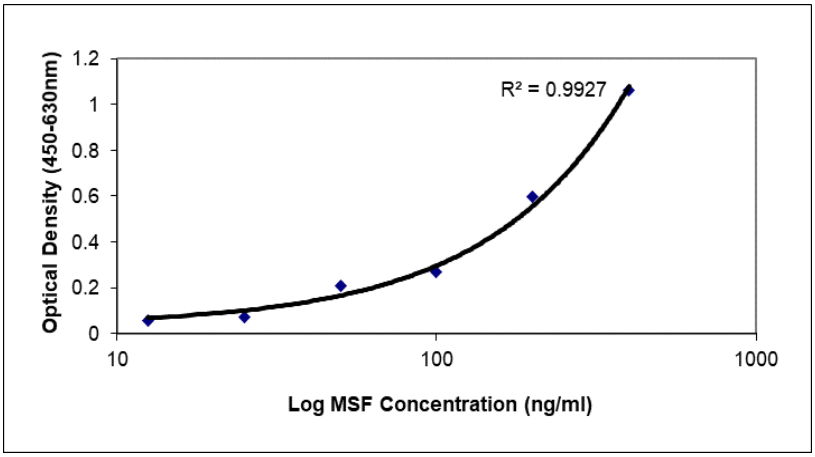
A number of optimisation assays were carried out to ascertain which diluent (i.e. PBS, PBS-Protease inhibitor, PBS-Tween (v/v 0.05%) or PBS-Tween-Protease inhibitor) resulted in optimal performance with the serum and stimulated saliva samples. Pooled serum and stimulated saliva samples were used in all the optimisation assays, however, there was insufficient unstimulated saliva available to produce a pooled sample. The pooled serum sample was diluted initially to either 1:50, 1:100 or 1:200 in the various diluents and plated in duplicate. The stimulated saliva sample was diluted to 1:10, 1:25, 1:50 and 1:100 dilutions in the different diluents. Each diluent was also plated in duplicate (blank wells) to assess the background noise of the assay. At this stage the ELISA protocol used 1% w/v dried milk-PBS-T as the blocking agent. Although the MSF standard curves for the initial assays produced trendlines with a high degree of fit (R^2 ranging from 0.94 to 0.99), the corrected optical densities ranges were small due to the high background noise of the assays. This issue resulted in the optical density scores for all the serum samples and the majority of the stimulated saliva samples being above those of the MSF standards, preventing calculation of the MSF content. In order to reduce the background noise of the assay further optimisation of the blocking stage was carried out.

In the subsequent assay the duplicate wells were blocked for one hour with either 1% w/v dried milk-PBS-T or 1% w/v BSA (200µl per well). Data from previous work carried out by Dr Katerina Kankova indicated that PBS-T-PI was likely to be the best diluent for the serum

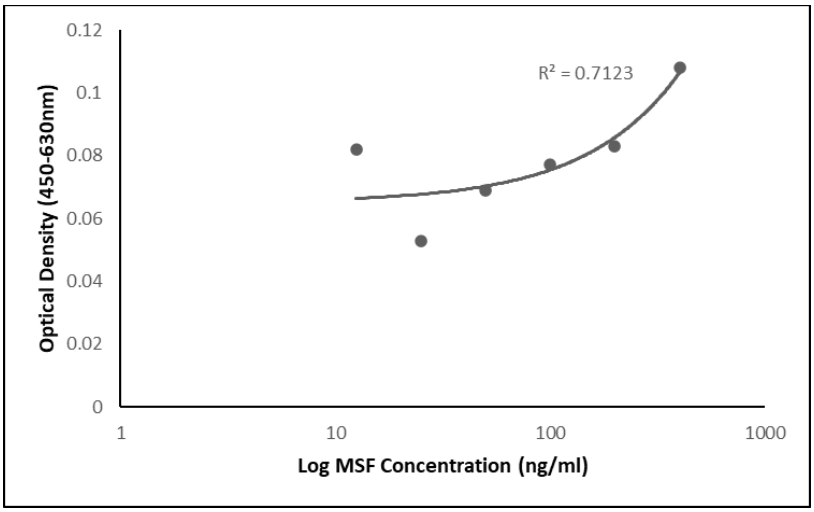
samples (personal communication Dr Sarah Jones). Therefore, the pooled serum sample was diluted in PBS-T-PI to 1:50, 1:100, 1:200, 1:400 and 1:800 to ascertain which resulted in corrected optical densities within the linear range of the MSF standard curve. Likewise, the pooled stimulated saliva sample was diluted in PBS-T-PI to 1:10, 1:50, 1:100. Blocking using 1% w/v BSA resulted in a good range of corrected optical densities between the lowest and the highest MSF standards, and a high degree of fit for the MSF standard curve (R^2=0.9927) (Figure 3.12a). The 1% dried milk-PBS-T block resulted in a MSF standard curve with a poor range of corrected optical density readings between the low and high MSF standards, and a poor level of fit to the standard curve (R^2=0.7123) (Figure 3.12b). Serial dilution of the pooled serum sample in PBS-T-PI (1:50 to 1:800) resulted in a linear dilution effect (trendline R^2=0.967). The serum dilution of 1:100 in PBS-T-PI resulted in a corrected optical density score at the mid-point of the linear range of the 1% w/v BSA blocked MSF standard curve, where the accuracy of the ELISA is most likely to be at its greatest. Likewise, serial dilution of the pooled stimulated saliva sample in PBS-T-PI (1:10 to 1:100) resulted in linear dilution effect (trendline R^2=0.9913). The stimulated saliva dilution of 1:10 in PBS-T-PI resulted in the best corrected optical density scores, albeit at the lower end of the BSA blocked MSF standard curve. It was not possible to use dilutions less than 1:10 due to saliva being too viscous to pipette accurately and difficulties in homogenising the samples (Chapter 2.2.3). Therefore, the final MSF ELISA protocol used a 1% w/v BSA block with the clinical samples diluted in PBS-T-PI to 1:100 and 1:10 for serum and saliva respectively (Chapter 2.2.2).

Figure 3.12 The effect of using (a) 1% w/v BSA and (b) 1% w/v dried milk-PBS-T for the blocking stage of the MSF ELISA. Results are shown as the corrected OD (450-630nm) scores versus log MSF standard concentration (ng ml[1]).

(a)



(b)



3.4.2.2 MSF ELISA optimisation: GCF

The GCF collection, processing and storage protocol is outlined in Chapter 2.1.8. Prior to the development of the MSF ELISA used in the present study, a migration assay study was carried out to estimate the percentage MSF recovery from the GCF elution protocol. In summary, there were three parts to this study. In the first part, equal quantities of recombinant MSF (rhMSF) (50ng ml^{-1}) were either pipetted (spiked) onto two PerioPapers (elution sample), to simulate the clinical collection of GCF samples, or kept as a solution (reference sample). To simulate the conditions of the clinical study both samples were kept at room temperature for 1 hour and then at 4^{o}C for one hour prior to the PerioPapers (elution sample) undergoing the standard GCF elution protocol stated Chapter 2.1.8. The resultant

samples both contained 10ng rhMSF in 90ul of PBS. Both samples and were then stored at -80^{o}C for 4 months. Prior to the migration assay, four sets of media were prepared using sterilised Eagle's Minimum Essential Medium (MEM): reference and elution samples (final serial dilution from 10pg ml^{-1} to 0.1pg ml^{-1}); GCF and stimulated saliva samples from subject KJD007 (Group 2b) (final serial dilution from 1:80 to 1:4000). It was important to sterilise the clinical samples using a 2µm Millipore filter to prevent contamination of the migration assay.

The results of the fibroblast migration assay (details of the protocol outlined in Schor et al. (2001)), carried out by Dr Ian Ellis, are shown in Table 3.10. The results suggested that at concentrations of rhMSF at 1pg ml^{-1} and above, the percentage recovery for the elution protocol was greater than 90%, assuming there was a linear relationship between migration activity and rhMSF concentration. This finding was consistent with Gustafsson (1996) who reported recovery rates of 90% for elastase using PerioPapers and a similar GCF elution protocol to that used in this thesis. However, the fibroblast migration activity generated by the reference samples was between 44%-86% of the equivalent control rhMSF samples used in the migration assay. This may indicate some degradation of rhMSF during the four month storage at -80^{o}C or binding of rhMSF to the non-siliconised Eppendorfs. Therefore, all clinical samples were stored in siliconised Eppendorfs. Interestingly, both the GCF and stimulated saliva samples for subject KJD007, a long-term smoker with severe periodontitis, showed migration activity in the assay. This was especially the case for the stimulated saliva sample, although it was not possible to ascertain whether this activity was due specifically to MSF. The addition of a MSF inhibitor would have given an indication of the extent, if any, of the migration activity in these clinical samples was due to MSF.

Table 3.10 Mean number of cells migrated (±SD) per 10 fields (Leica microscope high magnification) per migration assay for serial dilutions of rhMSF, eluted sample, reference sample and for the GCF and stimulated saliva samples from subject 007 (data supplied by Dr Ian Ellis).

Dilution.	rhMSF Control	Eluted rhMSF sample*[1]	Reference rhMSF sample*[1]	GCF KJD 007*[2]	Stimulated saliva KJD 007*[2]
SF-MEM	2.0±0.1	-	-	-	-
0.001pg (0.01ml)	2.7±0.1	1.7±0.1 (0.1pg)	1.2±0.4 (0.1pg)	1.8±0.2 (1:4000)	6.2±0.1 (1:4000)
0.01pg (0.05ml)	4.6±0.2	1.8±0.2 (0.5pg)	5.6±0.6 (0.5pg)	2.6±0.3 (1:800)	5.2±0.2 (1:800)
0.1pg (0.1ml)	6.5±0.2	5.0±0.1 (1pg)	5.6±0.5 (1pg)	6.4±0.8 (1:400)	4.8±0.1 (1:400)
1pg (0.5ml)	7.3±0.2	5.9±0.0 (10pg)	6.0±0.3 (10pg)	4.6±0.3 (1:80)	1.4±0.3 (1:80)

***[1]** Figures in brackets give the expected rhMSF concentration of the samples.
***[2]** Figures in brackets give the dilution factor of the elutant/GCF samples.

When the MSF ELISA was available, a number of optimisation assays were carried out using the GCF samples. In the first assay, ten GCF samples were harvested from a systemically and periodontally healthy subject (KJD002 Group 1a), eluted using the standard protocol (Chapter 2.1.8) and pooled prior to storage at -80°C. As a control, 1µl of rhMSF (30µg ml^{-1}) was pipetted onto PerioPapers (spiked sample) and eluted using the standard protocol, pooled and stored at -80°C. Both samples were assayed using the MSF ELISA with serial dilutions from undiluted to 1:64 in PBS-T-PI. Only the undiluted pooled GCF sample was found to have an optical density reading within the range of the MSF standard curve for the assay, however, this was at the lower end of the sensitivity of the assay (12.5ng ml^{-1}). As the pooled GCF sample was from a single systemically and periodontally healthy subject, GCF samples were subsequently assayed from all the clinical sub-groups, including a range of storage times. MSF was not detected in any of these samples. Subsequently, pooled samples from sub-groups 1a, 2a and 3a were assayed to ascertain whether data could be gained from pooling samples, however, no MSF was detected. As this point, a decision was made not to carry out any further MSF ELISAs with the GCF samples. Unfortunately, the ELISA kits for the other factors examined in this thesis were now out of date and there were insufficient GCF samples remaining to assay all the clinical cases.

3.4.3 Results

3.4.3.1 MSF concentration in serum

MSF was detected in the majority of serum samples and the results are summarised in Table 3.11 and are shown in Figure 3.13. MSF was quantified in 86 serum samples with mean levels for the study sub-groups ranging from 6937 to 17627ng ml^{-1}, although there were large variations in the levels detected. There was no evidence of serum MSF levels degrading during storage, with the MSF concentrations for samples stored the longest time prior to assaying (i.e. low KJD numbers) having similar ranges to those samples stored for the shortest time (i.e. highest KJD numbers). No significant differences were found between either the study groups (Two-way ANOVA test $F(2,80)=1.585$, $p=0.211$) or between periodontally healthy and severe periodontitis sub-groups (Two-way ANOVA test $F(1,80)=2.285$, $p=0.135$), and there was no significant interaction found between periodontal health status and the study groups (Table 3.12). A non-significant reduction was found in the smoking group (Group 2) in both periodontal health and severe periodontitis compared with the healthy control (Group 1) and the diabetes (Group 3) groups (Figure 3.14). Bonferroni post hoc tests (Table 3.13) confirmed that there were no significant differences between the study sub-groups. Diagnostic statistical analysis revealed that there were outliers in the data which had a significant influence on the overall statistical outcome (Studentized residual highest=3.67, lowest= -1.67; highest Cook's distance=0.22; samples KJD58 and KJD69 were deemed to be outliers). Bootstrapping analysis confirmed that the outcome of the Two-way ANOVA was not significantly influenced by the outliers. Sensitivity analysis, where data from the two outliers were removed from the dataset, confirmed there were no significant differences in the serum MSF levels between the study groups (Two-way ANOVA test $F(2,78)=0.521$, $p=0.596$; Table 3.12 and Figure 3.15).

Table 3.11 Descriptive statistics for MSF serum concentrations (ng ml^{-1}) for the study groups and sub-groups. Statistical data in brackets are the results following removal of extreme outliers (>3xSD) from the data set.

Group	Number Valid Cases*	Mean ± SD (ng ml^{-1})	Median (ng ml^{-1})	Interquartile Range	Range
1a	35	11723±9566	8352	17556	43958
1b	12	8338±9089	5080	12080	25829
Study Group 1	47	10859±9467	7802	14223	34958
2a	8	7630±9789	2950	15674	24210
2b	8	6937±5367	4922	10463	13145
Study Group 2	16	7284±7635	3692	10776	24210
3a	11 (9)	17627±20096 (9237±8311)	10784 (8367)	20240 (14043)	56205 (23680)
3b	12	9475±8710	6589	16748	23626
Study Group 3	23 (21)	13374±15454 (9373±8329)	8039 (6806)	17895 (14843)	56205 (24510)

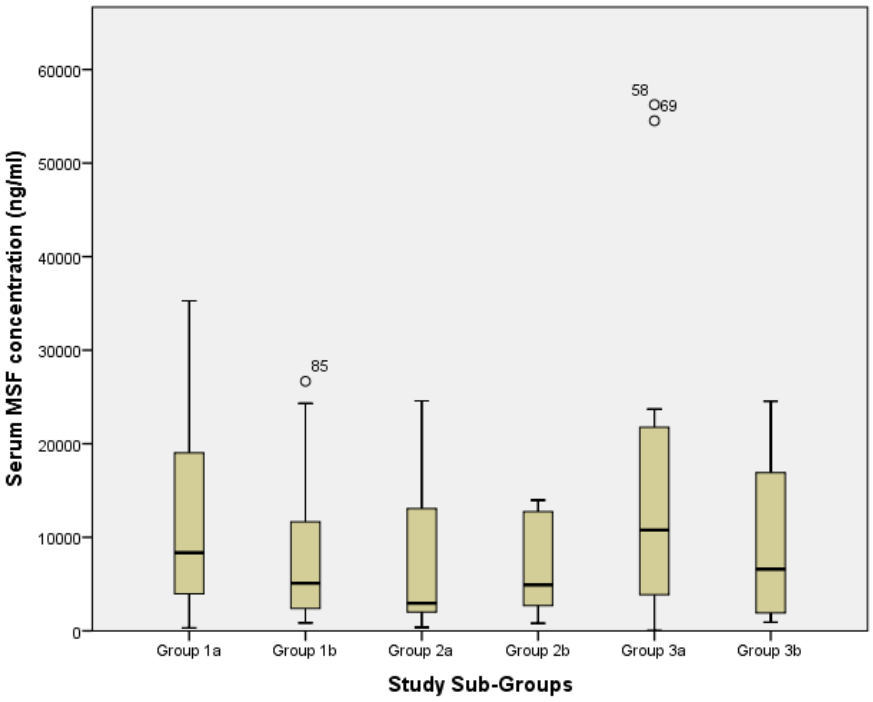
*Excluding either no sample available or factor not detected.

Table 3.12 Statistical comparison of the serum MSF concentrations of the study groups and periodontal health-severe periodontitis using Two-way ANOVA test. Data in brackets are the results following removal of outliers KJD058 and KJD069 from the data set.

Group	Degrees of Freedom (df)	F	Significance (p-value)
Periodontal Health – Severe Periodontitis	1	2.285 (0.329)	0.135 (0.568)
Study Groups (1, 2 and 3)	2	1.585 (0.521)	0.211 (0.596)
Periodontal Health – Severe Periodontitis Versus Study Groups (interaction)	2	0.593 (0.302)	0.555 (0.740)

Figure 3.13 Serum MSF concentrations (ng ml^{-1}) for (a) study sub-groups and (b) study groups. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

(a)



(b)

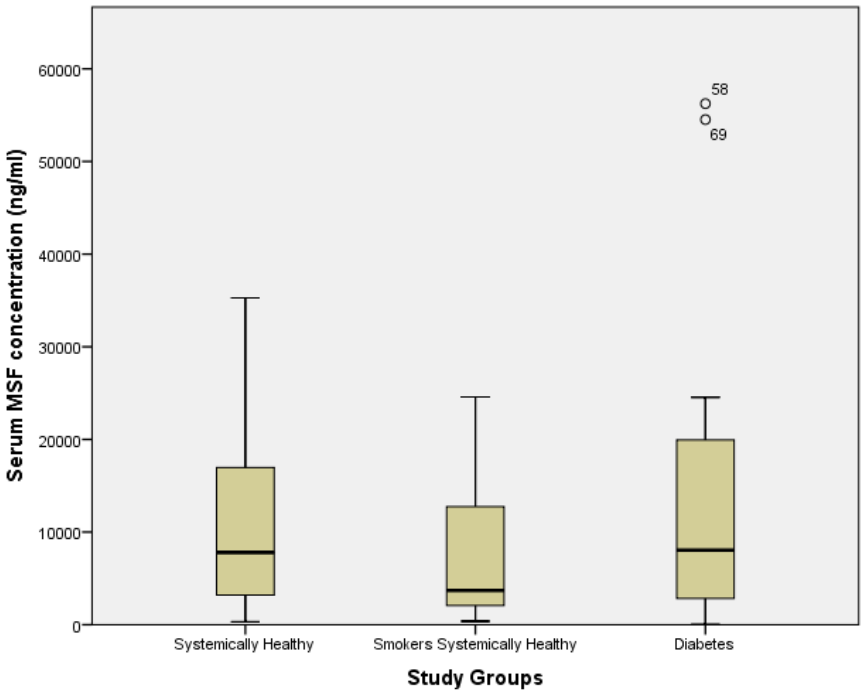


Table 3.13 Statistical comparison between study sub-groups for the serum MSF (Bonferroni test p-values). Data in brackets are the results following removal of outliers KJD058 and KJD069 from the data set.

Study Groups		Mean Difference	Significance (p-value)	95% Confidence Interval	
				Lower Bound	Upper Bound
Group 1	Group 2	3575.12 (3575.12)	0.801 (0.518)	-4244.81 (-2779.07)	11395.05 (9929.31)
	Group 3	-2515.06 (1485.68)	1.000 (1.000)	-9390.14 (-4276.61)	4360.02 (7247.97)
Group 2	Group 1	-3575.12 (-3575.12)	0.801 (0.518)	-11395.05 (-9929.31)	4244.81 (2779.07)
	Group 3	-6090.18 (-2089.44)	0.283 (1.000)	-14885.46 (-9374.45)	2705.11 (5195.57)
Group 3	Group 1	2515.06 (-1485.68)	1.000	-4360.02 (-7247.97)	9390.14 (4276.61)
	Group 2	6090.18 (2089.44)	0.283 (1.000)	-2705.11 (-5195.57)	14885.46 (9374.45)

Figure 3.14 Profile plot of periodontal health and severe periodontitis against estimated marginal mean serum MSF concentrations (ng ml^{-1}) for the three study groups.

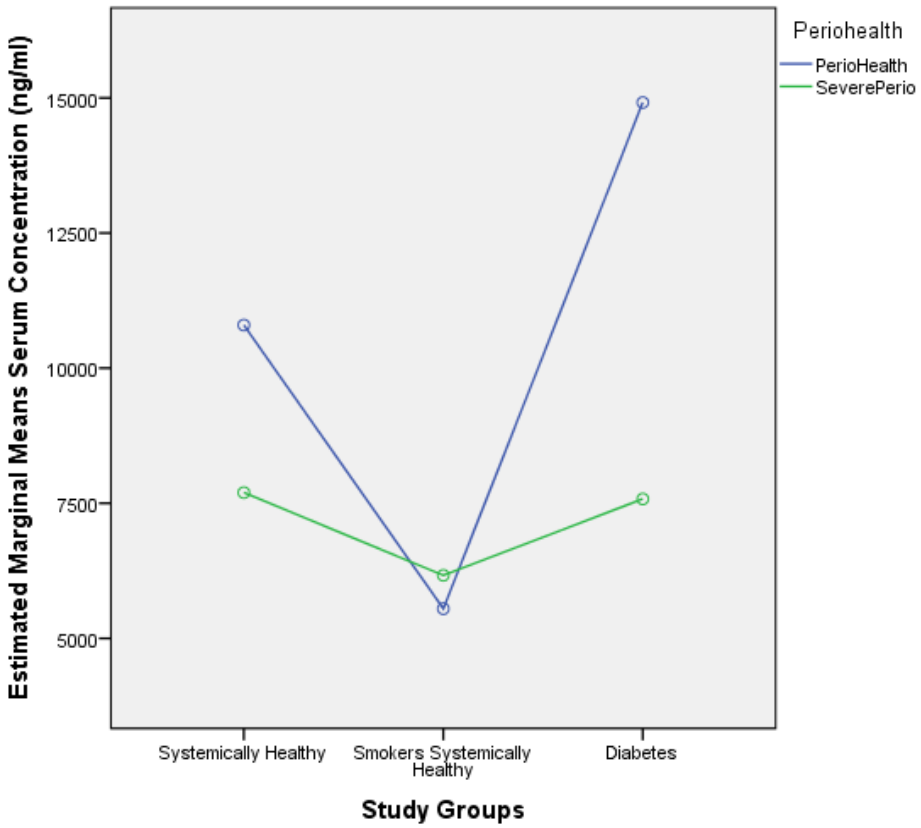
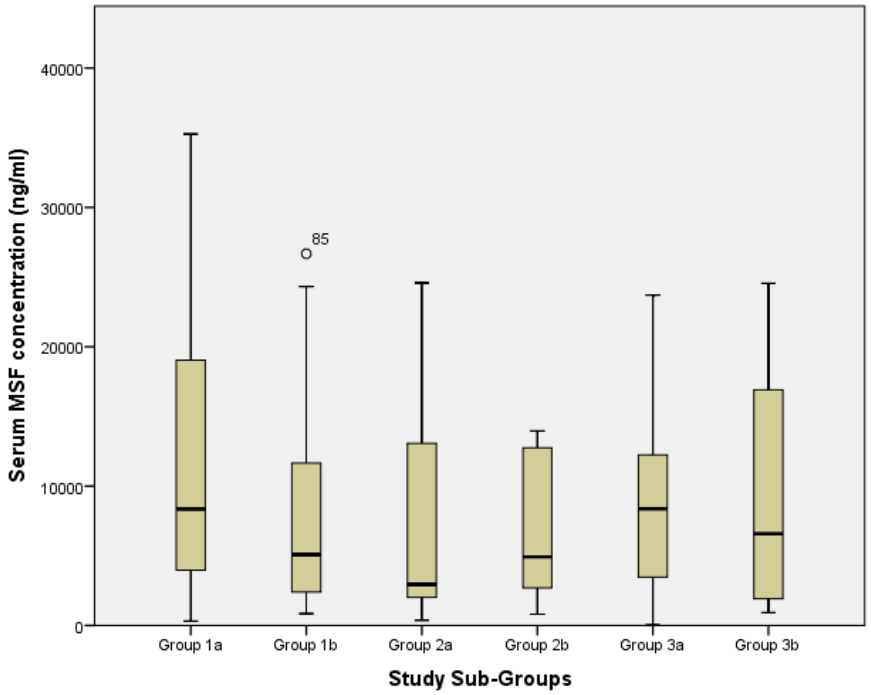
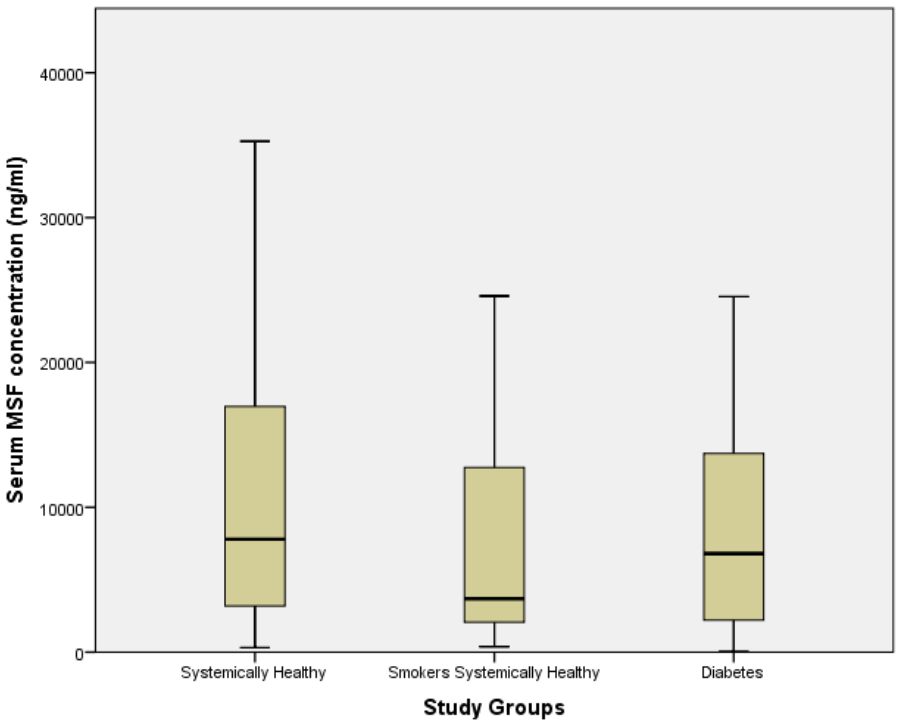


Figure 3.15 Serum MSF concentrations (ng ml^{-1}) with extreme outliers (>3xSD) removed for (a) study sub-groups and (b) study groups. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

(a)



(b)



There were no significant correlations between the serum MSF concentration with smoking dose (pack years) (Spearman rho=-0.076, p=0.487), glycaemic control (Spearman rho 0.098 p=0.655) or age (Spearman rho=0.142, p=0.193). No significant difference was found regarding gender and serum MSF concentration across the whole sample (Mann Whitney p=0.650).

3.4.3.2 MSF concentration in saliva

3.4.3.2.1 MSF concentration in stimulated saliva

Eighty five stimulated saliva samples were assayed to quantify the MSF concentration. MSF was detected in a minority of stimulated saliva samples (1a=1/26, 1b=1/13 and 3b=2/15), ranging from 58 to 2576ng ml^{-1}, and was not detected in any of the smoking sub-groups (2a and 2b) or the periodontally healthy diabetes group (3a). However, there were insufficient numbers of positive samples to allow statistical comparison of the MSF concentrations between each study sub-group.

3.4.3.2.2 MSF concentration in unstimulated saliva

Eighty eight unstimulated saliva samples were assayed to quantify the MSF concentration and the results are summarised in Table 3.11. MSF was detected in a minority of unstimulated saliva samples (1a=3/27, 1b=2/14, 2b=1/9 and 3a=1/13 and 3b=4/15), ranging from 75 to 1677ng ml^{-1} and was not detected in the healthy smoking sub-group (2a). However, there were insufficient numbers of positive samples to allow statistical comparison of the MSF concentrations between each study sub-group.

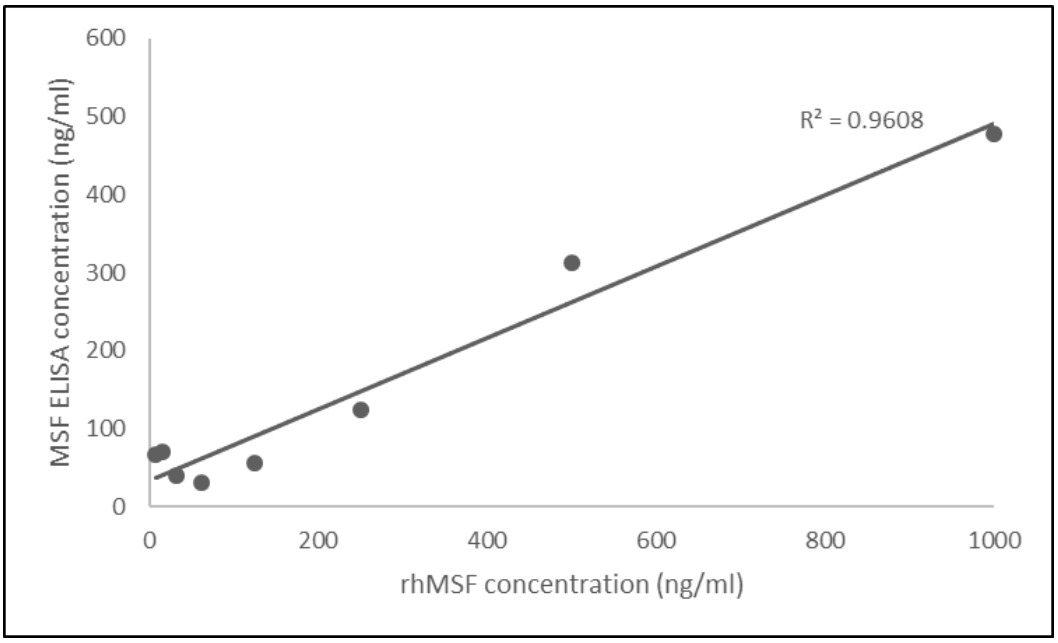
3.4.3.3 Efficiency of the MSF ELISA: rhMSF spiking analysis

During the optimisation of the MSF ELISA for use with saliva samples, it was noted that the optical density scores for the pooled stimulated saliva sample was close to the maximum sensitivity range for the assay. In all assays, the MSF standard dilutions resulted in a good range of optical density scores and very good standard curves ($R^2 \geq 0.99$), suggesting that the ELISA was functioning appropriately. Further attempts were made to use a 1:5 dilution of the saliva samples in PBS-T-PI, however, most of these had to be abandoned due to the saliva being too viscous to pipette, particularly the unstimulated saliva samples. It was possible to

assay one unstimulated saliva sample at 1:5 and 1:10 dilutions (KJD036), which resulted in a 38% difference in the final calculated MSF concentration. This difference was most likely due to pipetting inaccuracies and difficulties in homogenising the 1:5 dilution of the saliva sample.

In order to assess the efficiency of the MSF ELISA with regards to the clinical samples, a MSF negative serum sample (KJD098) was spiked with known quanitities of recombinant MSF (rhMSF) (serial dilution from 1000-6.25ng ml^{-1}). The resultant corrected optical density readings for each dilution was converted to a MSF concentration using the MSF standard curve to give a measure of the percentage recovery of the ELISA (Figure 3.16). The unspiked serum sample (KJD098) was found again to be negative for MSF. Apart from the highest rhMSF dilutions, there was a linear dilution effect with the significant correlation between rhMSF and the calculated (recovered) MSF concentration for the linear aspect of the dilution graph (Spearman rho 1.0 p=0.01 rhMSF 62.5-1000ng ml^{-1}; overall Spearman rho 0.619 p=0.102 for all rhMSF dilutions). The mean MSF recovery from the spiked serum sample was 51%. Possible explanations for the low percentage rhMSF recovery from the serum samples could be due to the presence of a MSF inhibitor and/or non-specific binding to other proteins in the serum sample, both of which would reduce the availability of the rhMSF to bind to the assay antibodies. It was noted that the background noise was increasing in the final assays, possibly indicating that the MSF ELISA antibodies were starting to degrade.

Figure 3.16 The effect of the serial dilution of a MSF negative serum sample (KJD098) spiked with known concentrations of rhMSF (ng ml^{-1}) against the MSF concentrations (ng ml^{-1}) calculated from the MSF ELISA.



3.4.4 Discussion

In this study, the pro-angiogenic factor MSF was measured directly for the first time in serum, unstimulated and stimulated saliva samples from three groups of subjects: (i) systemically healthy non-smokers, (ii) systemically healthy smokers and in (iii) subjects with diabetes. Each clinical group was divided into two sub-groups: (a) periodontally healthy and (b) subjects with severe periodontitis. Confidence in the assay methodology was demonstrated by the good assay standard curves, however, some issues were encountered with the final few MSF ELISA with regards to high background noise. MSF was detected in the majority of the serum samples assayed, at significantly higher levels than other factors studied in this thesis, however, it was undetectable in the majority of saliva samples and in all GCF samples assayed. With regards to MSF concentrations in serum, there were no significant differences or correlations were found.

MSF is produced by various cell types during foetal development and neoplasia, including fibroblasts, endothelial and epithelial cells, but is a generally not produced by adult cells in health (Schor et al., 1988a, Schor et al., 1988b, Schor et al., 2003) (Chapter 1.5.3.5). One

exception is in wound healing, where MSF is thought to have an important role through the induction of angiogenesis and its motogenic effect on various cell types, including fibroblasts, endothelial cells and pericytes (Ellis et al., 2010, Schor and Schor, 2010). *In vivo* evidence for the possible role of MSF in physiological wound healing was shown by Picardo et al. (1992) who reported that MSF activity was detected in wound fluid in 94% (n=18) of cases undergoing surgery for non-malignant conditions. However, MSF activity in serum was detected in 20% (n=5) of cases pre-operatively, and in no cases post-operatively, implying MSF was produced locally at the wound site, rather than systemically.

Like other angiogenic factors, MSF expression and production is dependent on context i.e. in relation to the presence of other growth factors and the nature of the underlying extracellular matrix (ECM). Schor et al. (2012) reported that human adult fibroblasts persistently expressed MSF when exposed to TGF-β_1 on a wound-like matrix, such as denatured collagen or fibrin. A subsequent exposure of the adult fibroblasts to TGF-β_1 resulted in the MSF expression being turned off, but only if the underlying matrix was type 1 collagen. This implies that the cytokine/growth factor milieu and nature of the ECM during wound healing, switches on the dermal fibroblasts to produce MSF, which is in turn switched off once the wound healing process is complete. Interestingly, TGF-β_1 has been shown to be significantly raised in serum, unstimulated whole saliva and in GCF in patients with periodontitis compared with age and gender matched periodontally healthy controls (Khalaf et al., 2014). To date, little is known regarding the role of MSF in oral healing. Irwin et al. (1994) reported that there are sub-populations of gingival fibroblasts, some of which display foetal fibroblast characteristics, which are permanently able to express and secrete MSF, in particular the papillary fibroblasts. This may indicate that gingival fibroblasts may effectively be permanently "switched on" with regards to MSF.

Other sources of MSF may also be important in oral wound healing, as discussed in Chapter 1.5, saliva has been shown to be a reservoir for a number of growth /angiogenic factors. Therefore, it was of interest to study MSF in both saliva and GCF, the latter being a minor constituent of saliva. Interestingly, a more recent study in the laboratory used immunohistochemistry to examine MSF expression in a variety of small benign and

malignant salivary gland tumours (Aljorani et al., 2011). This study also examined MSF expression in histologically normal peri-tumour salivary tissue as a control, similar to the methodology used in Chapter 4. Whilst MSF expression was found to be significantly higher in both benign and malignant tumours, low level MSF expression was reported in the peri-tumour normal tissue, particularly in the ductal and myoepithelial cells. There was very little MSF staining of the serous and mucous acinar cells, which would be the most likely source of MSF production if it was produced in salivary tissue, although one case did show MSF expression in the serous acini. There are several possible interpretations of these findings: (i) MSF is not produced in human salivary glands and the MSF staining found in the Aljorani et al. (2011) study was the result of the field cancerisation effect from the adjacent tumour; (ii) in health, MSF is only produced at very low concentrations; (iii) MSF is produced systemically and enters saliva in a similar way to steroid hormones.

In the present study, MSF was detected in a small number of stimulated and unstimulated saliva samples, suggesting it may be present in saliva, albeit it at lower concentrations than the limit of detection of the MSF ELISA. If this was the case, salivary MSF concentrations would potentially be in the range of motogenic activity for human fibroblasts (0.1pg ml^{-1} to 10ng ml^{-1}) (Ellis et al., 2010, Schor and Schor, 2010). Evidence from the migration assay study, carried out by Dr Ian Ellis as part of the initial GCF elution study, suggested that both stimulated saliva and GCF samples induced motogenic activity in a human fibroblast cell line. However, how much of this activity, if any, was due to the presence of MSF is unknown.

Regarding the analysis of the GCF samples, only a few samples resulted in optical densities within the MSF standard curves, however, these were at the extreme margin of the sensitivity for the assays. This may imply the presence of MSF in GCF, although this should be interpreted with caution and further research is required. A limiting factor was the high degree of dilution required to elute the small quantities of GCF from the PerioPapers, which was further limited by the low sensitivity of the MSF ELISA. Other potential limiting factors included degradation during storage and the protein recovery rate following the elution stage. Data from the migration assay, in the present study, suggested that between 44%-86% of rhMSF was retained following a four month storage at -80^{o}C in non-siliconised Eppendorfs.

This effect may have been due to the rhMSF binding to the Eppendorfs, so siliconised Eppendorfs were used subsequently, although further studies should have been undertaken to assess the effect of this change. The soak and centrifuge elution protocol, used in the present study (Chapter 2.1.8), has been widely reported in the literature and shown to result in protein recovery rates typically between 50%-100% (Gustafsson, 1996, Booth et al., 1998, Johnson et al., 1999b, Fentoglu et al., 2012, de Lima Oliveira et al., 2012, Wassall and Preshaw, 2016). In the present study, spiking PerioPapers with rhMSF resulted in an estimated recovery, measured indirectly using a migration assay, of up to 90%. However, there is wide variation in the elution techniques in the literature, even for the soak and centrifuge protocols, where different buffers, use of protease inhibitors, soaking times and centrifugation could all affect protein recovery. Furthermore, higher protein concentrations on PerioPapers have been shown to result in higher protein recovery following elution (Johnson et al., 1999b).

In a review of GCF sampling techniques, Barros et al. (2016) recommended PerioPapers should be snap frozen at the chairside using liquid nitrogen and then stored long-term in liquid nitrogen without buffer, as this significantly reduces oxidation of proteins increasing storage times. Unfortunately, due to health and safety reasons liquid nitrogen could not be used on the clinics in this institution, but it could be used in future studies for storing GCF. In retrospect, more detailed elution studies should have been carried out prior to the GCF collection from the study subjects to establish which protocol resulted in the best rhMSF recovery. Unfortunately, the MSF ELISA was not available until after the study clinical sampling had been completed.

MSF was detected in very high levels in serum (6937 to 17627ng ml^{-1}), significantly higher than the concentration range found to result in optimal migration of human foreskin fibroblasts in collagen gel assays (1pg ml^{-1} to 10ng ml^{-1}) (Ellis et al., 2010). Unexpectedly, no significant differences were found in the present study in the serum MSF concentrations between the study groups. It had been anticipated that the serum MSF concentrations would be increased in severe periodontitis, associated with increased inflammatory levels of MSF in the periodontium spilling over into the systemic circulation, whilst being reduced in

smoking and diabetes as these conditions are associated with reduced wound healing potential (Chapter 1.7 and 1.8 respectively). Interestingly in the present study, a non-significant reduction in serum MSF concentrations were found in smokers in both periodontal health and severe periodontitis. This may indicate that systemic levels of MSF may be reduced in smokers, however, further studies are required to ascertain whether this is significant and to determine the pathway for such an inhibitory effect.

Another possibility is that the high serum concentrations of MSF do not reflect the functional level of MSF activity due to the potential presence of a MSF inhibitor (MSFI). The presence of a MSF inhibitor may account for the unexpected low rhMSF recovery following the spiking of the known MSF negative serum sample, although it may indicate issues with the MSF ELISA, such as non-specific binding, low affinity of the antibodies or excessive blocking. The existence of MSFI was found when conditioned medium from keratinocytes inhibited rhMSF-induced migration in tissue culture studies, and was identified to be neutrophil gelatinase associated lipocalin (NGAL or lipocalin-2) (Jones et al., 2007). Levels of NGAL have been found to be raised in several inflammatory conditions, such as kidney diseases and in smokers with chronic obstructive pulmonary disease (Mori and Nakao, 2007, Bchir et al., 2017). Raised levels of NGAL have also been reported in periodontitis, compared with healthy controls, in whole unstimulated saliva, GCF and in urine (Westerlund et al., 1996, Tsuchida et al., 2013, Morelli et al., 2014, Pradeep et al., 2016, Nakajima et al., 2019). There has been interest in the study of NGAL in periodontitis as it is associated with matrix metalloproteinase-9 (MMP-9) released from neutrophils, high levels of which are released in periodontal inflammation (Soder et al., 2006). Furthermore, Bondy-Carey et al. (2013) reported that the presence of *Porphyromonas gingivalis* was associated with high levels of neutrophil induced MMP-9 and NGAL. NGAL has also been found to be significantly raised in GCF in obese patients with periodontitis, another area of interest currently in periodontal research, compared with both periodontally healthy and non-obese subjects with periodontitis (Pradeep et al., 2016).

The key limitation in the assessment of MSF in the study samples was the sensitivity of the MSF ELISA, which was in the range of ng ml^{-1}, whilst the commercial ELISA kits used for

Ang-1, Endostatin and VEGF were in pg ml^{-1} range. The sensitivity of the MSF ELISA was sufficient for measuring MSF concentrations in serum, but was insufficient to measure the MSF concentrations in saliva and GCF, if present. Further studies could investigate ways to increase the sensitivity of the MSF ELISA, for example, converting it from a colourimetric to a chemiluminescent assay. Furthermore, studies could examine the relationship between MSF and MSFI by examining MSF-NGAL ratios in serum, saliva and GCF, especially as commercial NGAL ELISA kits are available. This would establish to functional MSF level in serum and the oral fluids, which would be of particular interest with regards to levels in periodontal health and disease, and in smoking and poorly controlled diabetes.

3.5 Vascular Endothelial Growth Factor (VEGF)

3.5.1 Background

3.5.1.1 VEGF levels in serum and plasma

Serum and plasma concentrations of the pro-angiogenic factor VEGF (VEGF-A) have been reported in a wide variety of human pathological conditions, in comparison to healthy control subjects. Significantly raised serum VEGF concentrations have been reported in conditions such as polyarteritis nodosa (Kikuchi et al., 2005), oral/oropharyngeal squamous cell carcinoma (Shang et al., 2007, Polz-Dacewicz et al., 2016) and asthma (Gomulka et al., 2019) (Chapter 1.5.3.10 and Table 3.14). Likewise, significantly raised plasma VEGF concentrations have been reported in breast and prostate cancer (Caine et al., 2003), sickle cell disease (Mohan et al., 2005), pregnancy induced hypertension (Nadar et al., 2005), peripheral arterial disease and Type 2 diabetes (Atta et al., 2008).

Table 3.14 shows a representative selection of publications describing serum VEGF concentrations in healthy control subjects, which range from 8-1392pg ml^{-1}, and provides a baseline level to compare with the subjects examined in this thesis. Several studies have reported contradictory findings regarding serum VEGF concentrations in subjects with periodontitis in comparison with periodontally healthy controls (Chapter 1.5.3.10). Some studies reported significantly increased serum VEGF concentrations in periodontitis (Pradeep et al., 2011, Turer et al., 2017), while others have either reported no difference

Table 3.14 A representative selection of publications for VEGF concentrations in human serum and plasma.

SERUM	PLASMA
Hanatani et al. (1995) Healthy blood donors (n=30) • Mean=19 pg ml^{-1} (range 8-36pm ml^{-1})	Mohan et al. (2005) Systemically healthy adults (n=20) • Mean=11pg ml^{-1} (range 10-110) Sickle cell disease (n=56) • Mean=120pg ml^{-1} (range 72-780) Significantly increased VEGF concentration in Sickle cell disease compared with the controls ($p<0.001$)
Taichman et al.(1998) Systemically and periodontally healthy adults (n=17) • Mean=168±58(SD) pg ml^{-1}	Nadar et al. (2005) Systemically healthy females (n=30) • Mean=22pg ml^{-1} (range 10-162) Pregnancy induced hypertension (PIH) (n=64) • Mean=150pg ml^{-1} (range 14-175) Significantly raised VEGF concentration in PIH than healthy non-pregnant controls ($p<0.05$)
Kikuchi et al. (2005) Systemically healthy adults (n=20) • Mean=178± (SD) 41pg ml^{-1} Systemic Polyarteritis nodosa (PAN) (n=5) • Mean=484± (SD) 44pg ml^{-1} Cutaneous PAN (n=15) • Mean=228± (SD) 97pg ml^{-1} VEGF levels were significantly higher in both systemic ($p<0.005$) and cutaneous ($p<0.05$) PAN compared with the healthy controls	Caine et al. (2003) Breast cancer: Healthy female controls (n=12) • Mean= 30pg ml^{-1} (range 25-60pg ml^{-1}) Breast cancer (n=30) • Mean= 310g ml^{-1} (range 220-488pg ml^{-1}) Prostate cancer: Healthy male controls (n=12) • Mean= 26.5pg ml^{-1} (range 25-30pg ml^{-1}) Prostate cancer (n=30) • Mean= 210pg ml^{-1}(range 166-360pg ml^{-1}) Significantly higher VEGF concentration in breast and prostate cancers > controls ($p=0.0001$)
Shang et al. (2007) Systemically healthy adults (n=10) • Mean=149± (SD) 64pg ml^{-1} Oral squamous cell carcinoma (OSCC) (n=31) • Mean=568± (SD) 338pg ml^{-1} Significantly raised VEGF concentration in OSCC than healthy controls ($p<0.001$)	Atta et al. (2008) Systemically healthy adults (n=12) • Mean=30.5±(SD)3.1pg ml^{-1} Peripheral arterial disease (n=10) • Mean=51.6±(SD)5.2pg ml^{-1} Diabetes (n=15) • Mean=52.8±(SD)4.5pg ml^{-1} VEGF plasma concentration significantly higher in peripheral arterial disease and diabetes compared with control subjects.
Upile et al. (2009) Healthy controls (n=14) • Mean=1391.97(SE)199.36pg ml^{-1} Oral squamous cell carcinoma (OSCC) (n=7) • Mean=4451.01(SE)305.48pg ml^{-1} Significantly higher serum VEGF concentrations in OSCC group than healthy controls ($p<0.001$).	X
Turer et al. (2017) Periodontally healthy controls (n=20) • Mean 149.76±(SD)79.94pg ml^{-1} (median 158.88) Periodontitis group (n=20) • Pre-treatment - Mean 735.65±(SD)532.67pg ml^{-1} (median 599.00) • Post-treatment - Mean 253.12±(SD)306.18pg ml^{-1} (median 76.12) Significantly higher serum VEGF concentrations in periodontitis group compared with healthy controls. Non-surgical periodontal therapy significantly reduces serum VEGF concentration.	X
Gomulka et al. (2019) Systemically healthy adults (n=40) • Mean=246.6pg ml^{-1} Asthma with irreversible bronchoconstriction (n=42) • Mean=340.6pg ml^{-1} Asthma with reversible bronchoconstriction (n=40) • Mean=288.6pg ml^{-1} VEGF serum concentrations significantly raised in asthma compared with healthy controls, but no significant difference in asthma patients with or without irreversible bronchoconstriction.	X

Tayman et al. (2019) Periodontally healthy controls (n=20) • 7.87±(SD)7.10pg ml^{-1} Generalised Periodontitis (n=20) • 5.88±(SD)6.17pg ml^{-1} Generalised Aggressive Periodontitis (n=21) • 8.81±(SD)14.61pg ml^{-1} No significant differences between serum concentrations between the three groups.	X
Manufacturer (R&D Systems) Healthy adults (n=35) • Mean=239± (SD) 155 pg ml^{-1}	Manufacturer (R&D Systems) Healthy adults (n=35) • Mean=61± (SD) 45 pg ml^{-1}

(Widen et al., 2016, Tayman et al., 2019) or reduced levels (Zhu et al., 2015) in comparison with periodontally healthy controls. Although not directly relevant to the current study, serum VEGF concentrations have been reported to have significantly reduced following non-surgical periodontal treatment (Pradeep et al., 2011, Turer et al., 2017), suggesting that periodontal inflammation may influence systemic levels of pro-angiogenic factors such as VEGF.

3.5.1.2 VEGF levels in saliva

Salivary concentrations of VEGF have been reported in a number of oral human pathological conditions in relation to healthy controls. Raised salivary levels have been reported in oral and head-neck squamous cell carcinoma (Upile et al., 2009, Korostoff et al., 2011, Polz-Dacewicz et al., 2016) as well as in benign and malignant tumours of the parotid gland (Blochowiak et al., 2019). However, not all studies reported significant differences, for example, Andisheh-Tadbir et al. (2014) reported no significant difference in unstimulated salivary VEGF concentrations in head-neck squamous cell carcinoma, while Blochowiak et al. (2018) reported no significant difference in stimulated salivary VEGF concentration in both primary and secondary Sjögren's syndrome. Furthermore, reduced salivary levels of VEGF were reported in acute stages of recurrent major aphthous ulceration (Brozovic et al., 2002).

Table 3.15 shows a representative sample of publications which have reported VEGF concentrations in unstimulated, stimulated and fractionated saliva. Large ranges in the VEGF concentrations were reported in each study, although potential confounding factors were not taken into account in the selection criteria in some studies, such as smoking or previous

Table 3.15 A representative selection of publications for VEGF in unstimulated and stimulated saliva.

Unstimulated Saliva (US)	Stimulated Saliva (SS)
Booth et al.(1998) • Systemically and periodontally healthy adults (N=12) • Systemically healthy adults with periodontitis (N=20) Mean levels not stated in paper but VEGF was detected in all saliva samples and was significantly higher in periodontitis than healthy controls ($p<0.05$).	Pammer et al.(1998) Whole saliva from healthy adults (n=24) • Mean=1.4±0.77 (SD) ng ml^{-1} Parotid saliva from healthy adults (n=4) • Mean= 1.95ng ml^{-1} (range 1.26-2.58 ng ml^{-1})
Brozovic et al.(2002) Systemically healthy adults (n=27) • Mean=1652±(SD)567pg ml^{-1} Subjects with minor recurrent aphthous ulceration (n=20) • Acute stage mean=1417±(SD) 848pg ml^{-1} • Remission stage mean=1472± (SD) 867pg ml^{-1} Subjects with major recurrent aphthous ulceration (n=10) • Acute stage mean=341±(SD) 109pg ml^{-1} • Remission stage mean=1524± (SD) 784pg ml^{-1} Acute stages of major recurrent aphthous ulceration associated with significantly reduced salivary VEGF concentrations compared with the healthy controls ($p<0.01$) and the remission stage ($p<0.005$)	Taichman et al.(1998) Systemically and periodontally healthy adults (n=17) • Whole saliva mean =693±543(SD) pg ml^{-1} • Parotid saliva mean =424±470(SD) pg ml^{-1} • Submandibular + Sublingual saliva mean=131±(SD)100pg ml^{-1}
Upile et al. (2009) Healthy controls (n=14) • Mean=231.61(SE)113.97pg ml^{-1} Oral squamous cell carcinoma (OSCC) (n=7) • Mean=1148.88(SE)174.64pg ml^{-1} Significantly higher salivary VEGF concentrations in OSCC group than healthy controls (p=0.001).	Ribeiro et al. (2018) Control mothers with children without early childhood caries (n=19) • Median = 139.11pg ml^{-1} Mothers with children with early childhood caries (n=18) • Median = 187.97pg ml^{-1} Control children without early childhood caries (n=19) • Median = 153.83pg ml^{-1} Children with early childhood caries n=18) • Median = 245.44pg ml^{-1} • p=0.06 between groups.
Korostoff et al. (2011) Healthy controls (n=14) • Mean=32+(SD)8pg ml^{-1} Control smokers (n=14) • Mean=39+(SD)10pg ml^{-1} Control alcohol drinkers (n=14) • Mean=55+(SD)14pg ml^{-1} Control alcohol drinkers and smokers (n=14) • Mean=55+(SD)14pg ml^{-1} Exophytic squamous cell carcinoma of the tongue (n=8) • Mean=110+(SD)21pg ml^{-1} Endophytic squamous cell carcinoma of the tongue (n=10) • Mean=156+(SD)31pg ml^{-1} Significantly raised salivary VEGF concentrations in the both types of squamous cell carcinoma of the tongue compared with all the control groups ($p<0.0001$).	Blochowiak et al. (2019) Healthy controls (n=15) • Median 1001.69pg ml^{-1} [IQR 719.99] Malignant parotid tumours (n=7) • Median 2673.04pg ml^{-1} [IQR 2001.57] Pleomorphic adenoma (benign) (n=20) • Median 2447.23pg ml^{-1} [IQR 1334.76] Warthin's tumour (benign) (n=21) • Median 2176.17pg ml^{-1} [IQR 1964.30] VEGF salivary concentrations significantly higher in pleomorphic adenomas (p=0.007) and Warthin's tumours (p=0.016) compared with healthy controls.
Andisheh-Tadbir et al. (2014) Healthy controls (n=24) • Mean=149.58±(SD)101.88pg ml^{-1} Head and neck squamous cell carcinoma patients (n=30) • Mean=174.41±(SD)115.07pg ml^{-1} No significant difference between carcinoma and healthy control groups.	X
Wu et al. (2018) Non-periodontitis group (n=27) • Median 721.6pg ml^{-1} [IQR 373.4-1199.6] Periodontitis group (n=30) • Median 823.8pg ml^{-1} [IQR 562.4-1226.6] No significant difference in VEGF salivary concentrations between periodontitis and non-periodontitis groups (p=0.14).	X
Manufacturer (R&D Systems) Healthy adults (n=10) • Mean=1218±775 (SD) pg ml^{-1}	X

smoking (Pammer et al., 1998, Taichman et al., 1998) or time of day of saliva collection (Booth et al., 1998, Taichman et al., 1998). These factors were taken into account in the control group for the Brozovic et al. (2002) study, suggesting the mean unstimulated saliva VEGF concentration of 1652pg ml^{-1} (SD$\pm$567pg ml^{-1}) may be a more reliable indicator of the levels expected. Currently, no studies which have reported VEGF concentrations in both stimulated and unstimulated saliva from the same individuals, which will be addressed in the present study.

Although the majority of studies have examined mean VEGF concentrations in mixed whole saliva, evidence suggests that there are significant differences in VEGF concentrations in saliva derived from different salivary glands. Taichman et al. (1998) examined VEGF concentrations in both stimulated whole saliva and from individual major salivary glands (Table 3.15). Mean VEGF concentrations were highest in stimulated whole saliva (693pg ml^{-1}) compared with parotid saliva (424 pg ml^{-1}), and lowest in submandibular-sublingual saliva (131pg ml^{-1}). Conversely, Pammer et al. (1998) reported higher mean salivary VEGF concentrations in parotid saliva (1950pg ml^{-1}) compared with whole saliva (1400pg ml^{-1}), although parotid saliva was only examined in four cases. Few studies have compared VEGF concentrations in saliva with serum levels in the same subjects. Taichman et al. (1998) reported higher VEGF concentrations in whole saliva compared with serum, whilst Upile et al. (2009) reported the opposite finding. In the present study VEGF concentrations were compared in both unstimulated and stimulated saliva in comparison with serum.

Few studies have reported on the effect of smoking on salivary VEGF concentrations and those which have had significant issues related to the protocol. Although Booth et al. (1998) did not specifically examine the effect of smoking on salivary VEGF concentrations, they reported lower VEGF concentrations in unstimulated saliva in smokers with periodontitis compared with non-smokers with periodontitis, although this was not significant. A more recent study by Korostoff et al. (2011) reported raised levels of VEGF, although not significantly, in unstimulated saliva in control subjects who smoked compared with non-smoking controls. Furthermore, periodontal health was not accounted for in the study

criteria, which may have influenced the outcome. The present study will examine whether smoking influences salivary VEGF concentrations, both in periodontal health and disease.

Several recent studies have examined salivary VEGF concentrations in diabetes, although not in relation to periodontal health or disease. Significantly raised VEGF concentrations in unstimulated saliva have been reported in subjects with Type 2 diabetes in older subjects with denture stomatitis (Radovic et al., 2014) and in young pregnant subjects, in comparison with healthy controls (Surdacka et al., 2011). Salivary VEGF concentrations have also been positively correlated with elevated salivary glucose levels in obese adolescents (Hartman et al., 2016) and with maternal caries risk, adiposity and sugar intake in mothers of children with early childhood caries (Ribeiro et al., 2018).

Regarding periodontal health, Booth et al. (1998) first reported significantly raised VEGF levels in unstimulated saliva in small number of subjects with periodontitis compared with periodontally healthy controls. At the time of conducting the present study, Booth et al. (1998) was the only study which had reported salivary VEGF levels in periodontal healthy in comparison with periodontitis, and no studies at that time had aimed to investigate the effects of smoking and diabetes. More recently, there have been several studies which have examined these factors. Wu et al. (2018) reported elevated, but not significantly, salivary VEGF in unstimulated saliva in subjects with periodontitis compared with periodontally healthy controls. Interestingly, two recent studies have reported diametrically opposing findings in salivary VEGF concentrations in gingivitis. Afacan et al. (2019) found significantly raised VEGF concentrations in unstimulated saliva in gingivitis compared to both periodontal health and periodontitis (no significant difference between periodontal health and periodontitis groups). While Belstrom et al. (2017) replicated the classical experimental gingivitis studies where 29 dental students (mean age 24.7 years) ceased oral hygiene for 14 days, after which oral hygiene was reinstated. Significantly reduced stimulated saliva VEGF concentrations were found in the gingivitis group after 10 days, levels of which returned to normal 14 days after the oral hygiene was reinstated. There was no gingivitis group in the present study.

3.5.1.3 VEGF levels in GCF

Many studies have examined VEGF levels, expressed as either total amounts and/or as concentrations, in periodontal health compared with periodontal disease. VEGF levels in GCF have been widely reported to be significantly raised in subjects with periodontitis compared with healthy controls (Booth et al., 1998, Lee et al., 2003, Guneri et al., 2004, Prapulla et al., 2007, Pradeep et al., 2011, Padma et al., 2014, Sakallioglu et al., 2015, Zhu et al., 2015, Pannicker and Mehta, 2016, Turer et al., 2017, Romano et al., 2018, Afacan et al., 2019, Tayman et al., 2019), levels of which were found to be significantly reduced following periodontal therapy (Prapulla et al., 2007, Pradeep et al., 2011, Padma et al., 2014, Pannicker and Mehta, 2016, Turer et al., 2017, Romano et al., 2018). Several studies have also reported significant, albeit low to moderate, correlations between VEGF levels and periodontal parameters such as gingival index/bleeding on probing, pocket depths and loss of attachment (Lee et al., 2003, Turer et al., 2017, Tayman et al., 2019). Although most studies reported GCF level using VEGF concentrations, some studies only found significantly raised VEGF levels in periodontitis, compared with periodontally healthy controls, when the results were reported in total amounts of VEGF (30 second collection period) (Booth et al., 1998, Turer et al., 2017, Afacan et al., 2019, Tayman et al., 2019). In fact, two studies found significant higher VEGF GCF concentrations in the periodontally healthy subjects (Booth et al., 1998, Afacan et al., 2019). This latter finding may be due to significantly reduced GCF flow rates associated with periodontally healthy sites resulting in high VEGF concentrations (Turer et al., 2017) or errors in accurately measuring very small quantities of GCF in healthy sites resulting in falsely high concentration calculations (Wassall and Preshaw, 2016). Furthermore, several studies have reported significantly higher VEGF in GCF between subjects in sites with gingivitis compared with periodontally healthy sites (Lee et al., 2003, Pradeep et al., 2011, Padma et al., 2014), although this finding was not universal (Prapulla et al., 2007).

Currently few studies have examined the effect of smoking on VEGF levels in GCF. Sakallioglu et al. (2015) carried a split mouth study in smokers and non-smokers, who had both periodontally healthy and diseased sites. No significant differences were found between the smoking and non-smoking groups in terms of VEGF levels (concentration and total

amounts) in either the periodontally healthy sites or periodontitis sites. Similar findings were reported by Eren et al. (2015) between periodontally healthy smokers and non-smokers. Although these were two small studies, they infer smoking does not significantly affect VEGF levels in GCF.

Similar to smoking, there have only been a small number of studies which have examined the relationship between Type 2 diabetes and the levels of VEGF in GCF. Several studies have found no significant differences in GCF VEGF levels between subjects with Type 2 diabetes and systemically healthy subjects, either at periodontally healthy sites or at periodontitis sites (Guneri et al., 2004, Sakallioglu et al., 2007, Pannicker and Mehta, 2016). Pannicker and Mehta (2016) also found similar significant reductions in VEGF GCF levels following non-surgical periodontal treatment in both subjects with and without diabetes. One recent study reported significantly higher total VEGF in GCF in periodontitis subjects with diabetes compared with patients without diabetes once statistical adjustments were made for confounding factors, such as smoking, age, bleeding and plaque scores (Mohamed et al., 2015).

3.5.2 Results

3.5.2.1 VEGF concentration in serum

VEGF was detected in the majority of serum samples and the results are summarised in Table 3.16 and are shown in Figure 3.17. VEGF was quantified in 67 serum samples with mean levels for the study sub-groups ranging from 274 to 1602pg ml^{-1}, although there were large variations in the levels detected. No significant differences were found between either the study groups (Two-way ANOVA test $F(2,61)=0.387$, $p=0.681$) or between periodontally healthy and severe periodontitis sub-groups (Two-way ANOVA test $F(1,61)=0.691$, $p=0.409$), and there was no significant interaction found between periodontal health status and the study groups (Table 3.17). A non-significant reduction was found in the smoking group (Group 2) in periodontal health and increased levels in severe periodontitis compared with the healthy control (Group 1) and the diabetes (Group 3) groups (Figure 3.18). Bonferroni post hoc tests (Table 3.18) confirmed that there were no significant differences between the study sub-groups ($p=1.0$). Diagnostic statistical analysis revealed that there were outliers in the data which had a significant influence on the overall statistical outcome

(Studentized residual highest=5.74, lowest= -1.24; highest Cook's distance=1.10; samples KJD58, KJD89 and KJD41 were deemed to be outliers). Bootstrapping analysis confirmed that the outcome of the Two-way ANOVA was not significantly influenced by the outliers. Sensitivity analysis, where data from the three outliers were removed from the dataset, confirmed there were no significant differences in the serum VEGF levels between the study groups (Two-way ANOVA test F(2,61)=1.278, p=0.286; Table 3.17 and Figure 3.19). Bonferroni post hoc tests confirmed that there were no significantly differences in serum VEGF concentrations between the study groups (Table 3.18).

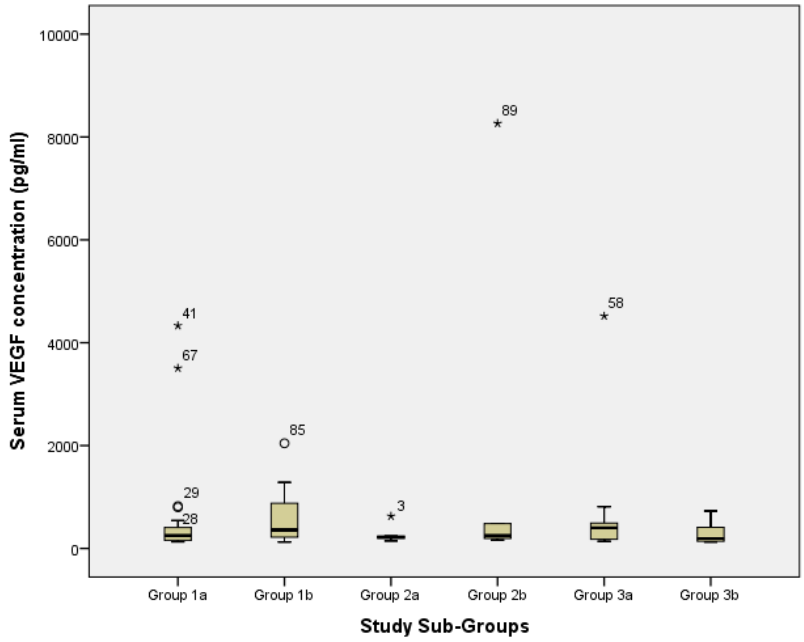
Table 3.16 Descriptive statistics for VEGF serum concentration (pg ml^{-1}) for the study groups and sub-groups. Statistical data in brackets are the results following removal of extreme outliers (>3xSD) from the data set.

Group	Number Valid Cases*	Mean ± SD (pg ml^{-1})	Median (pg ml^{-1})	Interquartile Range	Range
1a	30 (29)	543±942 (412±624)	251 (239)	273 (248)	4206 (3379)
1b	8	635±676	360	863	1920
Study Group 1	38 (37)	562±885 (460+632)	267 (263)	301 (269)	4207 (3380)
2a	6	274±176	218	158	482
2b	6 (5)	1602±3266 (269+125)	253 (243)	2240 (193)	8100 (318)
Study Group 2	12 (11)	938±2311 (272±148)	233 (223)	234 (69)	8119 (482)
3a	9 (8)	812±1406 (349±231)	400 (292)	477 (297)	4378 (672)
3b	8	293±212	193	280	604
Study Group 3	17 (16)	567±1039 (321±216)	221 (203)	299 (263)	4393 (687)

*Excluding either no sample available or factor not detected.

Figure 3.17 Serum VEGF concentrations (pg ml^{-1}) for (a) study sub-groups and (b) study groups. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

(a)



(b)

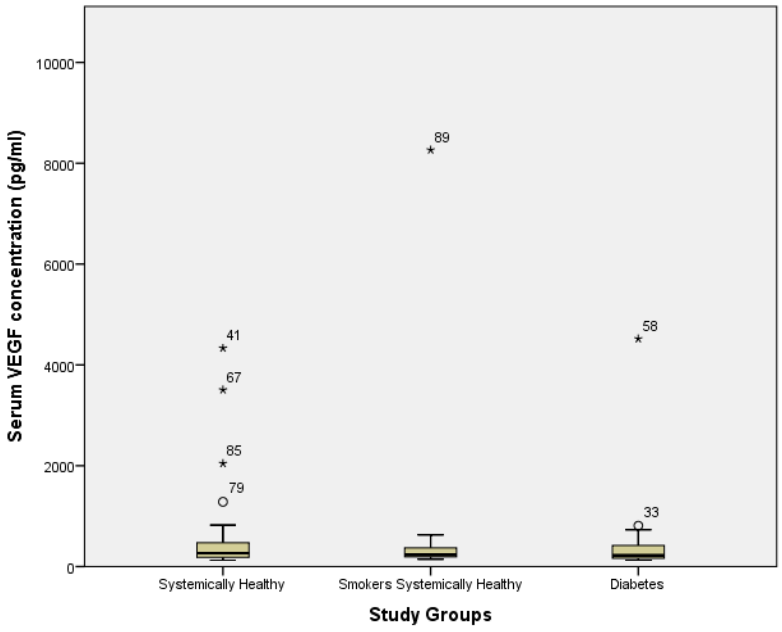


Table 3.17 Statistical comparison of serum VEGF concentrations of the study groups and periodontal health-severe periodontitis using Two-way ANOVA test. Data in brackets are the results following removal of outliers KJD41, KJD58 and KJD 89 from the data set.

Group	Degrees of Freedom (df)	F	Significance (p-value)
Periodontal Health – Severe Periodontitis	1	0.691 (0.132)	0.409 (0.717)
Study Groups (1, 2 and 3)	2	0.387 (1.278)	0.681 (0.286)
Periodontal Health – Severe Periodontitis Versus Study Groups (interaction)	2	1.878 (0.428)	0.162 (0.654)

Table 3.18 Statistical comparison between study sub-groups for serum VEGF (Bonferroni test p-values). Data in brackets are the results following removal of outliers KJD41, KJD58 and KJD 89 from the data set.

Study Groups		Mean Difference	Significance (p-value)	95% Confidence Interval	
				Lower Bound	Upper Bound
Group 1	Group 2	-375.78 (188.34)	1.000 (0.855)	-1411.98 (-242.06)	660.41 (618.74)
	Group 3	-5.42 (139.60)	1.000 (1.000)	-918.49 (-235.40)	907.65 (514.60)
Group 2	Group 1	375.78 (-188.34)	1.000 (0.855)	-660.41 (-618.74)	1411.98 (242.06)
	Group 3	370.36 (-48.74)	1.000 (1.000)	-809.48 (-539.62)	1550.20 (442.14)
Group 3	Group 1	5.42 (-139.60)	1.000	-907.65 (-514.60)	918.49 (235.40)
	Group 2	-370.36 (48.74)	1.000 (1.000)	-1550.20 (-442.14)	809.48 (539.62)

Figure 3.18 Profile plot of periodontal health and severe periodontitis against estimated marginal mean serum VEGF concentrations (pg ml^{-1}) for the three study groups.

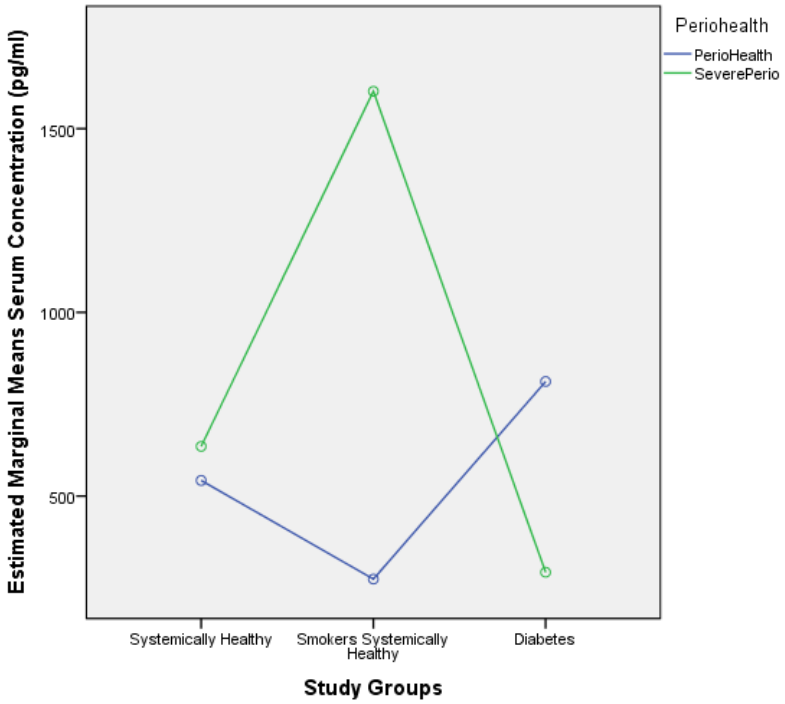
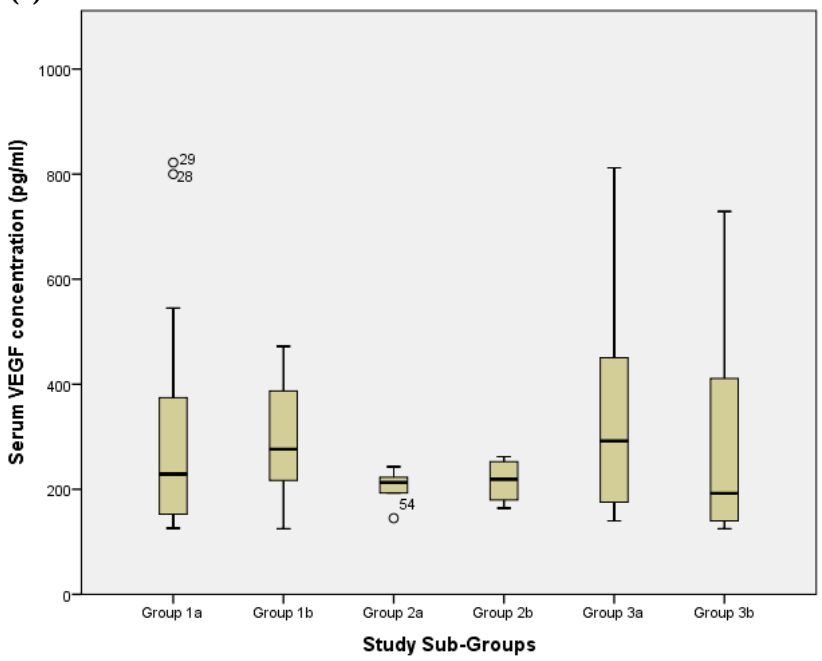
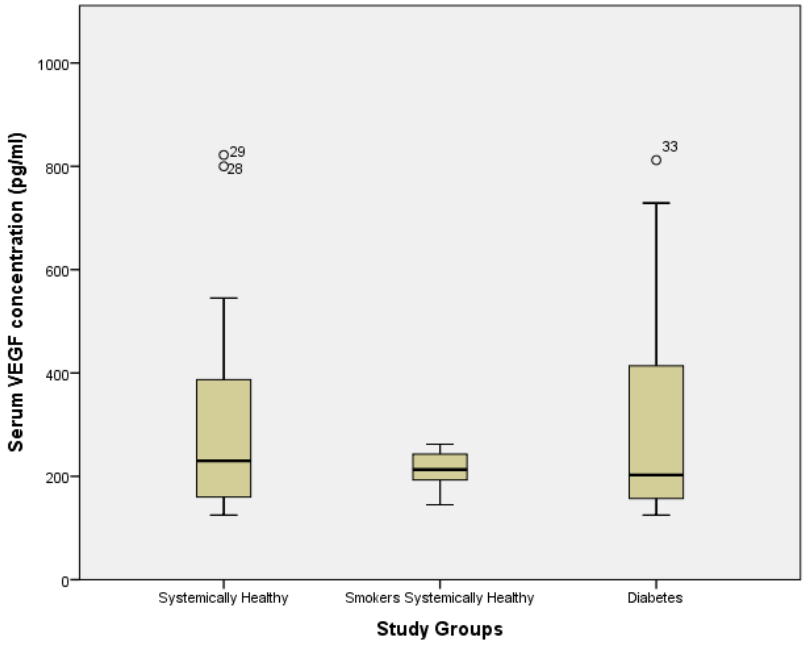


Figure 3.19 Serum VEGF concentrations (pg ml^{-1}) with extreme outliers (>3xSD) removed for (a) study sub-groups and (b) study groups. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

(a)



(b)



There were no significant correlations between the serum VEGF concentration with smoking dose (pack years) (Spearman rho=-0.090, p=0.481), glycaemic control (Spearman rho -0.119 p=0.649) or age (Spearman rho=0.146, p=0.246). No significant difference was found regarding gender and serum VEGF concentration across the whole sample (Mann Whitney p=0.712).

3.5.2.2 VEGF concentration in saliva

3.5.2.2.1 VEGF concentration in stimulated saliva

VEGF was detected in the majority of stimulated saliva samples and the results are summarised in Table 3.19 and shown in Figure 3.20. VEGF was quantified in 81 stimulated saliva samples with mean levels for the study sub-groups ranging from 465 to 961pg ml^{-1}, although there were large variations in the levels found. No significant differences were found between either the study groups (Two-way ANOVA test F(2,75)=0.791, p=0.457) or between periodontally healthy and severe periodontitis sub-groups (Two-way ANOVA test F(1,75)=0.241, p=0.626), and there was no significant interaction found between periodontal health status and the study groups (Table 3.20). A non-significant increase was found in the smoking group (Group 2) in periodontal health and reduced levels in severe periodontitis

compared with the healthy control (Group 1) and the diabetes (Group 3) groups (Figure 3.21). Bonferroni post hoc tests confirmed that there were no significant differences between the study sub-groups (Table 3.21). Diagnostic statistical analysis revealed that there was an outlier in the data which had a significant influence on the overall statistical outcome (Studentized residual highest=7.34, lowest=-1.93; highest Cook's distance=1.28; sample KJD54 was deemed to be an outlier; Figure 3.22). Bootstrapping analysis suggested the outcome of the Two-way ANOVA was not significantly influenced by the outlier. However, sensitivity analysis, where the outlier (KJD54) was removed from the dataset, revealed highly significant differences between the study groups (Two-way ANOVA test $F(2,75)=5.629$, $p=0.005$) but no significant difference between periodontally healthy and severe periodontitis sub-groups (Two-way ANOVA test $F(1,75)=2.575$, $p=0.113$). There was no significant interaction found between periodontal health status and the study groups (Table 3.20). Bonferroni post hoc tests (Table 3.21) revealed significantly higher stimulated saliva VEGF concentrations in the diabetes group (Group 3) compared with systemically healthy non-smokers (study group 1; 3>1 $p=0.012$) and smokers (Group 2; 3>2 $p=0.004$). No significant difference was found between the systemically healthy and smoking groups. These findings strongly suggested that stimulated saliva VEGF concentration was significantly raised in the diabetes study group.

Table 3.19 Descriptive statistics for VEGF stimulated (SS) and unstimulated saliva (US) concentrations (pg ml^{-1}) for the study groups and sub-groups. Statistical data in brackets are the results following removal of extreme outliers (>3xSD) from the data set.

	Group	Number Valid cases*	Mean ± SD (pg ml^{-1})	Median (pg ml^{-1})	Interquartile Range	Range
SS	**1a**	26	465±223	473	328	819
	1b	13	727±208	773	387	571
	Study Group 1	39	552±249	500	415	853
	2a	8 (7)	961±1482 (449±347)	429 (393)	755 (488)	4521 (1059)
	2b	9	481±226	478	228	788
	Study Group 2	17 (16)	707±1023 (467±275)	465 (456)	343 (279)	4521 (1059)
	3a	10	744±353	758	624	1053
	3b	15	778±352	765	410	1442
	Study Group 3	25	765±345	765	460	1449
US	**1a**	27	403±294	313	282	1413
	1b	14 (13)	838±577 (745±476)	625 (549)	845 (806)	2047 (1489)
	Study Group 1	41 (40)	552±456 (514±392)	450 (449)	449 (437)	2047 (1489)
	2a	9	311±197	224	290	492
	2b	9	481±259	599	504	648
	Study Group 2	18	396±240	275	442	648
	3a	13	721±386	623	451	1440
	3b	15 (14)	766±475 (678±343)	728 (701)	496 (475)	1772 (1270)
	Study Group 3	28 (27)	745±428 (699±358)	681 (673)	466 (467)	1772 (1496)

*Excluding either no sample available or factor not detected.

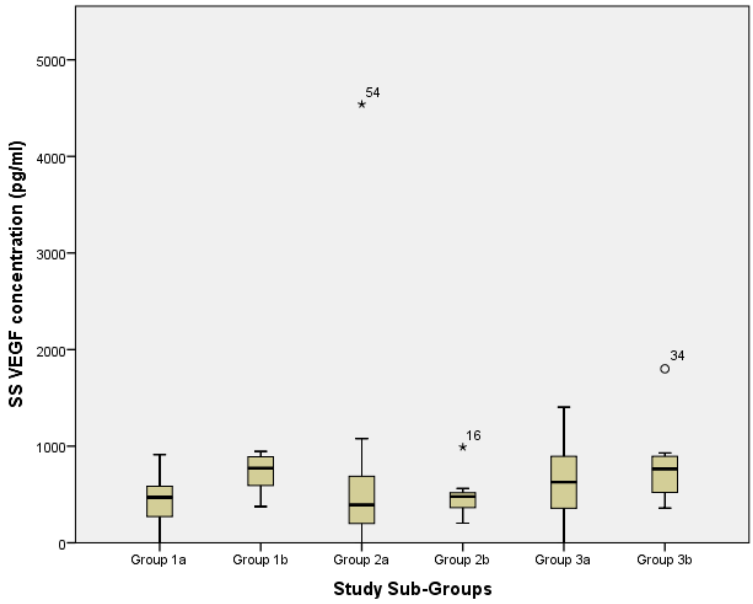
Table 3.20 Statistical comparison of the stimulated saliva VEGF concentrations of the study groups and periodontal health-severe periodontitis using Two-way ANOVA test. Data in brackets are the results following removal of outlier KJD54 from the data set.

Group	Degrees of Freedom (df)	F	Significance (p-value)
Periodontal Health – Severe Periodontitis	1	0.240 (2.575)	0.626 (0.113)
Study Groups (1, 2 and 3)	2	0.791 (5.629)	0.457 (0.005**)
Periodontal Health – Severe Periodontitis Versus Study Groups (interaction)	2	2.877 (1.557)	0.063 (0.218)

* - Significant difference <0.05
** - Significant difference <0.01

Figure 3.20 Stimulated Saliva VEGF concentrations (pg ml^{-1}) for (a) study sub-groups and (b) study groups. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

(a)



(b)

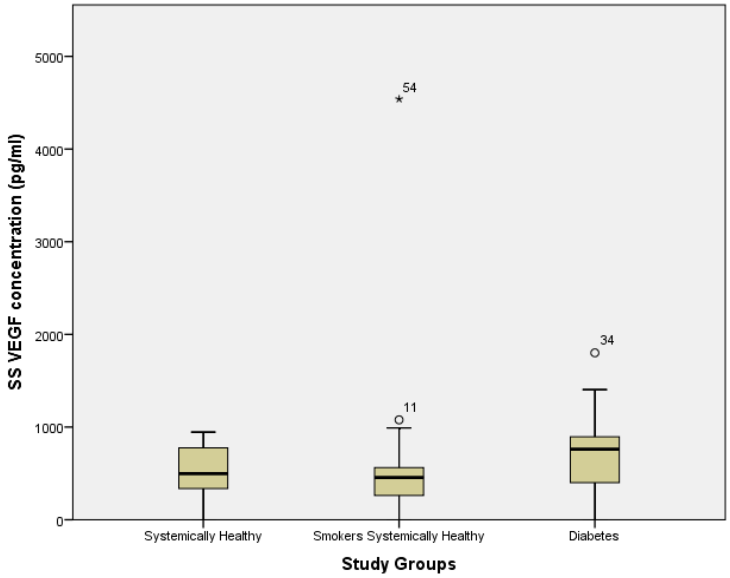


Table 3.21 Statistical comparison between study sub-groups for stimulated saliva VEGF (Bonferroni test p-values). Data in brackets are the results following removal of outlier KJD54 from the data set.

Study Groups		Mean Difference	Significance (p-value)	95% Confidence Interval	
				Lower Bound	Upper Bound
Group 1	Group 2	-154.45 (85.12)	0.934 (0.921)	-525.56 (-117.62)	216.66 (287.86)
	Group 3	-212.16 (-212.16)	0.349 (0.012*)	-539.32 (-387.12)	114.99 (-37.20)
Group 2	Group 1	154.45 (-85.12)	0.934 (0.921)	-216.66 (-287.86)	525.56 (117.62)
	Group 3	-57.72 (-297.29)	1.000 (0.004**)	-459.13 (-515.92)	343.70 (-78.66)
Group 3	Group 1	212.16 (212.16)	0.349 (0.012*)	-114.99 (37.20)	539.32 (387.12)
	Group 2	57.72 (297.29)	1.000 (0.004**)	-343.70 (78.66)	459.13 (515.92)

* - Significant difference <0.05
** - Significant difference <0.01

Figure 3.21 Profile plot of periodontal health and severe periodontitis against estimated marginal mean stimulated saliva (SS) VEGF concentrations (pg ml^{-1}) for the three study groups.

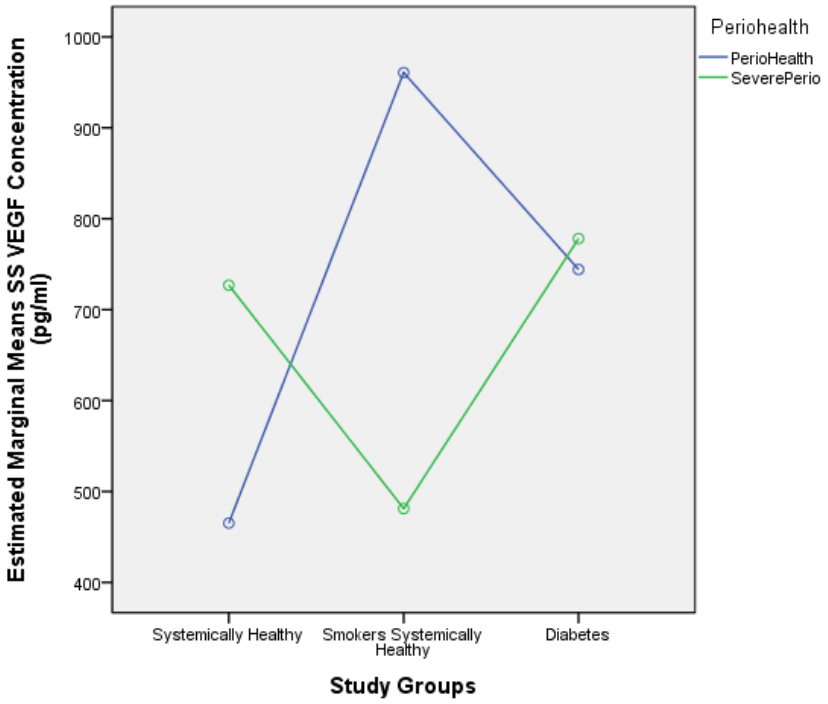
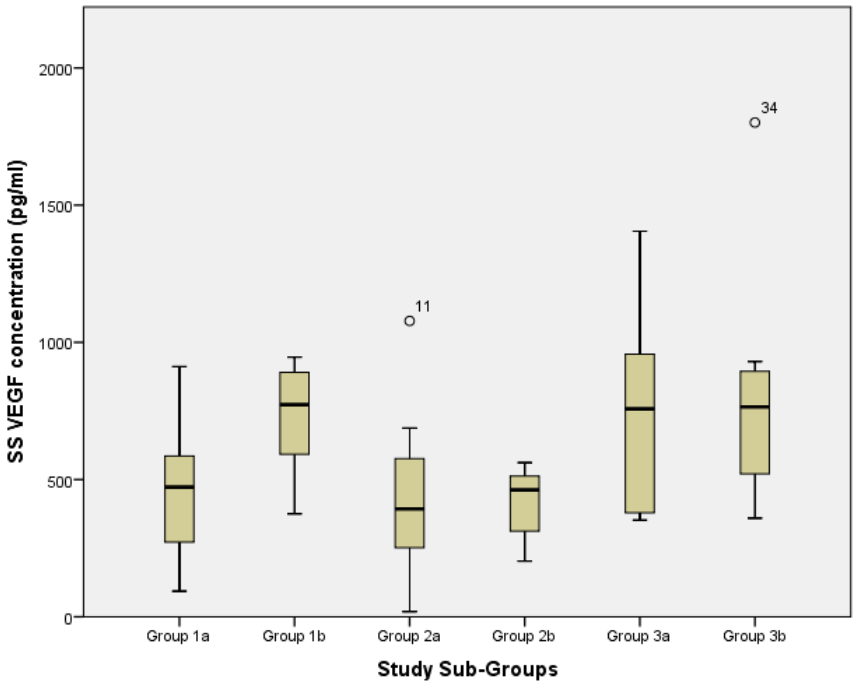
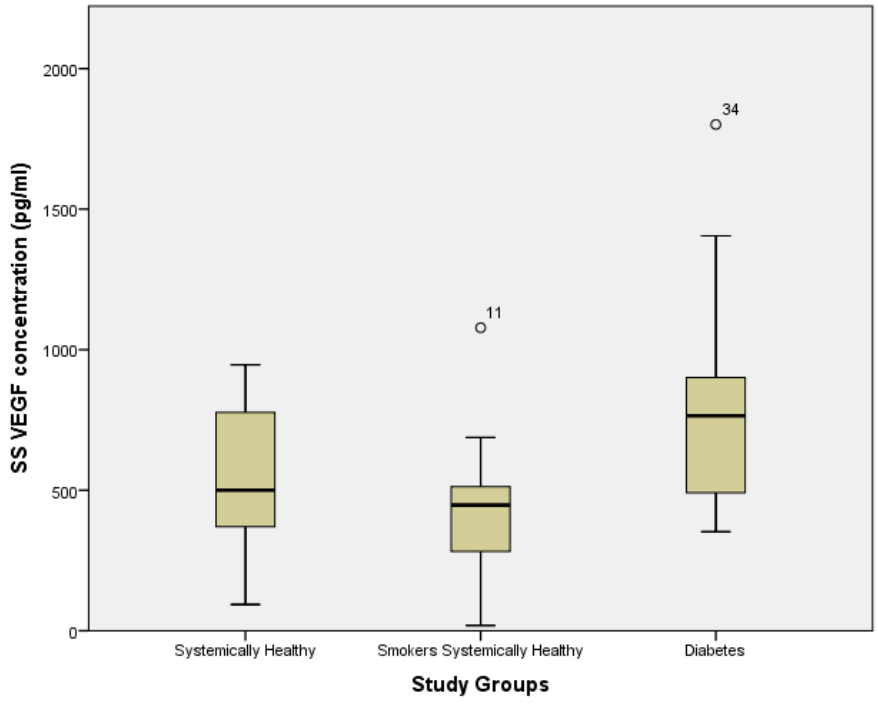


Figure 3.22 Stimulated saliva VEGF concentrations (pg ml^{-1}) with extreme outliers (>3xSD) removed for (a) study sub-groups and (b) study groups. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

(a)

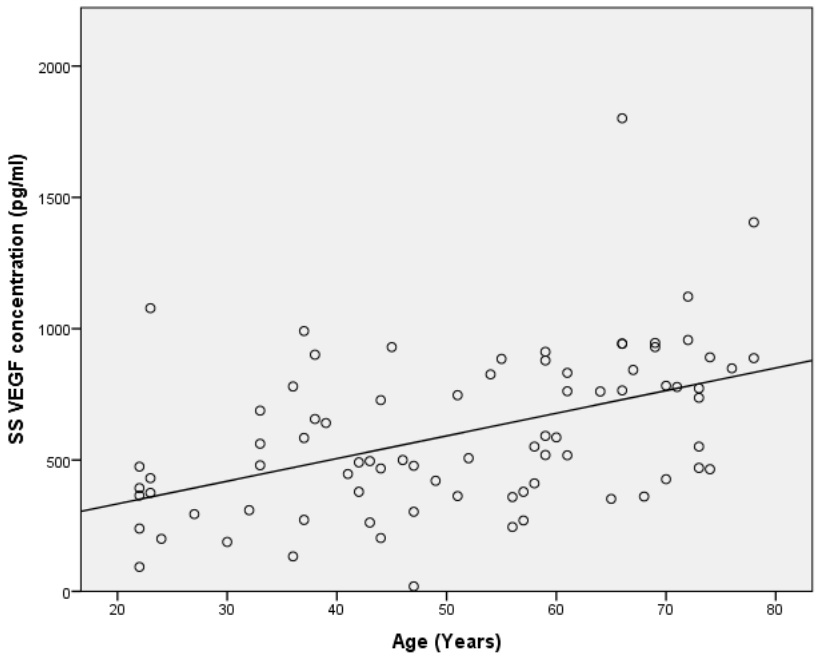


(b)



No significant correlations were found between the VEGF concentration in stimulated saliva with smoking dose (Spearman rho=-0.083, p=0.462) or glycaemic control (Spearman rho=-0.047, p=0.824). A highly significant moderate positive correlation was observed between age and VEGF concentration in stimulated saliva (Spearman rho=0.491, p<0.001; without outliers Spearman rho=0.486, p<0.001) (Figure 3.23). No significant difference was found regarding gender and stimulated saliva VEGF concentration across the whole sample (Mann Whitney p=0.916).

Figure 3.23 Scatterplot of the correlation between stimulated saliva VEGF concentration (pg ml^{-1}) with age (Years). Line of best fit shown*.



*Line of best fit R^2=0.223 (Spearman rho=0.491, p<0.001; without outliers Spearman rho=0.486, p<0.001)

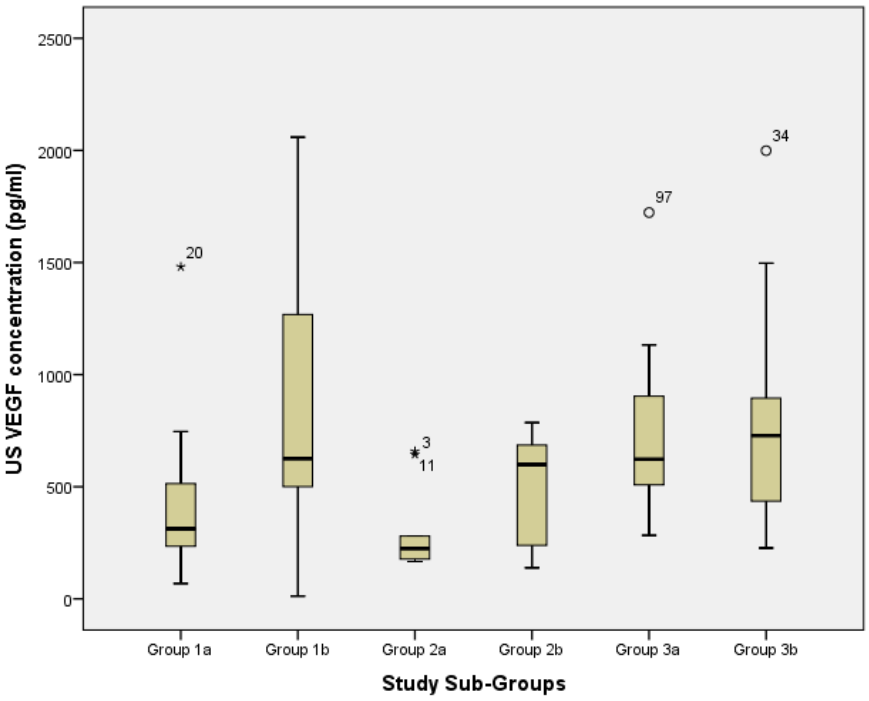
3.5.2.2.2 VEGF concentration in unstimulated saliva

VEGF was detected in the majority of unstimulated saliva samples and the results are summarised in Table 3.19 and shown in Figure 3.24. VEGF was quantified in 87 unstimulated saliva samples with mean levels for the study sub-groups ranging from 311 to 838pg ml^{-1}, although there were large variations in the levels found. Significant differences were found both between the study groups (Two-way ANOVA test F(2,81)=4.345, p=0.016)

and between periodontally healthy and severe periodontitis sub-groups (Two-way ANOVA test $F(1,81)=5.830$, $p=0.018$), and there was no significant interaction found between periodontal health status and the study groups (Table 3.22). A non-significant reduction was found in the smoking group (Group 2) in both periodontal health and severe periodontitis compared with the healthy control (Group 1) and the diabetes (Group 3) groups (Figure 3.25). Bonferroni post hoc tests revealed significantly higher VEGF concentrations in unstimulated saliva in the diabetes group (Group 3) compared with smokers (Group 2, 3>2 $p=0.012$) (Table 3.23). Diagnostic statistical analysis revealed that there were outliers in the data, however, their influence on the overall statistical outcome was low (Studentized residual highest=3.27, lowest= -2.19; highest Cook's distance=0.13; samples KJD34 and KJD85 were deemed to be outliers; Figure 3.26). Bootstrapping analysis confirmed that the outcome of the Two-way ANOVA was not significantly influenced by the presence of the two outliers. Sensitivity analysis, where the outliers (KJD34 and KJD85) were removed from the dataset, confirmed significant differences between the study groups (Two-way ANOVA test $F(2,81)=4.310$, $p=0.017$), however, a marginally significant difference was found between periodontally healthy and severe periodontitis sub-groups (Two-way ANOVA test $F(1,81)=3.906$, $p=0.052$). There was no significant interaction found between periodontal health status and the study groups (Table 3.22). Bonferroni post hoc tests confirmed significantly higher VEGF concentrations in the diabetes group (Group 3) compared with smokers (Group 2; 3>2 $p=0.013$) (Table 3.23). No significant difference was found between the systemically healthy and smoking groups. These findings suggested that unstimulated saliva VEGF concentration was significantly raised in the diabetes study group.

Figure 3.24 Unstimulated saliva VEGF concentrations (pg ml^{-1}) for (a) study sub-groups and (b) study groups. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

(a)



(b)

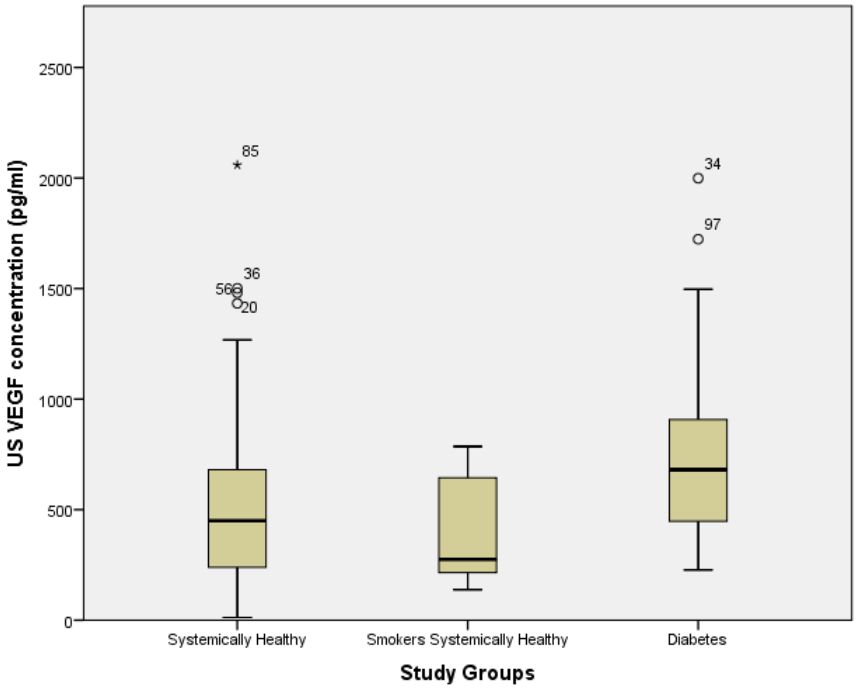


Table 3.22 Statistical comparison of unstimulated saliva VEGF concentrations of the study groups and periodontal health-severe periodontitis using Two-way ANOVA test. Data in brackets are the results following removal of outliers KJD34 and KJD85 from the dataset.

Group	Degrees of Freedom (df)	F	Significance (p-value)
Periodontal Health – Severe Periodontitis	1	5.830 (3.906)	0.018* (0.052)
Study Groups (1, 2 and 3)	2	4.345 (4.310)	0.016* (0.017*)
Periodontal Health – Severe Periodontitis Versus Study Groups (interaction)	2	2.093 (2.441)	0.130 (0.094)

* - Significant difference <0.05

Table 3.23 Statistical comparison between study sub-groups for unstimulated saliva VEGF (Bonferroni test p-values). Data in brackets are the results following the removal of outliers KJD34 and KJD85 from the data set.

Study Groups		Mean Difference	Significance (p-value)	95% Confidence Interval	
				Lower Bound	Upper Bound
Group 1	Group 2	155.75 (118.06)	0.487 (0.675)	-114.35 (-118.01)	425.85 (354.13)
	Group 3	-193.72 (-184.98)	0.139 (0.096)	-427.92 (-392.14)	40.47 (22.19)
Group 2	Group 1	-155.75 (-118.06)	0.487 (0.675)	-425.85 (-354.13)	114.35 (118.01)
	Group 3	-349.47 (-303.04)	0.012* (0.013*)	-638.06 (-556.13)	-60.87 (-49.94)
Group 3	Group 1	193.72 (184.98)	0.139 (0.096)	-40.47 (-22.19)	427.92 (392.14)
	Group 2	349.47 (303.04)	0.012* (0.013*)	60.87 (49.94)	638.06 (556.13)

* - Significant difference <0.05

Figure 3.25 Profile plot of periodontal health and severe periodontitis against estimated marginal mean unstimulated saliva (US) VEGF concentrations (pg ml^{-1}) for the three study groups.

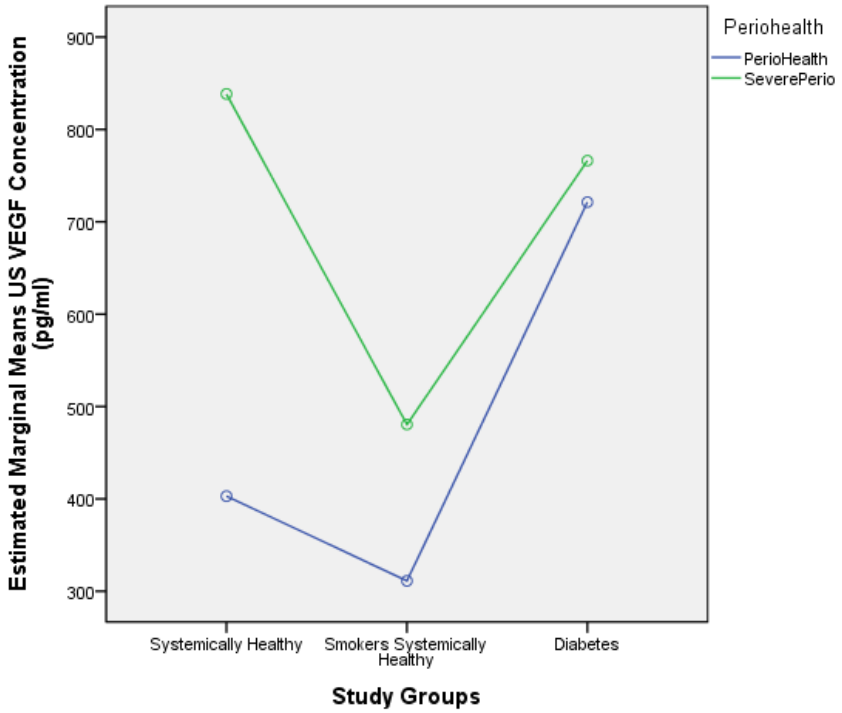
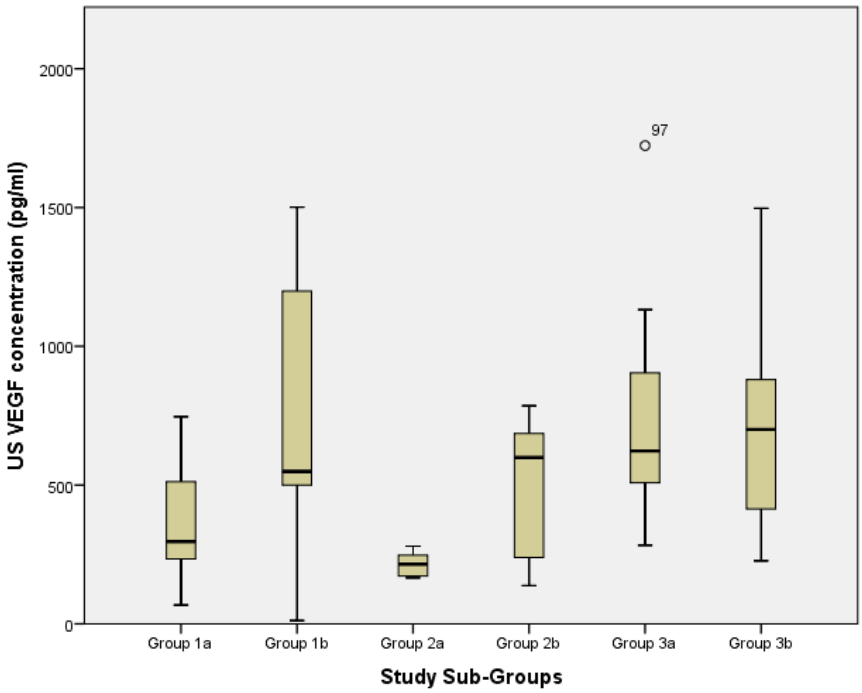
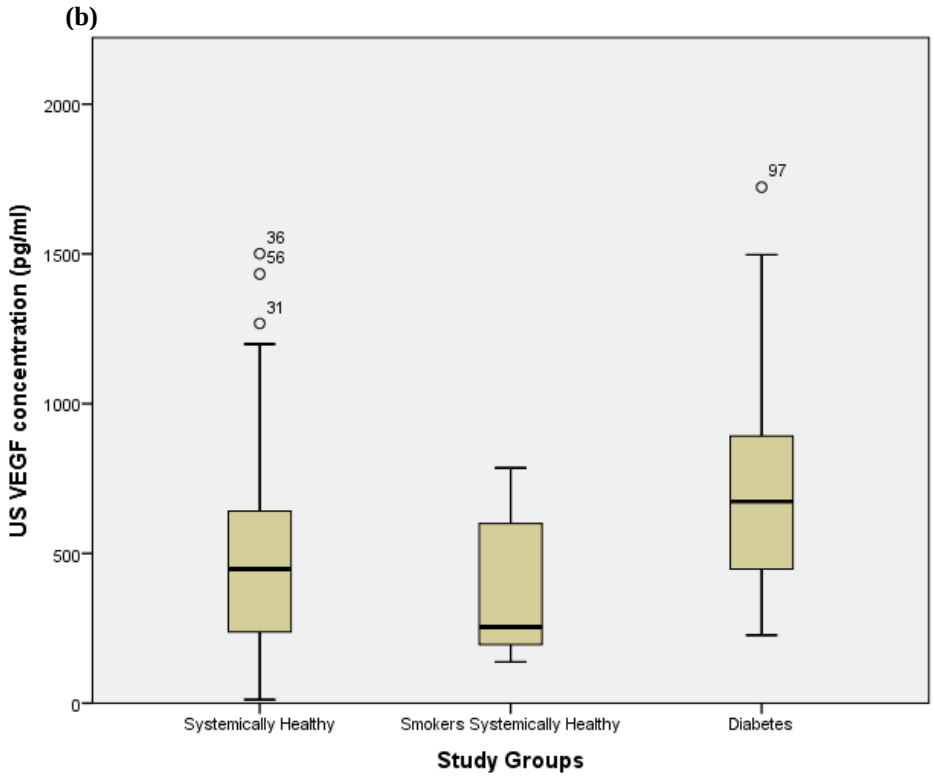


Figure 3.26 Unstimulated saliva VEGF concentrations (pg ml^{-1}) with extreme outliers (>3xSD) removed for (a) study sub-groups and (b) study groups. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

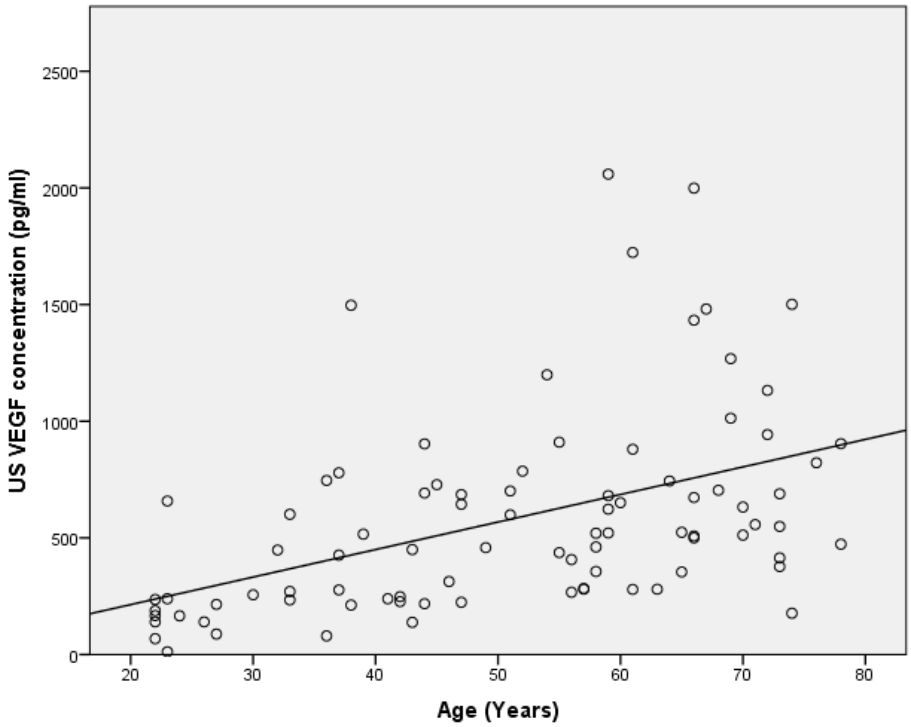
(a)





No significant correlations were found between the VEGF concentration in unstimulated saliva with smoking dose (Spearman rho=-0.097, p=0.374) or glycaemic control (Spearman rho=0.051, p=0.798). A highly significant moderate positive correlation was observed between age and the concentration of VEGF in unstimulated saliva (Spearman rho=0.549, p<0.001; without outliers Spearman rho=0.546, p<0.001) (Figure 3.27). No significant difference was found regarding gender and unstimulated saliva VEGF concentration across the whole sample (Mann Whitney p=0.344).

Figure 3.27 Scatterplot of the correlation between unstimulated saliva VEGF concentration (pg ml^{-1}) with age (Years). Line of best fit shown*.

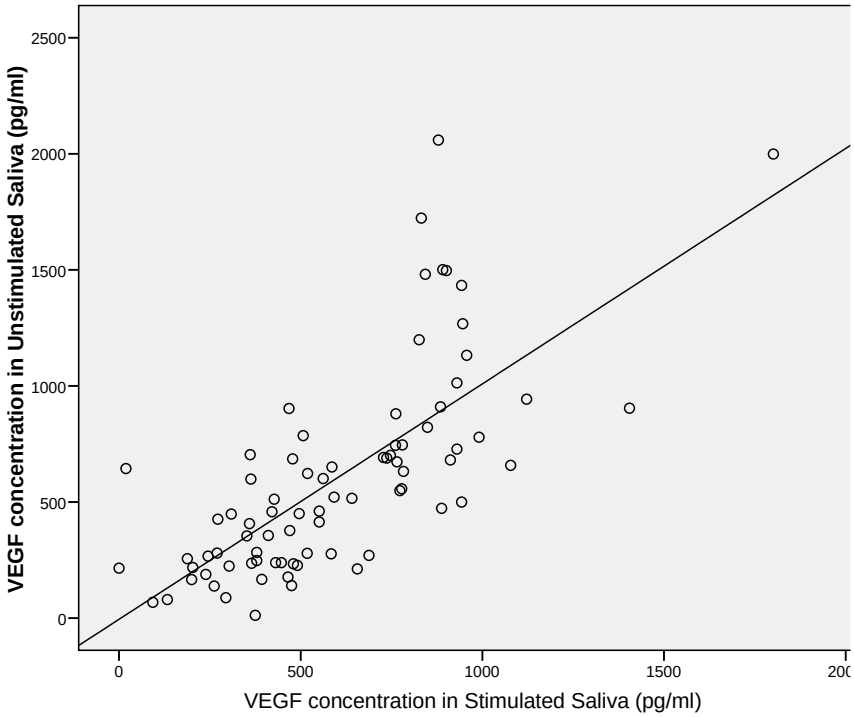


* Line of best fit R^2=0.232 (Spearman rho=0.549, p<0.001; without outliers Spearman rho=0.546, p<0.001)

3.5.2.3 Relationship between VEGF concentration in the serum and saliva

No significant correlations were found between serum VEGF concentrations with either the levels found in the unstimulated (Spearman rho=0.067, p=0.558) or stimulated saliva (Spearman rho=0.109, p=0.358) (data not shown). A highly significant strong positive correlation was found between paired unstimulated and stimulated saliva VEGF concentrations (Spearman rho=0.765, p<0.001; without outliers Spearman rho=0.748, p<0.001) (Figure 3.28).

Figure 3.28 Scatterplot of the correlation between the VEGF concentration (pg ml^{-1}) in unstimulated and stimulated saliva. Line of best fit shown*.



* Line of best fit R^2=0.473 (Spearman rho=0.765, p<0.001; without outliers Spearman rho=0.748, p<0.001)

3.5.3 Discussion

In this study, the concentration of the pro-angiogenic factor VEGF was measured in serum, unstimulated and stimulated saliva samples from three groups of subjects: (i) systemically healthy non-smokers, (ii) systemically healthy smokers and in (iii) subjects with diabetes. Each clinical group was divided into two sub-groups: (a) periodontally healthy and (b) subjects with severe periodontitis. Confidence in the assay methodology was demonstrated by the good assay standard curves, low assay background noise, acceptable levels of intra- and inter-assay variation. VEGF was detected in the majority of samples assayed and the mean concentrations, standard deviations and ranges were found to be within levels previously reported in the literature, especially after the effects of outliers were accounted for (Tables 3.14, 3.15 and 3.16). As far as can be ascertained, this is the first study to report VEGF concentrations in both stimulated and unstimulated saliva in relation to serum. Although no significant differences or correlations were found regarding serum VEGF

concentrations, VEGF concentrations in unstimulated and stimulated saliva were significantly raised in the diabetes group and there were highly significant positive correlations with age. Furthermore, a highly significant strong positive correlation was found between paired VEGF concentrations in unstimulated and stimulated saliva. Interestingly, VEGF was found to be marginally statistically higher in unstimulated saliva in subjects with severe periodontitis compared with periodontally healthy subjects ($p=0.052$). No significant correlations were found regarding gender, smoking (pack years) or glycaemic control (HbA1c) with VEGF concentrations in serum or saliva (unstimulated or stimulated).

There has been great interest in salivary VEGF as it has been postulated that it could have a significant contribution to enhanced oral healing as well as in the maintenance of oral soft tissues, salivary glands and the gastro-intestinal tract (Zelles et al., 1995, Booth et al., 1998, Pammer et al., 1998, Taichman et al., 1998, Parvadia et al., 2007, Keswani et al., 2013). Studies using animal wound healing models have provided good evidence to support this hypothesis. Parvadia et al. (2007) showed that removal of the submandibular glands, the primary source of salivary VEGF in mice (Mandel, 1987), resulted in reduced healing response following small bowel resection. The healing response was partially corrected by the administration of VEGF and was fully restored by the administration of both VEGF and Epidermal Growth Factor (EGF), suggesting that salivary VEGF plays a significant role in the maintenance and healing response of the gastro-intestinal tract in mice. The same research group later examined the effect of salivary VEGF on murine palatal wound healing through the removal of the submandibular glands and by using a specific VEGF inhibitor (VEGF-Trap protein) (Keswani et al., 2013). Removal of the submandibular glands resulted in significantly reduced salivary VEGF concentrations, delayed wound closure and re-epithelisation, reduced capillary density and VEGF receptor expression (VEGFR2/Flk-1) compared with the normal control animals. Similar results were found following the administration of the specific VEGF inhibitory protein in mice with intact submandibular glands when compared with controls. In animals where the submandibular glands had been removed addition of recombinant VEGF, at normal murine salivary concentration, restored wound closure, re-epithelisation, capillary density and VEGF receptor expression to normal

levels. This study provided good evidence, albeit in mice, of the pivotal role VEGF has in oral healing.

VEGF has been previously detected in unstimulated and stimulated whole saliva, as well as from individual major salivary glands, and mean VEGF concentrations found in the present study are within the range previously reported (Table 3.15). Evidence from immunohistochemistry and *in-situ* hybridisation studies suggests that VEGF is produced within human major and minor salivary glands (Pammer et al., 1998, Taichman et al., 1998, de Oliveira et al., 2002), in particular by serious acinar cells and accounts for the different concentrations of VEGF found in individuals in serum and saliva the present study. In the present study there was a highly significant correlation between VEGF concentrations in unstimulated and stimulated saliva concentrations in individuals. This implies that VEGF is likely to be produced by the submandibular gland, which produces approximately 70% of unstimulated saliva (de Almeida et al., 2008).

There have been surprisingly few studies which have examined salivary VEGF concentration in diabetes. At the time of carrying out the present study, there had been no studies in humans, although an immunohistochemistry study had reported increased VEGF expression in diabetic rat submandibular glands in comparison with healthy controls (Perrotti et al., 2007). Subsequently, several studies have reported significantly raised VEGF concentrations in unstimulated whole saliva in subjects with diabetes in pregnancy (Surdacka et al., 2011) and older subjects with denture stomatitis (Radovic et al., 2014), which correspond to the findings of the present study. However, the present study is the first to report raised VEGF levels in both unstimulated and stimulated saliva in diabetes. A possible explanation for the raised salivary VEGF concentrations in diabetes could be from VEGF derived from the periodontal tissues, especially in periodontitis, which has entered the saliva via the GCF. Although immunohistochemistry studies have reported increased tissue expression of VEGF in diabetics (Unlu et al., 2003, Guneri et al., 2004, Sakallioglu et al., 2007, Aspriello et al., 2009, Lucarini et al., 2009, Ramya and Kumar, 2014), in comparison with healthy controls, this did not correspond to increased VEGF levels in GCF (Guneri et al., 2004, Sakallioglu et al., 2007). Likewise, several studies have found no significant difference in VEGF GCF

concentrations in subjects with or without diabetes either in periodontal health and periodontitis (Guneri et al., 2004, Sakallioglu et al., 2007, Pannicker and Mehta, 2016). This may be explained by a quantitative real time PCR study which reported no significant difference in VEGF mRNA levels in gingival biopsies from subjects with periodontal health, gingivitis or periodontitis either with or without Type 2 diabetes (Keles et al., 2010). Although contrary to this, Mohamed et al. (2015) reported elevated GCF VEGF levels in both periodontally healthy and periodontitis patients with diabetes compared with healthy subjects with periodontitis.

Currently, there have been no publications which have examined VEGF concentrations in both saliva and serum in individuals with diabetes, although Ribeiro et al. (2018) speculated that VEGF would be raised in both in diabetes. This assumption was based on studies which have reported raised serum VEGF levels in Type 2 diabetes in comparison with healthy controls (Ozturk et al., 2009, Mahdy and Nada, 2011, Shao et al., 2016, Nalini et al., 2017), although some studies have reported no significant differences in serum levels (Blann et al., 2002, Gui et al., 2013). Likewise, serum VEGF concentrations have been reported to be higher in subjects with evidence of long-term poor glycaemic control who have diabetes related microvascular diseases, such as diabetic retinopathy (Ozturk et al., 2009, Mahdy and Nada, 2011, Nalini et al., 2017) or atherosclerosis (Blann et al., 2002), compared with diabetic subjects without complications. Some studies followed patients longitudinally and reported reduced serum (Mahdy and Nada, 2011) and plasma (Kakizawa et al., 2004) VEGF concentrations following improvement in glycaemic control. Although salivary levels of VEGF were found to be increased in subjects with diabetes in the present study, no correlation was with serum levels of VEGF. This may be due to the diabetes group in the present study having relatively good levels of glycaemic control (mean glycated haemoglobin Group 3a=6.8%$\pm$0.92(SD); Group 3b=7.69%$\pm$1.12(SD)), which would corresponds to studies which reported no significant difference in serum VEGF concentrations between diabetic subjects with good glycaemic control compared with healthy controls (Ruszkowska-Ciastek et al., 2014).

A further possible factor in the present study was the mean age of the diabetes study group (62±11 years) which was significantly higher ($p<0.05$) than the control (46±16 years) and smoking groups (42±14 years). The increased salivary VEGF concentrations found in the diabetes group could be result of age-related increase in VEGF levels found in the present study, as described above. Furthermore, Mata et al. (2004) found that unstimulated and stimulated salivary flow was significantly reduced in both well controlled Type 1 and Type 2 diabetes compared with age-matched controls. They also reported subjects with diabetes had significantly higher salivary protein concentrations compared with the healthy control groups. This implies that taking into account of salivary flow rate, in addition to VEGF concentration would be of interest. Therefore, further studies should take into account salivary flow rates and ensure all study groups are sufficiently age-matched to take into account of any potential age-related differences in VEGF salivary levels.

An interesting finding in the present study was the significant increase in VEGF concentration with age in both unstimulated and stimulated whole saliva. This finding corresponds to a small study which reported a significant correlation between VEGF concentration in unstimulated whole saliva with age in 14 healthy control and 7 cases of oral squamous cell carcinoma (Upile et al., 2009). Furthermore, this study found age was also significantly correlated with serum VEGF concentration and unstimulated saliva VEGF concentration was significantly correlated with serum levels. Neither of these findings were found in the present study. A possible explanation for correlation between salivary levels of VEGF and age, found in the present study, could be related to the reduced whole saliva flow rates in older subjects, resulting in the higher VEGF concentrations. However, this hypothesis assumes that there is a constant movement of VEGF from the salivary tissue into saliva, otherwise there would be a dilution effect during stimulation. Further work could investigate salivary VEGF concentration per unit flow.

There are however, inconsistencies in the literature regarding age-related changes to salivary flow in healthy subjects with some studies reporting either unaffected (Ben-Aryeh et al., 1986) or reduced unstimulated salivary flow (Heft and Baum, 1984, Navazesh et al., 1992, Percival et al., 1994), while stimulated parotid salivary flow is either unaffected (Heft and

Baum, 1984, Percival et al., 1994, Vissink et al., 1996) or increased with age (Navazesh et al., 1992). Wu et al. (1995) reported no significance difference in continuous parotid gland salivary flow with age, while submandibular flow rates reduced with age. The findings of the latter study are consistent with a meta-analysis of the literature which reported that the flow rate of both unstimulated and stimulated whole saliva reduced with age, while, parotid and minor salivary gland flow rates were not significantly affected (Affoo et al., 2015). However, older patients are more likely to be subject to the effects of polypharmacy with several common medications having negative effects on salivary flow rates. Evidence from histological studies showed acinar volume decreased by 20 to 40% in the submandibular, parotid and minor salivary glands with increasing age (Sreebny, 2000), suggesting that the parotid gland in particular has "secretory reserve" which maintains parotid saliva flow with increasing age (Scott et al., 1987, Affoo et al., 2015). Furthermore, evidence from a small immunohistochemistry study which examined VEGF expression in labial minor salivary glands in two age groups, under 20 and over 40 years old (n=15 in each group), reported no significant difference in VEGF expression with age (de Oliveira et al., 2002). Although this was a small study, albeit only in minor salivary glands, it does suggest salivary VEGF production probably does not increase with age and the results of the present study is due to reduced whole unstimulated and stimulated saliva flow leading to increased salivary VEGF concentration with age. Further immunohistochemistry and *in-situ* hybridisation studies would be required to ascertain whether VEGF production in the major salivary glands is significantly affected by increasing age.

No correlation was found in the present study regarding serum VEGF concentration and age, which corresponds with the findings of Meng et al. (2009) in healthy adults, although Okamoto et al. (2008) reported significantly higher serum VEGF in children compared with adults. With regards to the present study, which only involved adults, current evidence suggests serum VEGF concentrations do not significant change with age, although ideally measures should be taken to have age-matched control groups.

Another interesting finding in the present study was the raised VEGF unstimulated saliva concentration found in severe periodontitis compared with periodontal health, although this

was a marginally significant finding (p=0.052) once outliers were accounted for. This finding is consistent with three previous studies (Booth et al., 1998, Wu et al., 2018, Yilmaz Sastim et al., 2020), although Wu et al. (2018) reported a non-significant increase in the periodontitis group. However, out of these studies only Yilmaz Sastim et al. (2020) took into account diurnal variation during sampling, which may have affected the outcome of the other two studies. Booth et al. (1998) also reported significantly higher total VEGF in GCF in periodontitis compared with healthy controls and hypothesised that raised VEGF concentrations in unstimulated saliva from patients with periodontitis may be due to overspill of VEGF from the chronically inflamed periodontal tissue. Evidence from immunohistochemistry studies carried out on gingival tissue samples has shown increased VEGF and VEGFR2 expression in periodontitis compared with periodontal health (Johnson et al., 1999a, Artese et al., 2010, Vladau et al., 2016). Significantly raised GCF VEGF levels in periodontitis compared with periodontal healthy controls has been reported in the literature (Guneri et al., 2004, Sakai et al., 2006, Sakallioglu et al., 2015, Pannicker and Mehta, 2016, Turer et al., 2017).

Contrary to the finds in the present study, Afacan et al. (2019) reported no significant difference VEGF concentrations in unstimulated saliva between the periodontitis groups compared with healthy controls. This study took into account diurnal variation in the sampling protocol and there was good age and gender matching across the study groups. Interestingly, this group reported significantly raised salivary VEGF concentrations in gingivitis compared to both periodontal health and periodontitis. While Belstrom et al. (2017) reported significantly reduced VEGF concentrations in stimulated saliva following a 10 day period of cessation of oral hygiene in young adults. It is difficult to draw conclusions from these studies as the Afacan et al. (2019) study examined subjects with long-term gingivitis while Belstrom et al. (2017) examined VEGF levels in early stages of gingivitis in stimulated saliva in young adults. But these studies highlight further research is required to establish whether any changes occur in angiogenic factor levels in saliva, particularly VEGF, in the transition from periodontal health through gingivitis to periodontitis.

Currently there is great interest regarding the systemic overspill of various factors produced in periodontitis into the systemic circulation and their potential negative effects on systemic health (Beck et al., 2019, Falcao and Bullon, 2019). There was no evidence in the present study that periodontitis results in significantly raised serum levels of VEGF. Currently, there are inconsistencies in the literature regarding serum levels of VEGF in periodontitis, compared with periodontally healthy controls, with some studies reporting increased levels in periodontitis (Pradeep et al., 2011, Turer et al., 2017), while others reported either no difference (Widen et al., 2016, Tayman et al., 2019) or reduced VEGF concentrations (Zhu et al., 2015).

In the present study, no significant differences were found in VEGF concentrations in either serum or saliva in the smokers compared with the never smokers. Furthermore, no significant correlations were found between VEGF concentrations and smoking dose (pack years) in serum or saliva. These findings are consistent with several previous studies which reported no significant differences in VEGF levels between smokers and non-smokers in serum (Daloee et al., 2017, Alomari et al., 2018) or plasma (Belgore et al., 2000, Schmidt-Lucke et al., 2005). However, other studies have reported higher systemic levels of VEGF in smokers compared with healthy controls (Kimura et al., 2007, Ugur et al., 2018). Ugur et al. (2018) reported significantly raised serum concentrations of both VEGF and the pro-inflammatory cytokine IL-6 in smoker compared with non-smokers. Furthermore, this group found significantly reduced serum levels of the anti-inflammatory cytokine IL-10 in smokers compared with smokers, suggesting that smoking may increase systemic inflammation. Possible explanations for variation in the outcomes of these studies may reflect possible differences in laboratory protocols, smoking habits, age and gender of the study groups. For example, Kimura et al. (2007) reported significantly higher serum VEGF concentrations in males compared with female smokers. No such finding was found in the present study.

With regards to effect of smoking on periodontal health, no significant differences in VEGF concentrations in either serum or salivary levels were found between periodontal health and severe periodontitis in the present study. Although, there was an indication from the profile plots smoking may reduce serum and salivary VEGF concentrations, especially in

periodontal health in smokers. However, these were non-significant results and should be interpreted with caution. In terms of the literature, Sakallioglu et al. (2015) found no significant differences in VEGF concentration in GCF between smokers and non-smokers in patients with periodontitis. While Eren et al. (2015) reported no significant difference in both VEGF concentration and total VEGF in GCF between smokers and non-smokers in periodontally healthy subjects. One study has reported raised VEGF levels in unstimulated saliva in smokers, but this was only significant when combined with alcohol consumption (Korostoff et al., 2011). Currently there have been few studies which have examined VEGF expression in periodontal tissue in smokers and non-smokers. Jalayer Naderi et al. (2017) carried out such a study using periodontal surgical tissue and reported significantly less VEGF expression in smokers compared with non-smokers. Just like the situation in diabetes, there appears to be a discrepancy in the literature between VEGF expression in the periodontal tissues, as shown by immunohistochemistry and PCR studies, and VEGF levels in GCF. The common assumption being that GCF studies directly relate to the factor expression in the underlying periodontal tissue, however, this does not appear to be the case with VEGF. A potential issue with many studies related to smoking and periodontal research is that it is unclear whether previous smoking had been accounted for in the selection criteria. In the present study, the smoking group contained only never smokers to rule out the possible effects of previous smoking.

Although there is a large body of research with regards to VEGF, including in oral disease generally, there is significant scope for further research into conditions known to negatively impact upon oral wound healing and periodontal disease, namely smoking and diabetes. Ideally further studies involving serum and saliva samples should ideally have larger group sizes, although the power calculations for the present study were sufficient, and more closely age and gender matched study groups to reduce potential confounding effects, although no gender differences were found in the present study. It should be noted that there were a relatively large number of serum samples where VEGF was not detected, which may have impacted on the statistical power of the results. Since the acquisition of the ELISA kits used in this study, the sensitivity of kits available has increased due to the improved specificity of

the monoclonal capture antibody. Therefore, the expectation would be the VEGF would now be detected in a much higher proportion of the serum samples, if not all.

One of the issues encountered in the present study was the issue of measuring salivary VEGF concentrations in conditions associated with reduced salivary flow, namely diabetes and age, whilst comparing these results with healthy controls. This is analogous to comparing factor concentrations in GCF between periodontal disease and periodontal health, the latter being associated with extremely low flow rates. As a result it is now recommended that GCF studies report factor levels in total quantities per unit time (Wassall and Preshaw, 2016), usually 30 second sampling, although many recent studies also report factor concentration as well. In the case of studying factor levels in saliva, salivary flow rates could be taken into account in further studies, so that both factor concentration and total factor levels per unit time could be reported.

Currently, there is a gap in the literature regarding whether VEGF and its receptors levels in serum, GCF and saliva change in the transition between periodontal health, gingivitis and severe periodontitis. There is also scope for further studies into the potential effects of risk factors known to be associated with reduced oral healing, such as smoking and poorly controlled diabetes, on VEGF levels in oral fluids. This could include studying the effect of obesity and pre-diabetes on salivary and GCF levels in relation to periodontal health and disease. It would have been of great interest to examine VEGF GCF levels in the present study, however, due to the limited quantity of GCF it was decided to only assess these samples for MSF (Chapter 3.4).

Another significant modifiable risk factor for both oral healing and periodontal disease, as well as oral cancer, is alcohol consumption. Enberg et al. (2001) reported even a single high dose of alcohol significantly reduced stimulated saliva flow rate, but not unstimulated saliva flow, and significantly reduced various salivary electrolyte and total protein concentration. Furthermore, Korostoff et al. (2011) reported significantly raised VEGF concentration in unstimulated saliva in subjects who consumed at least one alcoholic drink per day during the

previous two years. Currently, there are no studies which have examined the acute and chronic effects on alcohol consumption on serum, salivary and GCF VEGF levels.

If it was possible to attain human salivary gland samples, it would be of interest to study VEGF protein and gene expression, using immunohistochemistry and PCR techniques, in smokers and subjects with diabetes in comparison with healthy controls. In reality it is likely that such studies would require the use of biopsies samples from subjects with various salivary pathologies, such as tumours, which runs the risk of field cancerisation effects.

3.6 Endostatin

3.6.1 Background

3.6.1.1 Endostatin levels in serum and plasma

Serum and plasma levels of the anti-angiogenic factor endostatin have been reported in a wide variety of human pathological conditions, in comparison to healthy control subjects. Significantly raised serum and plasma endostatin concentrations have been reported in coronary heart disease (Mitsuma et al., 2007), intracranial atherosclerosis (Arenillas et al., 2005), Type II diabetes (Atta et al., 2008) and in various tumours such as soft tissue sarcoma (Feldman et al., 2001), breast cancer (Teh et al., 2004), hepatocellular (Uematsu et al., 2005) and gastric (Woo et al., 2006) carcinomas. Interestingly, a recent study reported significantly reduced serum endostatin concentrations in oral squamous cell carcinoma, particularly when associated with metastasis, in comparison with healthy controls (Mardani et al., 2018). Currently, there have been no studies which have reported serum endostatin concentrations in periodontal health and disease.

Table 3.24 shows a representative selection of publications describing endostatin concentrations in serum and plasma. These studies also examined the serum endostatin concentrations in healthy control subjects, allowing comparison with the healthy control subjects examined in this thesis. It is apparent from the literature that a large range of serum endostatin concentrations have been reported in the healthy control subjects (17-176ng ml^{-1}).

3.6.1.2 Endostatin levels in saliva and GCF

Currently, there have been no publications which have reported endostatin concentrations in human saliva or gingival crevicular fluid in either oral health or disease. Furthermore, endostatin expression has not been reported in human periodontal tissues in either periodontal health or disease.

Table 3.24 A representative selection of publications for endostatin concentrations in human serum and plasma.

SERUM	PLASMA
Feldman et al. (2001) • Healthy controls (n=34) mean 25.8ng ml^{-1} • Soft tissue sarcoma (n=25) mean 43.0ng ml^{-1} • Significantly raised endostatin levels in Soft tissue sarcoma > controls (p=0.002) • Soft tissue sarcoma cases with endostatin levels >2 SD of the control mean were associated with aggressive tumour behaviour	Teh et al. (2004) • Healthy controls (n=7) mean 34.97±3.76 (SD) ng ml^{-1} • Breast cancer (n=17); mean pre-operative values 30.62±4.54ng ml^{-1}; post-operative values 60.59±7.7ng ml^{-1}. • Significantly raised endostatin levels in post-operative breast cancer both compared with healthy controls and pre-operative breast cancer (p=0.015)
Miyashita et al. (2003) • Healthy controls (n=15) mean 17.1±1.5 (SD) ng ml^{-1} • Liver tumours (n=12) mean 23.9±4.9ng ml^{-1} • Metastatic liver tumours (n=25) mean 18.8±1.5ng ml^{-1} • No significant differences between the groups	Gu et al. (2004) • Healthy male controls (n=7) mean 20.3±3.2 (SD) pg ml^{-1} • Plasma endostatin levels increased significantly 30 minutes after exercise to a mean 29.3±4.2pg ml^{-1}
Uematsu et al. (2005) • Healthy controls (n=13) mean 22.2±10.1(SD) ng ml^{-1} • Hepatocellular carcinoma (n=24) mean 31.5±15.8ng ml^{-1} • Significantly raised endostatin levels in Hepatocellular carcinoma > controls	Atta et al. (2008) • Healthy controls (n=12) mean 135±4 (SE) ng ml^{-1} • Type II diabetics (n=15) mean 189±2ng ml^{-1} • Significantly raised endostatin in Type II diabetics > controls (p<0.01)
Woo et al. (2006) • Healthy controls (n=23) mean 52.2±6.2 (SD) ng ml^{-1} • Metastatic gastric carcinoma (n=107) mean 70.1±16.6ng ml^{-1} • Significantly raised endostatin levels in metastatic gastric carcinoma > controls (p<0.001) • High endostatin levels were significantly associated with metastasis and poor prognosis (p<0.001)	Unpublished data from manufacturer • Healthy controls (n=7) mean 120±26 (SD) ng ml^{-1} (range 69-172 ng ml^{-1})
Mitsuma et al. (2007) • Healthy controls (n=15) median 49.6ng ml^{-1} (interquartile range 29.1 to 84.5) • Coronary heart disease (n=57) median 79.7ng ml^{-1} (interquartile range 46.2 to 130.3) • Endostatin levels significantly raised in coronary heart disease > control	X
Mardani et al. (2018) • Mean serum in oral squamous cell carcinoma (OSCC) (n=45) 68.8±85ng ml^{-1}. • Healthy controls (n=45) 175.6±73ng ml^{-1}. • Endostatin significantly lower in OSCC < healthy controls (p=0.001).	X
Unpublished data from manufacturer • Healthy controls (n=60) mean 122±30 (SD) ng ml^{-1} (range 58-232ng ml^{-1})	X

3.6.2 Results

3.6.2.1 Endostatin concentration in serum

Endostatin was detected in the majority of serum samples and the results are summarised in Table 3.25 and are shown in Figure 3.29. Endostatin was quantified in 97 serum samples with mean levels for the study sub-groups ranging from 32730 to 57256pg ml^{-1}. Highly significant differences were found between the study groups (Two-way ANOVA test $F(2,91) = 10.918$, $p<0.001$; Table 3.26). No significant difference was found between the periodontally healthy and severe periodontitis sub-groups, and there was no significant interaction found between periodontal health status and the study groups. A non-significant reduction was found in the smoking group (Group 2) in both periodontal health and severe periodontitis compared with the healthy control (Group 1) and the diabetes groups (Group 3) (Figure 3.30). Bonferroni post hoc tests (Table 3.27) revealed significantly lower serum endostatin concentrations in smokers (Group 2) compared with systemically healthy non-smokers (Group 1; 2<1 $p<0.001$) and subjects with diabetes (Group 3; 2<3 $p=0.001$). These findings strongly suggested that serum endostatin concentration was significantly reduced in smokers. Diagnostic statistical analysis revealed that there were no outliers in the data which had a significant influence on the overall statistical outcome (Studentized residual highest=1.90, lowest= -1.71; highest Cook's distance=0.05). Therefore, no further analysis regarding potential outliers was required.

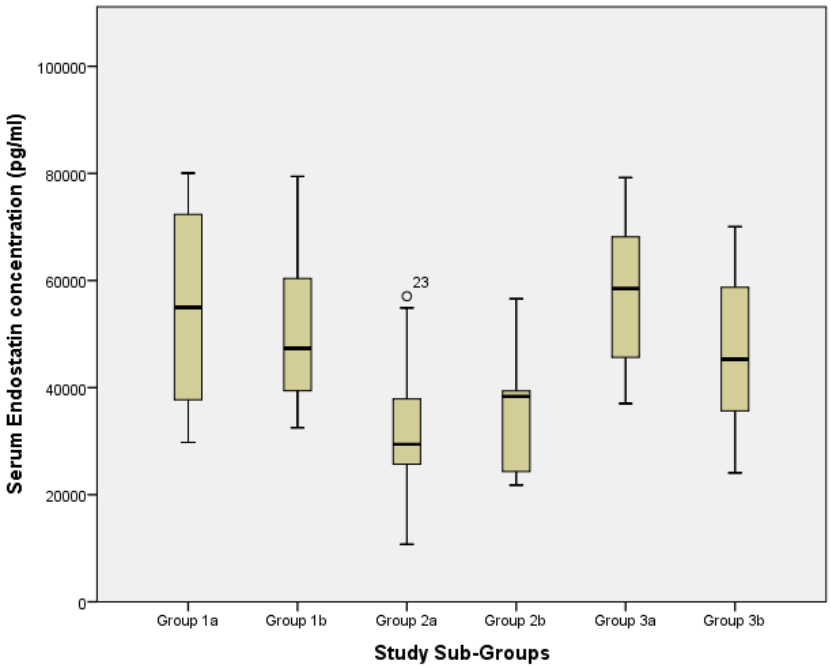
Table 3.25 Descriptive statistics for endostatin serum concentrations (pg ml^{-1}) for the study groups and sub-groups.

Group	Number Valid Cases*	Mean ± SD (pg ml^{-1})	Median (pg ml^{-1})	Interquartile Range	Range
1a	38	55752±17062	54990	35303	50257
1b	12	51362±15212	47340	22351	46940
Study Group 1	50	54698±16594	51615	33427	50257
2a	11	32730±13727	29414	14360	46316
2b	9	35969±12664	38356	21935	34826
Study Group 2	20	34188±13017	30079	15177	46316
3a	12	57256±14187	58500	28824	42210
3b	15	46422±14514	45316	29531	45998
Study Group 3	27	51237±15122	53135	25480	55164

*Excluding either no sample available or factor not detected.

Figure 3.29 Serum endostatin concentrations (pg ml^{-1}) for (a) study sub-groups and (b) study groups. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

(a)



(b)

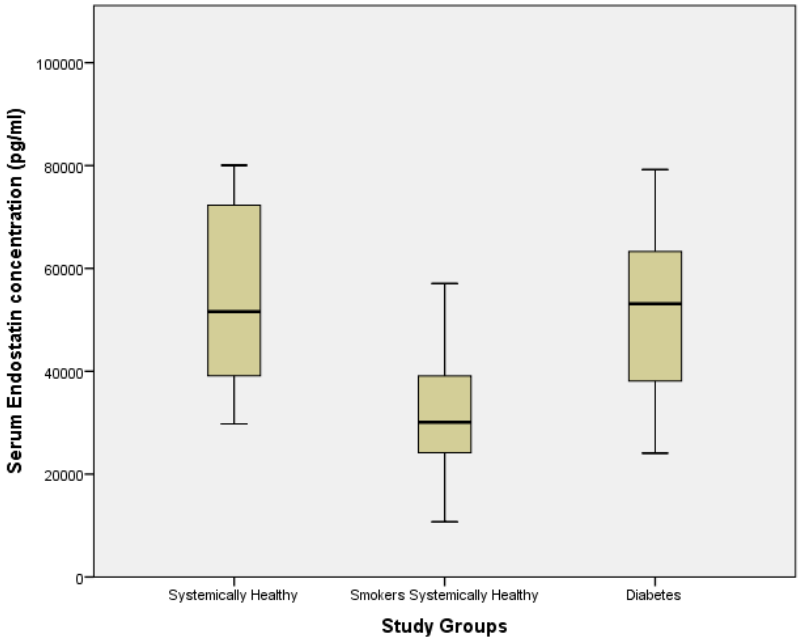


Table 3.26 Statistical comparison of serum endostatin concentrations of the study groups and periodontal health-severe periodontitis using Two-way ANOVA test.

Group	Degrees of Freedom (df)	F	Significance (p-value)
Periodontal Health – Severe Periodontitis	1	1.308	0.256
Study Groups (1, 2 and 3)	2	10.918	0.000***
Periodontal Health – Severe Periodontitis Versus Study Groups (interaction)	2	1.185	0.311

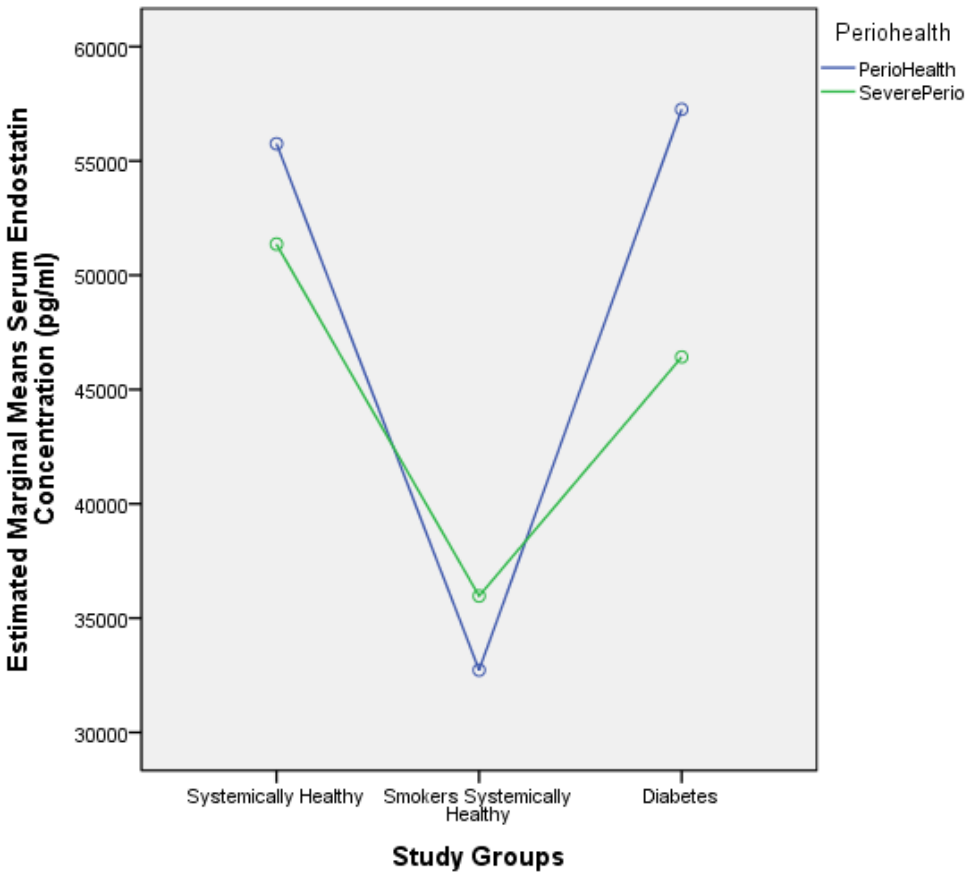
* - Significant difference <0.05
** - Significant difference <0.01
*** - Highly significant difference <0.001

Table 3.27 Statistical comparison between study sub-groups for serum endostatin (Bonferroni test p-values).

Study Groups		Mean Difference	Significance (p-value)	95% Confidence Interval	
				Lower Bound	Upper Bound
Group 1	Group 2	20510.75	0.000***	10557.89	30463.61
	Group 3	3460.86	1.000	-5523.31	12445.02
Group 2	Group 1	-20510.75	0.000***	-30463.61	-10557.89
	Group 3	-17049.89	0.001***	-28148.06	-5951.73
Group 3	Group 1	-3460.86	1.000	-12445.02	5523.31
	Group 2	17049.89	0.001***	5951.73	28148.06

* - Significant difference <0.05
** - Significant difference <0.01
*** - Highly significant difference <0.001

Figure 3.30 Profile plot of periodontal health and severe periodontitis against estimated marginal mean serum endostatin concentrations (pg ml^{-1}) for the three study groups.



No significant correlations were found between the serum endostatin concentration and the smoking dose (Spearman rho 0.230, p=0.330), glycaemic control (Spearman rho -0.230, p=0.249) or age (Spearman rho 0.163, p=0.112). Exploratory analysis indicated a significant difference with regards to gender and serum endostatin concentration across the whole sample (Mann Whitney male>female p=0.014). When re-analysed by sub-groups no significance differences were found with regards to gender once Bonferroni corrections were applied for running multiple statistical tests.

3.6.2.2 Relationship between serum pro-angiogenic factors and endostatin

The relationship between the pro-angiogenic factors Ang-1, MSF and VEGF, examined in the previous chapters, with endostatin was investigated using Spearman's correlation coefficient and the ratio between the pro-angiogenic factors and endostatin. A significant weak positive correlation was found between the serum concentrations of VEGF and

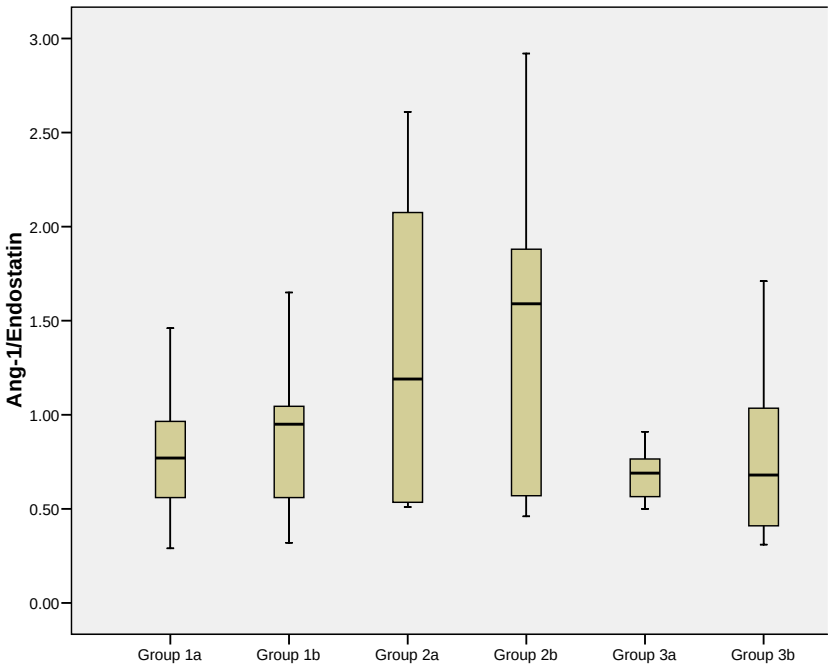
endostatin (Spearman rho 0.298, p=0.004). Otherwise no significant correlations were found between the serum levels of the other pro-angiogenic factors and endostatin (Ang-1 r=0.017, p=0.871; MSF r=0.075, p=0.498).

No significant differences were found between the study groups for either the serum MSF-endostatin ratios (Two-way ANOVA test F(2,77)=0.511, p=0.602) or the serum VEGF-endostatin ratios (Two-way ANOVA test F(2,59)=0.648, p=0.648). No significant differences were found between periodontally healthy and severe periodontitis sub-groups (MSF p=0.302; VEGF p=0.961), and there was no significant interactions found between periodontal health status and the study groups (MSF p=0.669; VEGF p=0.973).

A highly significant difference was found in the serum Ang-1-endostatin ratios between the study groups (Two-way ANOVA test F(2,85)=6.610, p=0.002; Figure 3.31 and Table 3.28). No significant difference was found between periodontally healthy and severe periodontitis sub-groups, and there was no significant interaction found between periodontal health status and the study groups. Bonferroni post hoc tests (Table 3.29) revealed significantly higher serum Ang-1-endostatin ratios in smokers (Group 2) compared with systemically healthy non-smokers (Group 1; 2>1 p=0.014) and subjects with diabetes (Group 3; 2>3 p=0.002). These findings strongly suggested that serum Ang-1-endostatin ratios were significantly increased in smokers. Diagnostic statistical analysis revealed that there were outliers in the data which potentially had a significant influence on the overall statistical outcome (Studentized residual highest=4.12, lowest= -1.77; highest Cook's distance=0.17; samples KJD01, KJD011 and KJD13 were deemed to be outliers). Bootstrapping analysis confirmed that the outcome of the Two-way ANOVA was unlikely to have been significantly influenced by the outliers. Sensitivity analysis, where data from the three outlying samples were removed, confirmed highly significant differences in the serum Ang-1-endostatin ratios between the study groups (Two-way ANOVA test F(2,82)=4.510, p=0.014).

Figure 3.31 Serum Ang-1-endostatin ratios for (a) the different study sub-groups and (b) the clinical study group. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

(a)



(b)

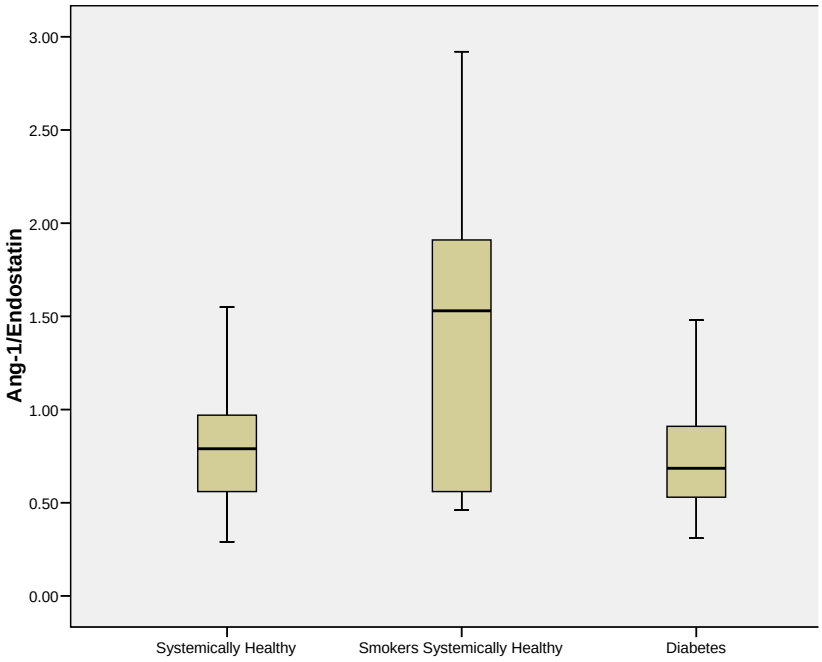


Table 3.28 Statistical comparison of Ang-1-endostatin ratios between the study groups and periodontal health-severe periodontitis using Two-way ANOVA test. Data in brackets are the results following the removal of outliers KJD001, KJD011 and KJD013 from the data set.

Group	Degrees of Freedom (df)	F	Significance (p-value)
Periodontal Health – Severe Periodontitis	1	0.005 (0.059)	0.942 (0.808)
Study Groups (1, 2 and 3)	2	6.610 (4.510)	0.002** (0.014*)
Periodontal Health – Severe Periodontitis Versus Study Groups (interaction)	2	0.021 (0.013)	0.979 (0.987)

* - Significant difference <0.05
** - Significant difference <0.01

Table 3.29 Statistical comparison between study sub-groups for serum Ang-1-endostatin ratios (Bonferroni test p-values). Data in brackets are the results following the removal of outliers KJD001, KJD011 and KJD013 from the data set.

Study Groups		Mean Difference	Significance (p-value)	95% Confidence Interval	
				Lower Bound	Upper Bound
Group 1	Group 2	-0.4625 (-0.3291)	0.014* (0.054)	-0.8515 (-0.6620)	-0.735 (0.0038)
	Group 3	0.1607 (0.1109)	0.721 (0.963)	-0.1712 (-0.1606)	0.4926 (0.3823)
Group 2	Group 1	0.4625 (0.3291)	0.014* (0.054)	0.0735 (-0.0038)	0.8515 (0.6620)
	Group 3	0.6232 (0.440)	0.002** (0.011*)	0.1977 (0.0794)	1.0487 (0.8005)
Group 3	Group 1	-0.1607 (-0.1109)	0.721 (0.963)	-0.4926 (-0.3823)	0.1712 (0.1606)
	Group 2	-0.6232 (-0.440)	0.002** (0.011*)	-1.0487 (-0.8005)	-0.1977 (-0.0794)

* - Significant difference <0.05
** - Significant difference <0.01

3.6.2.3 Endostatin concentration in saliva

Endostatin was detected in only 11 out of 86 stimulated saliva samples (1a=2/28, 1b=4/12, 3a=3/13 and 3b=2/15) and was not detected in any of the smoking sub-groups (2a and 2b). There were insufficient numbers of positive samples to allow statistical comparison of the endostatin concentrations between each study sub-group.

In order to ascertain whether unstimulated saliva contained significant levels of endostatin 12 samples were assayed using the same protocol as for the stimulated saliva. Endostatin was not detected in any of the unstimulated saliva samples (results not shown), and due to the relatively small volumes of unstimulated saliva collected, it was decided not to assay any further unstimulated saliva samples.

3.6.2.4 Effect of storage on salivary endostatin concentration

Degradation of endostatin during long-term storage at -80^{o}C may account for the lack of detection in the saliva samples. In order to investigate this, three sets of unstimulated and stimulated saliva samples from one individual (KJD001) were assayed: the first had been stored at -80^{o}C for approximately two years, the second was stored for four days at -80^{o}C and the third were fresh saliva samples (never frozen although stored for two hours at 4^{o}C). All samples were diluted 1:10 in reagent diluent and assayed using the standard protocol. Endostatin was not detected in either the fresh, recently frozen or long-term stored saliva (unstimulated and stimulated) samples, suggesting that either endostatin was not present in the samples or was at levels below the limit of detection of the ELISA. This experiment suggested that degradation of endostatin during storage was not the primary reason for the lack of detection of endostatin in the study samples, although ideally, further work should be carried out to investigate this issue.

3.6.3 Discussion

Angiogenesis is a complex process controlled by the balance between pro- and anti-angiogenic factors, which is further influenced by various components of the ECM. Although there have been many publications with regards to pro-angiogenic factors, such as FGF-2 and VEGF, there have been relatively few for angiogenic inhibitors, such as endostatin. Consequently, little is known regarding the inter-relationship between angiogenic factors and inhibitors, both in terms of physiological wound healing or in pathology, especially in oral health and disease.

In this study, the concentration of endostatin has been measured in serum, unstimulated and stimulated saliva samples from three groups of subjects: (i) systemically healthy non-

smokers, (ii) systemically healthy smokers and in (iii) subjects with diabetes. Each clinical group was divided into two sub-groups: (a) periodontally healthy and (b) subjects with severe periodontitis. High levels of endostatin were observed in all serum samples, however, there appeared to be a significant reduction in serum endostatin concentrations in subjects who were long-term smokers. As far as can be ascertained this is the first study to report this finding. There were no statistical differences found with regards to gender across the sub-groups and there were no significant correlations between smoking (pack years), glycaemic control (HbA1c) or age with serum endostatin concentration. Endostatin was undetectable in the majority of the stimulated saliva samples and was not detected in any of the limited number of unstimulated saliva samples assayed. No significant differences were found in the endostatin concentrations in either serum or saliva between the periodontally healthy or severe periodontitis sub-groups.

Confidence in the methodology was demonstrated by the good assay standard curves, low assay background noise, low intra- and inter-assay variation. Furthermore, the mean serum endostatin concentrations and standard deviations were all within the range previously reported in the literature for the systemically healthy control subjects (Table 3.24). Endostatin was detected in a minority of the stimulated saliva samples, however, the limit of detection of the assay may not have been sufficient to detect it in all of the samples, if present.

There have been few studies which have examined serum endostatin concentrations in smokers compared with healthy controls. Iribarren et al. (2006) reported serum endostatin concentrations were not significantly different in smokers, compared with non-smoker, or affected by a range of risk factors for myocardial infarction, such as age, body mass index (BMI), alcohol consumption or glycaemic control. Likewise, Liu et al. (2015) found no significant difference in serum endostatin levels between smokers and non-smokers in healthy subjects and those with non-small cell lung cancer. In the present study, smoking was found to result in significant reduction of serum endostatin concentrations, appearing to affect both periodontally healthy and subjects with severe periodontitis equally. However, with the exception of Ang-1, there was no change found in the relative levels of endostatin compared to MSF or VEGF across the study groups. A possible explanation for these

findings could be that the subjects in this study were generally not undergoing any significant systemic angiogenic events at the time of sampling, including the possibility that any angiogenic changes associated with severe periodontitis may have reached a chronic homeostatic stage. Therefore, the relative levels of systemic endostatin to VEGF and MSF could have reached low maintenance levels, effectively turning off the angiogenic switch. Interestingly, significantly raised serum Ang-1 to endostatin ratios were found in the smoking group. Nicotine in smoke has been shown to promote angiogenesis in both *in vitro* & *in vivo* models (Heeschen et al., 2001, Jacobi et al., 2002, Martin et al., 2009), which may account for this finding. However, due to the multiple toxic constituents within smoke (Chapter 1.7.1), it is thought that overall smoking results in anti-angiogenic conditions (Buduneli and Scott, 2018). In terms of the periodontal tissues, evidence suggests smoking causes anti-angiogenic effects, as shown by the lack of increased gingival vascularity in smokers with periodontitis compared with non-smokers (Chapter 1.7.3.3) (Rezavandi et al., 2002). Furthermore, smoking has been shown to have other detrimental effects in wound healing, such as toxic effects on endothelial cells and on gingival/periodontal fibroblast function (Silverstein, 1992, Tipton and Dabbous, 1995, Tanur et al., 2000) (Chapter 1.7.3.4). Another possible explanation is that although Ang-1 is considered to be a pro-angiogenic factor, especially in tumour growth, it also has anti-angiogenic activities due to its role in the final stages of angiogenesis, namely vessel stabilisation and maintenance of quiescent vessels (Wong et al., 1997, Brindle et al., 2006). Therefore, raised relative systemic levels of Ang-1 could be expected in maintenance conditions where there is little angiogenic activity.

The reduced systemic levels of endostatin and increased Ang-1-endostatin ratio found in this study, appears to be counter-intuitive in terms of the reported suppressed vascular reaction in the inflamed gingival tissues of smokers. However, the overall increase in the number of small calibre and tortuous gingival vessels in smokers (Chapter 1.7.3.3) may indicate suppressed vessel development. A possible explanation for this could be due to the reduced inflammatory cell infiltration into the gingival tissues in smokers (Pauletto et al., 2000, Rezavandi et al., 2002). This would lead to lower levels of pro-inflammatory mediators and angiogenic factors being released from the immune cells, such as VEGF, locally into the gingival tissue. Evidence for reduced angiogenic activity due to reduced PMN-induced

VEGF activity has been reported in a study in mice (Hao et al., 2007). These additional angiogenic factors released locally within the gingival tissue may be required for continued vascular development.

Few studies have examined endostatin concentrations in serum or plasma in subjects with diabetes ,and those which have, mainly examined its levels in various cardiovascular diseases (Arenillas et al., 2005, Atta et al., 2008). In the present study, no significant differences were found in the serum endostatin concentrations between healthy controls and those with diabetes. However, there are conflicting reports in the literature regarding the effect of diabetes on systemic endostatin levels. Atta et al. (2008) reported that endostatin concentrations in plasma were significantly higher in diabetics compared with healthy controls, although the age profile of the control group was significantly younger than the diabetes group, which may have affected the outcome. While Sponder et al. (2014) reported significantly lower serum levels of endostatin in middle-aged subjects with diabetes compared with aged-matched healthy controls. A possible explanation for the different outcomes of these studies could be related to differences in factor levels recorded in serum and plasma. Several studies have reported factor levels can be significantly different between serum and plasma, for example, McIlhenny et al. (2002) reported significantly higher concentrations of VEGF in serum than plasma. Furthermore, a recent meta-analysis study reported a significant correlation between serum VEGF concentration and the severity of diabetic retinopathy, however, no correlation was found in the corresponding plasma samples (Zhou et al., 2019).

In the present study, there were no significant differences found between the ratios of the angiogenic factors Ang-1, MSF or VEGF to endostatin between either the systemically healthy or diabetes study groups. Several animal studies have reported reduced expression of angiogenic factors Ang-1, Tie-2 and VEGF in diabetes, with increased expression of angiogenic inhibitors, such as angiostatin and endostatin (Boodhwani et al., 2007, Sodha et al., 2008). Whilst Atta et al. (2008) found raised plasma VEGF-endostatin ratios in human subjects with diabetes, compared with health controls, although not significantly so. The implications of these findings is that the molecular changes associated with chronic

hyperglycaemia alters the angiogenic switch leading to both impaired angiogenesis and wound healing. However, the exact mechanisms for this are still to be ascertained.

An unexpected finding, in the present study, was the similar serum endostatin levels in the systemically healthy and diabetes study groups. This may have been due to the high levels of glycaemic control in the diabetic subjects recruited to this study, who mostly had HbA1c levels close to target control levels (mean glycated haemoglobin Group 3a=6.8%$\pm$0.92(SD); Group 3b=7.69%$\pm$1.12(SD)). Another possible confounding factor was the diversity in the diabetes group in terms of their systemic health and medication, both of which could influence the levels of angiogenic factors and inhibitors. Further studies could examine whether there is a correlation between serum endostatin concentrations and glycaemic control in patients with more poorly controlled diabetes. In order to achieve statistical significance, such a study is likely to require significantly more participants than the present study.

There have been few studies which have explored whether there are any gender differences in systemic levels of endostatin. Sponder et al. (2014) reported significantly higher serum endostatin levels in both middle-aged healthy females and subjects with Type 2 diabetes, compared with the equivalent male cohorts. The authors hypothesised that the raised serum endostatin levels in middle-aged women was related to hormonal influences in that age group, although no physiological explanation was given. In the present study, there initially appeared to be significantly higher serum levels of endostatin in males compared with females, mainly driven by the systemically healthy group, however, no statistical differences were found between the subgroups. If there are age-related gender differences in systemic endostatin levels, there were insufficient numbers of cases across the age ranges in the present study to allow statistical analysis. A larger age stratified study would be required to investigate this hypothesis.

At the time of writing, there have been few studies which have reported angiogenic inhibitor levels in saliva. Furthermore, there have been no studies which have compared salivary levels of angiogenic factors and inhibitors in periodontal health and disease. Saliva is

considered to be a potential reservoir of wound healing/angiogenic factors important in the maintenance of the oral mucosa and the upper gastrointestinal tract (Zelles et al., 1995). It is therefore relevant to examine the possible role of angiogenic factors and inhibitors in human saliva, in relation to oral wound healing and periodontal disease. As far as can be ascertained, this is the first study to report the presence of endostatin in saliva, albeit in a minority of stimulated saliva samples, and suggests that the high serum concentrations of endostatin does not translate into high endostatin concentrations in either stimulated or unstimulated saliva. Whether endostatin is present in saliva in all cases has yet to be established and requires further investigation using more sensitive assays, such as chemiluminescent assays or microarrays. Immunohistochemistry studies could be used to confirm whether endostatin is produced within the salivary gland tissue and by which cell types.

One possibility is that endostatin is absent or at very low levels in human saliva, which may account for lack of previous publications. Interestingly, in the present study, endostatin was not detected in any of the saliva samples from the smoking group, which mirrors the significantly reduced levels of endostatin found in the serum in that group. However, there is insufficient data to draw any definite conclusions. Evidence that may support this hypothesis is that matrix metalloprotein-9 (MMP-9), which is important in the formation of endostatin through proteolytic cleavage of collagen XVIII (Heljasvaara et al., 2005), has been shown to be significantly reduced in the saliva of heavy smokers (Raitio et al., 2005, Nagler, 2007). However, further research is required to confirm this hypothesis.

Chapter 4

Assessment of Angiogenesis in Periapical Granulomas

4.1 Background

4.1.1 Assessment of angiogenesis

Immunohistochemistry techniques on biopsy material, both frozen and paraffin-embedded sections, have been used for many years to study angiogenesis in several pathological conditions, especially in tumours. A major limitation of immunohistochemistry is that it is a snapshot of the conditions present in the lesion at the time of biopsy, while angiogenesis is a dynamic process and so the conclusions from such studies are commonly inferential in nature (Davey et al., 2008). In order to deal with this inherent difficulty, it is important, whenever possible, to compare a lesion with its normal tissue counterpart, from where the lesion originates. Since angiogenesis is known to be controlled by the balance between various stimulators and inhibitors, the presence of angiogenic factors in the lesion does not necessarily indicate that angiogenesis is actively taking place (Carlile et al., 2001, Harada et al., 2001). Therefore, several studies have employed the quantification of blood vessels (vascularity) as an index of angiogenesis (Schor et al., 1998a, Schor et al., 1998b). In this case, angiogenesis is inferred by a significant increase in vascularity in the lesion, by comparison to the normal tissue.

In addition to *in-vitro* angiogenic assay techniques (Irvin et al., 2014), various non-invasive imaging techniques are currently undergoing development in order to study angiogenesis within a lesion on a longitudinal basis, including doppler ultrasound, dark-field imaging, contrast-enhanced magnetic resonance imaging (MRI), positron emission tomography (PET) and single-photon emission computed tomography (SPECT) (Cosgrove, 2003, Dobrucki and Sinusas, 2007, Ocak et al., 2007, Hu et al., 2017). Presently, these techniques require expensive and highly specialised equipment, but are likely to be important research and diagnostic tools in the future.

4.1.2 Endothelial cell markers

In order to assess vascularity of a tissue, specific endothelial cell markers (antibodies) are used to stain vessels that are then quantified using a variety of techniques outlined below. Endothelial cell markers are broadly divided into two groups: (i) pan-endothelial cell markers

which generally stain all vessels, both in terms of size and developmental stage, and (ii) markers specific for activated or proliferating endothelial cells (angiogenic vessels).

4.1.2.1 Pan-endothelial markers

A wide variety of pan-endothelial markers have been investigated, the most commonly used being CD31, CD34 and von Willebrand Factor (vWF) (Goncharov et al., 2017). These factors have been shown to stain both vascular and lymphatic endothelial cells in normal tissues (Miettinen et al., 1994) and neovessels in a wide range of tumours including breast carcinomas (Schor et al., 1998b, Teo et al., 2003), oral squamous cell carcinomas (OSCC) (Pazouki et al., 1997, Ascani et al., 2005) and lung tumours (Baillie et al., 2001a, Mineo et al., 2004). The use of these markers, combined with various methods of vascular quantification, has given conflicting results regarding their diagnostic and prognostic use in various tumours (Chapter 4.1.3). Many studies have used more than one pan-endothelial marker and the staining profiles of the markers often differ. For example, Mineo et al. (2004) found that CD34 expression had a significantly higher predictive value for poor long-term survival than CD31 in IB-IIA non-small-cell lung cancer. This study also found that the putative specific angiogenic vessel marker CD105 (endoglin) (Chapter 4.1.2.3) was a good predictor of poor outcome in these tumours, but not as good as CD34. Specificity problems have been reported with CD31 staining inflammatory cells, CD34 staining fibroblasts and vWF staining stromal cells (Miettinen et al., 1994, Vermeulen et al., 1996, Pazouki et al., 1997). Furthermore, it has been reported that such markers react strongly with endothelial cells in large blood vessels but their expression is weak or absent in microvessels in most normal tissue and in many tumours (Stashenko et al., 1994). Evidence also suggests that the expression of vascular markers may be affected by local conditions, for example FGF-2 and VEGF were found to up-regulate vWF mRNA expression in cultured endothelial cells (Zanetta et al., 2000), thus, suggesting that tumours that over express these factors may affect the intensity of the resultant vWF staining.

4.1.2.2 Angiogenic vessel markers

Various specific markers for activated or proliferating endothelial cells (angiogenic vessels) have been studied including endosialin (CD248) (Teicher, 2007), $\alpha_v\beta_3$ integrin (Brooks et al.,

1994) and CD105 (Burrows et al., 1995, Marioni et al., 2010). Specific markers of angiogenic vessels would provide a more accurate measure of angiogenesis than provided by pan-endothelial markers. Several studies have suggested that $\alpha_v\beta_3$ integrin preferentially binds to proliferating small vessels, and has potential prognostic value in breast (Gasparini et al., 1998) and colorectal carcinomas (Sato et al., 2001) where this marker was found to be highly expressed. However, $\alpha_v\beta_3$ integrin was found to be of limited diagnostic value in oral dysplasia and OSCC (Pazouki et al., 1997).

4.1.2.3 CD105 (Endoglin)

CD105 is a membrane protein involved in the TGF-β receptor signalling pathway (Barbara et al., 1999) and has been reported to be selectively expressed by proliferating endothelial cells (angiogenic vessels) in tumours (Burrows et al., 1995, Duff et al., 2003, Nassiri et al., 2011). However, like many current vascular markers CD105 does not appear to be truly specific for angiogenic vessels in all tissues. Minhajat et al. (2006) used double-immunofluorescence techniques and tissue microarrays to study a number of endothelial markers, including CD105, in a variety of human tumours and their adjacent normal peri-tumour tissue. Intense vascular CD105 expression was found in newly formed vessels in tumours of the brain, lung, breast, stomach and colon, whereas little CD105 expression was found in the adjacent normal tissues suggesting that angiogenesis had occurred in the development of these tumours. However, no difference was found in the CD105 expression between the tumours and the adjacent normal tissues in liver and renal cell carcinoma. This discrepancy in CD105 expression probably reflects the difference in vascular phenotypes in different types of tumour. CD105 has also been reported to stain normal tissue vessels and stromal cells in a variety of normal human tissues (Balza et al., 2001), although this may due to the specificity of the particular CD105 antibody used in this study.

Several studies have found high CD105 expression in tumours to be associated with poor prognosis, such as in non-small-cell-lung cancer (Tanaka et al., 2001) and in various types of squamous cell carcinomas including the breast (Kumar et al., 1999), stomach (Ding et al., 2006), and head and neck (Chien et al., 2006, Kyzas et al., 2006, Bellone et al., 2007). In these studies, CD105 was found to be more informative than pan-endothelial markers. In

OSCC CD105 expression has been shown to be significantly higher than in normal oral mucosa (Schimming and Marme, 2002, Schimming et al., 2004, Margaritescu et al., 2010, Nair et al., 2016, Silva et al., 2018) with tumours expressing high levels of CD105 being associated with poorer prognosis (Kyzas et al., 2006, Marioni et al., 2006, Marioni et al., 2010). Likewise, CD105 expression has been reported to be significantly higher in malignant salivary gland tumours compared with both benign salivary tumours and normal salivary tissue (Tadbir et al., 2012). Currently, there have been few studies which have examined CD105 expression in periapical lesions (Tasman et al., 2000, Davey et al., 2008, Lima et al., 2011, Estrela et al., 2019), with Davey et al. (2008), the publication relating to this chapter, being the first to quantify angiogenic activity using CD105 expression in periapical granulomas (PG).

4.1.3 Quantification of vascularity

Various methods have been used to quantify vascularity, the most common being the highest-microvascular density (h-MVD) in which only the most vascularised area of the section (hot spot) is assessed (Weidner et al., 1991). The number of vessels counted within the known area of a microscope eyepiece graticule is recorded, usually at x200 magnification, and the highest value is expressed as vessels per mm^2 (Chapter 2.3.8). An association between high h-MVD and poor tumour prognosis has been found in some studies (Weidner et al., 1991, Penfold et al., 1996, Pazouki et al., 1997), but not in others (Van Hoef et al., 1993, Zatterstrom et al., 1995, Chandrachud et al., 1997).

Alternative methods involve estimating average-microvascular density (MVD) or microvascular volume (MVV) in randomly selected areas of the sections (Chandrachud et al., 1997, Pazouki et al., 1997) (Chapter 2.3.8). Microvascular volume is a stereological point counting technique which uses a 100 point microscope eyepiece graticule. Stained vessel walls which coincide with the points are counted and the results are statistically proportional to the volume of the vessels (percentage volume).

Previous findings have demonstrated that different results may be obtained depending on the quantification method used (Pazouki et al., 1997, Schor et al., 1998b, Li et al., 2005). For

example, MVV increased significantly, in a step-wise fashion, with disease progression in oral lesions, from normal oral mucosa to dysplastic lesions and carcinomas, whereas h-MVD did not discriminate between dysplasia and carcinomas (Pazouki et al., 1997). Li et al. (2005) found that h-MVD in CD31 stained sections could not differentiate between normal oral mucosa and dysplastic lesions, but did significantly differentiate between normal oral mucosa and oral carcinoma. Therefore, in this thesis three different methods of vascular assessment have been carried out (MVV, a-MVD and h-MVD) in order ascertain whether any method was more discriminating in PG-PDL tissues.

It is clear that caution needs to be taken regarding the comparison between studies due to the variations in the methodology used. As well as the variation in the quantification methods, vascularity may also be affected by the different specificities of the markers used, tissue fixation method (formalin-fixed paraffin-embedded or frozen sections) (El-Gazzar et al., 2005b) and variations in the immunohistochemistry protocols, especially with regard to pre-treatments used to unmask epitopes (Schor et al., 1998b).

4.1.4 Quantification of vascularity in oral pathology

As stated above, several studies have reported significantly increased vascularity in OSCC compared with normal oral mucosa (NOM) (Pazouki et al., 1997, Carlile et al., 2001, Sheelam et al., 2018), with some studies showing no difference between NOM and oral dysplasia (Li et al., 2005). Several studies have reported this increased vascularity in OSCC to be associated with increased expression of angiogenic factors (positive correlation) such as FGF-2 (Li et al., 2005) PDGF (Li et al., 2005) and VEGF (Li et al., 2005, Shang and Li, 2005, Shang et al., 2007). Other studies, however, have found either no correlation or possibly reduced VEGF expression in OSCC (Artese et al., 2001, Carlile et al., 2001). This inconsistency may be due to the presence of the anti-angiogenic $VEGF_{xxx}b$ isoforms, generated by alternative splicing (Ladomery et al., 2007).

In addition to OSCC, several studies have directly measured vascularity in other oral lesions. Significantly raised angiogenic factor expression and various measures of vascularity, in comparison with appropriate control tissues, have been reported in oral conditions including

ameloblastomas (Kumamoto et al., 2002, Hande et al., 2011, Pereira et al., 2016), keratocysts (Gadbail et al., 2011), submucous fibrosis (Murgod et al., 2014, Tekade et al., 2017, Sharma et al., 2019), oral lichen planus (Tao et al., 2007, Mittal et al., 2012, Sheelam et al., 2018) and various salivary gland tumours (Tadbir et al., 2012). Vascularity and angiogenic factor expression has also been reported in various periapical lesions and these papers are discussed in more detail in Chapter 4.1.7 and 4.1.8.

4.1.5 Periapical granulomas (periradicular granulomas)

4.1.5.1 Aetiology

Periapical granulomas (PG) are benign growths believed to arise as a result of low grade chronic inflammation caused by the leakage of toxic products out of necrotic root canals into the periapical tissues (Yanagisawa, 1980, Nair, 2004). The aetiology involves the microbial invasion of the root canal space, leading to pulp necrosis, either as a result of dental trauma, caries, extensive dental treatment or microleakage around failing restorations (Mjor and Odont, 2001, Nair, 2004, Kirkevang et al., 2007). Pulpal exposures in gnotobiotic rats does not lead to periapical pathology, while similar pulpal exposures in normal rats leads to large periapical lesions suggesting that bacteria are an important aetiological factor (Kakehashi et al., 1966). Over time the endodontic bacterial flora becomes progressively more anaerobic in nature (Iwu et al., 1990, Wayman et al., 1992, Stashenko et al., 1994) and eventually the bacteria and/or their products leak into the periapical tissues. The resultant host response and associated periapical inflammation can result in either an acute or a chronic periapical lesion. Unfortunately, as the source of infection is the necrotic and now avascular root canals the host response is unable to totally eradicate the infection. An acute response leads to the formation of an acute apical periodontitis resulting in severe pain, especially on biting, due to hyperaemia and oedema of the periapical periodontal ligament (PDL). Histologically, these lesions are associated with progressive destruction of the periapical PDL and bone, and the ingress of large numbers of polymorphonuclear neutrophils (PMN). If untreated, suppuration occurs leading to the formation of an acute apical abscess. Alternatively, a chronic host reaction leads to the asymptomatic chronic apical periodontitis with the formation of a periapical granuloma, which may be detected as a radiolucency on periapical

radiographs (Nair, 2004). It is estimated that PG account for approximately 50% of periapical lesions (Ramachandran Nair et al., 1996).

4.1.5.2 Histopathological features of periapical granulomas

PG are anatomically circumscribed lesions which typically contain a heterogeneous collection of vessels, fibroblastoid cells, chronic inflammatory cells (macrophages, lymphocytes and plasma cells) and, occasionally, epithelial cells derived from the epithelial cells rests of Malassez (Nair, 2004). At their periphery, PG are surrounded by a dense fibrous capsule which is firmly attached to the root surface (Stern et al., 1982, Piattelli et al., 1991, Nair, 2004) and is continuous with the healthy PDL (Newman and Challacombe, 1995). Approximately 45% of PG have evidence of epithelial proliferation leading to the formation of palisades and arcades of epithelial cells which enclose areas of vascular granulation tissue (Ramachandran Nair et al., 1996). These epithelial cells are derived from the embryological remnants of Hertwig's Epithelial Root Sheath that lie dormant in the PDL as the Epithelial Cell Rests of Malassez (Lin et al., 2007, Padma Priya et al., 2015). Epithelial cell proliferation is thought to result from the up-regulation of various growth factors and receptors, such as FGF-2 (Moldauer et al., 2006), Keratinocyte Growth Factor (KGF) (Gao et al., 1996b) and Epidermal Growth Factor Receptor (EGFR) (Lin et al., 1996) due to the persistent low grade chronic inflammation.

The development of PG has been studied in rats, where surgical pulpal exposures lead to the rapid formation of periapical lesions (Stashenko et al., 1994, Yamanaka et al., 2012). In rats two clear developmental phases are apparent: initially there is an active phase associated with rapid expansion and bone destruction with high levels of prostaglandin E_2 (PGE_2) and IL-1α (over a period of 1 to 3 weeks) followed by a chronic phase associated with significantly reduced rate of expansion. Since research in human PG involves biopsy material, it is likely that only the chronic developmental stage can be studied. However, PGE_2 (McNicholas et al., 1991), IL-1β (Barkhordar et al., 1992, Lim et al., 1994) and TNFα (Ataoglu et al., 2002) are thought to be important mediators in the initiation and growth of human PG, although there is likely to be a complex web of factors involved (Walker et al., 2000).

Clinically, PG can remain seemingly dormant for significant periods where the host reaction is in equilibrium with the infective assault. PG may progress either into an acute periapical abscess or develop into cystic apical periodontitis (radicular cyst), with the formation of epithelial lined cavities. Although several hypotheses have been proposed regarding factors involved in the progression and growth of periapical cysts, it is likely that persistent inflammatory stimuli plays a significant role, causing increased local expression of inflammatory mediators and growth factors, such as IL-1, EGF, FGF-2, KGF and VEGF (Thesleff, 1987, Gao et al., 1996b, Leonardi et al., 2003, Moldauer et al., 2006, Lin et al., 2007). Approximately 15% of PG are thought to develop into cysts (Ramachandran Nair et al., 1996).

4.1.6 Vascular structure of periapical granulomas

PG have rich vascular networks, consisting mainly of arterioles and venules (Tasman et al., 2000), but few studies have quantified their vascularity. Bergenholtz et al. (1983) used morphometric analysis in conjunction with electron microscopy (EM) to measure the vascular volume of various elements of periapical lesions in root treated human teeth (Bergenholtz et al., 1983). The vascular volume was found to vary from 2.0$\pm$0.5% (SE) to 2.6$\pm$3.7%. It was not apparent from the paper whether these lesions were formally diagnosed as PG or whether the vascular element included lymphatic vessels.

4.1.7 Expression of angiogenic factors in periapical granulomas

Several studies have reported angiogenic factor expression in PG, often in comparison with other periapical lesions such as periapical cysts, including FGF-2, TNF-α, TGF-α, TGF-β_1 and VEGF, as well as Epidermal Growth Factor Receptor (EGFR) (Lin et al., 1996, Tyler et al., 1999, Danin et al., 2000, Leonardi et al., 2003, Moldauer et al., 2006, Nonaka et al., 2008, Andrade et al., 2013, Virtej et al., 2013, Fonseca-Silva et al., 2012, Vara et al., 2017, Alvares et al., 2018, Kudo et al., 2018). Many of these studies either related angiogenic factor expression to the extent of the inflammatory infiltrate, an important source of many angiogenic factors, and/or epithelial proliferation, which is commonly thought to provide evidence of maturation of PG and progression to periapical cysts (Alvares et al., 2018). For example, two studies reported that low VEGF expression in PG was associated with low

levels of inflammatory infiltrate (Leonardi et al., 2003, Nonaka et al., 2008). Similar findings were also reported by Andrade et al. (2013) for TGF-β_1 expression in PG. Leonardi et al. (2003) reported PG with little epithelial proliferation were associated with high percentage of strongly staining VEGF positive inflammatory cells, while the percentage of VEGF stained inflammatory cells decreased with increasing levels of epithelial proliferation. They postulated that this change in VEGF expression reflected the developmental changes, such as epithelial proliferation, that occur in PG as they mature into periapical cysts.

Interestingly, a recent paper by Kaneko et al. (2019) reported that inhibition of NF-κB, an important factor in several cellular pathways including VEGF, resulted in significant reduction in the size of induced periapical lesions in a rats and was associated with the inhibition of endothelial VEGFR2 mRNA and reduced VEGF expression. However, these findings contradict a dual-colour immunofluorescence and real-time PCR study by Kudo et al. (2018) which reported that Silent Information Regulator 2 Homologue 1 (SIRT1), which also suppresses the NF-κB pathway, resulted in significantly increased VEGF mRNA levels in 34 human PG compared with 10 control periodontal ligament (PDL) samples. Clearly, further research is required to ascertain the role of VEGF in the development of PG and the progression to periapical cysts in humans.

Two major difficulties arise from many of these studies. As stated previously, the expression of angiogenic factors alone may not necessarily mean that angiogenesis is actively taking place and that quantification of vascularity may provide more robust evidence of this. Another difficulty is that apart from Moldauer et al. (2006), Virtej et al. (2013) and Kudo et al. (2018), none of these studies compared the angiogenic factor expression with a suitable control tissue, such as the healthy PDL. Thus, it is difficult to ascertain whether the angiogenic factors are up-regulated in the PG compared with the normal tissue counterpart.

4.1.8 Vascularity as an index of angiogenesis in periapical granulomas

As with other oral granulomatous lesions, such as pyogenic granulomas, it is widely presumed that the PG capillary networks arise by angiogenesis (Bragado et al., 1999, Yuan et al., 2000b, Freitas et al., 2005, Seyedmajidi et al., 2015). Currently, there have been few

studies which have examined vascularity as an index of angiogenesis in human PG. Three studies examined vascularity and VEGF expression in relation to the inflammatory infiltrate in human periapical granulomas (Nonaka et al., 2008, Fonseca-Silva et al., 2012, Vara et al., 2017), although two similar studies were carried out on periapical cysts (Graziani et al., 2006, Zizzi et al., 2013a). However, since no comparable control tissue was used in any of these studies, such as healthy periapical PDL, there was no evidence of increased vascularity (angiogenesis) during the development of the periapical lesions. In essence, these are all comparative studies between different periapical lesions, or in the case of Vara et al. (2017) PG with oral pyogenic granulomas, with the assumption being that high VEGF expression provided evidence of angiogenesis had taken place.

Nonaka et al. (2008) used vWF expression as a vascular marker in conjunction with microvessel count (MVC) to assess vascularity in 20 PG, 20 periapical cysts and 10 residual radicular cysts. VEGF expression was found to be higher in PG and periapical cysts compared with residual radicular cysts, although there was no significant difference in vascularity found between the three lesions. Across the three types of lesion, there was a significant association between low inflammatory cell infiltration with low VEGF expression, while lesions with high levels of inflammatory cell infiltration were significantly associated with higher levels of vascularity. However, there was no correlation between VEGF expression and vascularity (MVC). Fonseca-Silva et al. (2012) carried out a similar study, which also examined mast cell numbers, using CD31 and h-MVD (Chapter 2.3.8 and Chapter 4.1.3) to assess vascularity in 28 PG and 40 periapical cysts. VEGF expression and vascularity were found to be similar in PG and periapical cysts, although the mean numbers of mast cells were significantly higher in periapical cysts than PG. A recent study by Vara et al. (2017), which used endothelial cell VEGF expression and MCV in 20 PG and 20 oral pyogenic granulomas, reported significantly higher vascularity and VEGF expression in oral pyogenic granulomas. However, the validity of the comparison between the two lesions is questionable as they form in totally different environments, especially when no appropriate control tissues were used (i.e. normal oral mucosa and healthy periodontal ligament). One interesting finding from this study was there was no significant correlation between the level

of inflammatory cell infiltration and VEGF expression in the PG, although this may simply reflect the low variation in the inflammatory cell infiltration the PG samples examined.

Currently, few studies have used CD105 as an angiogenic marker in human periapical lesions, although several studies have used CD105 as a mesenchymal stem cell marker in periapical lesions (Marrelli et al., 2013, Chrepa et al., 2015, Estrela et al., 2019). Tasman et al. (2000) first reported CD105 staining of PG vessels, although vascularity of the lesions was not examined. As far as can be ascertained, the present study was the first to use CD105 as an angiogenic marker in PG (Davey et al., 2008). Subsequently, Lima et al. (2011) examined the relationship between mast cell numbers and angiogenesis in human 24 PG and 24 periapical cysts. Angiogenesis was assessed using vascular CD34 and CD105 staining in conjunction with h-MVD and Microvascular Area (MVA), unfortunately, no healthy control periodontal ligament tissue was used. CD34 resulted in significantly higher vascular staining compared with CD105 in both PG and periapical cysts, from which they postulated that this provided evidence that angiogenesis had taken place, although no difference in the vascular (angiogenic) indices were found between the two lesions. As the extent of the inflammatory infiltrate in the PG is likely to affect angiogenic factor levels, as suggested by the literature discussed above, the level of inflammatory infiltrate will be examined in comparison with vascularity in the present study.

4.1.9 Periodontal ligament (PDL)

The PDL is a narrow (width 0.15-0.38mm) vascular fibrous connective tissue lying between the root cementum and the alveolar bone. Its principal function is to attach the tooth to the surrounding bone through the arrangement of characteristically orientated collagen fibres that insert into the root cementum and the alveolar bone. The PDL has important roles in tooth support, sensory perception, protective reflexes and tooth movement. The PDL is a highly active connective tissue which is constantly turning over and adapting to functional demands (Freezer and Sims, 1987). The principal cell type in the PDL is the fibroblast which produces and turns over collagen and other extracellular matrix (ECM) components (Everts et al., 1996). Similar to gingival fibroblasts, there is evidence of heterogeneity in the PDL fibroblast populations, which may have implications in PDL wound healing (Lekic et al.,

1997). PDL fibroblast populations differ from the gingival fibroblast populations by generally expressing higher levels of ECM components, such as collagen type I and fibronectin (Kuru et al., 1998). Other PDL cells include undifferentiated mesenchymal cells, synthetic cells (osteoblasts and cementoblasts), resorptive cells (osteoclasts and cementoclasts), Epithelial Cell Rests of Malassez and, in health, a small number of inflammatory cells (macrophages, lymphocytes, PMN and mast cells).

4.1.9.1 Vascular structure of the periodontal ligament

Electron microscopy studies during the 1960s (Griffin and Harris, 1967, Bevelander and Nakahara, 1968) described the fine structure of the blood vessels within the human PDL, but not their density. The majority of publications are in animal models (monkeys, rats, dogs and horses) where various labelling methods, such as perfusion of carbon or plastic spheres, or corrosive methods have been used to study the distribution of the blood vessels within the PDL (Castelli and Dempster, 1965, Folke and Stallard, 1967, Selliseth and Selvig, 1994, Masset et al., 2006, Nobuto et al., 2003). These studies showed significant species variation and caution needs to be applied in the interpretation of these studies in relation to the human PDL. These studies have demonstrated that there are three main sources of vascular (arterial and venous) supply to the PDL: (i) the apical vessels, (ii) vessels that enter the PDL through perforations in the tooth socket wall (Cribriform plate) and (iii) from the gingival vessels. The primary arterial supply within the PDL runs in an occlusal apical direction close to the alveolar bone with the PDL veins, unlike those in other connective tissues, generally not being closely associated with the lymphatic supply (Freeman, 1998).

PDL capillaries differ from those found in other fibrous connective tissues in that they have a high quantity of fenestrated capillaries, which are thought to be important in meeting the high metabolic demand of the PDL cells (Berkovitz, 2004). The most numerous vessel type in the human PDL is the post-capillary venule, which contain 69% of the total PDL blood volume and 49% of the luminal surface area (Foong and Sims, 1999). Animal studies have consistently demonstrated dense anastomosis between the PDL vascular network and those of the surrounding bone and gingiva (Castelli and Dempster, 1965, Carranza et al., 1966, Nobuto et al., 1989, Selliseth and Selvig, 1994). The close association between the PDL,

gingival and alveolar vascular networks may be important in wound healing. A study in beagle dogs found that angiogenesis in the PDL microvascular network was important in healing following mucoperiosteal flap surgery (Nobuto et al., 2003).

4.1.10 Periodontal ligament vascularity

Few studies have directly quantified vascularity in human PDL. Light microscope studies have found the PDL blood vessel volume in human premolar and maxillary molar teeth range from 1.63 to 3.5% (Gotze, 1976, Gotze, 1980) to as high as 11 to 20% (Sims, 1980). Foong and Sims (1999) used transmission electron-microscope techniques to investigate the human canine PDL blood vessel volume using material from a burns patient who had undergone jaw reconstruction. Their data suggested the mean PDL luminal blood vessel volume to be 9.52$\pm$2.28% (SE), which is similar to that found in the mouse and marmoset models of between 7.5 to 11.3% (Sims et al., 1996, Freezer and Sims, 1987, Parlange and Sims, 1993). The discrepancy between the data from the two types of microscopy may reflect the technical differences between the two methods and the different ways the data was presented. For instance, vascular volume can be measured in terms of luminal volume or vascular volume (including vessel walls) leading to significant discrepancies. Also, many of these studies involved small numbers of specimens which may not reflect the true population variation. Foong and Sims (1999) for example, only examined the PDL around two teeth from the same individual.

Evidence also indicates that the vascularity of the human PDL varies depending on which part of PDL and what type of tooth is being studied. Light and electron-microscopy (EM) studies on human PDL found that the vascular volume progressively increased from the coronal third to the apical third (Gotze, 1976, Foong and Sims, 1999). Foong and Sims (1999) found the total percentage vascular volume in human canine PDL to be 18.7%, 30.9% and 50.4% in the coronal, middle and apical thirds respectively. Similar differences were also found in the transverse distribution of the vessels across the PDL with the percentage vascular volume in human PDL to be 9% (0.85% of the total PDL volume), 78% (7.44%) and 13% (1.23%) for the cemental, middle and alveolar thirds respectively. Within the middle

circumferential third, 66% of the blood volume was contained in either venous capillaries or post-capillary-sized venules.

4.1.10.1 Age-related effects on periodontal ligament vasculature

Evidence from qualitative light microscopy studies initially suggested that age-related arteriosclerotic changes occurred in the human PDL (Grant and Bernick, 1970, Grant and Bernick, 1972). It was proposed that these changes led to the reduction in PDL vessel luminal size in older subjects resulting in "relative ischaemia", although, the actual impact of this on the PDL blood supply is likely to be greatly reduced due to anastomosis of vessels within the PDL. However, a more extensive histological study examined PDL vessel dimensions in a wide age range of cadavers (3^{rd} to the 10^{th} decades) and found no evidence of significant arteriosclerotic changes in the human PDL (Severson et al., 1978).

4.1.11 Vascularity as an index of angiogenesis in the periodontal ligament

Various angiogenic factors have been detected in human PDL tissue including EGF (Chang et al., 1996), FGF-2 (Gao et al., 1996a), TGF-β (Yamaji et al., 1995), TNF-α (Rossomando et al., 1990) and VEGF (Booth et al., 1998, Virtej et al., 2013). Currently, there have been few publications which have quantified angiogenesis using vascular indices in the healthy human PDL, although there have been several publications which have examined the change in vascularity (mainly vessel surface area) during orthodontic tooth movement in animal models (Krishnan and Davidovitch, 2006, Noda et al., 2009). A far as can be ascertained, this is the first study to use the vascular markers vWF and CD105 in combination with microvascular volume or density (MVV, a-MVD or h-MVD) and MSF expression to assess angiogenesis in the human PDL.

4.2 Aims of the study

The aims of this study, as stated in Davey et al. (2008), were:

1. "To quantify vascularity in PG using different endothelial markers and assess its value as an index of angiogenesis by comparing PG with healthy PDL.
2. To use oral squamous cell carcinomas (OSCC), compared with adjacent normal oral mucosa (NOM), as a positive angiogenic control."

Additional aims of this study were to:

3. Quantify MSF expression in PG and healthy PDL in comparison with a positive angiogenic control (OSCC-NOM).
4. Investigate whether there is a correlation between MSF expression in PG-PDL with three vascular indices.

4.3 Methods

Details of the materials and methods used in this study are outlined in Chapter 2.3. Optimisation of the staining protocols and vascular indices was required and are detailed in the following sections.

4.3.1 Optimisation of the endothelial marker staining protocols

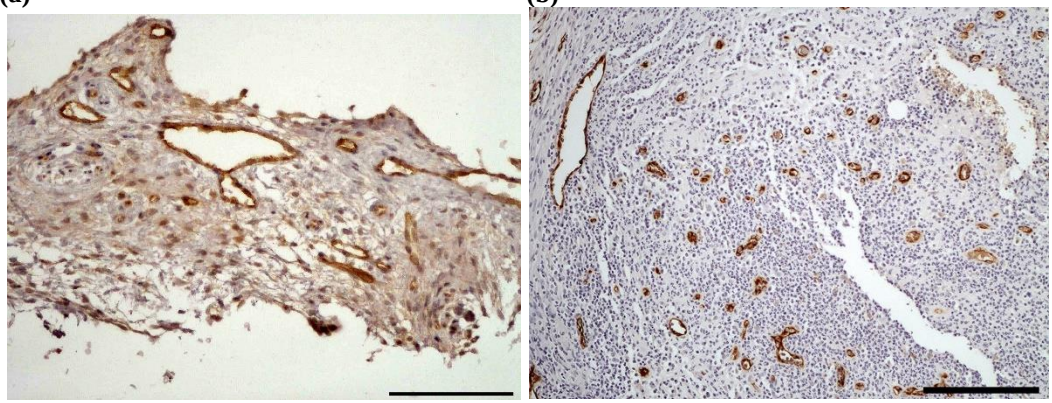
Prior to staining the study sections, the vWF and CD105 antibodies were optimised in order to achieve maximal antigen retrieval with minimal tissue damage and background staining. Optimisation was based on the primary antibodies manufacturer's recommendations, although there was evidence from other work within the laboratory that the staining profiles of these markers varied widely between different tissues. Hence the staining protocols were specifically optimised for human PG and PDL tissues. The two main variables affecting antigen retrieval were: (i) the dilution of the primary antibody and (ii) the type of pre-treatment used (if required). Chapter 2.3 details the final optimised staining protocols and the pre-treatment procedures.

4.3.1.1 von Willebrand Factor (vWF) antibody optimisation

Preliminary studies were carried out to optimise the vWF staining of the PG and PDL tissues. Various vWF antibody dilutions and pre-treatments (none, microwave in citrate buffer and two standard concentrations of Protease XXIV – 0.1 and 0.01%) were examined. An antibody dilution of 1:3000, with 0.01% Protease XXIV pre-treatment, was found to result in very good vessel staining with minimal background. This was the optimal protocol, adopted for the study sections (Chapter 2.3.5 and Figure 4.1). Although the microwave pre-treatment also resulted in good vessel staining, it was found to result in some staining of inflammatory cells in all of the sections including the negative controls, significant tissue damage and occasional tissue lifting.

Figure 4.1 vWF staining of (a) PDL and (b) PG sections (x200 magnification)*. Scale bar = 50μm.

(a) **(b)**



*Images from Davey et al. (2008)

Due to the very thin and fragile PDL tissue, all of the primary antibodies used in this study caused staining at the margins of the tissue. This staining was discounted in the vascular assessments.

4.3.1.2 Diffuse vWF staining

During the initial vascular assessment, it was noted that some PG sections contained diffuse areas of vWF staining. At high magnification it was apparent that these areas contained high levels of extra-vascular red blood cells probably the result of rupturing of immature blood vessels during the surgical removal and processing of the tissues. Diffuse perivascular vWF staining has been previously reported in conditions associated with vascular leakage, such as

cutaneous small cell vasculitis (Meijer-Jorna et al., 2002), leading to extracellular release of vWF. Vascular assessment of these diffusely stained areas was found to be more difficult due to the uncertainty of what was true vessel staining (Figure 4.2). Twenty random fields (25 point grid at x400 magnification) were assessed for each study section in order to gauge the number of sections affected and the extent of this staining. Seven PG and eight PDL sections were affected, although the number of fields containing diffuse staining was small (2 to 3 fields out of 20 per section) in the majority of cases. Only one PG and one PDL section contained significantly higher numbers of affected fields (50%). The three worst affected PG and PDL sections were then reassessed for MVV and MVD in order to study the effect of the diffuse staining on the counts. Twenty random fields were selected for each section in order to include a significant number of fields containing diffuse areas of staining. Each section was assessed using two variations of the standard protocol. In the first method (standard score), vessels were only counted if they could be absolutely identified as being stained vessels, whether they were in a diffuse area of staining or not. In the second method (diffuse score) any vessels in the non-diffuse staining areas were counted as normal; in addition, any possible vessels in the diffuse staining areas, that is any structure significantly more stained than the surrounding tissue, were also counted (Table 4.1). A comparison of "standard scores" and "diffuse scores" using the mean values showed that the latter were higher, with the difference being significant (Wilcoxon signed-rank test: MVV $p=0.03$) or marginally significant (MVD $p=0.06$). When the raw data was analysed (20 readings per specimen), the "diffuse scores" were significantly higher than the "standard scores" (Wilcoxon signed-rank test: MVV $p=0.001$ and MVD $p=0.008$). However, the diffuse and standard scores were directly and highly significantly correlated (Spearman's rank correlation: MVV $rho=0.978$, $p<0.001$; MVD rho 0.974, $p<0.001$) and did not affect the results regarding a comparison between PG and PDL tissues. For the final protocol, only absolutely definite vessels within the diffuse staining areas were counted (standard score). This reduced the dubiety in the counting and improved the intra- and inter-observer variation, although slight underestimations of the vascularity at the individual field level may occur.

Figure 4.2 Diffuse vWF staining in a PG (magnification x200). Scale bar 100µm.
⬅ Blood vessels and ◀ diffuse areas of vWF staining.

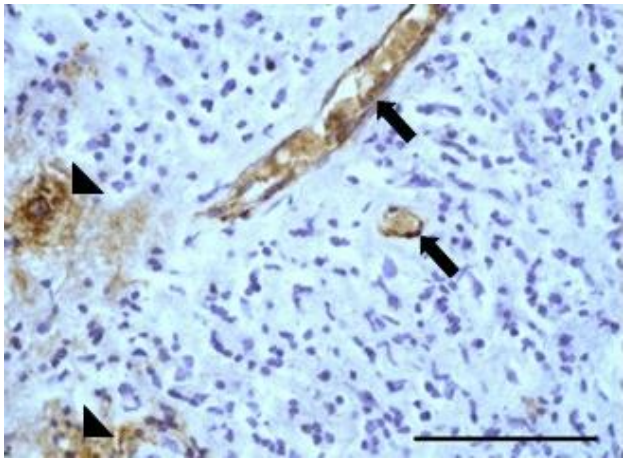


Table 4.1 The effect of diffuse vWF staining on the mean MVV and MVD scores (±SD) using standard vessel counting and diffuse vessel scoring.

Section	Tissue	Mean MVV standard score ± SD (%)	Mean MVV diffuse score ± SD (%)		Mean MVD ± SD (mm^{-2})	Mean MVD diffuse score ± SD (mm^{-2})
00/681GT	PG	3.6±5.0	4.6±6.28		115±93	125±97
00/0733GNT	PG	6.2±4.76	7.0±6.2		270±195	270±195
38764/00(13) GNT	PG	3.2±2.80	4.4±4.68		120±95	130±98
00/0685D HPDL	PDL	6.8±7.68	7.4±8.12		220±161	220±161
0084B HPDL	PDL	2.2±3.32	2.4±3.28		70±73	80±83
0088B HPDL	PDL	5.2±3.2	5.4±3.52		285±127	290±125
Group Mean (±SD)		**4.53±1.81**	**5.2±1.84**		**180±90**	**185±86**
***Wilcoxon test on raw data Standard vs diffuse score**		**0.001***			**0.008***	

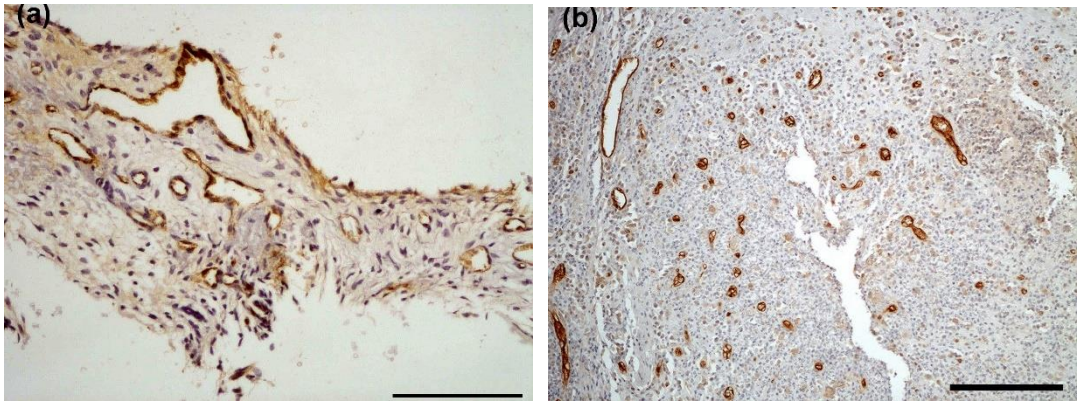
*Statistics generated on SPSS using all of the individual field scores for each section (n=120 individual counts).

4.3.1.3 CD105 (Endoglin) antibody optimisation

Preliminary studies were carried out to optimise the CD105 staining of the PG and PDL tissues. A 1:75 CD105 dilution combined with an autoclave pre-treatment resulted in good levels of blood vessel staining with minimal background staining. A comparison of citrate buffer microwave and autoclave pre-treatments was carried out at various CD105 dilutions (1:75, 1:100 and 1:150). Good levels of blood vessel staining with minimal background staining was found with the autoclave pre-treatment at all dilutions with marginally better

vessel staining at 1:75 dilution. This protocol was adopted for the study sections (Chapter 2.3.6 and Figure 4.3). Microwave pre-treatment resulted in good levels of vessel staining at 1:75 CD105 dilution but significantly reduced staining at 1:100 and 1:150 dilutions. In all these experiments it was noted that the majority of the background staining was associated with staining of immune cells and this finding was consistent with CD105 staining in breast tumours (Personal communication: Dr Stephane Perrier). CD105 has been found to stain normal stromal cells in several healthy human tissues (Balza et al., 2001). Unlike the vWF stained PG sections, there was no significant diffuse CD105 staining associated with extra-vascular red blood cells.

Figure 4.3 CD105 staining of (a) PDL and (b) PG sections (x200 magnification)*. Scale bar = 50μm.



*Images from Davey et al. (2008)

4.3.1.4 The effect of the length of slide storage on CD105 antigen retrieval

During the CD105 optimisation it was observed that the sections which had been cut previously and stored (light-tight boxes) for significant periods of time appeared to show different CD105 staining profiles to recently cut sections. This age effect was not found with the vWF stained sections. This possible age effect on CD105 antigen retrieval was studied by repeating the CD105 optimisation, as outlined above, using both old and recently cut sections. In order to maximise the possible effect of long-term storage, sections cut four years previously and stored in slide boxes (old sections) were compared with sections cut from the same blocks the day prior to staining. Similar good levels of blood vessel staining, with low background staining, were found with the autoclaved sections (1:75, 1:100 and 1:150 CD105 dilutions) in both the new and old sections. In the microwave pre-treatment

group, similar findings were found as before in the newly cut sections, however, very poor levels of blood vessel staining were found in the old sections at all CD105 dilutions. A possible explanation is oxidation which has previously been reported to cause some antigens to degrade with time, such as CD3 and chromogranin, in paraffin-embedded sections (Bertheau et al., 1998, Jackson, 2007). The autoclave pre-treatment appeared to overcome this age-related CD105 degradation or masking effect, although the exact mechanism for this has not been determined. Therefore, where possible the sections were cut immediately prior to staining. Due to the good reliability of staining, low background and resistance to loss of antigen retrieval in storage the final study sections were stained at a 1:75 CD105 dilution with an autoclave pre-treatment (Chapter 2.3.6).

4.3.2 Optimisation of the vascularity protocols

4.3.2.1 Adaptation of the standard assessment of vascularity

Preliminary MVV and MVD assessments of the sections were carried out using a 100 point (Mertz) eyepiece graticule at a magnification of x200 as described by Pazouki et al. (1997). Due to the narrowness of the PDL sections large areas of counting grid were empty of tissue at this magnification leading to problems with compatibility of the counts between the PG and PDL sections. Consequently, in order to compensate for this discrepancy, the area of the grid covered by the PDL tissue was recorded and the resultant vascular scores (MVV and MVD) were multiplied to convert to whole grid scores. However, this resulted in significant inter-observer variation of the PDL scores due to the subjective nature of estimating the percentage coverage of the grid, while there was excellent consensus of the PG scores. A quarter section of the 100 point counting grid (25 point square) at x400 magnification was found to consistently fit within the narrow PDL sections and was subsequently used to assess all of the tissue types. The 25 point grid scores could then be converted to give the final MVV (%) and MVD (mm^{-2}) scores. This change resulted in greatly reduced inter-observer variation of the preliminary PDL scores without significantly affecting the final MVV and MVD scores.

4.3.2.2 Intra-observer variation of standardised fields

Three vWF stained PG and PDL sections were selected and six randomly selected fields from each section were digitally imaged (standardised fields) at x400 magnification. A square grid similar to the light microscope eyepiece graticule was superimposed onto the images and the images were stored onto a computer. These standard images were assessed for MVV and MVD on two separate occasions seven days apart. Each section was allocated a number from 1 to 6 and the order in which the sections were assessed was randomly selected using a dice. Similarly, the order of assessment of the individual fields for each section was determined in the same fashion. This was carried out in order to reduce the chances of bias in the counting as there were relatively few sections/fields used. The consistency of the counts between the two occasions, both for MVV and MVD, was found to be very high (Wilcoxon signed-rank test $p=1.0$) suggesting very low intra-observer variation when examining standardised views. There was slightly more variation in the MVV scores and this was probably due to the difficulty in assessing whether the grid intersection was either on or very close to the vessel wall. No such dubiety was found using the MVD scoring.

4.3.2.3 The effect of sampling on intra-observer variation

It was important to establish the number of random fields per section required to compensate for tissue heterogeneity in order to achieve consistent vascularity scores (MVV and MVD). Previous studies from our laboratory have commonly used 10, 15 or 18 random fields per each section (Chandrachud et al., 1997, Schor et al., 1998a, Carlile et al., 2001). None of these studies involved PDL or PG tissues, so a small study was carried out to confirm the minimum number of random fields required to achieve consistent vascularity scores. Two PG and PDL vWF stained sections were randomly selected and each section was repeatedly recounted (MVV and MVD) with increasing number of random fields (minimum of 5 fields up to a maximum of 30 fields). The difference between the mean scores ($\pm$SD) for each field count was assessed using the Mann-Whitney U test (Table 4.2). The p-values for both the mean MVV and MVD scores generally increased and became consistent after 15-20 random fields, in both PG and PDL tissue, thus there was little benefit in counting more fields.

Table 4.2 Influence of the number of random fields counted per section on (a) MVV (%) and (b) MVD (mm^{-2}) scores for PG and PDL sections (25 point grid x400 magnification).

(a) MVV (%)

Block reference	Tissue	No fields counted	No of points	Mean (±SD) field counts	Mann-Whitney (p-value)	
38764/00 (12)	PG	5	125	3.2±4.8	0.557	-
		10	250	4.8±3.6		0.725
		15	375	5.2±4.0	0.588	
		20	500	6.0±3.6		0.711
		25	625	5.6±4.0	0.785	
		30	750	6.0±4.0		-
00/0672AG	PG	5	125	7.2±1.8	0.941	-
		10	250	7.2±2.5		0.419
		15	375	6.4±2.5	0.84	
		20	500	6.2±3.0		0.596
		25	625	6.7±3.2	0.885	
		30	750	6.5±3.2		-
0086A	PDL	5	125	5.6±2.0	0.629	-
		10	250	5.2±2.0		0.508
		15	375	5.2±2.0	0.736	
		20	500	5.6±2.4		0.788
		25	625	6.0±2.8	0.856	
		30	750	5.6±2.4		-
0088B	PDL	5	125	1.6±2.0	0.949	-
		10	250	2.4±2.8		0.704
		15	375	3.6±3.6	0.943	
		20	500	4.0±3.6		0.678
		25	625	4.0±3.2	0.962	
		30	750	4.0±3.2		-

(b) MVD (mm^{-2})

Block reference	Tissue	No fields counted	No of points	Mean (±SD) field counts	Mann-Whitney (p-value)	
38764/00 (12)	PG	5	125	80±110	0.791	-
		10	250	90±87		0.766
		15	375	80±86	0.656	
		20	500	100±110		0.876
		25	625	104±106	0.965	
		30	750	103±100		-
00/0672AG	PG	5	125	180±130	0.526	-
		10	250	140±107		1.0
		15	375	140±99	0.522	
		20	500	120±95		0.93
		25	625	116±90	0.719	
		30	750	127±98		-
0086A	PDL	5	125	100±71	0.948	-
		10	250	110±99		0.977
		15	375	113±106	0.821	
		20	500	125±116		0.694
		25	625	112±113	0.859	
		30	750	107±111		-
0088B	PDL	5	125	160±134	0.742	-
		10	250	180±132		0.681
		15	375	160±130	0.604	
		20	500	180±120		0.645
		25	625	164±115	0.951	
		30	750	167±118		-

4.3.2.4 The effect of sampling on inter-observer variation

Another observer (MM) independently repeated the above exercise in order to examine the effect of the number of random fields per section on the inter-observer variation (Table 4.3). The consistency between the observers, shown by the Mann-Whitney U test p-values, was found to generally improve with increasing numbers of random fields counted. Again, by the 15th random field the level of consistency did not greatly improve with further increase in the number of fields counted, thus confirming 15 random fields as being the minimum number of fields necessary for the PG-PDL sections. Generally, good levels of consistency were achieved for the PG sections, but significantly poorer levels were found with the PDL sections. This was probably due to the fine structure of the PDL with the resultant haemorrhaging in some sections that made assessment more difficult. In order to overcome these difficulties further training of three observers was carried out using computerised images of sections prior to the study sections being assessed for MVV and MVD. Following this training, differences in intra- and inter-observation were not significant.

Table 4.3 Influence of the number of random fields counted per section on inter-observer variation of MVV (%) scores (examiners - KJD and MM) for PG and PDL sections (25 point grid x400 magnification).

(a) 38764/00 (12) GNT (PG)

No. fields Counted	No. points Counted	Observer	Mean count ± SD	Difference Between Observers - Mann-Whitney (p-value)
5	125	MM	7.2±3.2	0.192
		KJD	3.2±4.8	
10	250	MM	5.6±2.0	0.449
		KJD	4.8±3.6	
15	375	MM	6.0±2.4	0.488
		KJD	5.2±4.0	
20	500	MM	7.2±3.2	0.437
		KJD	6.0±3.6	
25	625	MM	6.4±2.8	0.54
		KJD	5.6±4.0	
30	750	MM	6.8±3.2	0.504
		KJD	6.0±4.0	

(b) 00/0672AG (PG)

No. fields counted	No. points Counted	Observer	Mean count ± SD	Difference Between Observers - Mann-Whitney (p-value)
5	125	MM	8.0±5.6	0.824
		KJD	7.2±1.8	
10	250	MM	6.4±2.8	0.45
		KJD	7.2±2.5	
15	375	MM	6.4±3.3	0.982
		KJD	6.4±2.5	
20	500	MM	6.4±3.3	0.884
		KJD	6.2±3.0	
25	625	MM	5.8±2.6	0.28
		KJD	6.7±3.2	
30	750	MM	6.8±3.0	0.768
		KJD	6.5±3.2	

(c) 0086A HPDL (PDL)

No. fields counted	No. points Counted	Observer	Mean ± SD	Difference between Observers - Mann-Whitney (p-value)
5	125	MM	6.4±3.6	0.032
		KJD	1.6±2.0	
10	250	MM	7.2±3.2	0.004
		KJD	2.4±2.8	
15	375	MM	5.2±2.0	0.075
		KJD	3.6±3.6	
20	500	MM	6.0±2.4	0.026
		KJD	4.0±3.6	
25	625	MM	6.4±2.8	0.017
		KJD	4.0±3.2	
30	750	MM	6.0±2.0	0.020
		KJD	4.0±3.2	

(d) 0088B HPDL (PDL)

No. fields counted	No. points Counted	Observer	Mean ± SD	Difference Between Observers - Mann-Whitney (p-value)
5	125	MM	5.6±2.0	0.91
		KJD	5.6±4.4	
10	250	MM	5.6±2.0	0.934
		KJD	5.6±4.0	
15	375	MM	6.4±2.0	0.22
		KJD	5.2±3.6	
20	500	MM	6.0±2.0	0.334
		KJD	4.8±3.2	
25	625	MM	6.0±2.0	0.045
		KJD	4.8±2.8	
30	750	MM	6.0±2.0	0.053
		KJD	4.8±2.8	

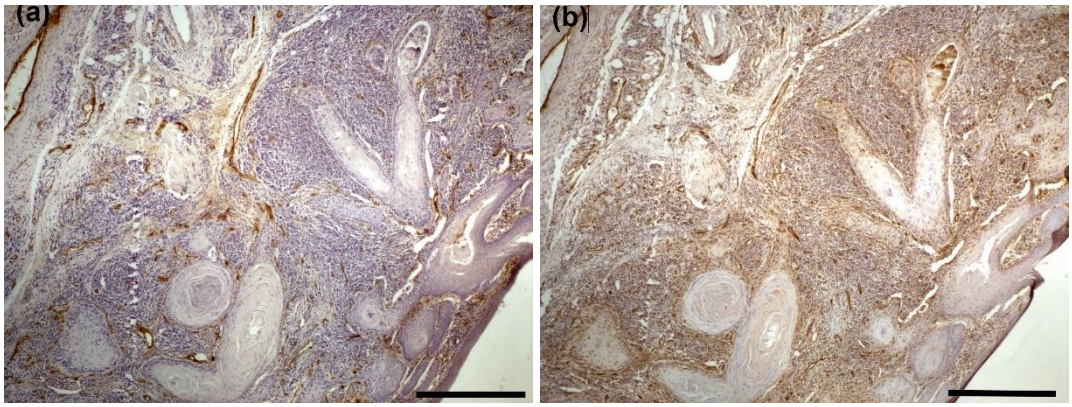
4.3.3 Oral squamous cell carcinoma (OSCC): positive angiogenic control

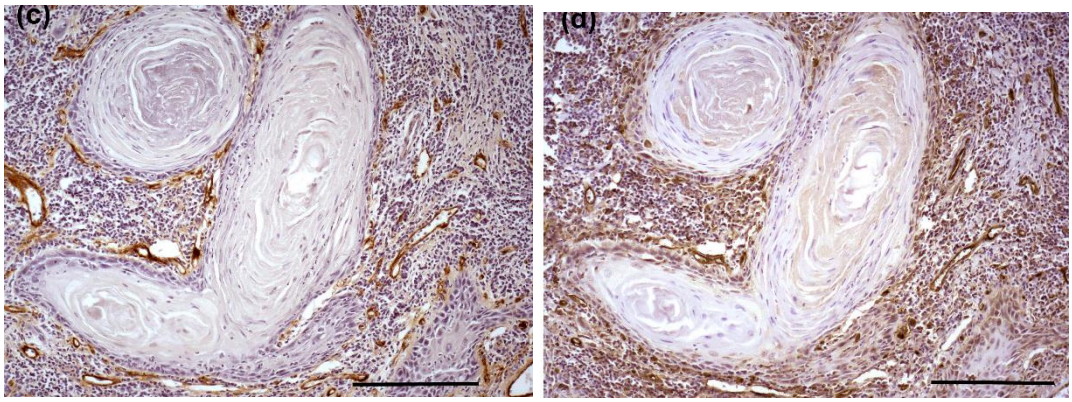
Initial examination of the MVV and MVD (vWF and CD105) scores showed no statistically significant differences between the PG and PDL sections. This finding was unexpected as it had been previously suggested that the formation of granulomatous lesions was accompanied by angiogenesis and so it was anticipated that the presumably angiogenic PG would have higher vascularity levels than the healthy PDL. Previous studies have indicated vascularity to be a good index of angiogenesis in OSCC (Pazouki et al., 1997, Schimming and Marme, 2002, Li et al., 2005). These studies found significantly higher vascularity in the OSCC compared to its histologically normal peri-tumour normal oral mucosa (NOM), suggesting that angiogenesis had occurred in the development of the OSCC. Hence, OSCC and its associated NOM were used as a positive angiogenic control in order to verify the robustness of the protocol and confirm the PG-PDL findings.

4.3.3.1 Optimisation of the OSCC sections

Optimisation studies confirmed that the staining protocols used to stain the PDL and PG sections for vWF and CD105 (Chapter 2.3.5 and 2.3.6 respectively) resulted in good levels of staining of the OSCC-NOM with minimal background staining. Fourteen OSCC sections, six of which contained histologically normal peri-tumour tissue (NOM), were stained with the vWF and CD105 markers (Figures 4.4 and 4.5). Positive (PG and PDL) and negative control sections were included to ensure conformity of staining. Control sections, stained with the corresponding non-immune IgG, were always negative.

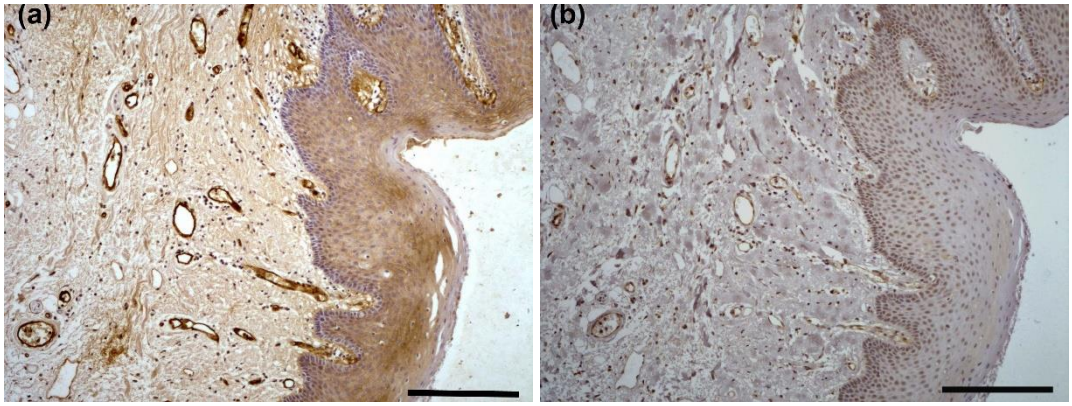
Figure 4.4 Consecutive sections of OSCC stained with vWF (a) and (c) and CD105 (b) and (d). Scale bar 100μm (a) and (b) (magnification x100). Scale bar 50μm (c) and (d) (magnification x200)*.





*Images from Davey et al. (2008)

Figure 4.5 Consecutive sections of Peri-tumour NOM (a) vWF and (b) CD105 (magnification x100)*. Scale bar = 100μm. Maximal contrast observed between vWF and CD105 staining in NOM is shown in this figure.



*Images from Davey et al. (2008)

4.3.3.2 Highest-Microvascular Density (h-MVD)

Highest-microvascular density (h-MVD), in which microvascular density is only assessed in vascular "hot spots" (Weidner et al., 1991), has been widely used in vascular assessment of tumour angiogenesis (Chapter 4.1.3). With the inclusion of the OSCC-NOM sections in the study, h-MVD counts were carried out to allow further comparison with the published data. In the OSCC-NOM sections three fields were counted in the most vascularised area and h-MVD was calculated as the mean of these scores (Chapter 2.3.8). Vessels were homogeneously distributed in the PG and PDL sections with no evidence of vascular hot spots. Therefore, h-MVD was calculated in the PG and PDL sections as the mean of the highest three MVD field values found per section.

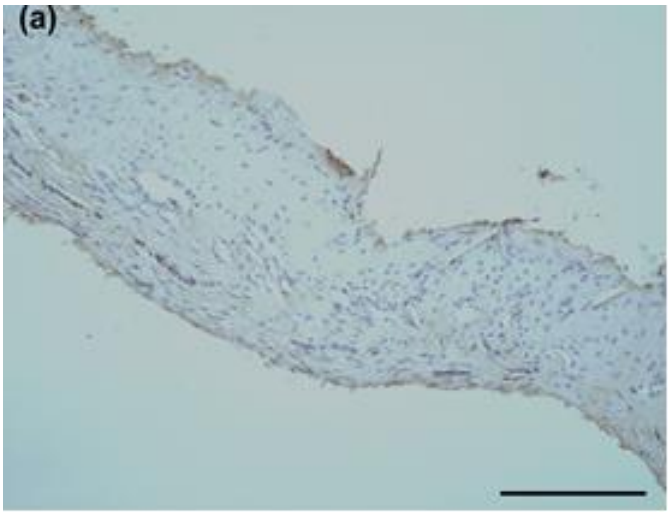
4.3.4 Optimisation of Migration Stimulating Factor (MSF) staining protocol

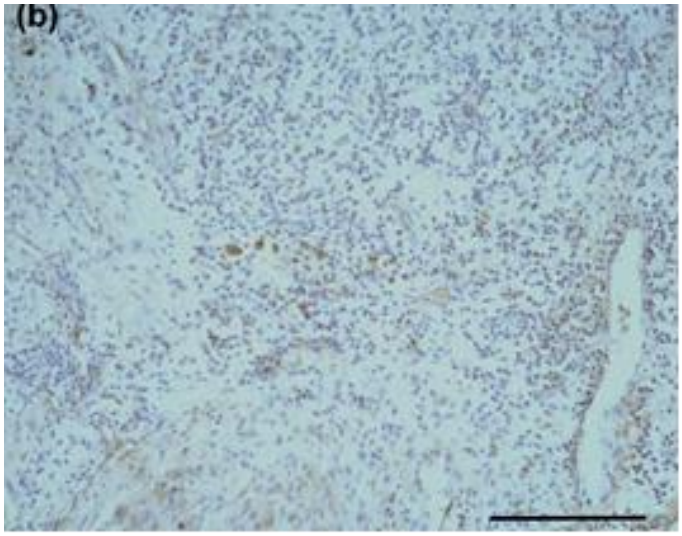
Prior to staining the study sections, the MSF antibody was optimised in order to achieve maximal antigen retrieval with minimal tissue damage and background staining. Negative control sections were used to ensure no significant cross reactivity of the reagents, this being shown by minimal/no background staining. MSF expression was initially localised using the MSF 7.31s ascites mouse anti-human polyclonal antibody, diluted 1:25 in PBS, combined with a citrate buffer microwave pre-treatment (Chapter 2.3.3) (Personal communication: Dr Teresa Estella). However, this antibody was superseded by the highly specific MSF 7.1AF (Ascites Fluid) mouse anti-human monoclonal antibody. As with other markers, the staining profile for this antibody was shown to vary depending on the type of tissue (oral mucosa, skin, breast, etc.) (Personal communication: Jacqui Cox), and so specific optimisation was carried out for the PG and PDL tissues. The two main variables affecting antigen retrieval were: (i) the dilution of the primary antibody and (ii) the type of pre-treatment used (if required). Chapter 2.3.2, 2.3.3 and 2.3.4 details the staining protocols and pre-treatment procedures. Various dilutions of the MSF 7.1AF antibody (1:700, 1:1000, 1:1200 and 1:1500 in PBS) with either no pre-treatment or a microwave pre-treatment in citrate buffer were investigated. Microwave pre-treatment caused high background staining at all dilutions, especially in the PDL sections. A further problem associated with the microwave pre-treatment was the high levels of inflammatory cell staining (especially plasma cells) even in the negative control sections. In the no pre-treatment group the 1:700 and 1:1000 dilutions of the MSF antibody caused significant overstaining in both PG and PDL sections. The 1:1200 dilution was associated with good levels of staining of isolated cell groups (fibroblasts, vascular endothelial cells and inflammatory cells), low background staining and good consistency between duplicate slides. The 1:1500 MSF antibody dilution resulted in a similar, but substantially weaker staining, compared to the 1:1200 dilution. Further dilutions studies (1:1200, 1:1500 and 1:2000 dilutions with no pre-treatment) found a rapid diminution in cellular MSF staining with increasing antibody dilution beyond 1:1200, with no cellular MSF staining by the 1:2000 dilution. The background MSF staining was minimal at the 1:1200 MSF antibody dilution and was affected little by further antibody dilution. Therefore, the study PG and PDL sections were stained in duplicate at a MSF antibody dilution of 1:1200 in PBS with no pre-treatment. Chapter 2.3.4 details of the final optimised MSF

staining protocol and Figure 4.6 shows representative samples of MSF stained PG and PDL sections. Several staining runs were required to stain all of the sections, so both positive and negative PG and PDL control sections were included in each run to ensure conformity of staining. In order to allow comparison with the vascular assessment of the PG and PDL sections, the same sections were used in this series of experiments. There was no evidence of diffuse MSF staining, as was found with the vWF staining (Chapter 4.3.1.2), and no evidence of reduced antigen retrieval with long-term storage as encountered with the CD105 staining (Chapter 4.3.1.4).

MSF staining intensity was scored using pre-determined calibration slides as either no staining (-), weak (±), moderate (+) or strong (++) staining. The final intensity of the MSF staining was assessed by two or three independent observers (KJD, JC and GO) and the final intensity grades were agreed by consensus.

Figure 4.6 MSF staining of (a) PDL and (b) PG sections (x100 magnification). Scale bar = 200μm.





4.3.5 Verification of the MSF staining protocol

4.3.5.1 Oral squamous cell carcinoma (OSCC): positive angiogenic control

Initial assessment showed the intensity of the MSF staining in the PG and PDL sections to be either weakly positive or negative (fibroblasts, vessels and inflammatory cells). In order to verify the robustness of the protocol and confirm the PG and PDL findings, OSCC and its associated histologically normal peri-tumour tissue NOM was used as a known positive angiogenic control. To allow direct comparison with the vascularity indices, consecutive sections from the same OSCC-NOM specimens were used in this study. Optimisation and final MSF staining of the OSCC-NOM sections was carried out by Dr Ohe as part of another study. Coincidently, the MSF protocol used to stain the OSCC-NOM sections was identical to that used to stain the PDL and PG, and these conditions resulted in good levels of staining of both the OSCC and NOM with minimal background staining. Unfortunately, two OSCC sections, one of which contained NOM tissue, were excluded from the study due to persistent overstaining with the MSF antibody. Although these sections could have represented particularly strongly MSF positive sections the staining was so intense that the assessment of the MSF expression of the individual cell types was impossible. Therefore, twelve OSCC sections, five of which contained histologically normal peri-tumour tissue (NOM), were stained for MSF.

4.3.5.2 Observer variation

Intra- and inter-observer variation of the MSF staining intensity was assessed in the individual tissues (PG, PDL, OSCC, paired-OSCC and NOM tissues) and in the specific cell types (Fibroblasts, Vascular Endothelial cells, Inflammatory cells and Epithelial cells) (Table 4.4).

Intra-observer variation was examined by one observer (KJD) reassessing all of the sections on two separate occasions (Table 4.4a). With the exception of the OSCC-Fibroblast and OSCC-Vessel Endothelial cell counts, the intra-observer variations were not significant with p-values ranging from 0.317 to 1 (Wilcoxon paired sample test). In the majority of tissues, the intra-observer variations were very low showing good consistency between the counts. When the OSCC-Fibroblast and OSCC-Vessel Endothelial cell intra-observer variations were examined there were very strong positive correlations found between the counts in each case (Spearman's rank correlation: fibroblasts rho=0.866, $p<0.001$; vessels rho=0.881, $p<0.001$). These differences were only found in the OSCC and were probably due to the heterogeneous nature of the OSCC sections.

Inter-observer variation was examined by two or three independent observers (KJD, JC and GO) (Table 4.4b). With the exception of the OSCC-Epithelial counts, the inter-observer variations were not significant with p-values ranging from 0.157 to 1 (Wilcoxon paired sample test). When the OSCC-Epithelial inter-observer variation was examined there was a nearly significant positive correlation between the counts (Spearman's rank correlation: rho=0.519, p=0.084) suggesting consistent differences in the observer's counts. One observer was significantly more experienced in the MSF staining assessment of OSCC sections and so this examiner provided further training and the final scores was agreed by consensus (Table 4.10).

Overall, both the intra- and inter-observer variations suggested there was good consistency between the counts, although further training was required in the assessment of some cell types in the OSCC-NOM tissues, and intra-observer variation was generally more consistent than the inter-observer variation.

Table 4.4 Assessment of (a) Intra- and (b) Inter-observer variation (Wilcoxon p-values) for MSF staining intensity of the cell types (Fibroblast, Blood Vessel Endothelial Cells, Inflammatory Cells and Epithelial Cells) in the different tissues.

(a) Intra-observer variation

Tissue (number)	TISSUE			
	Fibroblasts	**Blood Vessels**	**Inflammatory Cells**	**Epithelial Cells**
PG (13)	1	1	1	x
PDL (13)	1	1	1	x
OSCC (12)	0.046	0.025	0.317	1
Paired OSCC (5)	0.317	0.317	1	1
NOM (5)	1	1	1	1

(b) Inter-observer variation

Tissue (number)	TISSUE			
	Fibroblasts	**Blood Vessels**	**Inflammatory Cells**	**Epithelial Cells**
PG (13)	0.785	0.157	0.317	x
PDL (13)	0.317	0.317	0.317	x
OSCC (12)	0.317	0.317	0.317	0.002
Paired OSCC (5)	0.317	0.317	0.317	0.074
NOM (5)	1	1	1	0.257

4.4 Results

4.4.1 Final consensus assessment of vascularity

The final consensus MVV, MVD and h-MVD for all of the tissues were quantified by two or four independent observers and any sections with an inter-observer variation above 15% were recounted and agreed by consensus. Intra-observer variation was checked by one observer, (KJD), by recounting all of the sections on two separate occasions. A limited number (up to six) of random sections from other areas of the specimens were also examined for a small number (n=10) of specimens. No significant histological variation was observed between the replicate sections. The final consensus vWF-MVV, vWF-MVD, vWF-h-MVD, CD105-MVV, CD105-MVD and CD105-h-MVD scores for the all of the tissues are summarised in Table 4.5. The corresponding data for the six OSCC sections which contained histologically normal oral mucosa were referred to as “paired OSCC-NOM”.

Table 4.5 Overall summary of the mean vascularity indices (±SD) (MVV, MVD and h-MVD) in all of the tissue groups (PG, PDL, OSCC, paired OSCC and NOM) for both vascular markers (vWF and CD105).

	No	Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD		
		vWF MVV (%)	CD105 MVV (%)	vWF a-MVD (mm^{-2})	CD105 a-MVD (mm^{-2})	vWF h-MVD (mm^{-2})	CD105 h-MVD (mm^{-2})	% mean CD105 to vWF (MVV)	% mean CD105 to vWF (MVD)	% mean CD105 to vWF (h-MVD)
PG	**13**	2.64±1.1	2.7±1.0	113±42	109±27	220±70	226±41	**127±97**	**108±64**	**112±38**
PDL	**13**	3.58±2.0	3.37±1.6	122±63	115±40	221±84	228±45	**101±31**	**108±41**	**116±43**
OSCC	**14**	4.07±1.2	3.66±1.4	113±25	92±33	955±210	1150 ±233	**101±61**	**86±37**	***124±29**
OSCC	**6**	3.86±1.2	3.83±1.9	113±23	103±44	906±181	1122 ±252	**118±89**	**97±51**	****125±26**
NOM	**6**	2.84±0.9	1.87±0.5	78±19	55±9	594±180	389±55	**71±28**	**71±6**	**68±13**

* h-MVD OSCC > NOM (p=0.001)
** h-MVD Paired OSCC > NOM (p=0.002)

4.4.1.1 Visualisation of the blood vessels

Good positive staining of blood vessels, with minimal background, was achieved with both antibodies (Figures 4.1, 4.3, 4.4 and 4.5). The intensity of the staining was similar with both antibodies in all of the tissues, except for CD105 in NOM, which was generally lower. Maximal contrast observed between vWF and CD105 staining in NOM is shown in Figure 4.5 and further investigated in Chapter 4.4.1.6. Antibody to vWF stained only blood vessels, whereas CD105 antibody stained blood vessels and occasionally cells of the inflammatory infiltrate (Figure 4.4). These were easily distinguished from blood vessels and therefore did not affect the quantification of the latter. Control sections, stained with corresponding non-immune IgG, were always negative.

4.4.1.2 Observer variation

Intra- and inter-observer variations for each antibody and vascular index are shown in Table 4.6. With the exception of the h-MVD inter-observer variation, the intra- and inter-observer variations were not significant, with p-values ranging from 0.423 to 0.875 (Wilcoxon paired sample test). When the h-MVD inter-observer variations were examined there were moderate to strong positive correlations between the observer's counts (Spearman's rank correlation: vWF rho=0.503, p=0.009; CD105 rho=0.757, p<0.001) suggesting consistent differences in the observer's counts. This difference was only found in the OSCC and NOM tissues and was probably due to the examiners being less experienced in this assessment

method. Further training was carried out and the final h-MVD counts were agreed by consensus.

Table 4.6 Assessment of MVV, MVD and h-MVD intra- and inter-observer variation (Wilcoxon p-values).

Intra-observer variation Wilcoxon	MVV	MVD	h-MVD
vWF	0.875	0.637	0.665
CD105	0.820	0.720	0.571

Inter-observer variation Wilcoxon	MVV	MVD	h-MVD
vWF	0.864	0.423	0.01*
CD105	0.637	0.569	0.01*

* Significant difference between observers.

The overall high p-values, with the exception of h-MVD inter-observer variation, suggested that there was good consistency between the counts, although intra-observer variation was generally more consistent than the inter-observer variation. MVD p-values were generally lower than the MVV, especially for the vWF MVD inter-observer variation, although this was within acceptable limits. This shows the importance of independent observers in the assessment of the study sections.

4.4.1.3 Correlation between vascular indices

MVV, MVD and h-MVD are different indices of vascularity and could be expected to be correlated. However, it has been previously reported that different results may be obtained depending on the vascular index used (Pazouki et al., 1997) (Chapter 4.1.3). Volume and density vascularity scores were directly and significantly correlated for all tissues and antibodies, with the exception of the correlation between CD105 MVD and h-MVD (Table 4.7). This discrepancy may reflect the difference in methodology between the MVD and h-MVD counts. Overall, the high correlation between the vascular indices confirms the validity of the protocol.

Table 4.7 Correlation between the MVV, MVD and h-MVD vascular indices (Spearman rank correlation).

TISSUE	Primary Antibody	Vascular index	nº	Spearman's rho	P-value
All Tissues	vWF	MVV MVD	46	0.513	<0.001**
		MVV h-MVD	46	0.533	<0.001**
		MVD h-MVD	46	0.319	0.031*
All Tissues	CD105	MVV MVD	46	0.680	<0.001**
		MVV h-MVD	46	0.296	0.046*
		MVD h-MVD	46	-0.137	0.364

** Highly significant correlation.
* Significant correlation

4.4.1.4 Comparison of PG and PDL

Six parameters of vascularity (vWF-MVV, vWF-MVD, vWF-h-MVD, CD105-MVV, CD105-MVD and CD105-h-MVD) were assessed for each PG and PDL section (Table 4.5). Values tended to be higher in the PDL tissues compared to the PG, but the differences were not significant (Table 4.8). Results for MVV, MVD and h-MVD are shown in Figures 4.7, 4.8 and 4.9, respectively. CD105 values expressed as a percentage of vWF (Table 4.5) were not different, (approximately 100%) in PG and PDL samples.

Table 4.8 Comparison of PG-PDL and OSCC-NOM by different methods (Mann-Whitney U test p-values). Ranking was OSCC > NOM unless otherwise stated.

Tissues	Ab	Method		
		MVV	**MVD**	**h-MVD**
PG (13) vs PDL (13)	vWF	0.317	1.0	0.856
	CD105	0.367	0.837	0.840
	CD105 as % vWF	0.513	0.786	0.719
OSCC (14) vs NOM (6)	vWF	0.031*	0.007**	0.005**
	CD105	0.002**	0.014*	0.001**
	CD105 as % vWF	0.343	0.591	0.001**
OSCC (6) vs NOM (6)	vWF	0.08	0.01*	0.026*
	CD105	0.03*	0.10	0.002**
	CD105 as % vWF	0.522	0.335	0.002**

* Significant
** Highly significant

Figure 4.7 Estimate of microvascular volume (MVV, %) for PG and PDL (a) vWF and (b) CD105. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

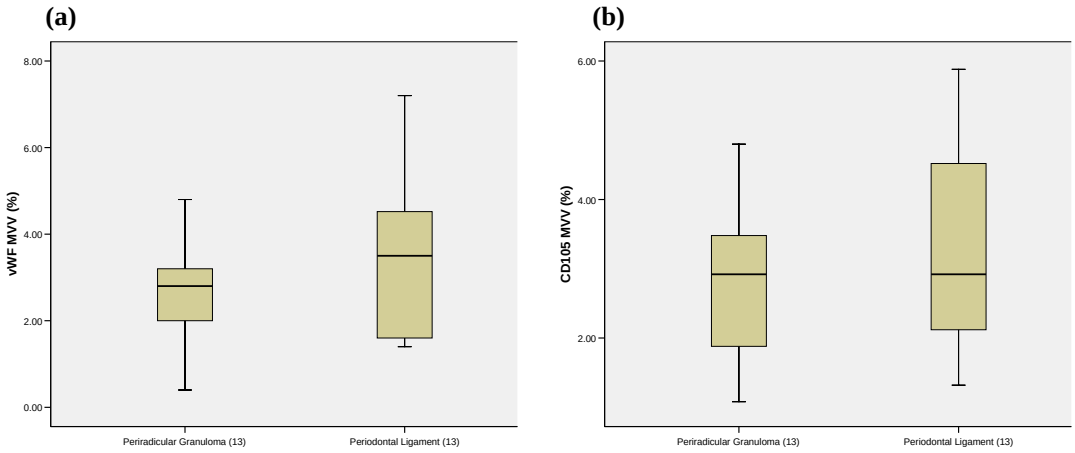


Figure 4.8 Estimate of microvascular density (MVD, mm^{-2}) for PG and PDL (a) vWF and (b) CD105. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

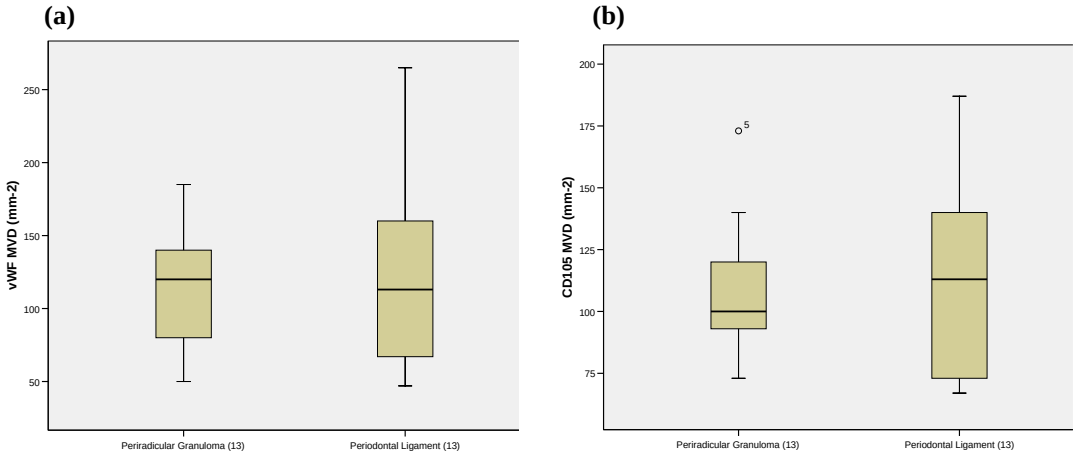
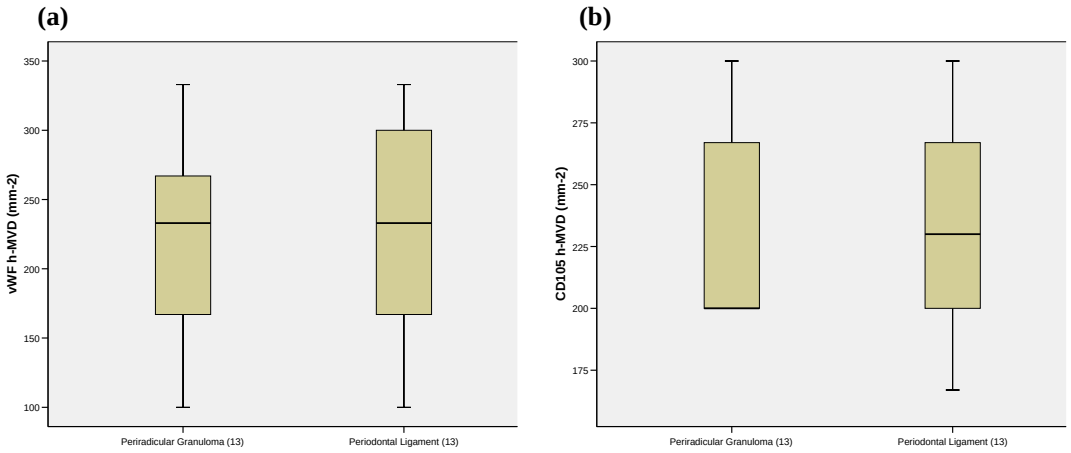


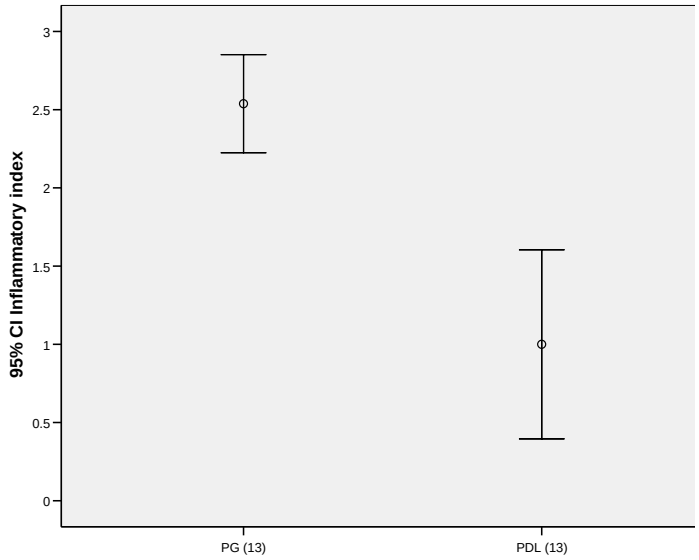
Figure 4.9 Estimate of highest-microvascular density (h-MVD, mm^{-2}) for PG and PDL (a) vWF and (b) CD105. The box represents the interquartile range, the horizontal line the median and the whiskers the range.



4.4.1.5 Assessment of inflammatory infiltration

Previous studies on angiogenic factor expression in periapical lesions have associated angiogenic factor expression with inflammatory infiltration (Chapter 4.1.7). The extent of inflammatory infiltration of each section was independently assessed by two examiners using four pre-determined PG and PDL calibration sections. The sections were scored either as Grade 0 (no inflammatory infiltrate), Grade 1 (mild degree of inflammatory infiltrate), Grade 2 (moderate degree of inflammatory infiltrate) or Grade 3 (severe inflammatory infiltrate). The final scores were agreed by consensus (Figure 4.10). As expected, statistically higher levels of inflammatory infiltration were found in the PG compared to the PDL tissue (Mann-Whitney U test p=0.001). No correlation was found between the inflammatory index of the PG and the vascularity scores.

Figure 4.10 Graph showing the mean (±SE) inflammatory index scores for the PG and PDL sections.



4.4.1.6 Comparison of OSCC and peri-tumour NOM tissues

Vascularity values were significantly higher in OSCC (n=14) than in the NOM tissues (n=6) (Table 4.5), irrespective of the antibody or quantification method used (p-values ranging from 0.031 to 0.001) (Table 4.8). Results for MVV (vWF and CD105) are shown in Figure 4.11, MVD in Figure 4.12 and those for h-MVD in Figure 4.13. When comparing only the six OSCC which contained NOM, the difference between paired OSCC and NOM groups was still significant for vWF-MVD (p=0.01), vWF-h-MVD (p=0.026), CD105-MVV (p=0.03) and CD105-h-MVD (p=0.002). In all cases, vascularity values for the six OSCC were not significantly different than those for the 14 OSCC (p values ranged from 0.51 to 0.93). When CD105 vascularity values were expressed as a percentage of vWF (Tables 4.5 and 4.8), the percentage was lower in NOM than in OSCC and highest in the "hot spot" of tumours (h-MVD). Consequently, the difference was statistically significant only for h-MVD (p=0.002).

These findings demonstrate, as previously reported, that angiogenesis is associated with the development of OSCC and verifies the use of OSCC-NOM as a good positive control model of angiogenesis for this protocol. Furthermore, the robustness of the methodology is further shown by the significant or near significant differences between tumour and normal tissues

being detectable even in very small experimental groups. These findings give a high level of confidence in the unexpected PG-PDL vascularity finding.

Figure 4.11 Estimate of microvascular volume (MVV, %) for OSCC and NOM sections using (a) vWF and (b) CD105. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

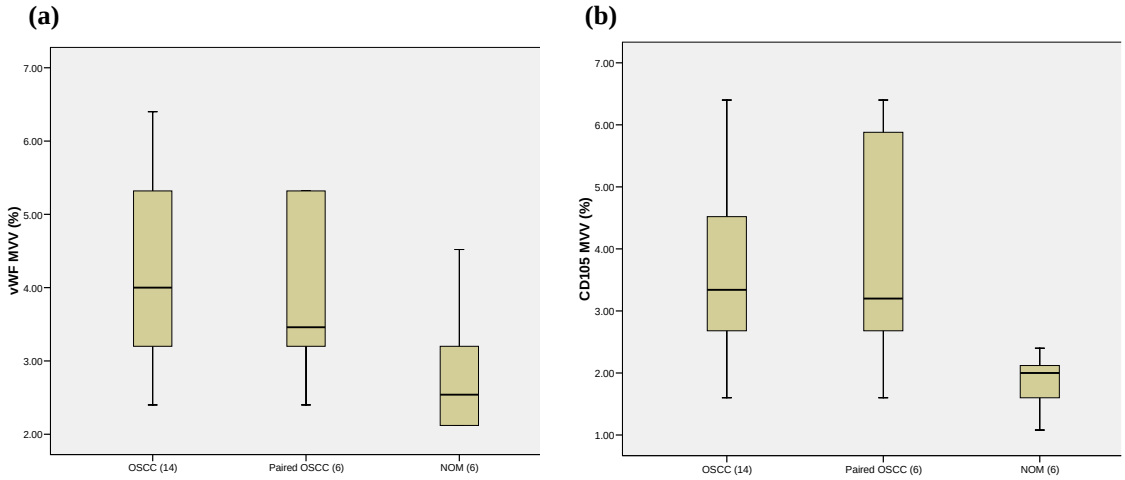


Figure 4.12 Estimate of microvascular density (MVD, mm^{-2}) for OSCC and NOM sections using (a) vWF and (b) CD105. The box represents the interquartile range, the horizontal line the median and the whiskers the range.

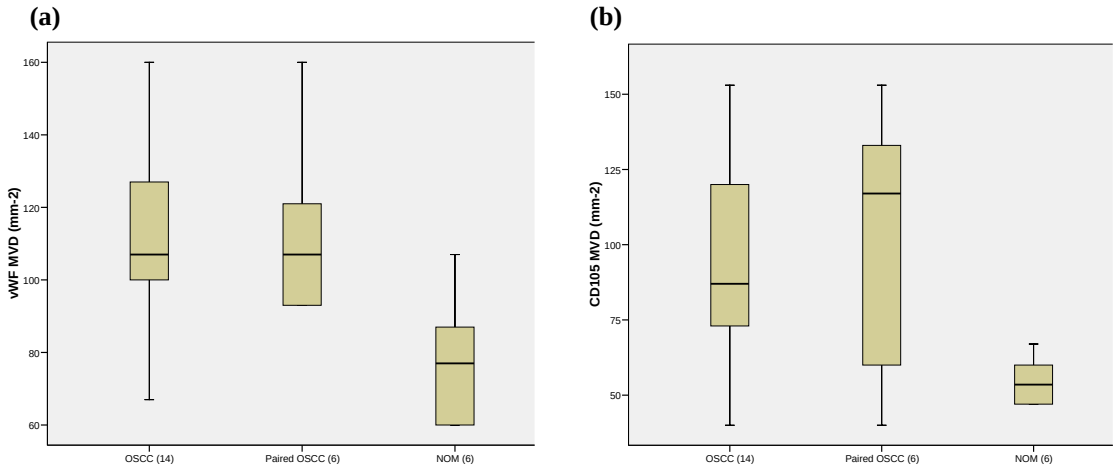
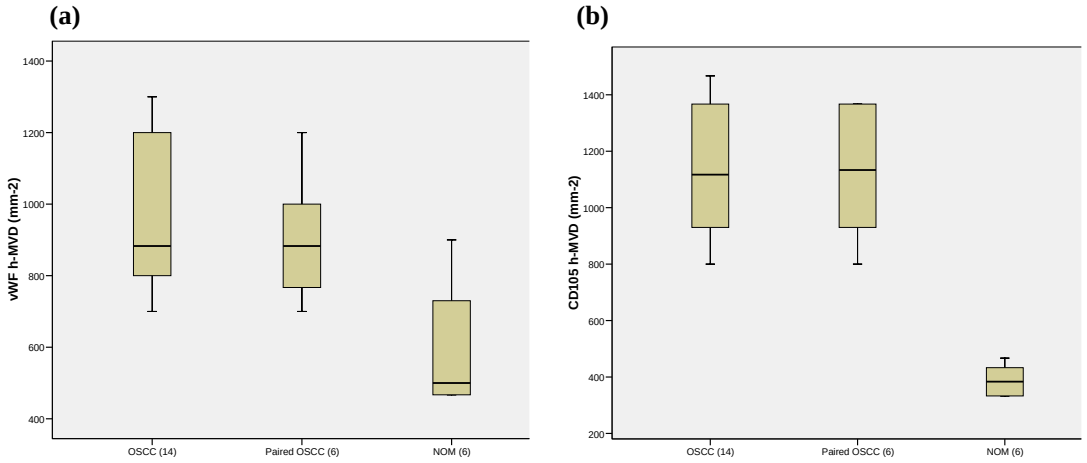


Figure 4.13 Estimate of highest-microvascular density (h-MVD, mm^{-2}) for OSCC and NOM sections using (a) vWF and (b) CD105. The box represents the interquartile range, the horizontal line the median and the whiskers the range.



4.4.1.7 Staining intensity

There was no difference between PG and PDL specimens in the intensity of the vWF and CD105 staining, however, the intensity of the staining with CD105 antibody was generally weaker in NOM than in OSCC (Figure 4.5 and Table 4.9). Analysis of the six matched specimens revealed that the intensity of vWF-positive vessels was similar in NOM and OSCC (p=0.29), whereas that of CD105-positive vessels was significantly lower in NOM (p=0.012).

Table 4.9 Staining intensity of vessels positive for vWF and CD105.

Vascular marker	Tissue	Staining intensity			Chi-square p-value
		+	++	+++	
vWF	**NOM**	2	3	1	0.29
	OSCC	0	4	2	
	PDL	0	0	13	1.0
	PG	0	0	13	
CD105	**NOM**	5	1	0	0.012*
	OSCC	0	4	2	
	PDL	0	0	13	1.0
	PG	0	0	13	

*Significant difference

Although there was a wide variation in the vascularity scores, especially in the PDL, it is of interest that all values were similar in OSCC, PG and PDL samples and lower in peri-tumour

NOM samples. This highlights the importance of comparing each lesion with its normal counterpart.

4.4.2 MSF antibody staining profile and consensus scoring

MSF staining, where present, was clearly defined in particular cell types (Fibroblasts, Vascular Endothelial Cells and Inflammatory Cells; Epithelial Cells in the OSCC-NOM sections), with low background staining and good agreement between duplicate sections. Differential MSF staining intensities were encountered both within tissues, in terms of cellular staining, and between tissues. Representative sections are shown in Figure 4.6. Two OSCC sections, one containing adjacent NOM, were excluded due to persistent overstaining with the MSF 7.1 AF antibody.

The final consensus MSF staining intensities were quantified by two or three independent observers (KJD, JC and GO) and the final score was agreed by consensus. The final consensus MSF staining intensity scores for the fibroblasts, vascular endothelial cells, inflammatory cells and epithelial cells for the PG, PDL, OSCC, paired-OSCC and NOM sections are shown in Table 4.10.

4.4.2.1 Comparison of PG and PDL

MSF staining intensity was assessed in fibroblasts, vascular endothelial cells and the inflammatory cell infiltrate for each PG and PDL section (summarised in Table 4.10 with statistical analysis shown in Table 4.11). MSF staining intensity was differential with many sections containing weak or no MSF staining in the different cell types. MSF staining was not statistically different for any of the cell types in the PG and PDL sections.

Table 4.10 MSF staining intensity for the cell types (Fibroblasts, Vascular Endothelial Cells, Inflammatory Cells and Epithelial Cells) for the PG, PDL, OSCC, Paired OSCC and NOM tissues.

Tissue (number)	MSF Staining Intensity											
	Fibroblasts			Vessels			Inflammatory Cells			Epithelial Cells		
	+	±	-	+	±	-	+	±	-	+	±	-
PG (13)	2 (15)	5 (39)	6 (46)	0 (0)	8 (61)	5 (39)	3 (23)	1 (7)	9 (60)	N/A		
	+/± 7			+/± 8			+/± 4					
PDL (13)	0 (0)	2 (15)	11 (85)	3 (23)	3 (23)	7 (54)	2 (15)	1 (7)	10 (78)	N/A		
	+/± 2			+/± 6			+/± 3					
OSCC (12)	8 (67)	4 (33)	0 (0)	12 (100)	0 (0)	0 (0)	5 (42)	3 (25)	4 (33)	8 (67)	0 (0)	4 (33)
	+/± 12			+/± 12			+/± 8			+/± 8		
Paired OSCC (5)	3 (60)	2 (40)	0 (0)	5 (100)	0 (0)	0 (0)	2 (40)	1 (20)	2 (40)	2 (40)	0 (0)	3 (60)
	+/± 5			+/± 5			+/± 3			+/± 2		
NOM (5)	2 (40)	2 (40)	1 (20)	4 (80)	1 (20)	0 (0)	1 (20)	2 (40)	2 (40)	2 (40)	0 (0)	3 (60)
	+/± 4			+/± 5			+/± 3			+/± 2		

Key:
+ Strong intensity of MSF staining (+, +± and ++).
± Weak intensity of MSF staining.
- No MSF staining.
( ) Percentage of total samples.
N/A Not applicable.

4.4.2.2 Comparison of OSCC and peri-tumour NOM tissues

Both the OSCC and NOM tissues generally showed high levels of MSF staining intensity, especially in the fibroblast and vascular endothelial cells. No significant differences were found in the cellular MSF staining intensities (Fibroblast, Vascular Endothelial Cells, Inflammatory and Epithelial cells) between the OSCC and the NOM sections (summarised in Table 4.10 with statistical analysis shown in Table 4.11). However, data from a larger study in the laboratory compared MSF staining in OSCC and peri-tumour NOM (unpublished data) found significantly higher MSF staining of vascular endothelial cells in OSCC compared to NOM (Chi-squared score $p<0.001$; Fisher's Exact Test $p=0.001$). A marginally significant difference was found in fibroblasts (OSCC>NOM; Chi-squared score $p=0.0434$; Fisher's Exact Test $p=0.0717$) but no significant difference in MSF staining of epithelial cells was seen (Chi-squared score $p=0.682$; Fisher's Exact Test $p=0.999$). This study quantified MSF staining in terms of intensity and area of staining (personal communication: Dr G Ohe).

Table 4.11 Statistical comparison (Fisher's Exact Test p-values) of the MSF staining of the comparable tissues (PG-PDL, OSCC-NOM, paired OSCC-NOM and OSCC-OSCC).

Tissues	Tissue			
	Fibroblasts	**Blood Vessels**	**Inflammatory Cells**	**Epithelial Cells**
PG (13) - PDL (13)	0.0968	0.6951	1	x
OSCC (12) - NOM (5)	0.2941	1	1	0.5928
OSCC (5) - NOM (5)	1	1	1	1
OSCC (12) - OSCC (5)	1	1	1	0.5928

Statistics: positive MSF staining (+/±) versus negative staining (-).

4.4.2.3 Comparison of the PG-PDL and the OSCC-NOM tissues

Although the PG-PDL and OSCC-NOM are not comparable tissues, the difference in MSF expression between the known positive angiogenic control tissues, OSCC-NOM, and the PG-PDL tissues was examined. Significantly higher MSF staining of the fibroblasts (PG<OSCC p=0.0149; PDL<OSCC p=0.0001) and vascular endothelial cells (PG<OSCC p=0.0391; PDL<OSCC p=0.0052) were found in the OSCC compared with the PG and PDL sections (Table 4.12). However, the only significantly difference in the MSF staining of the inflammatory cells was found in the PDL-OSCC sections (PDL<OSCC p=0.0472). Apart from the fibroblast MSF staining (PDL<NOM p=0.022) there were no significant differences found between the peri-tumour NOM and the PG and PDL sections. This may reflect the low number of NOM sections available.

Table 4.12 Statistical comparison (Fisher's Exact Test p-values) of the MSF staining of the PG and PDL sections with the OSCC and NOM angiogenic control sections (PG-OSCC, PDL-OSCC, PG-NOM and PDL-NOM).

	Tissues		
	Fibroblasts	**Blood Vessels**	**Inflammatory Cells**
PG (13) < OSCC (12)	0.0149*	0.0391*	0.1152
PDL (13) < OSCC (12)	0.0001**	0.0052**	0.0472*
PG (13) < NOM (5)	0.596	0.249	0.326
PDL (13) < NOM (5)	0.022*	0.101	0.268

Statistics: positive MSF staining (+/±) versus negative staining (-).
* Significant
** Highly significant

4.4.3 Correlation between MSF staining and vascular indices

MSF staining intensity of the cell types (Fibroblasts, Vascular Endothelial Cells, Inflammatory Cells and Epithelial Cells) for each tissue were examined in relation to the vascular indices (vWF-MVV, vWF-MVD, vWF-h-MVD, CD105-MVV, CD105-MVD and CD105-h-MVD). The results are shown in Table 4.13.

Table 4.13 Correlation between MSF staining and the Vascular Indices (vWF-MVV, vWF-MVD, vWF-h-MVD, CD105-MVV, CD105-MVD and CD105-h-MVD) for (a) Fibroblasts, (b) Blood Vessels, (c) Inflammatory Cells and (d) Epithelial Cells for all tissue groups (Spearman's rank correlation). P-values are shown with Spearman's rho values in brackets.

(a) Fibroblasts

Correlation	All (43)	PG (13)	PDL (13)	OSCC (12)	OSCC (5)	NOM (5)
vWF MVV vs MSF Fibroblasts	0.221 (0.18)	0.953 (0.018)	0.115 (0.458)	0.296 (-0.329)	0.498 (-0.406)	0.866 (0.105)
vWF MVD vs MSF Fibroblasts	0.599 (-0.078)	0.229 (-0.358)	**0.03* (0.599)**	0.208 (-0.392)	0.764 (-0.186)	0.727 (0.216)
vWF h-MVD vs MSF Fibroblasts	**<0.001** (0.621)**	0.484 (-0.213)	**0.05* (0.553)**	0.713 (-0.119)	0.866 (0.105)	0.897 (-0.081)
CD105 MVV vs MSF Fibroblasts	0.187 (0.194)	0.295 (0.315)	0.116 (0.457)	0.927 (-0.03)	0.361 (-0.527)	0.931 (0.054)
CD105 MVD vs MSF Fibroblasts	0.274 (-0.161)	0.425 (-0.242)	**0.041* (0.573)**	0.714 (-0.118)	0.866 (-0.105)	0.965 (0.028)
CD105 h-MVD vs MSF Fibroblasts	**<0.001** (0.669)**	0.995 (-0.002)	0.067 (0.523)	0.646 (0.148)	0.897 (-0.081)	0.668 (-0.264)

(b) Blood Vessel Endothelial Cells

Correlation	All (43)	PG (13)	PDL (13)	OSCC (12)	OSCC (5)	NOM (5)
vWF MVV vs MSF Blood vessels	0.222 (0.179)	0.486 (0.212)	0.423 (-0.243)	0.484 (-0.224)	0.437 (-0.459)	0.182 (0.707)
vWF MVD vs MSF Blood vessels	0.971 (0.005)	0.198 (0.382)	0.984 (-0.006)	0.782 (-0.09)	0.111 (-0.791)	0.165 (0.725)
vWF h-MVD vs MSF Blood vessels	**<0.001** (0.729)**	0.124 (0.449)	0.717 (-0.111)	0.379 (-0.28)	0.118 (-0.783)	0.343 (0.544)
CD105 MVV vs MSF Blood vessels	0.269 (0.163)	0.254 (0.341)	0.505 (-0.203)	0.943 (-0.023)	0.45 (0.447)	0.548 (0.363)
CD105 MVD vs MSF Blood vessels	0.443 (-0.113)	0.224 (0.362)	0.46 (-0.225)	0.504 (0.214)	**0.041* (0.894)**	0.327 (0.559)
CD105 h-MVD vs MSF Blood vessels	**<0.001** (0.727)**	0.07 (0.518)	0.243 (-0.349)	0.962 (-0.015)	0.102 (-0.803)	0.559 (0.354)

(c) Inflammatory Cells

Correlation	All (43)	PG (13)	PDL (13)	OSCC (12)	OSCC (5)	NOM (5)
vWF MVV vs MSF Inflammatory cells	0.110 (-0.234)	0.117 (-0.456)	0.146 (-0.427)	0.15 (-0.442)	0.794 (-0.162)	0.734 (0.211)
vWF MVD vs MSF Inflammatory cells	0.294 (-0.154)	0.167 (-0.408)	0.827 (-0.067)	0.734 (-0.110)	1 (0)	0.498 (0.406)
vWF h-MVD vs MSF Inflammatory cells	0.147 (0.213)	0.109 (-0.466)	0.365 (-0.274)	0.914 (-0.035)	0.8 (-0.158)	0.863 (0.108)
CD105 MVV vs MSF Inflammatory cells	**0.026* (-0.321)**	0.076 (-0.508)	0.095 (-0.482)	0.205 (-0.394)	0.252 (-0.632)	0.467 (-0.433)
CD105 MVD vs MSF Inflammatory cells	0.062 (-0.272)	1.173 (-0.403)	0.476 (-0.217)	0.557 (-0.188)	1 (0)	0.617 (0.306)
CD105 h-MVD vs MSF Inflammatory cells	0.088 (0.249)	0.716 (-0.112)	0.130 (-0.443)	0.343 (0.3)	0.594 (-0.324)	0.361 (-0.527)

(d) Epithelial Cells

Correlation	All (17)	PG (13)	PDL (13)	OSCC (12)	OSCC (5)	NOM (5)
vWF MVV vs MSF Epithelial cells	0.297 (0.233)	x	x	0.873 (0.052)	0.812 (0.148)	0.638 (0.289)
vWF MVD vs MSF Epithelial cells	0.387 (0.194)	x	x	1 (0)	0.495 (0.408)	0.628 (0.296)
vWF h-MVD vs MSF Epithelial cells	0.501 (0.152)	x	x	0.874 (-0.051)	1 (0)	0.812 (0.148)
CD105 MVV vs MSF Epithelial cells	0.333 (-0.217)	x	x	0.184 (-0.411)	0.058 (-0.866)	0.454 (0.444)
CD105 MVD vs MSF Epithelial cells	0.181 (-0.296)	x	x	0.156 (-0.436)	0.638 (-0.289)	0.807 (0.152)
CD105 h-MVD vs MSF Epithelial cells	0.701 (0.087)	x	x	0.937 (-0.026)	0.812 (-0.148)	0.638 (0.289)

* Significant correlation
** Highly significant correlation

4.4.3.1 Fibroblasts

When all tissues were examined highly significant moderate positive correlations were found between Fibroblast MSF staining with both vWF-h-MVD (rho=0.621, p<0.001) and CD105-h-MVD (rho=0.669, p<0.001). PDL Fibroblast MSF staining significantly correlated with vWF-MVD (rho=0.599, p=0.03), vWF-MVD (rho=0.553, p=0.05) and CD105-MVD (rho=0.573, p=0.041). No other significant correlations were found between Fibroblast MSF staining and the vascular indices in the different tissues.

4.4.3.2 Blood vessel endothelial cells

When all tissues were examined highly significant strong positive correlations were found between Vessel Endothelial Cell MSF staining with both vWF-h-MVD (rho=0.729, p<0.001)

and CD105-h-MVD (rho=0.727, p<0.001). Otherwise no significant correlations were found between Endothelial Cell MSF staining and the vascular indices in the different tissues.

4.4.3.3 Inflammatory cells

When all tissue were examined a significant weak negative correlation was found between Inflammatory cell MSF staining and CD105-MVV (rho=-0.321, p=0.026). Otherwise no significant correlations were found between Inflammatory Cell MSF staining and the vascular indices in the different tissues.

4.4.3.4 Epithelial cells

No significant correlations were found between the vascular indices and Epithelial MSF staining either overall or at individual cell type level, although a nearly significant negative correlation was found between CD105-MVV and MSF Epithelial Cell staining (rho=-0.866, p=0.058).

4.4.4 Correlation between inflammatory infiltration and MSF expression

Previous studies on angiogenic factor expression in periapical lesions have directly associated angiogenic factor expression with the extent of inflammatory infiltration (Chapter 4.1.8). The PG and PDL sections were previously graded for inflammatory cell infiltration using calibrated sections (Chapter 4.4.1.5 and Figure 4.10). MSF expression in the different cell types (Fibroblasts, Blood Vessels and Inflammatory cells) was examined in relation to the level of inflammatory cell infiltration in the PG and PDL tissues (Table 4.14). A significant moderate positive correlation was found between PDL-Blood Vessel MSF expression and the level of inflammatory infiltration (rho 0.623, p=0.023). A highly significant moderate positive correlation was found between PDL-Inflammatory Cell MSF expression and the level of inflammatory infiltration (rho 0.696, p=0.008). No other significant or near significant correlations were found.

Table 4.14 Correlation between Inflammatory Infiltration and the MSF Expression (Fibroblasts, Blood Vessel Endothelial cells and Inflammatory cells) in the PG and PDL tissues.

Correlation	All (26)		PG (13)		PDL (13)	
	p-value	rho	p-value	rho	p-value	rho
Inflammatory Index - Fibroblast MSF Staining	0.216	0.251	0.884	0.045	0.492	-0.209
Inflammatory Index - Blood Vessel MSF Staining	0.091	0.338	0.471	0.220	0.023*	0.623
Inflammatory Index - Inflammatory Cell MSF Staining	0.117	0.315	0.741	-0.102	0.008**	0.696

* Significant correlation
** Highly significant correlation

4.5 Discussion

Angiogenesis cannot be measured directly in human lesions, but can be inferred by measuring indices of angiogenesis (e.g. angiogenic factor expression, vascularity) in both the lesion of interest and its normal tissue counterpart. In this study, vascularity and the expression of the angiogenic factor Migration Stimulating Factor (MSF) were assessed in four different types of tissue: periapical granuloma (PG), normal periodontal ligament (PDL), oral squamous cell carcinoma (OSCC), and peri-tumour histologically normal oral mucosa (NOM). Vessels were stained with antibodies to von Willebrand factor (vWF) and CD105 and quantified by three methods that reflect vascular volume and density. These were average-microvascular volume (MVV), average-microvascular density (MVD) and highest-microvascular density (h-MVD). Furthermore, fibroblast, vascular endothelial cell and inflammatory cell MSF expression was assessed in each tissue and epithelial cell MSF expression was also assessed in the OSCC and NOM tissues. Each lesion was compared with its corresponding normal tissue, thus representing two different models (PG-PDL and OSCC-NOM) that can be compared to each other. Two main questions are addressed by this study: (i) is there evidence of angiogenesis, either measured using vascularity and/or by MSF expression, in chronic PG? and (ii) is CD105 a marker of angiogenic vessels?

Vascularity was significantly higher in OSCC than in NOM indicating that angiogenesis accompanies the development of the former. This finding confirms the validity of the OSCC-NOM model as a positive control (Pazouki et al., 1997) and was consistent with the concept of tumour growth being angiogenesis-dependent in order to meet the increased metabolic

requirements of the tumour (Folkman, 1971). In contrast, there was no difference in vascularity between PG and PDL tissues, irrespective of the antibody or methodology used. Therefore, there is no evidence of angiogenesis taking place in the PG-PDL model. This finding was unexpected as it had been previously suggested that the formation of granulomatous lesions is accompanied by angiogenesis (Bragado et al., 1999, Yuan et al., 2000b). Therefore, it was anticipated that a presumably angiogenic PG would have higher vascularity levels than the PDL. Information obtained from tissue sections is applicable to a fixed point in time. The possibility cannot be excluded, therefore, that angiogenesis may have occurred at earlier stages of PG development. This could be examined in animal models, but it is not possible to determine in humans, as only chronic lesions are clinically detectable and accessible for *ex-vivo* experimentation. Nevertheless, chronic oral lesions, such as oral lichen planus (Tao et al., 2007, Mittal et al., 2012, Sheelam et al., 2018), have been found to contain significantly higher vascularity than the corresponding normal tissue. Sampling error is another potential problem, given the heterogeneity of the tissues examined. However, no significant differences in vascularity values were found when up to six replicate sections of PG and PDL specimens were assessed. Furthermore, no significant differences in vascularity were found in the OSCC-NOM model, in spite of the well-known heterogeneity of the tumours.

Various methods have been used to quantify vascularity, the most common being the highest microvascular density (h-MVD) in which only the most vascularised area of the section (hot spot) is assessed (Weidner et al., 1991). Alternative methods involve estimating average-microvascular density (MVD) or volume (MVV) in randomly selected areas of the sections (Chandrachud et al., 1997, Pazouki et al., 1997). Previous findings have demonstrated that different results may be obtained depending on the quantification method used (Pazouki et al., 1997, Schor et al., 1998b, Li et al., 2005). For example, MVV increased significantly, in a step-wise fashion, with disease progression in oral lesions, from normal oral mucosa to dysplastic lesions and carcinomas, whereas h-MVD did not discriminate between dysplasia and carcinomas (Pazouki et al., 1997). Similarly, Li et al. (2005) found that h-MVD in CD31 stained sections could not differentiate between normal oral mucosa and dysplastic lesions,

but did significantly differentiate between normal oral mucosa and oral carcinoma. It is important, therefore, to assess vascularity by more than one method.

The vessels were homogeneously distributed in PG and PDL, and there was no evidence of vascular hot spots. Nevertheless, the highest-MVD, as well as the average-MVD and MVV were assessed in conjunction with vWF and CD105 staining to allow direct comparison between the OSCC-NOM and the PG-PDL models. Confidence in the methodology adopted was demonstrated by: (i) the lack of significant inter- and intra-observer variations, (ii) the significant correlation between h-MVD, MVV and MVD values and (iii) the significant difference between OSCC and NOM tissues. The latter results are in agreement with previous publications using larger numbers of specimens (Pazouki et al., 1997, Schimming and Marme, 2002, Li et al., 2005).

It is widely assumed that angiogenesis is involved in the formation of PG. Evidence supporting this assumption includes the presence of various angiogenic factors in these lesions (Lin et al., 1996, Tyler et al., 1999, Danin et al., 2000, Leonardi et al., 2003, Moldauer et al., 2006, Nonaka et al., 2008, Fonseca-Silva et al., 2012, Andrade et al., 2013, Virtej et al., 2013, Vara et al., 2017, Alvares et al., 2018, Kudo et al., 2018). Since angiogenesis cannot be measured directly in human tissues, expression of such factors is taken as an index of angiogenesis. In the present study, irrespective of the cell type examined, no significant difference in MSF expression was identified between PG and PDL tissues. Overall, MSF expression was weak or negative for all of the cell types examined in the PG and PDL tissues. It had been anticipated that the presumably angiogenic PG would show significantly higher levels of MSF expression than the healthy control PDL. The weak or negative MSF expression found in both the PG and PDL tissues mirrors the lack of evidence of active angiogenesis found in the same sections using the various measures of vascularity. However, it is not possible to rule out that angiogenesis, possibly associated with transiently increased MSF expression, as occurs in wound healing (Picardo et al., 1992), may have occurred at an earlier stage in PG development as only longstanding chronic lesions are clinically detectable in humans.

However, angiogenesis is regulated by a complex network of stimulators and inhibitors, including both soluble factors and insoluble extra-cellular matrix components. The expression of an angiogenic factor, therefore, does not necessarily indicate that angiogenesis has taken place, as the environment may not be permissive for angiogenic activity. In PG, the expression of angiogenic factors has been commonly studied in relation to the level of immune cell infiltration and/or the extent of epithelial proliferation, not to the vascularity of the lesions (Lin et al., 1996, Tyler et al., 1999, Danin et al., 2000, Leonardi et al., 2003, Moldauer et al., 2006, Andrade et al., 2013, Virtej et al., 2013, Alvares et al., 2018). In the present study, no significant correlations were found between MSF expression in the various cell types (Fibroblasts, Vascular Endothelial Cells and Inflammatory Cells) with the extent of the inflammatory infiltration of the PG. Significant correlations were found between the extent of inflammatory cell infiltration in PDL with both the Blood Vessel MSF expression and the Inflammatory Cell MSF expression. This suggests that although there was both little MSF expression and inflammatory cell infiltration in the majority of the PDL tissues, those sections which did have increased inflammatory cell infiltration were associated with raised MSF expression in both the vascular endothelial cells and in the inflammatory cells. This could suggest that MSF expression may be raised in the early stages of periodontal ligament inflammation.

Significant correlations were found between MSF expression of PDL fibroblasts with the measures of vessel density (MVD and h-MVD) with both markers (vWF and CD105). This suggests that although the MSF expression of the PDL fibroblast was generally negative, those sections which contained areas of increased vascular density, vascular hot spots, were associated with increased MSF expression in the PDL fibroblasts. It has been previously reported that gingival and periodontal fibroblasts exhibit foetal-like characteristics (Irwin et al., 1994, Moxham and Grant, 1995), with some gingival fibroblasts subpopulations producing detectable levels of MSF, while skin fibroblasts do not produce MSF (Irwin et al., 1994, Schor et al., 1988a). Therefore, the most likely source of MSF within the PDL is from the PDL fibroblasts and if MSF does play a significant role in angiogenesis within the PDL, it would be expected that MSF expression in the PDL fibroblasts would be increased in areas of high vascular density. However, further research is required to clarify this hypothesis.

No other significant correlations were found between the cellular MSF expression of the individual cell types with the measures of vascularity in the different tissues. When cellular MSF expression of the individual cell types (fibroblasts, vascular endothelial cells, inflammatory cells and epithelial cells) were examined in all of the tissues combined, significant correlations were found with some of the vascular indices (Table 4.13). However, these apparent correlations are likely to be simply due to statistical anomalies.

Quantification of vascularity represents another index of angiogenesis. Nokana et al.(2008) examined the differences in vascularity (using Microvessel Count (MVC): a vascular index not directly comparable with the vascular indices used in the present study) in PG, radicular and residual radicular cysts in comparison with the level of VEGF expression and the extent of the inflammatory infiltration. However, the vascularity of these tissues was not compared with the appropriate healthy tissue, the periodontal ligament, and so angiogenesis was assumed to have occurred due to the high levels of expression of VEGF in these tissues. The argument applies to the other similar studies which examined vascularity in PG (Lima et al., 2011, Fonseca-Silva et al., 2012, Vara et al., 2017). In all cases, it is important to test the robustness of an angiogenic index by comparing an assumed angiogenic tissue with its closest non-angiogenic counterpart. For example, expression of VEGF, a potent angiogenic factor, was found to be similar or higher in NOM than in OSCC, therefore it cannot be taken as a reliable index of angiogenesis in OSCC (Baillie et al., 2001b). This finding may be due to the presence of both pro-angiogenic and anti-angiogenic splice variants of VEGF, which cannot be distinguished by previously available antibodies (Ladomery et al., 2007). In contrast, vascularity appears to represent a good index of angiogenesis in oral tissues (Pazouki et al., 1997, Schimming and Marme, 2002, Li et al., 2005). It should be noted that, when using pan-endothelial markers, not all tumours have higher vascularity than the normal tissue from which they originate (Schor et al., 1998a, Schor et al., 1998b). In such cases it is not possible to determine whether angiogenesis has taken place.

Interestingly, in the present study, high levels of MSF expression were found in both the OSCC and NOM tissues, with no significant differences found in the cellular MSF staining intensities (Fibroblast, Vascular Endothelial Cells, Inflammatory and Epithelial Cells)

between the OSCC and the NOM sections. Although the PG-PDL and OSCC-NOM are not directly comparable tissues, significantly higher MSF staining was found in the OSCC compared with the PG and PDL sections. It was anticipated that MSF expression in the OSCC-NOM would mirror the differences in vascularity, with significantly higher MSF expression in the OSCC compared with the NOM. A possible explanation for the lack of significant difference in the MSF expression found in the OSCC-NOM model used in this study could be due to field change/cancerisation effects on the seemingly histologically normal mucosa adjacent to OSCC lesions. Several studies have shown peri-tumour NOM to have raised or significantly higher vascularity than true healthy NOM and so peri-tumour NOM cannot be regarded as being a truly normal tissue (Carlile et al., 2001, El-Gazzar et al., 2005a, Margaritescu et al., 2010). Likewise, it could be postulated that the expression of angiogenic factors, such as MSF, could be similarly increased in the peri-tumour NOM compared to true NOM. An alternative hypotheses for why the high level of MSF expression in the NOM did not result in angiogenic activity could be due to either some other cofactor not being present, such as another angiogenic factor or an extracellular matrix component, or due to the presence of an MSF inhibitor. A functional MSF inhibitor (MSFI), neutrophil gelatinase-associated lipocalin (NGAL), has been identified, although the exact mechanism for how it inhibits MSF has still to be identified (Jones et al., 2007).

However, data from a larger study carried out in the laboratory which specifically studied MSF staining in OSCC and peri-tumour NOM (unpublished data) found significantly higher vascular and fibroblast MSF expression in OSCC compared to NOM (personal communication: Dr G Ohe). The inconsistency between this study and the present study probably reflects the different number of OSCC-NOM sections assessed and the different methodologies used to quantify the MSF staining intensity. Therefore, the lack of significant difference found in the MSF expression between the OSCC and NOM tissues in the present study may simply be due to insufficient number of NOM specimens examined.

Pan-endothelial markers such as CD34, CD31 and vWF do not distinguish between mature and newly formed (or angiogenic) vessels. Furthermore, it has been reported that such markers react strongly with endothelial cells in large blood vessels but their expression is

weak or absent in microvessels in most normal tissue and in many tumours (Stashenko et al., 1994). Although no evidence was found in the present study of vessels not expressing vWF, the use of a marker specific for angiogenic vessels would be preferable to provide an accurate measure of angiogenesis. CD105 (endoglin) has been reported to be such a marker, being selectively expressed by proliferating endothelial cells *in vitro* and angiogenic vessels in tumours (Burrows et al., 1995, Nassiri et al., 2011), including OSCC (Schimming and Marme, 2002, Schimming et al., 2004, Margaritescu et al., 2010, Nair et al., 2016, Patil et al., 2018, Silva et al., 2018). Furthermore, high expression of CD105 in tumours has been associated with poor prognosis, being more informative than pan-endothelial markers (Kumar et al., 1999, Tanaka et al., 2001, Chien et al., 2006, Kyzas et al., 2006).

Unexpectedly, CD105 stained vessel endothelial cells in a similar fashion to vWF in PG and PDL and did not provide any additional information in these tissues. This differs from Lima et al. (2011) who reported significantly higher CD34 vascular staining compared with CD105 in PG and periapical cysts. They concluded that the differential staining between the two vascular markers provided evidence of angiogenesis taking place, probably resulting from hypoxic conditions in the periapical lesions leading to raised HIF-1 expression. However, as with the other studies which have examined vascularity in periapical lesions, healthy PDL was not used as a control.

In the OSCC-NOM model used in the present study, staining with CD105 added very limited value to staining with vWF. These results agree with those of Balza et al.(2001) who found CD105-positive vessels in a variety of normal (non-oral) human tissues. CD105 has been previously shown to stain vessels in periapical granulation tissue, although no attempt was made to quantify or compare this with other tissues (Tasman et al., 2000). In NOM, CD105 expression was weaker than in OSCC regarding intensity of staining. When expressed as a percentage of vWF-positive vessels, CD105-positive vessels were also significantly lower in NOM than in OSCC when measured by h-MVD, but not by MVV or MVD. Otherwise, there were no significant differences in the four tissues examined, although they tended to be similarly high in PG, PDL and OSCC and lower in NOM. The hypothesis that CD105 is a specific marker for angiogenic vessels has not been proved. If this hypothesis were correct,

the present results would indicate that angiogenesis is taking place in all the four tissues examined, at similar high levels in OSCC, PG and PDL and at lower levels in NOM. However, an alternative hypothesis can be proposed, namely that CD105 may be associated with high tissue turnover or some other intrinsic characteristic of these tissues, rather than with the formation of new blood vessels.

To date, although there have been a significant number of studies which have detected angiogenic factor expression in PG, there have been very few studies which have examined vascularity as a marker of angiogenesis. Unfortunately, there are difficulties in relating the findings of these studies due to disparities in the methodologies used, such as different vascular markers (vWF, CD31, CD34 or CD105), vascular and inflammatory indices, and the number of assessors. The unique feature regarding the present study was the inclusion of PDL as a tissue appropriate control to confirm whether or not angiogenesis had taken place. A further potential issue is that PG are assumed to be static lesions, but there is the possibility that some PG may be progressing towards periapical cyst formation. Further studies could include periapical cysts, in addition to the PDL and PG, to examine changes in vascularity. Epithelial and inflammatory markers may to help ascertain whether the PG are static or are likely to have been in the process of developing into periapical cysts.

Chapter 5

General Discussion, Conclusions and Further Investigations

5.1 General Discussion

In this thesis, a comparison of a number of angiogenic factors involved in the regulation of oral wound healing and the use of vascularity as an index of angiogenesis have been explored. Oral soft tissues are known to display features of enhanced wound healing, in comparison to dermal tissue, with reduced healing times and minimal scarring, despite the high microbial load and a wet environment (Sciubba et al., 1978, Schor et al., 1996, Szpaderska et al., 2003, Wong et al., 2009). Furthermore, oral wound healing is associated with reduced inflammation, faster re-epithelisation time and less angiogenic activity compared with dermal healing (Szpaderska et al., 2003, Szpaderska et al., 2005, DiPietro and Schrementi, 2018). Therefore, the study of oral wound healing may provide insight into improved wound care in general resulting in reduced scarring.

As discussed in Chapter 1.4, several theories have been proposed to account for the enhanced wound healing ability of oral tissues including angiogenic factors in saliva and factors intrinsic to oral soft tissues, including foetal-like fibroblast populations and high stem cell activity (Polverini, 1995, Schor et al., 1996, Izumi et al., 2007, Chen et al., 2010, Zhang et al., 2012, DiPietro and Schrementi, 2018, desJardins-Park et al., 2019). There is strong evidence that the angiogenic factor content of saliva is important in oral wound healing and maintenance of the upper gastro-intestinal tract (Zelles et al., 1995), as demonstrated by the studies by Bodner's group in rodent models (Chapter 1.5) (Bodner, 1991, Bodner et al., 1991a, Bodner et al., 1991b, Bodner et al., 1992, Dayan et al., 1992, Bodner et al. 1993, Bodner and Dayan, 1995). Furthermore, a study in mice by Noguchi et al. (1991) reported the addition of Epidermal Growth Factor (EGF) restored the healing capacity of incisional wounds on the tongue to normal following removal of the submandibular glands. However, the presence of saliva does not entirely account for all aspects of enhanced oral wound healing (Szpaderska et al., 2003, DiPietro and Schrementi, 2018). Therefore, it is likely that a number of intrinsic factors inherent to the oral soft tissues are also likely to be involved, although the exact mechanisms are poorly understood.

Wound healing is a complex process involving specific time critical interactions between various soluble mediators, blood cells, parenchymal cells and the extracellular matrix (ECM) (Gurtner et al., 2008). Angiogenesis is a key stage in oral wound healing and its disruption

(deficient or excessive) leads to delayed and ineffective healing (DiPietro, 2016). Angiogenic factors from both the systemic system (i.e. in serum) and those contained in saliva are likely to be involved in oral soft tissue healing. Although there is strong evidence that the angiogenic factor content of saliva is likely to contribute to enhanced oral wound healing, little is known regarding the relative concentrations of pro-angiogenic factors and inhibitors in saliva and how they compare with systemic levels. In this thesis, the concentration of the pro-angiogenic factors Ang-1, MSF and VEGF, in relation to the angiogenic inhibitor endostatin, have been assessed in serum and saliva. The broad aims of this aspect of the thesis are to investigate whether: (i) serum and salivary concentrations of the pro-angiogenic factors Ang-1, MSF and VEGF and the inhibitor endostatin are significantly different between periodontal health and severe periodontitis; (ii) serum and salivary concentrations of these factors are significantly altered in smokers and in subjects with diabetes compared with matched healthy controls.

Few studies have examined the levels of angiogenic factors in both unstimulated and stimulated whole saliva. The majority of the time the oral cavity is bathed in unstimulated saliva, mostly derived from the submandibular glands. Stimulated saliva differs from unstimulated saliva, both in terms of its lower viscosity, being mainly derived from the parotid glands and is mainly produced during mastication. The angiogenic factor content of stimulated saliva is also likely to have a role in oral wound healing, especially in the time period shortly following injury. As far as it can be ascertained, this is the first study to report concentrations of Angiopoietin-1 (Ang-1) and the anti-angiogenic factor Endostatin in saliva.

In the second aspect of this thesis, an immunohistochemistry study was carried out to examine whether various measures of vascularity can be used as direct evidence of angiogenesis taking place in periapical granulomas (PG) in comparison to healthy periodontal ligament (PDL). The broad aims were to: (i) quantify vascularity in PG using different endothelial markers and assess its value as an index of angiogenesis by comparing PG with healthy PDL; (ii) quantify MSF expression in PG and healthy PDL and investigate whether there is a correlation between MSF expression and vascularity.

Historically, the oral cavity has been regarded as being isolated from the systemic system, however, it is known that patients with generalised deep periodontal pocketing have large areas of ulcerated pocket walls potentially open to the systemic circulation (Offenbacher et al., 1996, Hujoel et al., 2001, Park et al., 2017). In essence, immediately adjacent to the ulcerated periodontal pocket walls is a large chronically inflamed soft tissue wound, i.e. the underlying periodontal connective tissue. Due to the highly vascular nature of the inflamed periodontal soft tissues, various inflammatory mediators, growth/angiogenic factors and bacteria can readily enter the systemic circulation. Currently there is great interest regarding the relationship between periodontal disease and systemic health, and there is increasing evidence that periodontal inflammation may contribute to systemic inflammation (D'Aiuto et al., 2013, Pink et al., 2015). For example, several studies have associated periodontitis with increased systemic levels of factors including C-reactive protein (CRP), IL-1β, IL-2, TNFα and IFN-γ (Noack et al., 2001, Gorska et al., 2003, Paraskevas et al., 2008) (Chapter 1.6.4). Therefore, systemic over spill from periodontal inflammation may acerbate various chronic systemic diseases, which are also known to have altered angiogenic-mediated responses, such as diabetes mellitus (Khader et al., 2006, Nascimento et al., 2018), cardiovascular disease (Genco et al., 2002, Janket et al., 2003, Bahekar et al., 2007, Carrizales-Sepulveda et al., 2018) and rheumatoid arthritis (Kaur et al., 2013, Fuggle et al., 2016).

In this thesis, each study group was sub-divided into periodontally healthy subjects (sub-group A) and those with severe periodontitis (sub-group B) to ascertain whether periodontal inflammation (periodontitis) resulted in significant changes in the serum and salivary concentrations of each angiogenic factor. Unexpectedly, no evidence was found that severe periodontitis resulted in significant changes to serum or salivary levels of Ang-1, MSF, VEGF or endostatin in comparison to periodontal health. The only exception was with the unstimulated salivary concentrations of VEGF, which were found to be marginal significantly higher in severe periodontitis ($p=0.052$), once outliers had been removed.

It had been anticipated that periodontal inflammation, associated with severe periodontitis, would result in the increased release of pro-angiogenic factors, such as Ang-1, MSF and VEGF, which would be reflected in significantly higher concentrations in serum and saliva. This hypothesis is based on several studies which have reported significantly increased

VEGF levels in serum (Pradeep et al., 2011, Turer et al., 2017), GCF (Booth et al., 1998, Lee et al., 2003, Guneri et al., 2004, Prapulla et al., 2007, Pradeep et al., 2011, Padma et al., 2014, Sakallioglu et al., 2015, Zhu et al., 2015, Pannicker and Mehta, 2016, Turer et al., 2017, Romano et al., 2018, Afacan et al., 2019, Tayman et al., 2019) and in unstimulated saliva (Booth et al., 1998) in periodontitis compared to periodontal health. Furthermore, studies have demonstrated that the reduction in periodontal inflammation following successful non-surgical periodontal therapy is associated with a significant reduction in serum VEGF levels (Pradeep et al., 2011, Turer et al., 2017). These findings support the view that periodontal inflammation could have a negative impact on systemic health and successful periodontal therapy may have some degree of systemic benefits. However, several studies have either reported no significant difference in serum VEGF concentrations in periodontitis compared to periodontal health (Widen et al., 2016, Tayman et al., 2019) or even reduced concentrations (Zhu et al., 2015). Furthermore, there is little data in the literature to indicate whether levels of angiogenic inhibitors, such as endostatin, are significantly changed in severe periodontitis compared with periodontal health. Therefore, the true relationship between periodontal and systemic health still requires further research.

In the present study, VEGF concentration in unstimulated saliva was found to be marginally significantly higher in severe periodontitis compared with healthy controls. This finding was consistent with two previous studies (Booth et al., 1998, Wu et al., 2018), with Wu et al. (2018) also reporting a non-significant increase in VEGF levels in unstimulated saliva in periodontitis. Booth et al. (1998) hypothesised that raised levels of VEGF produced in periodontal inflammation overspilled into the saliva via the GCF. This hypothesis has been supported by subsequent evidence from immunohistochemistry studies which reported increased VEGF and VEGFR2 expression in periodontitis, (Johnson et al., 1999a, Artese et al., 2010, Vladau et al., 2016). Currently, there is insufficient evidence from the data from this thesis or the literature to extend the assumption that all pro-angiogenic factors, such as Ang-1 and MSF concentrations in serum and saliva are raised in periodontitis. In fact, an ELISA study by Lester et al. (2009), which examined a range of inflammatory mediators and angiogenic factors in gingival tissue from extracted teeth, reported that tissue level Ang-1 concentrations were significantly lower in severe periodontitis compared with periodontal health. As expected, the concentrations of other factors, such as VEGF, were found to be

significantly raised in severe periodontitis. This group hypothesised that the reduced Ang-1 levels in periodontal disease promoted inflammation through reduced inhibition of VEGF and endothelin. Therefore, it is likely that there is a dynamic and time-related relationship between the concentrations of various pro-angiogenic factors in periodontal disease, which is influenced by the inflammatory state, the nature of the underlying extracellular matrix and the presence of inhibitors, such as endostatin.

Like diabetes, there is substantial evidence that smoking is a major risk factor for both reduced wound healing capacity (Silverstein, 1992, Towler, 2000) and increased risk of periodontal disease (Bergstrom, 1989, Grossi et al., 1994, Tomar and Asma, 2000, Bergstrom, 2003, Zeng et al., 2014) (Chapter 1.7). Smoking results in changes to the regulation of the periodontal vasculature (Chapter 1.7.3.3) (Bergstrom and Preber, 1986, Bergstrom et al., 1988, Mirbod et al., 2001, Scardina and Messina, 2005) and reduced periodontal tissue inflammation (Chapter 1.7.3.1) (Pauletto et al., 2000, Rezavandi et al., 2002) resulting in reduced bleeding on probing (Bergstrom and Floderus-Myrhed, 1983, Preber and Bergstrom, 1985, Dietrich et al., 2004). Furthermore, smoking has been shown to be associated with impairment of both gingival and periodontal ligament fibroblast function and has toxic effects on endothelial cell function (Silverstein, 1992, Tipton and Dabbous, 1995, Tanur et al., 2000). Although nicotine itself has been shown to have angiogenic characteristics, overall the multiple toxic constituents within smoke inhibit angiogenesis (Buduneli and Scott, 2018). Evidence of the inhibitory effect of smoking on angiogenesis in periodontal tissues has been suggested by the lack of increased gingival vascularity in smokers with periodontitis compared with non-smokers (Rezavandi et al., 2002) (Chapter 1.7.3.3). Contrary to the findings of these vascularity studies, significantly raised concentrations of VEGF in serum (Kimura et al., 2007, Ugur et al., 2018) and FGF-2 in unstimulated whole saliva (Ishizaki et al., 2000) have been reported in smokers compared with non-smokers.

In the present study, there were no significant changes in serum concentrations of Ang-1, MSF or VEGF in smokers compared with healthy never-smoking controls. However, there were indications that smoking may result in reduced angiogenic factor levels in periodontal health in both serum and saliva (non-significant). Interestingly, a significant reduction in

serum endostatin concentrations was found in the smokers. This finding differs from previous studies which reported no significant difference in serum endostatin concentrations between smoking and non-smokers (Iribarren et al., 2006, Liu et al., 2015). Furthermore, when the relative levels of the angiogenic factors (Ang-1, MSF and VEGF) were compared with endostatin, which may give an indication of the angiogenic conditions, the only significant finding was the relative Ang-1 to endostatin concentration was significantly raised in the smoking group. A possible explanation for these findings could be that the subjects in this study were generally not undergoing any significant systemic angiogenic events at the time of sampling, including the possibility that any angiogenic changes associated with severe periodontitis may have reached a chronic homeostatic stage and were not significantly affecting the systemic levels of these factors. Therefore, the relative levels of systemic endostatin to VEGF and MSF could have reached low maintenance levels, effectively turning off the angiogenic switch. The reduced systemic levels of endostatin and increased Ang-1-endostatin ratio found in this study, appears to be counter-intuitive in terms of the reported suppressed vascular reaction in the inflamed gingival tissues of smokers. However, the overall increase in the number of small calibre and tortuous nature of the gingival vessels in smokers may indicate suppressed vessel development (Mirbod et al., 2001, Scardina and Messina, 2005). A possible explanation for this could be due to the reduced inflammatory cell infiltration into the gingival tissues in smokers (Pauletto et al., 2000, Rezavandi et al., 2002). This would lead to lower levels of pro-inflammatory mediators and angiogenic factors, such as VEGF, being released from the inflammatory cells locally into the gingival tissue. Evidence for reduced angiogenic activity due to reduced PMN-induced VEGF activity has been reported in a study in mice (Hao et al., 2007). These additional angiogenic factors released locally within the gingival tissue may be required for continued vascular development. Essentially, the results of the present study provide further evidence that smoking leads to dysfunctional regulation of angiogenesis, which may contribute to increased risk of periodontal disease progression and reduced oral wound healing.

Altered angiogenesis is widely thought to be one of the mechanisms by which chronic hyperglycaemia in diabetes results in poor wound healing (Chapter 1.8.3.3) (Goodson and Hung, 1977, Bohlen and Niggl, 1979, Rasul et al., 2012). In the present study, no significant

differences were found in serum concentrations of Ang-1, MSF, VEGF or endostatin between the diabetes group and healthy controls, which is consistent for previous studies regarding serum concentrations of Ang-1 and VEGF (Gui et al., 2013, Zeng et al., 2013, Yeboah et al., 2016). However, several studies have reported significantly raised serum VEGF levels in Type 2 diabetes in comparison with healthy controls (Ozturk et al., 2009, Mahdy and Nada, 2011, Shao et al., 2016, Nalini et al., 2017). Potential reasons for the difference in the findings in the VEGF levels may relate to the size of the study cohorts, differences in glycaemic control, genetic factors, age-related factors, co-morbidity, poly-pharmacy and differences in laboratory protocols.

Interestingly in the present study, once an extreme outlier was removed from the dataset, serum Ang-1 concentration was found to be significantly lower in the diabetes group compared with the healthy controls. If this is the case, a possible hypothesis for this outcome could be related to Ang-1 being an angiogenic factor principally involved in the later stages of angiogenesis and in vessel maintenance in quiescent vessels (Chapter 1.5.3.2). Therefore, as diabetes is associated with prolonged and dysfunctional angiogenesis, associated with exacerbated inflammation, it would be expected that there would be a low requirement for Ang-1. Alternatively, systemic Ang-1 concentrations may be unchanged compared to healthy controls, but is reduced in relation to other factors, such as Ang-2 and VEGF, systemic levels of which have been shown to be raised Type 2 diabetes (Lim et al., 2005). Ang-2 is thought to be a competitive inhibitor for Ang-1, through binding to the Tie2 receptor, and is important in vessel disruption required early in angiogenesis (Maisonpierre et al., 1997, Gale et al., 2002, Bogdanovic et al., 2006). Indeed, increased ratios of Ang-1 to Ang-2 have been reported in subjects with diabetes, compared with healthy controls, in plasma (Lim et al., 2004, Lim et al., 2005, Yeboah et al., 2016) and in serum (Chen et al., 2015, Li et al., 2015). Similar findings have been reported in diabetic mouse wound healing models, where diabetic wounds are associated with prolonged periods of raised Ang-1 to Ang-2 ratios, suppressed levels of VEGF and reduced endothelial cell numbers (Kampfer et al., 2001). Similar studies have also shown that therapeutic use of Ang-1 results in enhanced wound healing associated with faster re-epithelialisation, increased levels of angiogenesis and blood flow (Cho et al., 2006, Balaji et al., 2015). This raises the possibility of using

Ang-1 to promote wound healing in patients with diabetes, although there are currently concerns regarding potential side effects and the risk of promoting tumour progression (Koh, 2013).

The Ang-1 and VEGF serum concentrations in the diabetes group, in the present study, did not reflect the corresponding salivary concentrations. Unfortunately, it was not possible to quantify salivary concentrations of MSF and endostatin. Although no significant differences were found in salivary concentration of Ang-1, significantly raised salivary VEGF concentrations (stimulated and unstimulated saliva) were found in the diabetes group. This finding is consistent with other studies, which have reported significantly raised VEGF concentrations in unstimulated whole saliva in subjects with diabetes in pregnancy (Surdacka et al., 2011) and older subjects with denture stomatitis (Radovic et al., 2014). However, the present study is the first to report raised VEGF concentrations in both unstimulated and stimulated saliva in diabetes.

Currently, there have been surprisingly few studies which have examined angiogenic factor levels in human saliva in diabetes. Significant raised salivary concentrations of epidermal growth factor (EGF) and transforming growth factor-beta1 (TGF-β1) have been reported in subjects with diabetes (Astaneie et al., 2005, Bernardi et al., 2018), although Oxford et al. (2000) reported significantly reduced salivary concentrations of EGF in diabetes. Whilst a recent study reported no significant differences in salivary concentrations of nerve growth factor (NGF) in diabetes (Tvarijonaviciute et al., 2017). Therefore, work is required to ascertain to what extent the salivary angiogenic profile is altered in diabetes. Another consideration is whether the reduced salivary flow in diabetes (Lopez-Pintor et al., 2016), which could influence salivary concentrations levels, and other confounding factors, such as side effects of medication, account for any differences found.

Another risk factor associated with reduced healing capacity is age (Guo and Dipietro, 2010), although in healthy, older subjects this manifests as delayed healing without affecting the quality of healing (Gosain and DiPietro, 2004). However, medical conditions and medication are more likely to negatively affect oral wound healing in older subjects than age itself. One possible reason for delayed oral wound healing in older people could be due to changes to

the angiogenic content of saliva, in addition to reduced flow rates associated with advanced age and medication (Affoo et al., 2015). Age related changes in salivary concentrations of angiogenic factors have previously been reported, for example, stimulated saliva levels of FGF-2 were found to be significantly higher in non-smoking young individuals compared to middle-aged, who in turn had significantly higher levels than the elderly (Westermark et al., 2002). A significant but weak negative correlation with NGF and age in stimulated saliva has also been reported (Nam et al., 2007). In the present study, significant positive correlations were found between age and salivary concentrations of Ang-1 (unstimulated) and VEGF (both stimulated and unstimulated). A similar finding was previously reported in a small study which examined unstimulated saliva concentration in VEGF (Upile et al., 2009). With regards to VEGF, it has been shown that it is produced in both major and minor salivary glands (Taichman et al., 1998). Evidence from a small immunohistochemistry study found VEGF expression in human minor salivary glands was not significantly affected with increasing age (de Oliveira et al., 2002). Currently, there are no studies which provide evidence to whether Ang-1 is produced in healthy human salivary glands, if so, whether its production is influenced by increasing age. With regards to the findings of the present study, one possibility is that the increased unstimulated saliva concentrations of Ang-1 and VEGF may reflect the reduced submandibular and sublingual salivary flow rates associated with old age (Affoo et al., 2015). However, this does not account for the raised concentrations of VEGF in stimulated saliva with age as evidence shows that stimulated saliva production is not significantly reduced with increasing age. A possible explanation is that the diabetes study group accounts for a larger proportion of the older subjects in the study, who may also be on multiple medications, which could reduce the saliva flow and hence increase the Ang-1 and VEGF concentrations in saliva. Furthermore, other factors which potentially affect saliva flow could also influenced the results such as depression, stress and anxiety which were not formally taken into account in the study criteria (Bergdahl and Bergdahl, 2000, Tan et al., 2018). Further work could assess salivary flow rates and total protein levels to allow these factors to be taken into account when assessing the concentrations of angiogenic factors in saliva.

Angiogenesis is a complex and dynamic process influenced by the presence of angiogenic factors, inhibitors and by the context of the underlying extracellular matrix. A common

assumption is that the detection of angiogenic factors, in itself, provides evidence of angiogenesis taking place at that time. Angiogenesis cannot be measured directly in human lesions, but can be inferred by measuring indices of angiogenesis (e.g. angiogenic factor expression and vascularity) in both the lesion of interest and its normal tissue counterpart (Davey et al., 2008). PGs are chronic inflammatory lesions, partially derived from the PDL, and in common with other oral granulomatous lesions, such as pyogenic granulomas, it is widely presumed that the PG capillary networks arise by angiogenesis (Bragado et al., 1999, Yuan et al., 2000b, Freitas et al., 2005, Seyedmajidi et al., 2015). Several studies have reported angiogenic factor expression in PG, often in comparison with other periapical lesions such as periapical cysts, but not with the healthy PDL, including FGF-2, TNF-α, TGF-α, TGF-β_1 and VEGF, as well as Epidermal Growth Factor Receptor (EGFR) (Lin et al., 1996, Tyler et al., 1999, Danin et al., 2000, Leonardi et al., 2003, Moldauer et al., 2006, Nonaka et al., 2008, Andrade et al., 2013, Virtej et al., 2013, Fonseca-Silva et al., 2012, Vara et al., 2017, Alvares et al., 2018, Kudo et al., 2018).

In this thesis, three different vascular indices (MVV, a-MVD and h-MVD) were used to investigate whether the combination of PG along with its healthy counterpart, the PDL, could provide direct evidence of angiogenic activity in the same way as the established NOM-OSCC (positive control) model (Davey et al., 2008). However, no significant differences in vascularity and similarly low levels of expression of MSF were found in PG and PDL tissues, suggesting that little angiogenic activity was taking place in the PG lesions at the time of biopsy. In contrast, significantly higher vascularity indices were found in OSCC compared to NOM, indicating that angiogenesis had occurred in the development of the OSCC. Interestingly, high levels of MSF staining were found in both OSCC and NOM tissues, probably due to field cancerisation/change effects in the seemingly histologically normal oral mucosa adjacent to OSCC. Evidence from several studies suggest that peri-tumour NOM does not always reflect the findings in true healthy oral mucosa, for example, peri-tumour NOM from oral cancers has been reported to have raised or significantly higher vascularity than healthy oral mucosa (Carlile et al., 2001, El-Gazzar et al., 2005a, Margaritescu et al., 2010).

A further element of this study was the use of CD105 (endoglin), which had been reported to be a specific angiogenic vessel marker in tumours (Burrows et al., 1995, Duff et al., 2003, Nassiri et al., 2011), including OSCC (Schimming and Marme, 2002, Schimming et al., 2004, Margaritescu et al., 2010, Nair et al., 2016, Patil et al., 2018, Silva et al., 2018). Furthermore, high expression of CD105 in tumours has been associated with poor prognosis, being more informative than pan-endothelial markers (Kumar et al., 1999, Tanaka et al., 2001, Chien et al., 2006, Kyzas et al., 2006). CD105 expression has previously been reported in periapical lesions (Tasman et al., 2000, Davey et al., 2008, Lima et al., 2011, Estrela et al., 2019), with Davey et al. (2008), the publication relating to this thesis, being the first to quantify angiogenic activity using CD105 expression in periapical granulomas (PG).

In the present study, the staining profile of CD105 in PG-PDL tissues was very similar to the pan-endothelial marker von Willebrand Factor (vWF) suggesting that either there was high angiogenic activity in both the PG and PDL tissues or that CD105 does not act as a specific angiogenic marker in these tissues. This finding is consistent with Balza et al. (2001) who reported positive CD105 vascular staining in various types of normal human tissue. This is in contrast to Lima et al. (2011) who reported significantly higher CD34, another pan-endothelial marker, vascular staining compared with CD105 in PG and periapical cysts. They concluded that the differential staining between the two vascular markers provided evidence that angiogenesis had taken place, despite the fact there was no differences in the vascular indices used (h-MVD and microvascular area) and healthy PDL had not been used as a control tissue. Therefore, there is some dubiety as to whether CD105 is a true angiogenic marker in PGs and further work is required. A possible hypothesis to explain the findings of the present study is that CD105 staining is associated with high tissue turnover or some other intrinsic characteristic of PGs rather than angiogenic activity (Davey et al., 2008).

A possible explanation for the low angiogenic activity found in the present study could be due to PG being chronic static lesions and angiogenesis had occurred at an earlier stage in their formation. Whether angiogenesis is reactivated in PG which subsequently progress to form periodontal cysts, has yet to be ascertained. In reality, such developmental studies could only be carried out in animal models where periapical lesions can be induced. But, the findings of this thesis raises the question as to whether the expression of angiogenic factors

in periapical lesions alone, which is commonly used in the literature, is sufficient evidence of angiogenic activity. For example, the expression of angiogenic factors may be counter-balanced by angiogenic inhibitors or the context of the ECM.

Overall, the protocols and strict patient selection criteria used in this thesis have attempted to counteract issues such as previous smoking and diurnal variation when collecting saliva samples, which were not accounted for in several papers in the literature. However, several issues and limitations were encountered in this thesis. With regards to limitations of the methodologies used in this thesis, perhaps the most significant was the assumption that the detection of various angiogenic factors, either using ELISAs or by immunohistochemistry methods, indicates that angiogenesis is taking place. This is mainly due to the complex time-critical and environment-related nature of angiogenic activity, where the relative levels of angiogenic factors, presence of inhibitory factors and the nature of the extracellular matrix could have an impact on angiogenic activity. As shown in this thesis, the use of vascular indices in immunohistochemistry studies can be used, in conjunction with angiogenic factor expression, to provide direct evidence of angiogenesis. However, it would be extremely difficult to use such techniques directly in relation to ELISA studies using clinical samples.

A logistical limitation encountered during this study was the time taken to recruit sufficient numbers of subjects, approximately 2 years, taking into account the strict selection criteria (Chapter 2.1.1, 2.1.2 and 2.1.3) and the requirements of the ethics committee approval. Furthermore, difficulty was encountered in engaging colleagues to help recruit patients, which compounded the time taken to collect the clinical samples. Therefore, it was necessary to store samples in non-stick Eppendorf tubes at -80^{o}C for a significant period of time prior to assaying. Although, there was no evidence of significant degradation of factor levels during storage, it cannot be ruled out that this may have taken place to a certain extent. Another issue related to the recruitment was the initial intention to have sufficient numbers of subjects with diabetes to allow statistical analysis both in terms of the entire diabetes group and in terms of Type 1 and Type 2 diabetes. Unfortunately, only a very small number of Type 1 diabetics were recruited, so statistical analysis could only be performed on the entire diabetes group. In future studies, measures would be taken to have better staff engagement in recruitment and perhaps in taking the clinical samples. Although, calibration procedures

would need to be implemented to ensure consistency in approach, especially regarding the recording of the periodontal clinical data.

Another consequence of the difficulties in recruiting subjects for this study, was the uneven size of the study groups. For example, the periodontally healthy control sub-group was significantly larger than the other sub-groups due to the availability of these subjects within the department. However, the size of the study sub-groups were all above the sample sizes required by the power calculation to allow statistical analysis (Chapter 2.1.4). As far as possible factors such as age and gender were accounted for during recruitment, however, there was a bias for older men (>60 years old) in the diabetes group, due to the nature of the condition. Furthermore, due to the demographics of the local population the majority of subjects were white Scottish, with few subjects from ethnic minorities. Studies have suggested the ethnic/genetic variation may affect systemic angiogenic factors levels, for example, systemic levels of angiogenin were found to be significantly higher in healthy subjects of black or South Asian origin compared with white Europeans (Bennett et al., 2013). No significant differences were reported in a similar study for systemic levels of VEGF, Ang-1 and Ang-2 between subjects of South Asian origin and white Europeans (Jaumdally et al., 2007). Currently, there is no evidence to suggest that ethnicity is likely to have affected the angiogenic factor levels studied in this thesis, although further research is required in this area. Another consideration is even though strict selection criteria were applied, other confounding factors may have influenced the angiogenic factor levels, such as stress, other medication or alcohol, although basic alcohol histories were recorded. The influence of such confounding factors could be investigated in future studies. Furthermore, it has to be recognised that the accuracy of the patient data regarding their smoking and alcohol intake is likely to be unreliable (Rebagliato, 2002, Stockwell et al., 2004).

With regards to the use of ELISAs for detecting angiogenic factors in clinical samples, there are a number of limitations. Firstly, as stated above, the detection of factors assumes that these factors are biologically active, when this may not be the case. Secondly, the antibodies in the ELISA kits may not detect all the angiogenically active isoforms and breakdown products of the parent angiogenic factor, and so, underestimating the true angiogenic levels. For example, fragments of MSF have been shown to have biological activity in tissue culture

studies, with regards fibroblast migration, but may include angiogenic activity (Ellis et al., 2010).

A significant limitation with the endostatin and MSF ELISAs used in the present study was the limit of detection (LOD) was insufficient to detect these factors, assuming these factors were present, in the saliva and GCF samples. There was some evidence to suggest that these factors were likely to be present in these fluids because detectable levels, albeit at the limit of detection of the ELISAs, were found in some samples. Furthermore, GCF samples did result in positive fibroblast activity in the migration assay, however, it was not confirmed that this was specifically due to MSF, which would require the use of a MSF inhibitor. Furthermore, the commercial endostatin ELISA kit had been optimised for use with a wide range of human fluids, including saliva, this was not fully the case with the in-house MSF ELISA. Although the MSF ELISA did undergo optimisation prior to use with the saliva samples, there was an continued issue of relatively high background noise, which was not encountered with the commercial ELISA kits. This may indicate an issue with high levels of non-specific binding, which has previously been reported with commercial ELISA kits which had not been specifically optimised for use with saliva (Matin et al., 2016). In the Matin et al. (2016) paper, high levels of non-specific binding in a commercial NGF ELISA kit was thought to be due to the presence of a heterophile antibody in the saliva samples. Non-specific binding issues have also been reported as being a common issue potentially affecting many ELISAs, especially when assaying serum samples for subjects with autoimmune conditions, such as rheumatoid arthritis (Terato et al., 2016). Another issue with the MSF protocol was the necessity to use an unaliquoted stock sample of MSF capture antibody, which is likely to have degraded during storage leading to increasing background noise and limiting detection.

GCF samples are often studied as a relatively non-invasive method to ascertain factor levels in the underlying periodontal tissues (Wassall and Preshaw, 2016). In this study, it had been planned to assess the relationship between salivary and GCF concentrations of MSF in periodontal health and severe periodontitis, and to study the effect of smoking and diabetes. Furthermore, the relationship between MSF levels in GCF and MSF expression in healthy PDL samples would also be examined. To date, there have been no studies which have

reported MSF concentrations in either GCF or saliva. One of the most disappointing issues related to this thesis was that the LOD of the MSF ELISA was insufficient to detect MSF in the GCF samples, if present, which was made worse by the high dilution required as part of the eluting process. Significant time and effort was taken to collect the GCF, calibrate the Periotron 8000, calculate GCF volumes, process and store the GCF samples. Although there was some indication that there may be MSF present in saliva, as shown by a small number of positive samples, it is uncertain whether MSF is any present in GCF. Unfortunately, too much of the valuable GCF samples had been used and the other factor ELISA kits were out of date, so it was not possible to assay the remaining samples. Furthermore, the detailed periodontal data required for the GCF element of this study could not be utilised beyond confirming that the study subgroups were either periodontally healthy (sub-group A) or severe periodontitis (sub-group B). Previous studies have indicated that human gingiva contains sub-populations of MSF-producing gingival fibroblasts (Irwin et al., 1994), so it would be of interest to ascertain whether MSF was detectable in periodontal health and disease. In order for such a study to be carried out in the future the limit of detection of the MSF ELISA would need to be significantly improved, probably by converting the assay from the current chromogenic ELISA to either fluorescence, or even better, to chemiluminescence. Further optimisation of the assay would also be required to ensure there was no cross reactivity with other proteins within saliva, and so, reduce the background noise.

One of the challenges encountered in analysing the angiogenic factor levels in the clinical samples was determining whether there were any extreme outliers in the dataset, these are often defined as three or more times the standard deviation (Southworth, 2012). As expected from previous studies, there were large variations in the individual angiogenic factor levels found. However, where previous data was available the angiogenic factors levels (mean and standard deviations) in the present study were consistent with the literature. The issue of identifying extreme outliers in the data, which may have a disproportionate effect on the outcome, and determining whether such data should be either retained or removed was made in conjunction with a statistician. Outliers can result from laboratory or data transcription errors, or from patients who genuinely have unusual levels of factors, for example, as the result of an undiagnosed condition (Southworth, 2012). In order to comply with research integrity protocols the full dataset was used throughout the present study, only when extreme

outliers were identified using diagnostic statistical methods (Studentized residuals of $\geq$3 and/or Cook's distance $\geq$1), were a small number of samples removed (Field, 2018). Although a potential issue with the removal of extreme outliers is that they may represent a genuine proportion of subjects in the wider population, which may warrant their inclusion, however, to do so would significantly affect the overall statistical analysis.

Following the outcome of this thesis, many questions still remain. Further studies could include the further development of the MSF ELISA, as previously discussed, by converting the assay to chemiluminescent ELISA to increase the sensitivity. This would allow verification of whether MSF is present in saliva and GCF. Furthermore, the relative levels of MSF in serum and oral fluids could be compared with the MSF inhibitor Neutrophil Gelatinase-Associated Lipocalin (NGAL) (Jones et al., 2007), especially as commercial ELISA kits for NGAL are now widely available. In the intervening time since the laboratory work for this thesis was completed, the sensitivity of commercial ELISAs has improved, which may allow verification of whether endostatin is present in saliva. Furthermore, the development of multiplex immunoassays systems now facilitate simultaneous assaying of several pro- and anti-angiogenic factors, as well as inflammatory markers, using small volumes of clinical samples. Such studies would allow the relative comparison of these factors to be investigated, which may provide additional insight into the enhanced healing capacity of oral soft tissues and the changes associated with the development of periodontal disease. Furthermore, this work could ascertain whether the angiogenic balance is significantly affected by risk factors such as poorly controlled diabetes, smoking and alcohol consumption. Currently, there have been very few studies which have examined the relative levels of angiogenic factors in periodontal health and disease, especially with regards to the relative levels of Ang-1, Ang-2 and endostatin in comparison with known key angiogenic factors such as VEGF. Unfortunately, multiplex immunoassays are expensive, so such work would require substantial grant funding and the collection of further clinical samples. However, such studies do not take into account the nature of the extra-cellular matrix, which would require cell culture studies, which were beyond the scope of this thesis.

Currently, there are significant gaps in the literature regarding the relative expression of factors such as Ang-1, Ang-2, MSF and endostatin in both periodontal health and

periodontitis, and in normal human salivary gland tissue. Currently, there have been few studies which have related angiogenic factor expression in oral tissues to the levels detected in serum and in oral fluids. Immunohistochemistry studies, including the use of vascular indices to provide direct evidence of angiogenic changes, could be carried out if it was possible to obtain sufficient numbers of gingival and normal salivary gland tissue biopsies. *In-situ* hybridisation studies could establish whether these factors are manufactured in normal salivary gland tissue and, if so, by which cells.

Although the power calculations for the current study showed that there was sufficient number of subjects, and the group sizes were comparable with similar studies in the literature, there were non-significant trends found in the data, such as reduced angiogenic factor levels in several of the factors studied in periodontally healthy smokers. Further studies could specifically examine angiogenic factor levels in serum, saliva and GCF between smokers and never smokers in periodontal health and severe periodontitis in larger numbers of subjects to help to establish whether there are any significant findings.

In this thesis several factors, both pro- and anti-angiogenic, thought to be important in the regulation of oral wound healing, have been examined in periodontal health and in severe periodontitis. Evidence from this thesis has shown that risk factors known to cause dysfunctional angiogenesis, namely diabetes and smoking, can affect both pro- and anti-angiogenic factor concentrations both systemically and in saliva. However, the study of angiogenesis is complex involving the time-related interaction of multiple angiogenic, growth and inflammatory factors, as well as contextual factors related to the underlying extracellular matrix. Saliva is a complex fluid containing a vast array of other biologically active molecules, which may also play an important part in oral wound healing, in addition to factors intrinsic to the oral soft tissues (Chapter 1.5). This thesis has also raised the issue that the expression or measurement of angiogenic factors levels does not provides evidence that angiogenesis has taken place and proposes that, where possible, vascular indices can be used to provide direct evidence of angiogenesis by comparing pathological tissue with its closest normal tissue. Furthermore, caution needs to be taken when using supposed specific angiogenic markers, such as CD105, when using them to stain vessels in normal or non-neoplastic lesions.

5.1.1 Summary of conclusions

- No significant differences were found in serum and salivary concentrations of Ang-1, MSF, VEGF and endostatin between periodontal health and severe periodontitis.
- Salivary levels of VEGF were significantly raised in diabetes.
- Smoking significantly reduced serum endostatin levels.
- Quantification of vascularity can provide evidence of angiogenesis in addition to the expression of angiogenic factors.
- No evidence of angiogenesis was found in PGs.
- CD105 did not act as a specific angiogenic marker in PGs or in healthy PDL tissues.

5.1.2 Summary of further studies

- Development of a chemiluminescent MSF ELISA.
- ELISA studies to investigate the relationship between MSF and NGAL in serum and oral fluids in periodontal health and severe periodontitis.
- Multiplex immunoassay studies to examine the complex relationship between pro-angiogenic factors, inhibitors and inflammatory markers in serum and oral fluids in periodontal health and severe periodontitis.
- Immunohistochemistry studies to examine the relative expression of angiogenic factors and inhibitors in oral tissues and correlate this to the levels in serum and oral fluids in periodontal health and severe periodontitis.
- *In-situ* hybridisation studies to investigate whether angiogenic factors and inhibitors are manufactured in normal salivary gland tissue and, if so, by which cells.

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Appendices

Appendix 1

Clinical study forms

1) Tayside committee on medical research ethics approval.

2) Clinical research study poster.

3) Patient/volunteer information sheet.

4) Clinical study – staff information sheet.

5) Clinical study – consent form.

6) Clinical study – data collection sheet.

1)

Tayside Committee on Medical Research Ethics

Our Ref: 129/02

Level 9
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DUNDEE
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Enquiries to: Mr N F Brown
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Dr Kevin Davey
Room 190
8th Floor
Dundee Dental Hospital & School

NFB/FB

23 October 2002

Dear Dr Davey

Ref: 129/02 Quantification of Migration Stimulating Factor (MSF) in oral fluids and serum

Thank you for your letter of 14 October 2002 with the amended Patient/Volunteer Information Sheet. This is satisfactory and I am pleased to confirm approval, but what is currently the second sentence under the heading 'How many visits will it take?' is, in fact, two sentences and you will note that there is a small typing error at the beginning of the first one. No doubt you will wish to correct this, but there is no need to send me another copy.

Yours sincerely

Secretary

2)

Clinical Research Study.

Are you interested in taking part in a clinical study which could help towards improving our understanding of how wound healing occurs within the mouth?

If you are interested then please contact **Kevin Davey** on the 3rd floor Periodontology clinic for details.

The study would **only** require **a single one hour visit** on top of any periodontal treatment you may already be receiving.

An inconvenience fee of £5 will be paid (+ travel expenses if applicable).

This study is open to all students, staff & members of the public.

Many thanks,

Kevin Davey.
Lecturer in Periodontology (Principal investigator),
3rd Floor Periodontology clinic,
Ext 35973 (clinic).
Ext 35826 (office).
k.j.davey@dundee.ac.uk
Tayside Committee on Medical Ethics approval Ref 129/02.

3) Ed.140605

Patient/Volunteer information sheet.
Quantification of angiogenic factors in oral fluids and serum.

We invite you to participate in a research project. We believe it to be of potential importance. However, before you decide whether or not you wish to participate, we need to be sure that you understand firstly why we are doing it, and secondly what it would involve if you agreed. We are therefore providing you with the following information. Read it carefully and be sure to ask any questions you have, and, if you want, discuss it with outsiders. We will do our best to explain and to provide any further information you may ask for now or later. You do not have to make an immediate decision.

1) Background:-

What are angiogenic factors?
These are substances produced by the body, which are thought to be very important in the healing of wounds. We currently do not fully understand how these factors work in the body.

Why are we looking for the amounts of angiogenic factors in the mouth?
Nobody has previously measured the actual amounts of many of the angiogenic factors found in the mouth. It has been known for many years that the mouth has exceptional powers of healing, often without scarring. If we knew the amount of these angiogenic factors found in the mouths of healthy individuals it would allow us to compare it to the levels found in people we know to have poor healing such as diabetics & smokers. Eventually it may be possible to produce treatments to improve the healing in such patients. The results from this study will be important in reaching this goal.

What do we mean by oral fluids?
Oral fluids includes:-
i) Saliva - This is produced by salivary glands and is very important in the protection of the mouth, keeping the mouth moist, helping swallowing and in the digestion of our food. A small amount of saliva is produced all the time, but it is produced in greater amounts during eating. We would like to take samples of saliva at both times to see if it is an important source of angiogenic factors in the mouth.
ii) Gingival crevicular fluid (GCF) - This is the fluid which continuously flows around the gum pockets and like saliva GCF is important in the protection of the gums from disease. We would like to take samples of GCF also to see if it is an important source of angiogenic factors in the mouth.

What is serum & why is it needed?
Serum is the liquid part of normal blood after all the red & white blood cells have been removed from it in the laboratory. We would like to see if the levels of the angiogenic factors in serum match the levels found in the oral fluids. If this study found that this was the case then future studies could use oral fluid samples only to look at the levels of these angiogenic factors.

Why have I been chosen for this study?
As part of your normal periodontal examination we have found that you have fallen into one of two groups of patients which we are interested in for this study. One group has people who are resistant to periodontal disease & have healthy gums. The other group of people have active gum disease. We are also interested in whether you are a smoker or a non-smoker & if you are healthy or suffer from diabetes. Any differences found in the levels of the angiogenic factors between each group of people may allow us to understand more about how smoking and diabetes affects healing. This may lead to improved treatments for these diseases in the future. This study could also improve our understanding of how gum disease occurs & how we could improve the treatment of it.

2) What does the study entail?

How are the samples taken?

There are 2 types of saliva sample needed. The first only involves spitting saliva into a small bottle for a short while. The second saliva sample is the same but you would be also asked to move a small sterile marble around the mouth to increase the amount of saliva produced.

Samples of GCF are taken by placing a small piece of special blotting paper into the gum crevice, between the gum & the teeth. This is left there for 30 seconds. This may be repeated a few times in several parts of the mouth.

The dentist or a specially trained nurse would take about 2-3 tablespoons of blood from your forearm.

After the samples have been taken a thorough examination of your gums will be carried out to accurately assess the condition of your gums. This will be followed by a short questionnaire on how you thought the oral examination was done.

Will the procedures be uncomfortable?

The taking of the saliva & gingival crevicular fluid samples should be pain free with no after effects. You may feel a little short term discomfort when the blood sample is taken. It is also common to have a little bruising around the area where the blood was taken from. This usually disappears after about a week or so with no long-term problems.

As with any gum examination we would record your gum pockets using special gum probes & some patients may find this a little uncomfortable. Any discomfort caused is minor & very short lasting. We are interested in how comfortable you feel the gum probing procedure to be & we would ask you to fill in a short questionnaire so we can assess this.

How many visits will it take?

These samples would be taken during any additional appointments made for your routine periodontal treatment. If you do not require to return for further treatment, your travel expenses would be paid for the extra appointment to collect the samples. These samples would be collected by a clinical member of staff & not by the students.

Will I need to do or not do anything immediately before the visit when the samples would be taken?

Apart from not eating or chewing gum within 2 hours before the visit, there are no other restrictions. After the visit you can carry on with your day as normal.

Will this affect the dental treatment I receive?

No, once these samples are taken normal routine periodontal treatment can be performed as required.

Who do I contact if I have a problem?

In the very unlikely event of a problem you should contact the Periodontology department directly on **(01382) 635973** & ask to speak to one of the dentists involved in the study.

Why are diabetic patient's Medical Practitioners/Diabetes clinic contacted for Glycosylated Haemoglobin ($HbAC_1$) scores?

It is known that the effects of diabetes tend to be more severe if your diabetic control is poor. This includes your risk of having gum disease & poor wound healing. By requesting your most recent Glycosylated Haemoglobin score it allows us to readily gauge your long term diabetic control which is important when we analyse the results of this study.

3) Confidentiality.

Who will have access to my records?

Your personal details will be kept strictly confidential. Any personal details will be kept either in a locked cupboard or on a password-secured computer. Only the clinical staff involved in the research will have access to your personal details, as would be the case during normal routine treatment. Any published information would be coded & personal information will not be released to any external body.

4) What are my rights?

How can I obtain more information?

You can contact the Periodontology department & ask for more information from the clinical staff involved in the study. We would be happy to answer any questions that you may have.

Can I refuse to take part or change my mind later even if I agree to take part now?
Yes, you are free to withdraw from the study at any point. This will not affect the routine treatment which you normally receive in the department.

Who has given permission for this study to go ahead?
The Tayside Committee on Medical Research Ethics, which has responsibility for scrutinising all proposals for medical research on humans in Tayside, has examined the proposal and has raised no objections from the point of view of medical ethics.

Participation in this study is entirely voluntary and you are free to refuse to take part or to withdraw from the study at any time without having to give a reason and without this affecting your future dental care or your relationship with the dental staff looking after you.

Thank you for spending the time to read this information sheet. Please ask any further questions you wish about any aspect of the study.

Yours Sincerely,
Dr Kevin Davey BDS BMSc MFDS RCS (Edin) (Dentist)
Clinical Lecturer in Periodontology,
University of Dundee Dental Hospital & School.

4)

Quantification of Migration Stimulating Factor (MSF) in oral fluids and serum.

Kevin Davey.

(i) Aims of the study:

- To quantify the levels of MSF in the oral fluids (Saliva/GCF) and in serum in periodontal health and disease.
- To compare the levels of MSF found in smokers and non-smokers.
- A preliminary study into the effects of diabetes on the levels of MSF.

This study would: -

i) Determine if the levels of MSF found in saliva/GCF change significantly between periodontal health & disease. This could further our understanding of the role MSF in the maintenance of oral health and the changes that occur in periodontal disease.

ii) If the levels of MSF found in subjects with known reduced healing potential, such as smokers & diabetics, is significantly changed this could lead to the therapeutic use of MSF to enhance periodontal healing.

iii) If it is found that the levels of MSF in saliva/GCF correlates well with the levels of MSF found in serum, this could allow for large population studies of MSF using oral fluids rather than taking blood.

(ii) Study groups / Inclusion criteria:

- Three cohorts of patients will be studied. Two groups (Groups 1 & 2) will contain systemically healthy patients. Group 1 will also contain patients who have never smoked & Group 2 will contain patients who are current long-term smokers ($\geq$10 per day). The third group (Group 3) will contain diabetic subjects.

Medical criteria for Groups 1 & 2:

- Clear medical/drug histories - including no:
    - NSAIDs / steroid creams.
    - Diabetes.
    - No recent antibiotics (last 3 months).
- No recent periodontal treatment (within the last 6 months) – Will consider 3 months.

Medical criteria for Group 3:

- Patients with diabetes. Each type of diabetes (Type 1 & 2) will be further divided into the Periodontally healthy & the Severe Chronic Periodontitis groups as above.
    - Type 1 diabetes
        - Periodontally healthy.
        - Severe Chronic Periodontitis.
    - Type 2 diabetes
        - Periodontally healthy.
        - Severe Chronic Periodontitis.

- The level of long-term diabetic control will be assessed by the glycosylated haemoglobin.

Exclusion criteria:

- No pregnant subjects will be accepted into the study:
    - Woman of child bearing age are to be specifically asked if they are likely to be pregnant.
- Previous or irregular smoker.
- Medical history where the recording of the clinical data / taking of the clinical samples would require extra pre-cautions
    - Patients requiring Antibiotic cover.
    - Patients with known infectious disease
    - Hepatitis B/C
    - HIV
- Immuno-compromised patients.

Sub-groups: -

- Within each Group there will be two sub-groups (**a** and **b**). Sub-group **(a)** will contain periodontally healthy patients & Sub-group **(b)** will contain patients with severe active periodontal disease (Chronic Periodontitis). The sub-groups are defined as follows:-

Sub-group 1: Periodontally healthy patients with no loss of attachment (LOA), periodontal probing depths ≤3mm, minimal full mouth plaque scores (<15%) & minimal full mouth bleeding on probing scores (BOP) (<15%)[11].

Sub-group 2: Patients with evidence of severe Chronic Periodontitis (CP) with high full mouth plaque scores (>40%), significant LOA (≥5mm), ≥ 5mm periodontal probing depths (true pocketing) & generalised BOP (>40% full mouth bleeding scores)[11].

Summary of the study groups:-

Group 1 - Systemically healthy / non-smokers.
- 1a - Periodontally healthy.
- 1b - Severe Chronic Periodontitis.

Group 2 - Systemically healthy / Smokers.
- 2a - Periodontally healthy.
- 2b - Severe Chronic Periodontitis.

Group 3 - Diabetic subjects (smokers & non-smokers including any medications)

Type 1 diabetes
- 3a - Periodontally healthy.
- 3b - Severe Chronic Periodontitis.

Type 2 diabetes
- 3a - Periodontally healthy.
- 3b - Severe Chronic Periodontitis

Quantification of angiogenic factors in oral fluids and serum.

CONSENT FORM

NB. ***This form must be completed and signed by the research subject in the presence of someone with knowledge of the research designated by the Principal Investigator. This may be a doctor, nurse, clinical research assistant or other member of the research team who must countersign the form as witness to the subject's signature***

Please tick (✓) appropriate box

Have you read and understood the Subject Information Sheet?
Yes ☐ No ☐

Have you been given an opportunity to ask questions and further discuss this study?
Yes ☐ No ☐

Have you received satisfactory answers to all of your questions?
Yes ☐ No ☐

Have you now received enough information about this study?
Yes ☐ No ☐

Who have you spoken to? Dr/Mr/Mrs/Miss ……

Do you understand that your participation is entirely voluntary?
Yes ☐ No ☐

Do you understand that you are free to withdraw from this study:

At any time?
Yes ☐ No ☐

Without having to give a reason for withdrawing?
Yes☐ No ☐

Without this affecting your present or future medical care?
Yes ☐ No ☐

Do you agree that your records in this research and supporting medical records be made available for inspection by monitors from:

NHS Tayside monitors?
Yes ☐ No ☐

Do you agree to take part in this study?
Yes ☐ No ☐

Do you agree to any tissue (Blood, Saliva & Gingival Fluid) used in this study being retained for use in future research? Yes ☐ No ☐
Not applicable ☐

Diabetic patients only – Do you consent that your doctor / diabetic clinic can be contacted in order to obtain your glycosylated haemoglobin results?

Yes ☐ No ☐

Subject's signature ……. Date ……..……

Subject's name in block capital letters ……

Telephone contact (Subject) ……(Home) ……..(Work)

Signature witnessed by …… Date ..……

Witness name in block capital letters ……..

Study nº ……

6)

Patient details for the clinical Angiogenesis study.

Clinician:……..
Date:…./…./….
Time samples taken …… am/pm.
Patient filled in consent form □

Patient details:
Age:……years.
Sex: M/F.

Smoking history:

Patient has never smoked: □
Current smoker □
How many packets smoked per day ……
How long for …… years.
Pack years ……

Checklist:

- Medical history taken □
- Confirm not in exclusion category □
- Periodontal measurements:
  - Full mouth plaque/bleeding scores □
  - Mobility chart □
  - Periodontal pocket chart □

Study group category:

Group 1 - Systemically healthy / non-smoker
- **1a** - Periodontology healthy □.
- **1b** - Severe chronic adult periodontitis □.

Group 2 - Systemically healthy / smokers
- **2a** - Periodontally healthy □.
- **2b** - Severe chronic adult periodontitis □.

Group 3 - Diabetic smokers/non-smokers
- **3a** - Periodontally healthy □.
- **3b** - Severe chronic adult periodontitis □.

Type 1 diabetic □
Type 2 diabetic □

Non-smoker □
Smoker □.

- **Periodontal diagnosis (1999)**

……
……
……

- **<u>Clinical samples</u>:**
  - 10ml blood sample + labelled □
  - Unstimulated saliva sample + labelled □
  - Stimulated saliva sample + labelled □
  - GCF samples taken from:

<u>i) Healthy sites</u> (x4)

Site	Tooth (FDI)	Site.	Periotron reading
1			
2			
3			
4			
5			
6			
7			
8			

<u>ii) Diseased sites</u> (x4)

Site	Tooth (FDI)	Site.	Periotron reading
1			
2			
3			
4			

Time GCF samples taken ……..
Time after samples taken before freezing …… hrs.

Comments:

- Relevant additional clinical information:

……
……
……
……
……

- Problems encountered in taking the clinical samples:

……
……
……
……
……

- Problems encountered in the laboratory procedures:

……
……
……
……
……

Contact Kevin Davey:

Office extension	35826
8th Floor lab	35827
Microscope room	35825
10th Floor lab	35883

Appendix 2

Summary of the demographic data for the clinical study

		Total	Group 1			Group 2			Group 3		
			Grp 1 Total	Grp 1a	Grp 1b	Grp 2 Total	Grp 2a	Grp 2b	Grp 3 Total	Grp 3a	Grp 3b
Number		102	53	39	14	20	11	9	29	13	16
Age - years	**Mean (±SD)**	49.53 (±16.365)	45.55 (±16.015)	41.41 (±14.65)	57.07 (±14.291)	42.1 (±14.345)	39.36 (±17.761)	45.44 (±8.457)	61.93 (±11.016)	65.77 (±7.27)	58.81 (±12.692)
	Min-Max (range)	22-78 (56)	22-74 (52)	22-73 (51)	23-74 (51)	22-74 (52)	22-74 (52)	33-61 (28)	38-78 (40)	55-78 (23)	38-78 (40)
Gender M/F (%)		M= 38 (37) F= 64 (63)	M= 32 (60) F= 21 (40)	M= 23 (59) F= 16 (41)	M= 9 (64) F= 5 (36)	M= 9 (45) F= 11 (55)	M= 4 (36) F= 7 (64)	M= 4 (44) F= 5 (56)	M= 23 (79) F= 6 (21)	M= 10 (77) F= 3 (23)	M= 13 (81) F= 3 (19)
Smoking (Pack Years)	**Mean (±SD)**		N/A	N/A	N/A		14.64 (±12.824)	21.22 (±11.155)		N=1/13	N=7/16
Glycaemic Control ($HBA1_c$)	**Mean (±SD)**		N/A	N/A	N/A	N/A	N/A	N/A	7.34 (±1.082)	6.9 (±0.883)	7.69 (±1.123)
Number of teeth	**Mean (±SD)**	23.72 (±4.270)	24.68 (±4.035)	26.04 (±2.63)	21.45 (±5.007)	24.56 (±4.176)	25.00 (±5.099)	24.11 (±3.257)	21.85 (±4.176)	21.85 (±4.180)	21.86 (±4.330)
	Min-Max (range)	12-32	12-32	20-32	12-28	13-30	13-30	20-30	14-28	15-28	14-28
Plaque Scores (%)	**Mean (±SD)**	34.76 (±30.908)	29.46 (±31.041)	15.42 (±20.510)	62.64 (±26.345)	34.28 (±29.714)	14.33 (±14.739)	54.22 (±27.64)	42.33 (±34.76)	21.00 (±15.083)	62.14 (±29.025)
	Min-Max (range)	0-100 (100)	0-96 (96)	0-77 (77)	0-96 (96)	0-88 (88)	0-40 (40)	7-88 (81)	3-100 (97)	3-51 (48)	13-100 (87)
Bleeding Scores (%)	**Mean (±SD)**	10.94 (±12.818)	13.35 (±16.014)	7.50 (±6.243)	27.18 (±22.943)	11.94 (±10.729)	6.67 (±3.775)	17.22 (±12.950)	6.96 (±7.491)	3.54 (±5.206)	10.14 (±8.037)
	Min-Max (range)	0-66 (66)	0-66 (66)	0-30 (30)	0-66 (66)	0-34 (34)	1-12 (11)	0-34 (34)	0-26 (26)	0-17 (17)	0-26 (26)

Note:

- Not possible to collect all types of clinical samples from some subjects.
- A small number of the Group 3 patients also smoked.

Appendix 3

Data Tables

Table 1: Raw data and Ang-1 concentrations (pg ml^{-1}) in serum, unstimulated and stimulated saliva.

(a) Group 1a:

Study no	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	Ang-1 concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
001	33	M	0	-	**95872**	**1077**	**4188**
002	36	M	0	-	-	-	-
004	22	F	0	-	-	**28509**	**28306**
006	27	F	0	-	**74156**	**27390**	**41852**
008	44	F	0	-	**36375**	**90371**	**64101**
009	37	M	0	-	**53567**	**4497**	**2854**
012	32	F	0	-	**42782**	**38100**	**44071**
014	36	M	0	-	**47389**	**22932**	**30371**
015	30	M	0	-	**42551**	**8918**	**4569**
017	26	F	0	-	**56918**	**25843**	-
018	22	M	0	-	**62838**	**27519**	**91754**
019	43	M	0	-	**44115**	**2045**	**3632**
020	67	M	0	-	**18634**	**115549**	**117061**
021	22	M	0	-	**39767**	**45120**	**53055**
027	46	M	0	-	**21179**	**0**	**1887**
028	70	M	0	-	**69202**	**76423**	**53734**
029	39	M	0	-	**58517**	**71468**	**80469**
030	23	M	0	-	**27977**	**44751**	**22409**
032	36	F	0	-	**46602**	**111115**	**67569**
038	57	M	0	-	**49760**	**52874**	**65366**
040	37	F	0	-	**57611**	**64266**	**52741**
041	38	F	0	-	**47060**	-	-
042	45	M	0	-	**70930**	-	-
043	34	M	0	-	**60060**	-	-
044	33	M	0	-	**43200**	-	-
045	40	M	0	-	**41770**	-	-
046	31	F	0	-	**29590**	-	-
047	46	F	0	-	**48670**	-	-
048	48	F	0	-	**46030**	-	-
050	50	M	0	-	**46030**	-	-
051	22	M	0	-	**31719**	**50557**	**93096**
055	70	M	0	-	**30222**	**91649**	**110415**
057	58	M	0	-	**45916**	**104285**	**67759**
063	56	M	0	-	**30506**	**1356**	**0**
067	60	F	0	-	**42624**	**0**	**2023**
073	73	F	0	-	**43493**	**34698**	**61365**
080	59	F	0	-	**25243**	**35968**	**37115**
099	33	F	0	-	**57657**	-	-
101	45	F	0	-	**36204**	-	-
N=39	**Mean=41±15yrs**	**M=23/F=16**			**N=37**	**N=27**	**N=26**

(b) Group 1b:

Study no	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	Ang-1 concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
024	23	F	0	-	**-**	**28306**	**34946**
031	69	M	0	-	**24771**	**17905**	**13996**
036	74	M	0	-	**54267**	**22693**	**28222**
039	54	F	0	-	**62829**	**12552**	**10226**
056	66	M	0	-	**31511**	**91615**	**76103**
060	58	M	0	-	**60806**	**31601**	**20889**
064	51	M	0	-	**42586**	**8997**	**8851**
074	59	M	0	-	**33445**	**5871**	**6457**
075	44	M	0	-	**50818**	**7391**	**6223**
076	73	F	0	-	**15290**	**45801**	**34513**
079	38	F	0	-	**36342**	**54212**	**37556**
084	66	M	0	-	**60844**	**79167**	**51414**
085	59	M	0	-	**50244**	**16684**	**20027**
092	65	F	0	-	**-**	**6223**	**-**
N=14	**Mean=57±14yrs**	**M=9/F=5**			**N=12**	**N=14**	**N=13**

(c) Group 2a:

Study no	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	Ang-1 concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
003	47	F	30	-	**11943**	**7340**	**23771**
010	24	F	1	-	**53724**	**44425**	**34949**
011	23	M	4	-	**71487**	**73660**	**77615**
022	22	F	3	-	**54966**	**40395**	**30874**
023	33	F	2	-	**68014**	**1605**	**8258**
054	63	M	15	-	**58585**	**0**	**2843**
077	47	F	30	-	**29506**	**88**	**46392**
095	27	M	11	-	**18935**	**22366**	**24351**
098	74	M	30	-	**27787**	**42256**	**45510**
100	48	F	30	-	**29414**	**-**	**-**
102	25	F	5	-	**10744**	**-**	**-**
N=11	**Mean=37±17yrs**	**M=4/F=7**	**Mean=14.6±13.5**		**N=11**	**N=9**	**N=9**

(d) Group 2b:

Study no	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	Ang-1 concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
007	61	F	40	-	**63878**	**60944**	**45293**
013	33	M	17	-	**71108**	**2187**	**2679**
016	37	F	3	-	**46869**	**36121**	**42140**
059	51	M	30	-	**42624**	**29962**	**37510**
083	41	F	25	-	**46069**	**29797**	**40426**
089	47	M	15	-	**45156**	**43988**	**46037**
093	43	M	25	-	**22392**	**39121**	**46712**
094	44	F	26	-	**26201**	**53344**	**59333**
096	52	M	10	-	**18438**	**31127**	**28772**
N=9	**Mean=47±10yrs**	**M=5/F=4**	**Mean=20.6±10.7**		**N=9**	**N=9**	**N=9**

(e) Group 3a:

Study no (Type of DM)	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	Ang-1 concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
026 (II)	72	F	0	6	**35280**	**47558**	**46537**
033 (II)	73	M	0	5.9	**71941**	**759#**	**10655**
037 (II)	59	M	0	6.7	**39270**	**3856**	**4472**
058 (II)	78	F	0	7.5	**92926**	**98625**	**99237**
065 (II)	66	M	20	5.2	**40152**	**77757**	**52220**
069 (I)	71	M	0	7	**25824**	**101846**	**84322**
070 (II)	55	M	0	7.4	**30399**	**39935**	-
071 (II)	58	M	0	6.5	**34114**	**74688**	**65421**
072 (II)	72	F	0	7.4	**53175**	**43479**	**54689**
082 (II)	68	M	0	7.4	-	**40595**	**40391**
088 (II)	65	M	0	8.7	**34150**	**42971**	**43348**
090 (II)	57	M	0	7.2	**40355**	**70817**	**59369**
097 (II)	61	M	0	6.8	**28863**	**9184**	**8131**
N=13	**Mean=66±7yrs**	**M=10/F=3**		**Mean=6.9±0.9**	**N=12**	**N=13**	**N=12**

(f) Group 3b:

Study no (Type of DM)	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	Ang-1 concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
005 (I)	45	M	36	8.1	**54112**	**4366**	**11844**
025 (I)	69	M	71	7.5	**21260**	**60214**	**127784**
034 (II)	66	M	0	8.7	**25617**	**10632**	**11189**
035 (II)	61	M	0	9.5	**28225**	**14695**	**10643**
049 (I)	61	M	0	8.7	**48548**	-	-
052 (II)	78	M	0	5.8	**52794**	**48471**	**50455**
053 (II)	55	M	0	6.6	**55160**	**30109**	**28216**
061 (II)	42	M	20	6.8	**29506**	**33915**	**49832**
062 (II)	56	M	0	7.6	**11913**	**38108**	**41438**
066 (II)	76	M	33	8.8	**24247**	**67167**	**68498**
068 (II)	73	M	0	8.1	**23365**	**15667**	**4776**
078 (II)	66	M	22	6.6	**23420**	**53767**	**52129**
081 (II)	64	F	51	7.1	**39902**	**99514**	**87679**
086 (I)	42	F	7	9.4	**35902**	**60691**	**72997**
087 (II)	38	F	24	7.5	-	**8276**	**6808**
091 (II)	49	M	0	6.3	**19675**	**7971**	**6252**
N=16	**Mean=59±13yrs**	**M=13/F=3**		**Mean=7.7±1.1**	**N=15**	**N=15**	**N=15**

Table 2: Raw data and MSF concentrations (ng ml^{-1}) in serum, unstimulated and stimulated saliva (UD – undetected).

(a) Group 1a:

Study no	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	MSF concentration (ng ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
001	33	M	0	-	**8152**	**UD**	**UD**
002	36	M	0		**-**	**-**	**-**
004	22	F	0	-	**22661**	**UD**	**UD**
006	27	F	0	-	**4496**	**UD**	**UD**
008	44	F	0	-	**3313**	**UD**	**UD**
009	37	M	0	-	**12480**	**UD**	**UD**
012	32	F	0	-	**8770**	**UD**	**UD**
014	36	M	0	-	**4039**	**UD**	**UD**
015	30	M	0	-	**16378**	**UD**	**UD**
017	26	F	0	-	**3071**	**UD**	**-**
018	22	M	0	-	**22077**	**UD**	**UD**
019	43	M	0	-	**1316**	**1091**	**UD**
020	67	M	0	-	**29142**	**UD**	**UD**
021	22	M	0	-	**6152**	**UD**	**UD**
027	46	M	0	-	**3879**	**1676**	**225**
028	70	M	0	-	**21435**	**UD**	**UD**
029	39	M	0	-	**23906**	**UD**	**UD**
030	23	M	0	-	**309**	**UD**	**UD**
032	36	F	0	-	**11295**	**UD**	**UD**
038	57	M	0	-	**383**	**UD**	**UD**
040	37	F	0	-	**1270**	**UD**	**UD**
041	38	F	0	-	**21674**	**-**	**-**
042	45	M	0	-	**16634**	**-**	**-**
043	34	M	0	-	**7809**	**-**	**-**
044	33	M	0	-	**6030**	**-**	**-**
045	40	M	0	-	**35267**	**-**	**-**
046	31	F	0	-	**7482**	**-**	**-**
047	46	F	0	-	**8352**	**-**	**-**
048	48	F	0	-	**12543**	**-**	**-**
050	50	M	0	-	**8649**	**-**	**-**
051	22	M	0	-	**7802**	**UD**	**UD**
055	70	M	0	-	**UD**	**UD**	**UD**
057	58	M	0	-	**2910**	**UD**	**UD**
063	56	M	0	-	**UD**	**UD**	**UD**
067	60	F	0	-	**1727**	**1677**	**UD**
073	73	F	0	-	**28851**	**UD**	**UD**
080	59	F	0	-	**26339**	**UD**	**UD**
099	33	F	0	-	**13698**	**-**	**-**
101	45	F	0	-	**UD**	**-**	**-**
N=39	**Mean=41+15yrs**	**M=23/F=16**			**N=38**	**N=27**	**N=26**

(b) Group 1b:

Study no	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	MSF concentration (ng ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
024	23	F	0	-	**-**	**UD**	**UD**
031	69	M	0	-	**1791**	**352**	**UD**
036	74	M	0	-	**2389**	**1546**	**2576**
039	54	F	0	-	**UD**	**UD**	**UD**
056	66	M	0	-	**5222**	**UD**	**UD**
060	58	M	0	-	**845**	**UD**	**UD**
064	51	M	0	-	**2399**	**UD**	**UD**
074	59	M	0	-	**6002**	**UD**	**UD**
075	44	M	0	-	**4937**	**UD**	**UD**
076	73	F	0	-	**24321**	**UD**	**UD**
079	38	F	0	-	**17294**	**UD**	**UD**
084	66	M	0	-	**5598**	**UD**	**UD**
085	59	M	0	-	**26674**	**UD**	**UD**
092	65	F	0	-	**2595**	**UD**	**-**
N=14	**Mean=57±14yrs**	**M=9/F=5**			**N=13**	**N=14**	**N=13**

(c) Group 2a:

Study no	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	MSF concentration (ng ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
003	47	F	30	-	**UD**	**UD**	**UD**
010	24	F	1	-	**3259**	**UD**	**UD**
011	23	M	4	-	**2641**	**UD**	**UD**
022	22	F	3	-	**3996**	**UD**	**UD**
023	33	F	2	-	**374**	**UD**	**UD**
054	63	M	15	-	**1835**	**UD**	**UD**
077	47	F	30	-	**24584**	**UD**	**UD**
095	27	M	11	-	**UD**	**UD**	**UD**
098	74	M	30	-	**UD**	**UD**	**UD**
100	48	F	30	-	**22139**	**UD**	**UD**
102	25	F	5	-	**2212**	**-**	**-**
N=11	**Mean=37±17yrs**	**M=4/F=7**	**Mean=14.6±13.5**		**N=11**	**N=10**	**N=10**

(d) Group 2b:

Study no	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	MSF concentration (ng ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
007	61	F	40	-	**12695**	**UD**	**UD**
013	33	M	17	-	**5921**	**UD**	**UD**
016	37	F	3	-	**813**	**UD**	**UD**
059	51	M	30	-	**13958**	**UD**	**UD**
083	41	F	25	-	**3922**	**UD**	**UD**
089	47	M	15	-	**3461**	**UD**	**UD**
093	43	M	25	-	**UD**	**UD**	**UD**
094	44	F	26	-	**1927**	**UD**	**UD**
096	52	M	10	-	**12800**	**75**	**UD**
N=9	**Mean=47±10yrs**	**M=5/F=4**	**Mean=20.6±10.7**		**N=9**	**N=9**	**N=9**

(e) Group 3a:

Study no (Type of DM)	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	MSF concentration (ng ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
026 (II)	72	F	0	6	**19787**	**UD**	**UD**
033 (II)	73	M	0	5.9	**10784**	**UD**	**UD**
037 (II)	59	M	0	6.7	**4274**	**UD**	**UD**
058 (II)	78	F	0	7.5	**56239**	**UD**	**UD**
065 (II)	66	M	20	5.2	**8367**	**UD**	**UD**
069 (I)	71	M	0	7	**54524**	**UD**	**UD**
070 (II)	55	M	0	7.4	**464**	**UD**	-
071 (II)	58	M	0	6.5	**3474**	**UD**	**UD**
072 (II)	72	F	0	7.4	**UD**	**UD**	**UD**
082 (II)	68	M	0	7.4	**23714**	**UD**	**UD**
088 (II)	65	M	0	8.7	**UD**	**UD**	**UD**
090 (II)	57	M	0	7.2	**12237**	**UD**	**UD**
097 (II)	61	M	0	6.8	**34**	**431**	**UD**
N=13	**Mean=66±7yrs**	**M=10/F=3**		**Mean=6.9±0.9**	**N=13**	**N=13**	**N=12**

(f) Group 3b:

Study no (Type of DM)	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	MSF concentration (ng ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
005 (I)	45	M	36	8.1	**2211**	**UD**	**UD**
025 (I)	69	M	71	7.5	**24544**	**UD**	**58**
034 (II)	66	M	0	8.7	**UD**	**255**	**UD**
035 (II)	61	M	0	9.5	**23012**	**616**	**456**
049 (I)	61	M	0	8.7	**6372**	-	-
052 (II)	78	M	0	5.8	**918**	**UD**	**UD**
053 (II)	55	M	0	6.6	**5087**	**UD**	**UD**
061 (II)	42	M	20	6.8	**13721**	**UD**	**UD**
062 (II)	56	M	0	7.6	**20106**	**1329**	**UD**
066 (II)	76	M	33	8.8	**UD**	**UD**	**UD**
068 (II)	73	M	0	8.1	**1612**	**UD**	**UD**
078 (II)	66	M	22	6.6	**8039**	**414**	**UD**
081 (II)	64	F	51	7.1	**UD**	**7**	**UD**
086 (I)	42	F	7	9.4	**1270**	**UD**	**UD**
087 (II)	38	F	24	7.5	-	**UD**	**UD**
091 (II)	49	M	0	6.3	**6806**	**UD**	**UD**
N=16	**Mean=59±13yrs**	**M=13/F=3**		**Mean=7.7±1.1**	**N=15**	**N=15**	**N=15**

Table 3: Raw data and VEGF concentrations (pg ml^{-1}) in serum, unstimulated and stimulated saliva (UD – undetected).

(a) Group 1a:

Study no	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	VEGF concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
001	33	M	0	-	**493**	**234**	**480**
002	36	M	0	-	**-**	**-**	**-**
004	22	F	0	-	**-**	**68**	**93**
006	27	F	0	-	**392**	**88**	**294**
008	44	F	0	-	**140**	**692**	**728**
009	37	M	0	-	**193**	**426**	**272**
012	32	F	0	-	**160**	**448**	**309**
014	36	M	0	-	**239**	**80**	**133**
015	30	M	0	-	**319**	**256**	**188**
017	26	F	0	-	**UD**	**140**	**-**
018	22	M	0	-	**357**	**140**	**475**
019	43	M	0	-	**192**	**450**	**496**
020	67	M	0	-	**126**	**1481**	**843**
021	22	M	0	-	**195**	**188**	**239**
027	46	M	0	-	**UD**	**313**	**500**
028	70	M	0	-	**822**	**632**	**783**
029	39	M	0	-	**800**	**516**	**641**
030	23	M	0	-	**UD**	**239**	**431**
032	36	F	0	-	**UD**	**746**	**780**
038	57	M	0	-	**263**	**280**	**270**
040	37	F	0	-	**145**	**277**	**584**
041	38	F	0	-	**4332**	**-**	**-**
042	45	M	0	-	**207**	**-**	**-**
043	34	M	0	-	**545**	**-**	**-**
044	33	M	0	-	**182**	**-**	**-**
045	40	M	0	-	**408**	**-**	**-**
046	31	F	0	-	**345**	**-**	**-**
047	46	F	0	-	**324**	**-**	**-**
048	48	F	0	-	**219**	**-**	**-**
050	50	M	0	-	**144**	**-**	**-**
051	22	M	0	-	**140**	**236**	**365**
055	70	M	0	-	**UD**	**512**	**427**
057	58	M	0	-	**UD**	**461**	**551**
063	56	M	0	-	**140**	**267**	**245**
067	60	F	0	-	**3505**	**651**	**586**
073	73	F	0	-	**545**	**377**	**470**
080	59	F	0	-	**132**	**681**	**912**
099	33	F	0	-	**271**	**-**	**-**
101	45	F	0	-	**0**	**-**	**-**
N=39	**Mean=41+15yrs**	**M=23/F=16**			**N=37**	**N=27**	**N=26**

(b) Group 1b:

Study no	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	VEGF concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
024	23	F	0	-	**-**	**12**	**375**
031	69	M	0	-	**221**	**1268**	**946**
036	74	M	0	-	**472**	**1501**	**891**
039	54	F	0	-	**125**	**1199**	**826**
056	66	M	0	-	**UD**	**1433**	**943**
060	58	M	0	-	**UD**	**356**	**411**
064	51	M	0	-	**UD**	**701**	**747**
074	59	M	0	-	**387**	**521**	**592**
075	44	M	0	-	**217**	**903**	**468**
076	73	F	0	-	**UD**	**549**	**773**
079	38	F	0	-	**1284**	**212**	**656**
084	66	M	0	-	**332**	**500**	**943**
085	59	M	0	-	**2045**	**2059**	**879**
092	65	F	0	-	**-**	**524**	**-**
N=14	**Mean=57±14yrs**	**M=9/F=5**			**N=12**	**N=14**	**N=13**

(c) Group 2a:

Study no	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	VEGF concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
003	47	F	30	-	**627**	**644**	**19**
010	24	F	1	-	**UD**	**166**	**200**
011	23	M	4	-	**193**	**658**	**1078**
022	22	F	3	-	**213**	**167**	**393**
023	33	F	2	-	**223**	**270**	**688**
054	63	M	15	-	**145**	**280**	**4540**
077	47	F	30	-	**UD**	**224**	**303**
095	27	M	11	-	**UD**	**215**	**UD**
098	74	M	30	-	**243**	**177**	**465**
100	48	F	30	-	**UD**	-	-
102	25	F	5	-	**UD**	-	-
N=11	**Mean=37±17yrs**	**M=4/F=7**	**Mean=14.6±13.5**		**N=11**	**N=9**	**N=9**

(d) Group 2b:

Study no	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	VEGF concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
007	61	F	40	-	**482**	**279**	**518**
013	33	M	17	-	**262**	**601**	**562**
016	37	F	3	-	**UD**	**779**	**991**
059	51	M	30	-	**164**	**599**	**363**
083	41	F	25	-	**UD**	**239**	**447**
089	47	M	15	-	**8264**	**686**	**478**
093	43	M	25	-	**243**	**138**	**262**
094	44	F	26	-	**195**	**218**	**203**
096	52	M	10	-	**UD**	**786**	**507**
N=9	**Mean=47±10yrs**	**M=5/F=4**	**Mean=20.6±10.7**		**N=9**	**N=9**	**N=9**

(e) Group 3a:

Study no (Type of DM)	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	VEGF concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
026 (II)	72	F	0	6	**184**	**1132**	**957**
033 (II)	73	M	0	5.9	**812**	**689**	**737**
037 (II)	59	M	0	6.7	**409**	**623**	**519**
058 (II)	78	F	0	7.5	**4518**	**904**	**1405**
065 (II)	66	M	20	5.2	**UD**	**508**	**UD**
069 (I)	71	M	0	7	**492**	**557**	**778**
070 (II)	55	M	0	7.4	**174**	**437**	-
071 (II)	58	M	0	6.5	**UD**	**520**	**UD**
072 (II)	72	F	0	7.4	**400**	**943**	**1122**
082 (II)	68	M	0	7.4	-	**704**	**361**
088 (II)	65	M	0	8.7	**UD**	**354**	**352**
090 (II)	57	M	0	7.2	**177**	**283**	**263**
097 (II)	61	M	0	6.8	**140**	**1723**	**832**
N=13	**Mean=66±7yrs**	**M=10/F=3**		**Mean=6.9±0.9**	**N=12**	**N=13**	**N=12**

(f) Group 3b:

Study no (Type of DM)	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	VEGF concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
005 (I)	45	M	36	8.1	**UD**	**728**	**930**
025 (I)	69	M	71	7.5	**UD**	**1013**	**930**
034 (II)	66	M	0	8.7	**UD**	**1999**	**1801**
035 (II)	61	M	0	9.5	**UD**	**880**	**762**
049 (I)	61	M	0	8.7	**419**	-	-
052 (II)	78	M	0	5.8	**164**	**473**	**888**
053 (II)	55	M	0	6.6	**729**	**910**	**885**
061 (II)	42	M	20	6.8	**UD**	**227**	**491**
062 (II)	56	M	0	7.6	**UD**	**407**	**359**
066 (II)	76	M	33	8.8	**221**	**822**	**849**
068 (II)	73	M	0	8.1	**150**	**414**	**551**
078 (II)	66	M	22	6.6	**130**	**673**	**765**
081 (II)	64	F	51	7.1	**403**	**744**	**761**
086 (I)	42	F	7	9.4	**125**	**248**	**379**
087 (II)	38	F	24	7.5	-	**1497**	**901**
091 (II)	49	M	0	6.3	**UD**	**458**	**421**
N=16	**Mean=59±13yrs**	**M=13/F=3**		**Mean=7.7±1.1**	**N=15**	**N=15**	**N=15**

Table 4: Raw data and endostatin concentrations (pg ml^{-1}) in serum, unstimulated and stimulated saliva.

(a) Group 1a:

Study no	Age (years)	Gender (M/F)	Pack Years	HbA1c (%)	Endostatin concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
001	33	M	0	-	**29781**	**0** **0** **0**	**0** **0** **0**
002	36	M	0	-	-	-	**0**
004	22	F	0	-	**47020**	-	**0**
006	27	F	0	-	**33015**	-	**0**
008	44	M	0	-	**69550**	-	**530**
009	37	M	0	-	**36720**	-	**0**
012	32	F	0	-	**72293**	-	**0**
014	36	M	0	-	**76732**	-	**0**
015	30	M	0	-	**80038**	-	**0**
017	26	F	0	-	**64046**	-	**0**
018	22	M	0	-	**79998**	-	**0**
019	43	M	0	-	**76880**	-	**0**
020	67	M	0	-	**43781**	-	**0**
021	22	M	0	-	**72362**	-	**0**
027	46	M	0	-	**74007**	-	**0**
028	70	M	0	-	**54140**	**0**	**0**
029	39	M	0	-	**60260**	-	**0**
030	23	M	0	-	**46420**	-	**0**
032	36	F	0	-	**37720**	-	**530**
038	57	M	0	-	**65122**	-	**0**
040	37	F	0	-	**33505**	-	**0**
041	38	F	0	-	**45040**	-	-
042	45	M	0	-	**77740**	-	-
043	34	M	0	-	**55840**	-	-
044	33	M	0	-	**44860**	-	-
045	40	M	0	-	**49180**	-	-
046	31	F	0	-	**47600**	-	-
047	46	F	0	-	**65520**	-	-
048	48	F	0	-	**67300**	-	-
050	50	M	0	-	**50440**	-	-
051	22	M	0	-	**75561**	-	**0**
055	70	M	0	-	**36665**	-	**0**
057	58	M	0	-	**35004**	-	**0**
063	56	M	0	-	**76322**	-	**0**
067	60	F	0	-	**31898**	-	**0**
073	73	F	0	-	**79893**	-	**0**
080	59	F	0	-	**32462**	**0**	**0**
099	33	F	0	-	**57657**	-	-
101	45	F	0	-	**36204**	-	-
N=39	**Mean=41±15yrs**	**M=23/F=16**			**N=38**		**N=28**

(b) Group 1b:

Study no	Age	Gender (M/F)	Pack Years	HbA1c (%)	Endostatin concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
024	23	F	0	-	-	-	**0**
031	69	M	0	-	**76831**	-	**776**
036	74	M	0	-	**57873**	-	**1124**
039	54	F	0	-	**38126**	-	**0**
056	66	M	0	-	**48440**	**0**	**0**
060	58	M	0	-	**39136**	-	**0**
064	51	M	0	-	**42400**	-	**0**
074	59	M	0	-	**79432**	-	**0**
075	44	M	0	-	**52789**	-	**0**
076	73	F	0	-	**32492**	-	**290**
079	38	F	0	-	**39700**	-	**0**
084	66	M	0	-	**62880**	-	**0**
085	59	M	0	-	**46240**	-	**1492**
092	65	F	0	-	-	-	-
N=14	**Mean=57±14yrs**	**M=9/F=5**			**N=12**		**N=12**

(c) Group 2a:

Study no	Age	Gender (M/F)	Pack Years	HbA1c (%)	Endostatin concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
003	47	F	30	-	**21329**	-	**0**
010	24	F	1	-	**23980**	-	**0**
011	23	M	4	-	**27401**	-	**0**
022	22	F	3	-	**28750**	**0**	**0**
023	33	F	2	-	**57060**	-	**0**
054	63	M	15	-	**38340**	-	**0**
077	47	F	30	-	**30744**	-	**0**
095	27	M	11	-	**37403**	-	**0**
098	74	M	30	-	**54867**	-	**0**
100	48	F	30	-	**29414**	-	-
102	25	F	5	-	**10744**	-	-
N=11	**Mean=37±17yrs**	**M=4/F=7**	**Mean=14.6±13.5**		**N=11**		**N=9**

(d) Group 2b:

Study no	Age	Gender (M/F)	Pack Years	HbA1c (%)	Endostatin concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
007	61	F	40	-	**38806**	-	**0**
013	33	M	17	-	**24345**	-	**0**
016	37	F	3	-	**27696**	-	**0**
059	51	M	30	-	**52780**	**0**	**0**
083	41	F	25	-	**21777**	-	**0**
089	47	M	15	-	**23961**	-	**0**
093	43	M	25	-	**39395**	-	**0**
094	44	F	26	-	**56603**	-	**0**
096	52	M	10	-	**38356**	-	**0**
N=9	**Mean=47±10yrs**	**M=5/F=4**	**Mean=20.6±10.7**		**N=9**		**N=9**

(e) Group 3a:

Study no (Type of DM)	Age	Gender (M/F)	Pack Years	HbA1c (%)	Endostatin concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
026 (II)	72	F	0	6	**59040**	-	**0**
033 (II)	73	M	0	5.9	**79240**	-	**5200**
037 (II)	59	M	0	6.7	**55320**	-	**0**
058 (II)	78	F	0	7.5	**63000**	-	**3023**
065 (II)	66	M	20	5.2	**57960**	-	**0**
069 (I)	71	M	0	7	**37030**	-	**0**
070 (II)	55	M	0	7.4	**38065**	-	**0**
071 (II)	58	M	0	6.5	**38157**	**0**	**0**
072 (II)	72	F	0	7.4	**73300**	-	**1197**
082 (II)	68	M	0	7.4	-	**0**	**0**
088 (II)	65	M	0	8.7	**59140**	-	**0**
090 (II)	57	M	0	7.2	**73689**	-	**0**
097 (II)	61	M	0	6.8	**53135**	-	**0**
N=13	**Mean=66±7yrs**	**M=10/F=3**		**Mean=6.9±0.9**	**N=12**		**N=13**

(f) Group 3b:

Study no (Type of DM)	Age	Gender (M/F)	Pack Years	HbA1c (%)	Endostatin concentration (pg ml^{-1})		
					Serum	Unstim Saliva	Stimult Saliva
005 (I)	45	M	36	8.1	**31591**	-	**0**
025 (I)	69	M	71	7.5	**45316**	-	**0**
034 (II)	66	M	0	8.7	**38479**	-	**0**
035 (II)	61	M	0	9.5	**41664**	-	**778**
049 (I)	61	M	0	8.7	**64186**	-	-
052 (II)	78	M	0	5.8	**50118**	**0**	**0**
053 (II)	55	M	0	6.6	**53903**	-	**431**
061 (II)	42	M	20	6.8	**24076**	-	**0**
062 (II)	56	M	0	7.6	**34014**	**0**	**0**
066 (II)	76	M	33	8.8	**45567**	-	**0**
068 (II)	73	M	0	8.1	**66424**	-	**0**
078 (II)	66	M	22	6.6	**70074**	-	**0**
081 (II)	64	F	51	7.1	**30067**	-	**0**
086 (I)	42	F	7	9.4	**37311**	-	**0**
087 (II)	38	F	24	7.5	-	-	**0**
091 (II)	49	M	0	6.3	**63545**	-	**0**
N=16	**Mean=59±13yrs**	**M=13/F=3**		**Mean=7.7±1.1**	**N=15**		**N=15**

Appendix 4

Periotron 8000 calibration protocol

Periotron 8000 Calibration

Introduction

A Periotron model 8000 machine (1995) (Oraflow Incorporated, Plainview, New York, USA) was used to measure the minute volumes of GCF harvested on the PerioPapers (Proflow Incorporated, Amityville, New York, USA). A single batch of PerioPapers was used throughout the study to ensure consistency (batch number 6178). The Periotron works by measuring electrical capacitance across the PerioPaper, which is placed between the jaws of the machine. The jaws have an opposing electric charge which polarises the molecules on the papers leading to a reduced electrical potential difference and an increased capacitance between the jaws (Ciantar and Caruana, 1998). The capacitance of a wet PerioPaper increases in proportion to the volume of the fluid it, which is reflected in the Periotron reading (Periotron Units), and the relationship has been shown not to be linear but is best described by a fourth order polynomial (Chapple et al., 1999). Thus, the machine has to be calibrated in order to convert the digit output from the machine (Periotron units) into volumes of GCF (microlitres) by producing a standardised calibration curve.

Periotron Calibration

The method used was based on the protocol described by Preshaw et al.(1996). Prior to use the Periotron 8000 must be switched on for ten minutes, making sure it is set to the "Perio" setting required for GCF recording. This model of Periotron is also designed to record volumes of saliva obtained from minor salivary glands when switched to the "Sialo" setting. Ideally GCF should be used as the standard calibration fluid but it is impractical to collect it in sufficient quantities, so serum or distilled water is usually used for calibration. Evidence has shown that serum is the most accurate as it has similar characteristics to GCF (Chapple et al., 1999). A standardised stock of serum was produced, the blood being taken from myself, using the same protocol used for the serum collection in the study (Chapple et al., 1997) (Chapter 2.1.8). This was stored in the -20^{o}C freezer prior to use. A 1 in 5 dilution of the serum in sterile distilled water was used for the calibration to reduce the clogging of the Hamilton syringes (Hamilton Company, Reno, Nevada, USA) (Personal communication: Dr Gareth Brook). Chapple et al. (1999) showed that this dilution of serum had little effect on

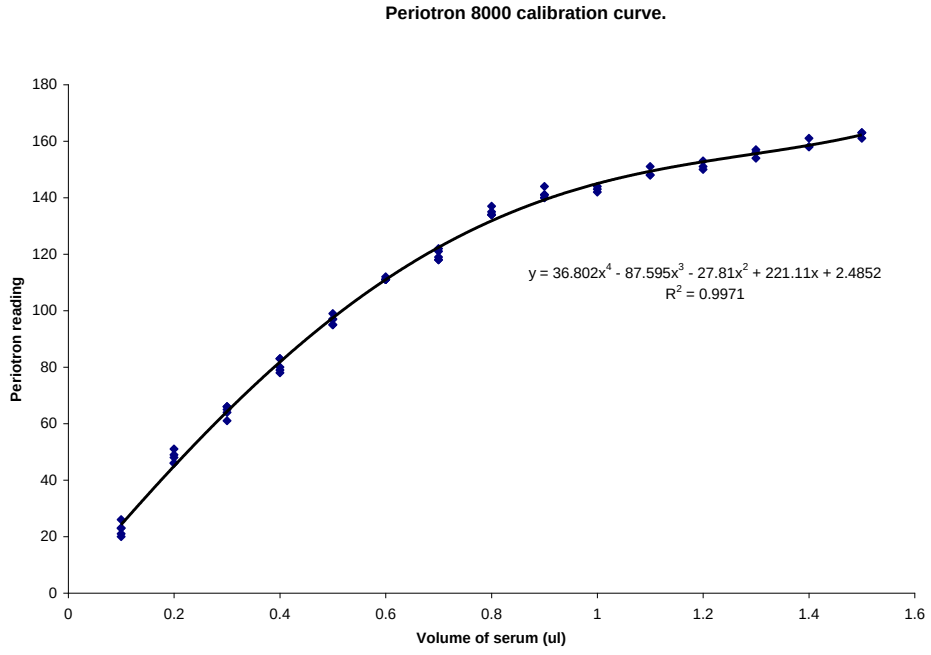
the accuracy of the GCF volume determination above 0.1µl. This paper also showed that the Periotron 8000 was accurate over time and only required recalibration every few months. Blunt-ended positive pressure displacement Hamilton 7000 syringes, sizes 1µl (gauge 22) and 2µl (gauge 23), were used to place accurate volumes of the diluted serum onto PerioPapers prior to reading in the Periotron. The PerioPaper was held parallel to the floor and the serum was expelled from the syringes at 90^{o} onto the centre of the white part of the PerioPapers in one movement. Each PerioPaper was immediately placed between the jaws of the Periotron to within 0.5mm of the orange area with the PerioPaper being perpendicular to the main body of the machine. Exact positioning of the PerioPaper needs to be consistent to reduce variations in the readings (van der Bijl et al., 1986). Immediate reading of the PerioPapers has been shown to be important as evaporation, particularly with small volumes of fluids, can lead to significant sampling errors (Garnick et al., 1979). Tozum, et al. (2004) found no significant fluid losses due to evaporation with transfer times of 5 seconds and 10 seconds compared to the baseline test volumes. Transfer times in the order of 30 seconds resulted in significant reductions in Periotron readings and subsequent volume readings were detected. Other potential environmental sources of error include room temperature and humidity (Tozum, et al. (2004). These environmental factors have been shown to result in an error range between 5% to 11% in earlier models of the Periotron (Garnick et al., 1979, Offenbacher et al., 1984).

It is also important to ensure that no metal objects, such as rings or watches, are in close proximity to the Periotron, as this has a marked effect on the Periotron readings. The final Periotron reading is displayed on the LED screen after 16 seconds. The Periotron was recalibrated to zero between readings using a blank PerioPaper. It is important that clean dry college tweezers were used to move the PerioPapers, using only the orange part of the papers, as any moisture contamination can affect the readings. Periotron readings were recorded for volumes of serum in 0.02µl increments between 0.02µl and 1µl. Each volume was pipetted five times and the Periotron readings were averaged for each volume, any outliers were rejected and the reading repeated. Triplicates readings for each 0.02µl increment were recorded for the volumes between 1µl and 2µl. Due to the minute volumes used, care was taken to prevent air bubble formation in the Hamilton syringes, which would greatly affect

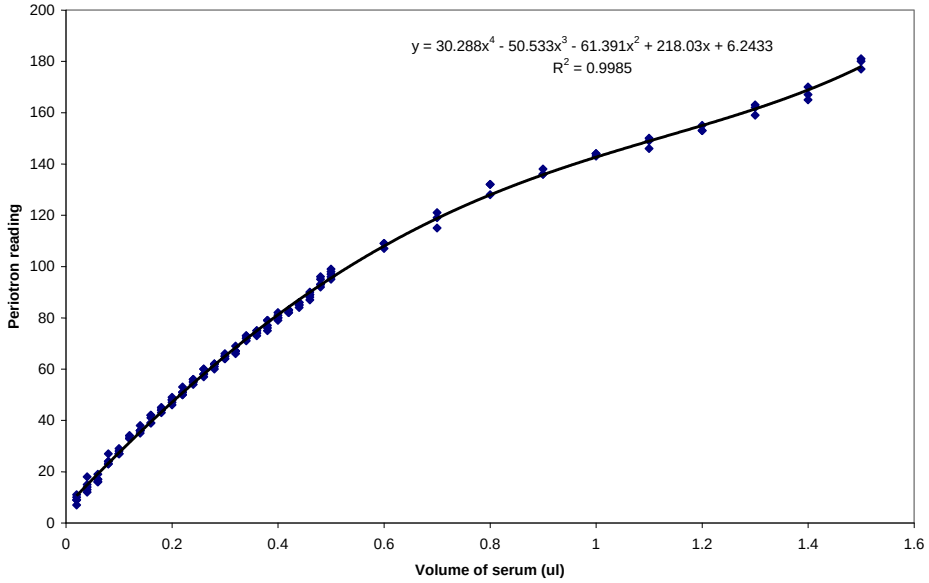
the accuracy, when measuring the diluted serum. A good way to prevent air bubble formation was to draw too much fluid into the syringe and forcefully expel the volume and repeat this multiple times prior to drawing up the desired volume. Any additional droplets of serum at the tip of the syringe were removed by wiping the syringe tip against a dry side of the Universal container holding the diluted serum. The readings were carried out immediately after pipetting to prevent evaporation of the small volumes of fluid used (Tozum et al., 2004). These potential errors have a greater percentage effect on the Periotron readings at small volumes, and so, it has been proposed that the accuracy limit of the Periotron should be regarded as around 0.1µl. The manufacturer states that each machine should read about 100 Periotron units for 0.5µl of serum, but the actual readings will vary between individual machines. A single Periotron 8000 machine was used throughout this study and was calibrated at the start of the study, and every 3 to 4 months subsequently.

A calibration curve for the Periotron was produced by plotting the Periotron readings (units) against the volumes of diluted serum (µl) and plotting a fourth order polynomial grid line (Chapple et al., 1999). The mathematical formula of this gridline was then used to calculate the GCF volumes of the clinical study samples using an Excel spreadsheet.

Figure 1: Examples of Periotron 8000 calibration curves from the clinical study.
(a)



(b)


y = 30.288x4 - 50.533x3 - 61.391x2 + 218.03x + 6.2433
R2 = 0.9985
Periotron reading
Volume of serum (ul)
0
20
40
60
80
100
120
140
160
180
200
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6

Appendix 5

Haematoxylin and Eosin (H and E) staining protocol

The slides used for the immunohistochemistry studies were sectioned from formalin-fixed paraffin-embedded tissue blocks. One slide from each tissue block was stained with H and E to allow the histology of each sample to be studied and then compared to complimentary slides stained with various antibodies e.g. anti-vWF and anti-CD105 antibodies. A standard protocol was used (NHS Tayside Oral Pathology Service).

The slides were placed into a metal slide rack and immersed sequentially into staining troughs for set times as outlined below. The sections were initially dewaxed and rehydrated prior to staining. The dewaxing stage involved using xylene, so a metal slide rack was used as xylene damages plastic. The staining protocol was as follows:

1) Dewaxing and rehydration stage

Xylene (BDH)	5 minutes x 2
100% Ethanol	2 minutes
95% Ethanol	2 minutes
Distilled Water	2 minutes

2) H and E Staining stage

Haematoxylin (Mayers)	5 minutes
Distilled Water	2 minutes
Blueing agent (Thermo Shandon)	4 minutes
Distilled Water	2 minutes
Eosin (aqueous) (Thermo Shandon)	3 minutes
Distilled Water	2 minutes
96% Ethanol	$1^1/_2$ minutes
100% Ethanol	1 minute
100% Ethanol	$1^1/_2$ minutes
Xylene (BDH)	3 minutes

The slides were remounted using DPX microscopy mountant (BDH Laboratory Supplies) and glass cover slips were applied and the slides were dried at 37^{o}C for 3-4 hours.

Haematoxylin and Eosin stains most cells and organelles in a standard staining pattern. Haematoxylin stains negatively charged nucleic acids, DNA and RNA, blue (basophilic). Eosin stains positively charged structures such as most organelles (except ribosomes), muscle cells, fibrin and keratin bright red (eosinophilic). Eosin also stains collagen pink and red blood cells orange/red (Wheater et al., 1987).