C-REACTIVE PROTEIN, PERIODONTITIS AND SYSTEMIC INFLAMMATION

A report submitted to the University of Adelaide in partial fulfillment of the requirements of the Degree of Doctor of Clinical Dentistry (Periodontology)

Emma Dominique Megson BSc (UNSW) BDent (Hons) (USyd)

Table of Contents

Abstract ... iii

Declaration ... iv

Acknowledgements ... v

Chapter One. Literature Review of C-reactive Protein ... 1

1.1 Introduction ... 1

1.2 What is C-reactive Protein? ... 1

1.3 Source ... 2

1.4 Structure ... 3

1.5 Forms of CRP ... 4

1.6 Ligand Binding ... 5

1.7 Receptors ... 5

1.8 Functional properties ... 6

1.9 Acute Phase Response ... 8

1.10 Normal levels of CRP ... 10

1.11 Measurement of CRP ... 11

1.12 Genetics ... 11

1.13 CRP is Associated with Systemic Disease ... 13

1.13.1 Intervention Studies ... 15

1.13.2 Biological Considerations ... 16

1.14 Systemic Disease is Associated with Periodontal Disease ... 18

1.14.1 Intervention Studies ... 19

1.14.2 Biological Plausibility ... 21

1.15 Periodontal Disease is Associated with CRP ... 23

1.15.1 Cross Sectional Studies of CRP in Saliva and GCF ... 24

1.15.2 Cross Sectional Studies for Serum CRP and Periodontitis ... 26

1.15.2.1 No Correlation with CRP ... 26

1.15.2.2 Insufficient Adjustment for Confounders ... 26

1.15.2.3 Systemic Medical Conditions ... 26

1.15.2.4 Non-traditional Measures of Periodontal Disease ... 27

1.15.2.5 Epidemiological Studies ... 27

1.15.2.6 Systemically Healthy Subjects ... 28

1.15.2.7 Edentulousness ... 29

1.15.3 Serum CRP and Periodontal Therapy Longitudinal Studies ... 29

1.15.3.1 Time Course of Changes in Serum CRP Following Periodontal Therapy 29

1.15.3.2 Non-surgical Therapy 30
1.15.3.3 Surgical and Non-surgical Therapy 31
1.15.3.4 Adjunctive Local Antibiotics....................................31
1.15.3.5 Adjunctive Systemic Antibiotics 32
1.15.3.6 Host Modulation 32
1.15.3.7 Dental Clearance....................................32
1.15.3.8 Summary....................................33
1.15.4 Longitudinal Studies of Serum CRP and Periodontal Disease Activity..........35
1.15.5 Biological Plausibility....................................36
1.16 Conclusion 38
1.17 References....................................40
Chapter Two. C-reactive Protein in Gingival Crevicular Fluid may be Indicative of Systemic Inflammation 73
2.1 Introduction....................................74
2.2 Methods 75
2.2.1 Study Population....................................75
2.2.1.1 Patients....................................75
2.2.2 Sample Collection, Processing and Analysis....................................75
2.2.2.1 Gingival Crevicular Fluid (GCF) Collection and Analysis 75
2.2.2.2 Immunohistochemistry for C-reactive protein....................................76
2.2.2.3 Real-Time Polymerase Chain Reaction for CRP gene detection 77
2.2.3 Statistical Analysis....................................78
2.3 Results....................................79
2.3.1 Subject demographics and sample collection 79
2.3.2 Detection of CRP in GCF samples 79
2.3.3 Immunohistochemical staining for CRP in gingival tissue 81
2.3.4 Real-time CRP analysis of gingival tissue for CRP mRNA gene expression 81
2.4 Discussion....................................81
2.5 References....................................85
Tables and Figures 91
Chapter Three. Addendum....................................94
3.1 Addendum....................................94
3.2 References....................................96

Abstract

Background and Aim: Periodontitis is associated with elevated C-reactive protein (CRP) in both serum and gingival crevicular fluid (GCF). CRP is an acute phase protein, the levels of which closely follow inflammatory disease activity. CRP is used as a risk predictor for cardiovascular events, including myocardial infarction. Periodontitis is associated with an increased risk cardiovascular disease. The nature of the relationship between periodontitis and cardiovascular disease is unclear, but may involve systemic inflammation as measured by CRP. Although the liver is the primary source of CRP, extra-hepatic production of CRP has been reported. Local production of CRP in the periodontal tissues may contribute to serum levels. This study aimed to determine whether CRP in GCF is produced locally in the gingivae.

Materials and Methods: Gingivae and GCF were collected from non-periodontitis and periodontitis sites. Presence of CRP in gingivae was assessed by immunohistochemistry. CRP in GCF was measured using ELISA. Gene expression for CRP in gingivae was determined using real-time polymerase chain reaction.

Results: CRP was found in both the gingivae and GCF. No gingivae had detectable amounts of CRP mRNA. Not all patients with periodontitis had detectable levels of CRP in the GCF. Some non-periodontitis patients had detectable levels of CRP in the GCF.

Conclusion: CRP in the GCF appears to be of systemic origin, and therefore may be indicative of systemic inflammation from either a periodontal infection or inflammatory disease elsewhere. CRP in the GCF may be a substitute measure for serum CRP. The correlation between levels of CRP in GCF and serum requires validation in future studies.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Emma Dominique Megson and, to the best of my knowledge and belief, contains no material previously published or written by another person except where due reference has been made in the text.

The input of the co-authors to this work was mainly advisory and I carried out the bulk of the laboratory procedures and all the writing of this manuscript.

I give consent to this copy of the thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis (as listed below) resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on he web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Published works:
Megson E, Fitzsimmons T, Dharmapatni K, Bartold PM. C-reactive protein in gingival crevicular fluid is indicative of systemic inflammation. *Journal of Clinical Periodontology* 37:797-804; 2010.

Declared by: Dr Emma Megson　　Witnessed by: ________________________

Date:　　Date:

Acknowledgements

This study was supported by a grant from the National Health and Medical Research Council of Australia (Project Grant # 565341).

I wish to thank my supervisor Professor Mark Bartold for his guidance and feedback.

I was very lucky to be expertly supported in my laboratory work by Dr Tracy Fitzsimmons and Dr Kencana Dharmapatni. Their knowledge and enthusiasm is much appreciated.

Thank you to Mr Dale Caville for his help with imaging the histological sections.

Thank you to Ms Catherine Offler for her assistance in the final editing of this manuscript.

I am very grateful for the excellent help from my postgraduate colleagues, Dr Raymond Chan, Dr Chris Bates, Dr Geoff Harvey, Dr Kere Kobayashi and Dr Tina Choo in sample collection, and also for their friendship over the course of the program.

I could not have withstood the rigors of the program without support from my partner Dr Raymond Chan. His dedication to excellence has been an inspiration for me both personally and professionally.

Chapter One. Literature Review of C-reactive Protein

1.1 Introduction

Oral fluids are a substrate for a new generation of non-invasive diagnostic tools through the development of ultra- and high-sensitivity detection systems and the fact that they contain many serum analytes that reflect normal function or disease status (Tanke 2007). Within the oral cavity C-reactive protein (CRP) has been detected in both gingival crevicular fluid (GCF) and saliva, and is considered to be an important biomarker for systemic disease. Traditionally, CRP was thought to be produced exclusively by the liver, but recent research suggests localised production in neurons (Yasojima *et al* 2000), smooth muscle cells (Yasojima *et al* 2001, Jabs *et al* 2003b), macrophages of atherosclerotic plaques (Yasojima *et al* 2001), epithelial cells (Jabs *et al* 2003a, Jabs *et al* 2005), and adipose tissue (Ouchi *et al* 2003). This then leads to the question of what is the source of CRP in oral fluids – is it local from the periodontal or oral tissues, or delivered by the serum as a result of systemic inflammation? If systemic, then GCF levels could be used to replace blood tests as an easy measure of systemic CRP to evaluate risk/prognosis in other diseases. If local, then this may be indicative of active inflammation in periodontal tissues, with the potential to act as a marker of disease activity.

1.2 What is C-reactive Protein?

CRP is a plasma protein involved in the acute phase response. CRP was first discovered in 1930, as a protein that could precipitate the “C” polysaccharide derived from the pneumococcal cell wall (Tillet and Francis 1930). The specific ligand was later found to be phosphocholine (Volanakis and Kaplan 1971). CRP is of interest as a biomarker in a number of diseases and is currently used as a marker of disease activity (Shrive *et al*. 1996). However, research findings suggest that CRP is not merely a marker of inflammation, but also has complex functions that may directly modify the inflammatory process (Pasceri *et al* 2000). CRP has since been found to interact with various other ligands, activate the classical complement pathway, stimulate phagocytosis and bind to FcγR immunoglobulin receptors (Black *et al* 2004).

CRP is a member of a conserved, phylogenetically ancient super-family of oligomeric calcium-binding proteins exhibiting high sequence homologies, known as pentraxins (Osmand *et al* 1977a, Shrive *et al* 1996). Pentraxins have been found in

mammals, birds, amphibians, fish and the primitive horseshoe crab (Pepys *et al* 1978, Robey and Liu, 1981). The evolutionary conservation of this protein and its binding specificity over a diverse range of species suggests that CRP has important biological functions (Pepys *et al* 1978, Shrive *et al* 1996), which are likely to be beneficial to survival (Pepys *et al* 2006).

1.3 Source

Historically, it was believed CRP was produced primarily, if not exclusively, by liver hepatocytes (Miller *et al* 1951, Hurlimann *et al* 1966, Heinrich *et al* 1990, Steel and Whitehead 1994). However, the first report of extra-hepatic synthesis of CRP, by human lymphocytes, was in 1986 (Kuta and Baum 1986), and since then several reports have outlined a variety of extra-hepatic sources of human CRP. These include the neurons of patients with Alzheimer's disease (Yasojima *et al* 2000), epithelial cells from nasal polyps (Gould and Weiser 2001), inflamed and non-inflamed secretions from the respiratory tract (Gould and Weiser 2001), normal arterial smooth muscle (Yasojima *et al* 2001), smooth muscle cells and macrophages of atherosclerotic plaques (Yasojima *et al* 2001), normal saphenous veins in patients with atherosclerosis (Jabs *et al* 2003b), smooth muscle cells of diseased coronary artery venous bypass grafts (Jabs *et al* 2003b), aortic aneurysmal tissue (Vainas *et al* 2003), renal cortical tubular epithelial cells of renal allografts with acute rejection (Jabs *et al* 2003a), adipose tissue from patients with coronary artery disease (Ouchi *et al* 2003), and renal cell carcinoma epithelium and normal renal tubular epithelium from patients with renal cell carcinoma (Jabs *et al* 2005). There is accumulating evidence that the acute-phase reaction or its components do not always represent a systemic response pathway, but may take place in the local environment (Jabs *et al* 2003a, Jabs *et al* 2005). However, it is unlikely that these extra-hepatic sources substantially increase serum levels of CRP (Black *et al* 2004).

1.4 Structure

NOTE:
This figure is included on page 3 of the print copy of
the thesis held in the University of Adelaide Library.

Figure 1. Structure of C-reactive protein (Black *et al* 2004)

CRP has a cyclic pentameric structure, consisting of five identical subunits of 206 amino acids (Shrive *et al* 1996). These are noncovalently arranged around a central pore (Shrive *et al* 1996). Each protomer is composed of two antiparallel β strands (Shrive *et al* 1996) arranged in a flattened jellyroll topology (Thompson *et al* 1999).

The concave recognition face contains the phosphocholine-binding site and two calcium ions adjacent to a hydrophobic pocket (Thompson *et al* 1999, Black *et al* 2004). Phe-66 and Glu-81 are believed to mediate the binding of CRP to phosphocholine by hydrophobic interactions with methyl groups and positively charged choline nitrogen respectively (Thompson *et al* 1999, Agrawal *et al* 2002), although Phe-66 is the major determinant of the CRP-phosphocholine interaction (Agrawal *et al* 2002).

The opposite face is the effector face, in which a deep cleft is believed to mediate binding with complement C1q and Fcγ receptors (Agrawal *et al* 2001, Black *et al* 2004). Conformational changes in the C1q binding site of CRP are required for binding to C1q (Gaboriaud *et al* 2003), and these changes differ depending on the ligand to which CRP is bound (Black *et al* 2003). Each individual subunit is capable of interacting with phosphocholine and C1q, resulting in five phosphocholine binding sites per CRP molecule (Black *et al* 2003).

1.5 Forms of CRP

Native CRP exists in a pentamer of five identical subunits (Osmand 1977). However, in the absence of calcium or in a denaturing environment, native CRP may undergo structural changes, dissociating from its pentameric arrangement (Potempa *et al* 1983, Motie *et al* 1996, Wang and Sui 2001). Modified forms of CRP (mCRP) express different epitopes compared to native CRP (Ying *et al* 1989), and have distinct properties (Potempa *et al* 1987, Egenhofer *et al* 1993, Motie *et al* 1996, Zouki *et al* 2001).

Naturally occurring antigens cross-reactive with modified forms of CRP have been detected in normal human peripheral blood lymphocytes (Samberg *et al* 1988), human NK and B cells (Bray *et al* 1988), rat hepatocytes and Kupffer cells (Egenhofer *et al* 1993), normal human blood vessel intima (Diehl *et al* 2000), human monocytes (Kolb-Bachofen *et al* 1995), rat islet cells (Fehsel *et al* 1997), epithelial cells of the human respiratory tract (Gould and Weiser 2001), the plasma of rheumatoid arthritis patients (Potempa *et al* 1987), and inflamed rabbit liver and muscle (Rees *et al* 1988), suggesting mCRP is a naturally occurring tissue-associated molecule (Diehl *et al* 2000, Heuertz *et al* 2005).

Several forms of CRP have been reported *in vitro* (Wang *et al* 2002): native pentameric CRP was found on ligand-containing membranes in a calcium dependent manner; small globulin-like monomers were found on negatively charged membranes and were structurally stable in the absence of calcium; and freshly purified CRP formed fibril-like structures by the face-to-face stacking of pentameric CRP, which continued to grow in length with increasing duration of storage (Wang *et al* 2002). CRP pentamers in solution under calcium-free conditions undergo spontaneous dissociation into CRP subunits (Myles *et al* 1990). In neutral buffer and in the absence of calcium fresh CRP pentamers slowly and continuously dissociate, while in the presence of calcium dissociation is rare (Wang *et al* 2002). Similarly, in acid conditions, dissociation is greatest in the absence of calcium (Wang *et al* 2002).

Therefore, CRP stored for prolonged periods in the absence of calcium may undergo spontaneous formation of mCRP, and this may affect experimental results. Under laboratory conditions, mCRP can be formed from native CRP by treatment with acid, urea-chelation and heating in the absence of calcium (Potempa *et al* 1987). It is proposed that conformationally altered forms of CRP are created from native CRP at the inflammatory site due to the conditions of the local environment (Motie *et al* 1996), and contribute to the inflammatory process through their pro-inflammatory properties (Shields 1993). As the local conditions favouring conversion of native CRP to modified CRP subside, native CRP

becomes the predominant form, and assists resolution of the lesion (Shields 1993). In contrast, some authors believe that mCRP is not a naturally occurring molecule and that it is unlikely to persist *in vivo* long enough to have biologically significant effects (Pepys and Hirschfield 2003).

1.6 Ligand Binding

CRP can bind to a wide variety of ligands (Shrive *et al* 1996, Black *et al* 2004). The primary ligand of CRP is the phosphate monoester, phosphocholine (Volanakis and Kaplan 1971). Phosphocholine is universally found in the phospholipids of cell membranes and in plasma proteins, and is commonly found in complex polysaccharides of plants, fungi and bacteria (Thompson *et al* 1999). However, in normal cells the phosphocholine is inaccessible to CRP and therefore binding only occurs in damaged and apoptotic cells when disruption of the membrane exposes the phosphocholine (Volanakis and Wirtz 1979, Hack *et al* 1997, Chang *et al* 2002).

CRP binding to ligands occurs in two ways, through calcium-dependent binding and polycation binding (Du Clos 1996). Calcium-dependent binding is strongly inhibited by phosphocholine (Potempa *et al* 1981). In contrast, polycation binding is not inhibited by phosphocholine, but is inhibited by calcium (Siegel *et al* 1975, DiCamelli *et al* 1980, Black *et al* 2003), an effect reversed by the presence of phosphocholine (Potempa *et al* 1981).

CRP binds to other phosphate monoesters (Gotschlich and Edelman 1967), chromatin (Robey *et al* 1984, Du Clos *et al* 1988), histones (Du Clos *et al* 1988), fibronectin (Salonen *et al* 1984), small nuclear ribonucleoproteins (Du Clos 1989, Pepys *et al* 1994), phosphorylated polysaccharides (Culley *et al* 2000), laminin (Swanson *et al* 1989), and oxidized LDL (Chang *et al* 2002) in a calcium-dependent manner.

As such, the presence of phosphocholine and calcium may have important effects on CRP binding, and therefore activity, *in vivo*.

1.7 Receptors

CRP binds to the receptors FcγRI (Mold *et al* 2001, Mold *et al* 2002) and FcγRII on phagocytic cells of mice (Mold *et al* 2002) and FcγRIIa, also known as CD32, in humans (Bharadwaj *et al* 1999, Stein *et al* 2000, Chi *et al* 2002). In contrast, binding of mCRP on neutrophils is inhibited by anti-CD16 antibodies, suggesting it binds to an alternative receptor, FcγRIII (CD16), compared to native CRP (Heuertz *et al* 2005). However, the

findings of a specific receptor for CRP have not been reproducible by other authors (Hundt *et al* 2001, Saeland *et al* 2001). The enhancement of phagocytosis by CRP is likely to occur by signaling through these receptors (Black *et al* 2004). Differential activation of the Fc receptors by the different forms of CRP may explain the pro- and anti-inflammatory effects of CRP (Mold *et al* 2002).

1.8 Functional properties

Although CRP is most commonly used as a marker of disease activity, the exact physiological role of CRP is unclear (Shrive *et al* 1996). *In vitro* research suggests CRP has both pro- and anti-inflammatory effects (Black *et al* 2004). An explanation for the seemingly conflicting pro- and anti-inflammatory actions of CRP is that distinct species of CRP are formed during inflammation (Shields 1993), and have different properties.

A wide variety of properties have been attributed to CRP, including complement activation, cytokine release, enhancement of phagocytosis, inflammatory cell recruitment and activation, removal of material from necrotic cells and promotion of atherogenesis. Disappointingly almost all the findings from this research are potentially questionable due to the possibility of contamination of commercial CRP preparations with biologically active constituents, such as sodium azide (Liu *et al* 2005) and lipopolysaccharide (LPS) (Pepys *et al* 2005, Taylor *et al* 2005), and much of the research on CRP has been undertaken without the use of suitable azide and endotoxin controls (Turu *et al* 2008). It has been suggested that the proinflammatory effects of human CRP preparations on cells *in vitro* were actually due to the effects of contaminants and not CRP itself (Pepys *et al* 2005, Taylor *et al* 2005).

Commercial CRP is derived from recombinant human CRP produced in *Escherichia coli* and has sodium azide added as a preservative. As such it is potentially contaminated with both LPS and sodium azide (Pepys *et al* 2005), unlike CRP isolated from human malignant ascites fluid or produced recombinantly in mammalian cells (Pepys *et al* 2005). In fact, even when purified by dialysis, recombinant CRP produced by *E. coli* does not give equivalent results to ascites-fluid derived CRP (Pepys *et al* 2005).

Pure preparations of CRP applied to human endothelial cells have no effect on apoptosis, migration, or proliferation, unlike contaminated preparations of CRP or sodium azide alone (Liu *et al* 2005). Similarly, LPS, sodium azide and commercial CRP are able to induce changes in cell proliferation, cell morphology, apoptosis, expression of endothelial nitric oxide synthase and intercellular adhesion molecule (ICAM) -1, and increase

interleukin (IL) -8 and monocyte chemotactic protein (MCP)-1 secretion, whereas pure CRP does not (Taylor *et al* 2005). *In vivo* and *in vitro*, pure CRP is not proinflammatory, with no effect on acute phase proteins after 24 hours, nor on inflammatory cytokines (Pepys *et al* 2005). This has serious implications for the validity of previously published research findings using contaminated CRP preparations. However, it is still acknowledged that CRP complexed to a ligand, as occurs in damaged tissues, is indeed proinflammatory (Pepys *et al* 2005).

Recent research addressing the possibility of contamination by the use of appropriate controls and purification, still implicates CRP as an active contributor to atherosclerosis and inflammation (Dasu *et al* 2007, Ho *et al* 2008, Turu *et al* 2008). In a study using purified LPS, sodium azide and unpurified commercial CRP as controls and anti-CRP antibodies to confirm results, gel filtration-purified recombinant CRP stimulates angiogenesis *in vitro* and *in vivo* (Turu *et al* 2008). Gel filtration-purified native CRP derived from human pleural or ascites fluid stimulates IL-1β, IL-6, IL-8, and plasminogen activator inhibitor (PAI)-1 *in vitro* from Toll-like receptor 4 knockout human aortic endothelial cells unlike LPS controls, indicating LPS contamination was not responsible for these effects (Dasu *et al* 2007). Dialysis-purified recombinant native CRP has no effect on human saphenous vein smooth muscle cell viability or proliferation, but did enhance chemotaxis to PDGF (Ho *et al* 2008). CRP from purified, dialyzed human ascites or pleural fluid decreases endothelial cell nitric oxide synthase in human aortic endothelial cells, resulting in increased superoxide production, and decreased nitric oxide production (Singh *et al* 2007), and decreased IL-10 secretion from activated human macrophages (Singh *et al* 2006). However, strictly controlled studies confirming the proinflammatory properties of CRP are both few and unconfirmed.

The potentially pro-atherogenic/pro-inflammatory *in vitro* properties of CRP are not wholly supported by *in vivo* studies on mice (Scirica *et al* 2006). Male apolipoprotein E-deficient mice, transgenic for human CRP, exhibited larger atherosclerotic lesions than non-transgenic mice, but the differences were not statistically different for female mice (Paul *et al* 2004). Other studies have found no relationship between CRP and atherosclerosis in transgenic mice (Hirschfield *et al* 2005, Trion *et al* 2005). Mice transgenic for CRP are predisposed to arterial thrombosis following vascular injury (Danenberg *et al* 2003). However, when this thrombosis is controlled with aspirin and heparin at the time of injury, these mice exhibited less cellular proliferation and apoptosis in the damaged vessel intima than wild-type mice treated with aspirin and heparin, which suggests CRP is a complex regulator of vascular biology (Danenberg *et al* 2008).

However, the mouse model may have severe limitations for the study of CRP. This is because CRP is not naturally an acute phase reactant in mice (Torzewski 2005), only increasing modestly to a maximum of 2 mg/L (Pepys and Hirschfield 2003), and does not induce systemic inflammation in mice (Hirschfield *et al* 2005). Transgenic mice produce supra-physiological concentrations of CRP (concentrations up to 123 mg/L) (Paul *et al* 2004), and human CRP is a foreign protein in mice with uncertain functions in these animals (Torzewski 2005). This may in part explain the mixed results in CRP transgenic mice (Scirica *et al* 2006). Similarly in rats, CRP is not considered a typical acute phase protein, being present at much higher basal levels than humans, approximately 300-500 mg/L (Nunomura 1990) and only increasing a maximum of two-fold in response to injury (Giffen *et al* 2003, Cray *et al* 2009). Watanabe heritable hyperlipidemic rabbits may be a better animal model to study CRP as these rabbits produce a CRP that is 70% homologous to humans (Osmand *et al* 1977b), responsive to inflammatory stimuli (Kushner and Feldmann 1978), elevated in the presence of high cholesterol, and present in levels which correlate with aortic atherosclerosis lesion size (Sun *et al* 2005).

Some authors dispute the likelihood that CRP has detrimental pro-inflammatory effects, because as an evolutionarily preserved protein it is more likely to have conferred a survival advantage to the host. However, this does not preclude the possibility that CRP has damaging effects that only manifest in the post-reproductive years, as such traits would not have been diluted by natural selection (Pepys *et al* 2006).

1.9 Acute Phase Response

The systemic changes that accompany inflammation are referred to as the acute-phase response (Kushner 1982), even though they accompany both acute and chronic inflammation (Gabay and Kushner 1999). Understanding of the acute phase response is incomplete and continues to develop (Gabay and Kushner 1999). There are two arms to the acute phase response (Gabay and Kushner 1999): changes in the concentrations of plasma proteins; and behavioural, physiologic, biochemical and nutritional changes

Acute phase proteins are those proteins whose plasma concentration is altered (increases or decreases) by at least 25% in response to inflammation (Kushner 1982, Morley and Kushner 1982). The liver produces many of these proteins. Acute-phase proteins have both pro- and anti-inflammatory properties (Gabay and Kushner 1999), and include CRP, serum amyloid A, plasminogen activator-1, the complement proteins C3, C4 and C9 and fibrinogen. In fact, CRP and serum amyloid A both increase by 1000% in

response to inflammation, while other proteins may only increase by 50%, as in the case of ceruloplasmin (Kushner 1982).

Acute phase reactants are an early, non-specific response to bacterial, viral or parasitic infection, mechanical or thermal trauma, ischemic necrosis or malignant growth (Kushner 1982, Koj 1989, Baumann and Gauldie 1994, Gabay and Kushner 1999). Additionally changes occur following surgery (Craig *et al* 2001) and in unmedicated psychiatric illness (Maes *et al* 1997). The role of these reactants is to ensure survival during the period immediately following injury (Ebersole and Cappelli 2000), by assisting in the removal of the inciting agent and promoting tissue repair (Cid *et al* 1993, Steel and Whitehead 1994). The acute phase response acts to restore the normal homeostasis disturbed by the injury, but excessive production of inflammatory mediators may have negative effects (Koj 1996), contributing to tissue damage (Steel and Whitehead 1994).

Production of acute phase proteins by hepatocytes is stimulated by cytokines released by macrophages and monocytes at sites of inflammation, in particular interleukin-6 (IL-6) and IL-1β (Ganter *et al* 1989, Kushner *et al* 1995, Zhang *et al* 1995, Weinhold and Ruther 1997), but also transforming growth factor-β (TGF-β) (Mackiewicz *et al* 1990) and IL-8 (Wigmore *et al* 1997). These cytokines act as a cascade and network to regulate the production of other cytokines and mediators: IL-1 induces IL-6 (Smith *et al* 1992) and tumour necrosis factor (TNF) (Ikejima *et al* 1990); IL-6 inhibits expression of TNF-α (Xing *et al* 1998); and TNF-α induces IL-1 (Brennan *et al* 1989). Advanced glycation end products such as those produced in diabetes also stimulate IL-6 and IL-1β production by human monocytes, and thereby enhance CRP production (Li *et al* 2007). Overall, the effect on a cell depends on the combination of cytokines to which it is exposed (Mackiewicz *et al* 1991).

Other changes associated with the acute phase response include fever, anorexia, somnolence, lethargy, anemia of chronic disease, and cachexia (Gabay and Kushner 1999). Extreme forms of the acute phase responses such as septic shock can be fatal, and the persistent acute phase response seen in advanced cancer and AIDS is responsible for the marked cachexia associated with these conditions (Gabay and Kushner 1999).

Measuring acute phase proteins provides information about the presence and intensity of an inflammatory process, thus helping to differentiate inflammatory from non-inflammatory conditions, giving an indication of the need for and response to therapy (Gabay and Kushner 1999). In some diseases, such as rheumatoid arthritis, serial measurements of C-reactive protein are used in prognosis (Van Leeuwen *et al* 1994).

1.10 Normal levels of CRP

CRP is normally present in ng/ml quantities in human serum, but may increase 100 to 1000-fold within 72 hours of tissue injury (Claus *et al* 1976). CRP concentrations closely follow the course of the acute-phase response to inflammation or tissue necrosis, and so can theoretically provide a valuable measurement for many disease processes (Ebersole and Cappelli 2000, Gould and Weiser 2001). CRP has several analytical properties that make it attractive as a clinical marker (Libby and Aikawa 2002), in particular: a long half-life with no observable circadian rhythm (Meier-Ewert *et al* 2001); rapid changes follow the course of the inflammatory process (Gabay and Kushner 1999); a broad range of abnormal values (Gabay and Kushner 1999); levels are not affected by the age of the patient (Gabay and Kushner 1999); easy measurement by blood test; and commercially available high-sensitivity assays that provide similar results in fresh, stored and frozen plasma (Rifai *et al* 1999).

Large population-based studies suggest CRP levels have a log-normal distribution, with a mean of approximately 1.6 mg/L and a wide variation (Koenig *et al* 1999). Most normal human subjects have CRP concentrations of less than 1 mg/L, and up to 10 mg/L has historically been regarded as clinically unimportant (Morley and Kushner 1982). However, concentrations well below 10 mg/L have more recently been associated with progressive joint damage in osteoarthritis patients (Spector *et al* 1997), and have also been predictive of subsequent coronary events (Haverkate *et al* 1997, Ridker *et al* 1997). As such, even mildly elevated serum CRP levels are evidence of an active tissue-damaging process, and serum CRP levels can be used as a sensitive measure of disease activity and response to therapy (Pepys 1981). The American Heart Association made a statement that the weight of evidence supports the use of serum CRP levels to measure cardiovascular disease (CVD) risk as follows: Low <1 mg/L, Average 1-3 mg/L, and High >3.0 mg/L (Pearson *et al* 2003). These thresholds are based on data from Western populations and may be different for other populations (Arima *et al* 2008).

Baseline levels of CRP are significantly influenced by a wide variety of factors. As such, these factors need to be taken into account in any study investigating the association between elevated CRP and a disease or intervention. CRP appears to be significantly influenced by socioeconomic status, age, diabetes, smoking, body mass index (BMI), ethnicity, hormone replacement therapy in women, waist circumference, total cholesterol and triglycerides, gender, level of physical activity, the use of aspirin or statins, pregnancy and chronic infections (Mendall *et al* 1996, Tracy *et al* 1997a, Tracy *et al* 1997b, Cushman

et al 1999, Danesh *et al* 1999, Ikonomidis *et al* 1999, Visser *et al* 1999, Albert *et al* 2001, Ridker *et al* 2001, Ford *et al* 2003, Jousilahti *et al* 2003, Albert *et al* 2004, Ford *et al* 2004, Cicarelli *et al* 2005, Khera *et al* 2005, Albert *et al* 2006, Pollitt *et al* 2007).

Notably there are significant interactions between many of these variables (Tracy *et al* 1997a, Tracy *et al* 1997b). For example, socioeconomic status indicators may act partly through their influence on BMI, smoking and HDL cholesterol (Pollitt *et al* 2007). It is also likely that genetic factors are responsible for part of the inter-individual variability in CRP (Pankow *et al* 2001).

1.11 Measurement of CRP

Previously, the methodology used in studies investigating CRP has been criticized for using standard CRP assays as opposed to high sensitivity CRP (hsCRP) assays (Paraskevas *et al* 2008). It should be pointed out that hsCRP is not a different molecule from CRP and only refers to the type of assay that has been used, although this is infrequently emphasised in the literature. Low thresholds of detection are necessary for determining the small changes in serum CRP that may be indicative of a future risk of cardiovascular events (Pearson *et al* 2003). The US Food and Drug Administration released a report in 2005 clearly outlining the criteria for the use of “high sensitivity” as a descriptor of the assay (US Food and Drug Administration 2005). In this document they highlighted that hsCRP assays should have a lower limit of detection of <1.0 mg/L ($<10^6$ pg/mL) in order to detect inflammatory conditions in the serum of otherwise healthy individuals, compared to 10 mg/L for conventional CRP assays used for measuring infection, tissue injury and inflammatory disorders (historically, <10 mg/L was regarded as a normal level of serum CRP (Pepys 1981). The use of high sensitivity assays therefore refers to a difference in the ELISA assay designed to increase the sensitivity of the test for detecting CRP at low levels. Additionally, the use of high sensitivity assays is regarded as essential in measuring serum levels of CRP for the assessment of cardiovascular risk, and is not necessarily critical for testing GCF samples in a non-clinical situation.

1.12 Genetics

The single copy of the human CRP gene is located on the proximal arm of chromosome 1 (Whitehead *et al* 1983). Single nucleotide polymorphisms (SNP) of the

CRP gene and other genes significantly influence serum levels of CRP, and contribute evidence to support the hypothesis of a hyperinflammatory phenotype.

Various single nucleotide polymorphisms are associated with either high or lower serum CRP (Miller *et al* 2005, Suk Danik *et al* 2006). The effect of any particular SNP also appears to be modified by race (Lange *et al* 2006). Based on a meta-analysis of 26 studies containing 32,802 subjects, the CRP SNPs +1444C>T, +2302G>A, and -286C>T/A have the strongest independent association with plasma CRP concentration (Verzilli *et al* 2008).

Genetic variations of the IL-1 and IL-6 genes may also influence on CRP levels, as hepatic CRP synthesis is dependent on these cytokines. IL-1β polymorphisms C/C at IL-1B(-511) and C/T at IL-1B(-511) and C/C at IL-1B(-3737) are associated with raised serum CRP (P=0.02) (Rogus *et al* 2008). Homozygosity for IL-6 (-6106A) polymorphism is significantly associated with increased CRP levels in multivariate analysis including age, gender, ethnicity, smoking, BMI, and number of gingival pockets, in systemically healthy patients with generalised severe periodontitis (Nibali *et al* 2008).

However, none of the polymorphisms associated with higher CRP are associated with risk of myocardial infarction or ischemic stroke, indicating the relationship is likely to be complex, involving both genetic and environmental interactions (Miller *et al* 2005).

Gene-CVD risk studies require samples of at least 15,000 cases and 15,000 controls (Danesh *et al* 2008). Proof of causality by Mendelian randomization analysis requires positive associations at three levels: gene-CRP level; CRP level-CVD risk; and gene-CVD risk (Danesh *et al* 2008). Such a study is currently underway, with plans for analysis of 37,000 coronary heart disease cases and 120,000 controls (Danesh *et al* 2008), as are Mendelian randomization analyses of gene-CRP risk (Casas *et al* 2006, Lange *et al* 2006, Verzilli *et al* 2008), and CRP level-CVD risk (Danesh *et al* 2007).

Even so, knowledge of genetic variants may not be particularly useful for prediction of disease as common multi-factorial diseases may not actually be strongly associated with specific genetic variants (Ebrahim and Davey Smith 2008). Indeed, there is currently no evidence linking genetic polymorphisms of CRP with the risk of CVD events (Shen and Ordovas 2009). This highlights the complex nature of systemic inflammatory diseases, and the role that multiple gene polymorphisms are likely to play (Kinane and Hart 2003). However, genetic association studies will lead to better understanding of the mechanisms underlying particular disease processes (Ebrahim and Davey Smith 2008), and the underlying genetics may impact on the effectiveness of treatments aimed at lowering CRP.

In the future such knowledge may help to guide appropriate therapy (Shen and Ordovas 2009).

1.13 CRP is Associated with Systemic Disease

CRP has been implicated as a risk predictor and prognostic indicator in a multitude of diseases, but despite strong statistical evidence, there continues to be controversy about the usefulness of CRP and the validity of these findings.

CRP is used as a marker of disease activity and outcomes in inflammatory diseases such as atherosclerosis (Ridker *et al* 1997, Danesh *et al* 1998, Blake and Ridker 2001, Blake *et al* 2003), neonatal sepsis (Lam and Ng 2008), rheumatoid arthritis (van Leeuwen *et al* 1997, Aletaha *et al* 2007, Emery *et al* 2007), acute pancreatitis (Rau 2007, Schutte and Malfertheiner 2008), Crohn's disease (Vermeire *et al* 2005), community acquired pneumonia (Bruns *et al* 2008, Chalmers *et al* 2008) and prediction of treatment response in rheumatoid arthritis (Heidari *et al* 2007).

However, the primary systemic disease associated with CRP is cardiovascular disease. Literally thousands of publications present findings of a positive association between elevated CRP and incidence of future cardiovascular events. Biomarkers of cardiovascular risk are of great interest, as 15-20% of cardiovascular disease (CVD) cases have none of the currently accepted risk factors of smoking, diabetes, hyperlipidemia, and hypertension (Greenland *et al* 2003, Khot *et al* 2003). Many population cohorts support the usefulness of CRP as a predictor of myocardial infarction, ischemic stroke, and cardiovascular death, particularly in otherwise healthy subjects (Koenig *et al* 1999, Curb *et al* 2003, Ballantyne *et al* 2004, Koenig *et al* 2004, Pai *et al* 2004, Best *et al* 2005, Cushman *et al* 2005, Laaksonen *et al* 2005, Boekholdt *et al* 2006).

A meta-analysis of four prospective studies published since 2000 which comprised 4107 cases of coronary heart disease in a population of over 25,000 asymptomatic individuals found that those with CRP in the highest tertile had a significantly higher risk of future coronary heart disease compared to those individuals with CRP concentration in the lowest tertile (OR 1.49, 95% CI: 1.37-1.62) (Danesh *et al* 2004). However, this article pointed out that smoking, total cholesterol, and systolic blood pressure were more useful predictors of coronary heart disease than CRP (Danesh *et al* 2004).

In contrast, results from other studies suggest that CRP is the strongest predictor of cardiovascular events in apparently healthy subjects. A prospective, nested case-control study of 28,263 apparently healthy post-menopausal women with a mean follow-up of 3

years showed that although elevations of other biomarkers such as, homocysteine, IL-6, total cholesterol, serum amyloid A, apolipoprotein B-100, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and the ratio of total cholesterol/HDL cholesterol also correlated with the risk of cardiovascular events, CRP was the strongest predictor of the risk of cardiovascular events (Ridker *et al* 2000). CRP was a stronger predictor of cardiovascular events and death than LDL cholesterol in 27,939 apparently healthy American women followed for a mean of eight years (Ridker *et al* 2002). Women not taking hormone-replacement therapy but with high CRP/low LDL were at a higher risk of cardiovascular events than those with low CRP/high LDL (Ridker *et al* 2002). Newly developed risk algorithms using a combination of traditional and novel risk factors (age, systolic blood pressure, HbA1c, smoking, total and HDL cholesterol, CRP and parental history of myocardial infarction) more accurately predicted cardiovascular events than existing classification systems (Ridker *et al* 2007). In fact, CRP and LDL cholesterol measurements appear to identify different high-risk groups for cardiovascular events, as although they both predict cardiovascular events the two measurements were poorly correlated at baseline (Ridker *et al* 2002). Further, subjects with high CRP/high LDL experienced the highest rate of cardiovascular events, suggesting an additive prognostic usefulness of CRP to LDL measurements (Ridker *et al* 2002).

Even though the characteristics used to identify metabolic syndrome: upper-body obesity, hypertriglyceridemia, low HDL, hypertension, and abnormal glucose, are all associated with increased levels of CRP, CRP levels add significant prognostic information at all levels of severity of metabolic syndrome in predicting the risk of cardiovascular events (Ridker *et al* 2003), indicating its use as an additional independent risk factor.

While some studies do not strongly support the use of CRP as a marker of cardiovascular risk (Wang *et al* 2006), these have been criticised for using poorly designed statistical analysis and unclear cardiovascular endpoints such as coronary insufficiency (Ridker *et al* 2007).

CRP may also be a predictor of developing diabetes. Over 4 years of follow-up, in 27,628 women initially free of diagnosed diabetes, cardiovascular disease and cancer, CRP was a significant predictor of developing type 2 diabetes (RR 15.7, 95% CI, 6.5-37.9) (Pradhan *et al* 2001). Similarly, a meta-analysis of six studies showed a positive association between serum levels of CRP and incident diabetes (Dehghan *et al* 2007). In the highest category of serum CRP concentration (≥2.6 mg/L), the risk ratio was 2.37 (95% CI: 1.57-3.58), after adjusting for age, sex, and BMI (Dehghan *et al* 2007). A prospective study of 2052 initially non-diabetic men followed for an average of 7 years showed that

elevated CRP at baseline was associated with the risk of developing type 2 diabetes (Thorand *et al* 2003). CRP may also give information about the risk of diabetic complications. For example, a cross-sectional study of Type 1 diabetic patients found a significant association between elevated CRP and the presence of diabetic microvascular complications (Devaraj *et al* 2007).

Overall, the evidence for CRP as a risk predictor for future cardiovascular events is quite strong. In fact, even extreme CRP levels, either very low (<0.5 mg/ml) or very high (>10 mg/ml), have clinical significance and correlate with the lowest and highest risk of future cardiovascular events (Ridker and Cook 2004). Still under investigation is the hypothesis that CRP is an active contributor to the disease process and therefore a possible therapeutic target.

1.13.1 Intervention Studies

A variety of interventions can apparently lower serum CRP. The effect may be indirect, as suggested by a meta-analysis of 27 randomized control trial intervention studies that showed that statin therapy has minimal direct effect on lowering CRP, and that the apparent lowering of CRP by statin therapy is mediated by lowering of LDL (Kinlay 2007). Aspirin use results in a lowered risk of myocardial infarction in men, but only for those men in the highest quartile of CRP at baseline (Ridker *et al* 1997). Lowering CRP alone may also be a valid target since patients with lower CRP following statin therapy, regardless of LDL level, had lower risk of myocardial infarction or death due to cardiac causes (Ridker *et al* 2005).

The recent Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) trial (Ridker *et al* 2008) has reignited debate as to the role of CRP as a marker and therapeutic endpoint for CVD therapy (Kones 2009, Lavie *et al* 2009, Bajpai *et al* 2010), and is the most important pharmaceutical intervention study targeting CRP to date. This trial enrolled 17,802 apparently healthy men >50 years and women >60 years without hyperlipidemia but with CRP>2 mg/L. Treatment with placebo or 20 mg daily rosuvastatin was randomised, and subjects were monitored for the occurrence of myocardial infarction, stroke, arterial revascularization, hospitalization for unstable angina, or death from cardiovascular causes (Ridker *et al* 2008). Although a follow-up period of 5 years was planned, the trial was stopped early by an independent data and safety monitoring board after a median period of 1.9 years, due to the clear reduction in major cardiovascular events in the treatment group, which was nearly half that

of the placebo group. In the treatment group, LDL cholesterol was reduced by 50% and CRP by 37%, and the reduction in cardiovascular events was consistent across all subgroups of age, race and gender. The results of this study suggest that reduction of serum CRP is beneficial, even in a population considered to be at low risk of cardiovascular events.

1.13.2 Biological Considerations

When considering the possible effectiveness of lowering CRP, consideration needs to be given to the significance of an elevated serum CRP level.

Serum CRP may reflect an underlying chronic infection such as *Chlamydia pneumoniae* or *Helicobacter pylori*, or may be an indicator of classical vascular disease risk factors such as smoking and obesity or of the extent of pre-existing vascular disease (Danesh *et al* 2000). In either case, CRP is thought to be acting as a marker and not as a causal factor. However, it may be that CRP is itself causal by contributing to inflammation. In the case of cardiovascular disease, the association of elevated CRP with future disease suggests that inflammation precedes the onset of cardiovascular events (Ridker and Silvertown 2008), and this is also likely to be the case for other disease processes.

Based on the strong epidemiological evidence of an association between elevated levels of CRP and cardiovascular events (Ridker *et al* 1997, Danesh *et al* 1998, Ridker *et al* 2002, Pearson *et al* 2003, Danesh *et al* 2004, Pai *et al* 2004, Cushman *et al* 2005), the experimental data implicating the presence of CRP within atherosclerotic lesions (Torzewski *et al* 1998, Zhang *et al* 1999, Torzewski *et al* 2000), and the findings that existing preventive therapies also lower CRP (Chrysohoou *et al* 2004, Esposito *et al* 2004, Kinlay 2007, Pearson *et al* 2007), it has been hypothesized that CRP is not merely a marker of inflammation but an active participant (Jialal *et al* 2004, Nilsson 2005, Torzewski 2005, Scirica *et al* 2006). If CRP is indeed an active participant in the pathogenic process, then it could be considered a modifiable risk factor worthy of specific targeting by therapeutic intervention (Scirica *et al* 2006).

Proof of causality requires evidence that lowering CRP can prevent disease occurrence (Kuo *et al* 2005). Indeed, until the JUPITER trial (Ridker *et al* 2008), one of the key criticisms of the use of CRP as a prognostic marker was that there was no evidence that such knowledge can improve treatment outcomes (Di Napoli *et al* 2005). Even now, the use of CRP as a therapeutic target is vigorously debated.

In order to establish conclusively that CRP is a direct modulator of atherosclerosis, research should satisfy each of the “modified” Koch’s postulates (Scirica *et al* 2006):

- Identification of CRP in related stages of atherosclerotic lesions
- That the activation of CRP ligands promotes atherosclerosis
- That the addition of purified, exogenous CRP promotes atherosclerosis
- That the disruption or blockade of CRP or its actions inhibits the development of atherosclerosis in animal and human studies.

Research must address whether inhibition of CRP delays or prevents atherothrombosis, and whether it can reduce cardiovascular risk (Scirica *et al* 2006).

Others argue that CRP is a marker only, because atherosclerotic plaques are immunologically active, producing inflammatory cytokines such as IL-1, TNF and interferon (INF)-γ that induce the production of IL-6, which in turn acts on the liver to stimulate production of acute phase proteins, including CRP (Hansson 2005). Therefore CRP acts as a downstream marker of inflammation within the vasculature and other tissues such as adipose tissue (Hansson 2005). Since multiple inflammatory markers with different properties contribute to the statistical risk of cardiovascular events it is unlikely that these markers are contributing to the disease process (Hansson 2005). Reductions in CRP achieved by therapeutic interventions therefore represent reduction in total systemic inflammation (Hansson 2005).

Indeed, observational studies have a strong potential for residual confounding, even after adjustment for all relevant variables, and as a result cannot reliably establish whether CRP is a causal risk factor, risk marker for other cardiovascular risk factors, or risk marker for subclinical cardiovascular disease (Danesh *et al* 2008). This is because accurate adjustment for potential confounders is extremely difficult, and these models do not sufficiently allow for the possibility of reverse confounding (subclinical disease causing an elevation in CRP) (Danesh *et al* 2008). Even intervention studies of CRP-lowering agents will be unable to firmly establish causality due to the multiple effects of these agents (Danesh *et al* 2008).

The interpretation of evidence for a possible risk factor actually being causal for a disease is based on six possible lines of evidence (Paquette 2002):

1. A risk factor is more likely to be causal if most, if not all, studies dealing with the relationship produce similar positive results, particularly if studies involve different populations, methods or time periods.
2. The association should be strong, as measured by the odds ratios and risk ratios, as a stronger association is less likely to be due to error.

3. The association should be specific or significant after adjusting for known risk factors of the disease.
4. The exposure to the potential risk factor should precede the onset of the disease.
5. The risk of developing the disease should be related to the degree of exposure to the risk factor.
6. The association should be biologically plausible and should be supported by experimental evidence in animal models or human intervention trials.

Based on the preceding evidence, CRP appears to satisfy the first five criteria but currently does not satisfy the sixth.

1.14 Systemic Disease is Associated with Periodontal Disease

CRP is clearly associated with cardiovascular disease. There is now substantial evidence that cardiovascular disease is also significantly associated with periodontal disease.

One of the earliest published studies reporting a possible association between cardiovascular disease and oral health was in two separate case-control studies of 100 patients with acute myocardial infarction and 102 controls (Mattila *et al* 1989). Poor dental health, as measured by caries and periodontal disease, was associated with acute myocardial infarction, even after controlling for known risk factors (Mattila *et al* 1989), and the search for a mechanism of association began.

The results of a large number of published studies examining the possible association between periodontal disease and cardiovascular disease have been assessed in several recent systematic reviews (Dave and Van Dyke 2008, Persson and Persson 2008). Large-scale trials, including the Atherosclerosis Risk in Communities Study (ARIC) (Desvarieux *et al* 2005a, Engebretson *et al* 2005), and the Oral Infections and Vascular Disease Epidemiology Study (INVEST) (Beck *et al* 2001, Beck *et al* 2005), also support an association. However, while there is clearly an association, a definitive etiologic relationship has not been established (Dave and Van Dyke 2008).

Results from meta-analyses emphasise that the association is modest (Persson and Persson 2008) at approximately 15-20% increase in risk of CVD events in periodontitis patients. Periodontal disease appears to be associated with a 19% increase in risk (relative risk (RR) = 1.19, 95% CI: 1.08-1.32) of future cardiovascular events, including fatal and nonfatal myocardial infarction, admission to the hospital with diagnosis of coronary heart disease, and stroke. However, this increase in risk is doubled (RR 1.44, 95% CI: 1.20-1.73)

in subjects aged ≤65 years (Janket *et al* 2003). Similarly, a significant dose-dependent association was found between periodontitis and coronary heart disease incidence among men <60 years, independent of established risk factors, but no relationship was found for men ≥60 years (Dietrich *et al* 2008). Analysis based on five cohort studies calculated patients with periodontal disease had a 1.14 times (RR 1.14, 95% CI: 1.074-1.213, P<0.001) higher risk of developing coronary heart disease than the controls, while analysis based on five cross-sectional studies calculated patients with periodontal disease had a 1.22 times (OR 2.22, 95% CI 1.59-3.117) higher risk of developing coronary heart disease than the controls (Bahekar *et al* 2007). The reported risk ratio between periodontal disease and stroke is even stronger (RR 2.85, 95% CI: 1.78-4.56) (Janket *et al* 2003).

Early stage measures of cardiovascular disease, including carotid intimal-media thickness, flow-mediated dilation and endothelial dysfunction, are also associated with periodontal infection. According to a recent meta-analysis, periodontal disease measured by elevated markers of systemic bacterial exposure (bacterial burden, serology, CRP) is strongly associated with mean carotid intimal-medial thickening compared to subjects without periodontal disease, with an odds ratio of 1.75 (95% CI: 1.32-2.34) (Mustapha *et al* 2007). Severe periodontitis has also been linked to carotid intimal thickness in systemically healthy young (≤40 years) patients (Cairo *et al* 2008). Increased carotid intimal thickness has previously been associated with cardio- and cerebrovascular events in middle aged subjects (Burke *et al* 1995). The severity of periodontal disease has also been correlated with angiographic extent of coronary artery disease, independent of systemic inflammation (Amabile *et al* 2008). Periodontitis has been associated with endothelial dysfunction in both systemically healthy and hypertensive patients, and this may be partly mediated by systemic inflammation (Higashi *et al* 2008). Similarly, flow mediated dilation is impaired in periodontitis patients compared to matched controls (Amar *et al* 2003, Seinost *et al* 2005), and is also believed to be a result of systemic inflammation.

1.14.1 Intervention Studies

There is no current evidence that treatment of periodontal disease will prevent atherosclerotic events. In fact, evidence to the contrary is suggested by the systemic inflammation induced by periodontal therapy.

Within 24 hours after intensive periodontal treatment (full-mouth subgingival debridement with adjunctive minocycline microspheres), there are significant elevations of CRP, IL-6 and endothelial-activation markers soluble E-selectin and von Willebrand

factor, indicating an acute systemic inflammatory response and a transient impairment of endothelial function (Tonetti *et al* 2007). By day 7 and up to 6 months however, the intensive periodontal treatment group has equal or lower circulating inflammatory biomarkers than the control group of community-based care. It is unknown if the transient effect of treatment on systemic inflammation is more harmful than daily bacteremias in untreated patients induced by mastication and tooth brushing or vice versa (Beck *et al* 2008).

Current evidence on the effect of periodontal treatment rests on surrogate measures of cardiovascular disease such as endothelial dysfunction and flow-mediated dilation, which are also predictors of cardiovascular events (Neunteufl *et al* 2000, Modena *et al* 2002, Gokce *et al* 2003). Traditional cardiovascular risk factors (age, hyperlipidemia, diabetes, smoking) are associated with reduced flow-mediated dilation, while modification of these risk factors improves flow-mediated dilation (Modena *et al* 2002, Kwang *et al* 2004). Following successful periodontal therapy consisting of debridement and systemic antibiotics, flow-mediated dilation is significantly improved in the periodontitis group, along with a reduction in serum CRP (Seinost *et al* 2005).

The addition of local adjunctive antibiotics to mechanical debridement results in greater improvement in flow-mediated dilation and lower levels of soluble E-selectin than the debridement only group of periodontitis patients, and this difference persisted over 6 months of follow-up (Tonetti *et al* 2007). Notably the degree of improvement correlated with the amount of reduction in full mouth bleeding scores and number of periodontal pockets ≥5 mm, suggesting the importance of periodontal therapy directed at reducing the inflammatory and infectious burden.

Similarly, surgical and non-surgical periodontal therapy reducing the percentage of PD ≥5 mm from 20% to 2%, resulted in a significant improvement in flow-mediated dilation (Elter *et al* 2006). Carotid intima-media thickness, is also significantly reduced following periodontal therapy (Piconi *et al* 2009)

Recent findings from the PAVE pilot study report that there are no differences in adverse events in patients receiving treatment for periodontitis compared to those receiving community dental care (Beck *et al* 2008). However, more recent analysis of the data from this pilot study suggests that the periodontal intervention failed to improve periodontal health, in that there were no significant differences in bleeding on probing, clinical attachment levels or the extent of subgingival calculus between the intervention and community care groups at 6 months (Offenbacher *et al* 2009). This may be because 48% of the control community dental care group received preventive or periodontal treatment.

Further there were no significant differences in serum CRP reduction between the intervention and community care groups. As a result, the data from this study do not clearly show the effect of periodontal therapy on cardiovascular disease outcomes. When analysed according to *any treatment* received, there was a significant reduction in the percentage of people with elevated serum CRP>3 mg/L at 6 months, but this effect was only significant in non-obese individuals.

There are presently no other studies published on the effect of periodontal therapy on the risk of cardiovascular events.

1.14.2 Biological Plausibility

The nature of the relationship between periodontal disease and cardiovascular disease is currently unknown, however there are several proposed explanations:

- Confounding of common risk factors that are difficult to fully adjust for in statistical analysis or are as yet unknown
- These patients exhibit a genetic propensity for hyper-responsiveness to inflammatory stimuli that predisposes the patient to both periodontal disease and atherosclerosis (Haynes and Stanford 2003, Meurman *et al* 2004)
- Inflammation in the periodontal tissues may stimulate humoral and cell-mediated inflammatory pathways and potentiate the atherosclerotic process
- Bacteremias associated with periodontal infection may lead to inoculation of atherosclerotic plaques with periodontal pathogens resulting in inflammation and plaque instability (Haynes and Stanford 2003).

Determining the relationship between periodontal disease and atherosclerosis is crucial so that appropriate screening and treatment recommendations can be made (Haynes and Stanford 2003).

Four key aspects are used to explain the biological plausibility of a periodontal infection-systemic disease link (Paquette 2002):

1. Infection in general is implicated in the pathogenesis of atherosclerosis.

Systemic introduction of Gram-negative bacteria and LPS in animal models can induce inflammatory cell infiltration into major blood vessels, smooth muscle cell proliferation, vascular fatty degeneration and intravascular coagulation – all events similar to those of atherosclerosis (O'Connor *et al* 2001). Therefore it has been proposed that the presence of infection could influence the formation and progression of atheromas (Epstein 2002).

However, systemic disease is not found in all patients with infections and as such the relationship, if it truly exists, is believed to be complex (Epstein 2002). This relationship is likely to be influenced by: the host's ability to suppress pathogen-induced inflammatory activity, the pathogen burden, the ability of pathogens to influence systemic inflammatory and immune responses, and the resultant increases in circulating cytokines (Epstein 2002).

2. Periodontal infection causes transient and low-grade bacteraemias and endotoxaemias.

The size of the ulcerated epithelial surface of periodontal pockets in untreated patients with moderate to severe periodontitis can range from 8-20 cm^2 (Hujoel *et al* 2001), and through contact with the plaque biofilm they act as a portal of entry for periodontal pathogens into the circulation and allow development of bacteremia after mastication, tooth brushing and scaling in some, but not all, periodontitis patients (Forner *et al* 2006). Bacterial shedding of LPS can also similarly cause chronic low-grade endotoxaemia following mastication (Geerts *et al* 2002). Periodontal pathogens have been identified in atheromatous plaques (Chiu 1999, Haraszthy *et al* 2000), confirming that a bacteraemia has taken place.

Other studies have failed to show bacteraemia in untreated periodontitis patients following chewing (Murphy *et al* 2006) and normally bacteraemia does not pose a threat to healthy hosts as it is eliminated by the host's immune system within minutes (Zaremba *et al* 2007). As such, the presence of bacteraemia alone may be insufficient to cause disease. However, in atherosclerosis-susceptible animals, both oral and intravenous inoculation with *Porphyromonas gingivalis* accelerates the development of atherosclerosis (Li *et al* 2002, Jain *et al* 2003, Lalla *et al* 2003, Brodala *et al* 2005).

3. Periodontal infection promotes systemic inflammation and immune responses that may modify the course of other diseases.

Locally produced proinflammatory mediators such as IL-1, TNF-α, IL-6 and prostaglandin E_2 (PGE_2) may be released from inflamed periodontal tissues and exert systemic effects (Loos 2005). White blood cell counts are elevated in periodontitis compared to controls but still within normal limits, and increase in a dose-response relationship with increasing severity and extent of periodontitis (Loos *et al* 2000). A dose-dependent relationship with periodontitis has also been reported for levels of CRP (Paraskevas *et al* 2008), IL-6 (Mengel *et al* 2002), and increased fibrinogen (Sahingur *et al* 2003). This suggests that systemic inflammation is one of the mechanisms by which periodontal disease influences cardiovascular disease.

Periodontal disease significantly impairs branchial artery flow-mediated dilation in otherwise healthy non-smokers compared to controls (Amar *et al* 2003), and this may be a result of the effect of circulating cytokines on the vascular endothelium.

4. Periodontal pathogens express specific virulence factors that can invoke specific immune responses.

Elevated antibody titers are detected in periodontitis patients in response to specific bacteria: *Aggregatibacter actinomycetemcomitans* (Listgarten *et al* 1981, Tsai *et al* 1981, Ebersole *et al* 1982, Ebersole *et al* 1987); *Porphyromonas gingivalis* (Haffajee *et al* 1995, Califano *et al* 1997, O'Brien-Simpson *et al* 2000). Antibody levels to periodontal pathogens have been associated with atherosclerosis (Yamazaki *et al* 2007). In a concept referred to as 'molecular mimicry', the development of anti-*P. gingivalis* antibodies may be important in the progression of atherosclerosis due to the possibility of their cross-reaction with heat shock proteins on human endothelium (Tabeta *et al* 2000). This would result in an auto-immune attack on host cells, damaging the endothelium.

Recent reviews argue that systemic inflammation rather than direct bacterial colonisation of atherosclerotic plaques is the basis for the relationship between periodontal disease and cardiovascular disease (Dave and Van Dyke 2008). This is supported by the evidence that traditional risk factors for cardiovascular disease can all be linked to inflammatory processes (Libby and Aikawa 2002).

1.15 Periodontal Disease is Associated with CRP

It is generally accepted that there is a significant association between periodontal disease and cardiovascular disease, and separately that there is a significant association between cardiovascular disease and serum CRP level. It must be emphasised that the nature of these associations is still unclear. One interpretation of these findings is that the link between periodontal disease and cardiovascular disease is in some way related to serum CRP levels (Ebersole and Cappelli 2000, Paraskevas *et al* 2008).

The earliest report of an association between CRP and oral disease is from 1967 (Boucher *et al* 1967). Serum from systemically healthy patients diagnosed with inflammatory oral conditions was tested and found to be positive for CRP in 90% of acute alveolar abscess, 31% of chronic alveolar abscess, 16% of acute pericoronitis, 33% of acute necrotizing gingivitis and 22% of simple periodontitis cases (Boucher *et al* 1967).

1.15.1 Cross Sectional Studies of CRP in Saliva and GCF

Investigation into the components that comprise gingival crevicular fluid (GCF) and saliva have been undertaken in the hope that these may provide information on disease activity locally in the periodontium and/or systemically. Gingival crevicular fluid (GCF) is an ultrafiltrate of blood that accumulates elements of bacterial and host metabolism from the gingival crevice environment (Goodson, 2003). In health, GCF is a transudate of gingival tissue interstitial fluid arising due to an osmotic gradient from the connective tissue to the sulcus, but in the presence of inflammation (gingivitis and periodontitis) GCF is a true inflammatory exudate resulting from an increase in vessel permeability of the vasculature underlying the junctional and sulcular epithelium (Griffiths, 2003). As such, GCF is composed of serum, cells from the gingival crevice and locally generated materials such as tissue breakdown products, inflammatory mediators and antibodies (Armitage, 2004).

CRP has been found in the gingival crevicular fluid and saliva of patients with periodontitis (Sibraa *et al* 1991, Pederson *et al* 1995, Christodoulides *et al* 2005, Hirasaki *et al* 2005, Tuter *et al* 2007a, Fitzsimmons *et al* 2009, Floriano *et al* 2009, Fitzsimmons *et al* 2010).

However, reports concerning the detection of CRP in the GCF of periodontitis subjects have been inconsistent. Levels of CRP in the gingival crevicular fluid do not vary significantly between healthy and diseased sites taken from the same patient, and CRP is not detected at all in a large number of sites (Sibraa *et al* 1991).

Serum CRP levels correlated with all clinical parameters of periodontitis (PD, plaque index, gingival index), but GCF CRP levels did not correlate with the clinical parameters of periodontitis. Further, there were no significant differences in GCF CRP levels between periodontitis and periodontally healthy groups, and GCF CRP levels did not correlate with serum CRP (Tuter *et al* 2007a). However, this is likely to be because some GCF samples did not have any detectable CRP (70% of chronic periodontitis + coronary artery disease, 65% of chronic periodontitis only, and 53% of the control group) and therefore there was insufficient data to be able to detect any correlation, if one exists.

In contrast, in other studies on GCF, CRP levels did correlate with periodontal disease measurements (Fitzsimmons *et al* 2009, Fitzsimmons *et al* 2010). The first paper in the series analysed GCF from 1073 subjects: 511 periodontitis cases and 562 controls (Fitzsimmons *et al* 2009). However, there was no controlling for medical history or other factors that may affect CRP. Even so, similar to the findings of Tuter *et al* (2007a), only a

certain proportion of subjects had detectable CRP in the GCF: 48% periodontitis cases, and 34% of non-cases. Elevated CRP in the GCF correlated with deeper PD, increased clinical attachment loss, and a higher gingival index. Another analysis of the same data set found that detection of CRP in the GCF increased the odds of having periodontitis (OR = 1.9, 95% CI 5 1.5–2.5) (Fitzsimmons *et al* 2010).

In saliva, CRP is detected more consistently and appears to correlate with periodontal status. In the whole saliva of patients without obvious systemic conditions, levels of CRP correlate with severity of the patient's periodontal status (P=0.0427), although the most obvious differences were between healthy (mean 6.7 pg/ml) and gingivitis (75.1 pg/ml) and between edentulousness (10.2 pg/ml) and gingivitis, with some overlap between gingivitis, mild to moderate periodontitis (43.4 pg/ml), and severe periodontitis (80.4 pg/ml) (Pederson *et al* 1995).

Salivary CRP has been reported to be significantly higher in patients with both heart disease and periodontal disease (PD>4 mm), compared to periodontally healthy controls (4.5±2.7 vs 0.1±0.3 mg/L, P<0.01) (Hirasaki *et al* 2005). Although there was no significant difference in serum CRP between systemically healthy periodontal disease subjects and periodontally healthy subjects, there was a clear numerical difference.

Periodontitis subjects were found to have significantly higher whole salivary CRP compared to edentulous and periodontally healthy subjects (mean 2,001 vs 92 and 65 pg/mL, P-value not given), using an ultra-sensitive ELISA (detection threshold 10 pg/mL = 10^{-11} mg/L) and lab-on-a-chip system (Christodoulides *et al* 2005). However, no adjustment was made of other possible influences on CRP as this study was a pilot study trialing a new method for CRP detection. When tested with a standard high sensitivity ELISA, only 2 of the 30 samples tested had any detectable CRP, which clearly has significant implications for the validity of findings using high sensitivity ELISA and not ultra-sensitive ELISA (Christodoulides *et al* 2005). Similarly, salivary lab-on-a-chip tests for biomarkers including CRP are being tested as a screening method for cardiac events (Floriano *et al* 2009).

In many of these studies, non-detection of CRP may be due to an inadequate threshold of detection by ELISA, enzymatic degradation of CRP in GCF/saliva, or CRP not diffusing into GCF/saliva due to its molecular weight (Tuter *et al* 2007a).

1.15.2 Cross Sectional Studies for Serum CRP and Periodontitis

1.15.2.1 No Correlation with CRP

Several papers have found no association between periodontal disease and serum CRP. No association is found in treated periodontitis patients with low levels of inflammation (Fredriksson *et al* 2002), and in experimental gingivitis (Norman *et al* 1979).

Studies using indices that may not give an accurate assessment of the periodontal condition are not associated with serum CRP: Silness and Löe plaque index, CPITN (Schillinger *et al* 2006, Genctoy *et al* 2007), self-reported diagnosis of periodontal disease (Mochari *et al* 2008), and periodontal bacterial burden based on subgingival plaque sample analysis (Desvarieux *et al* 2005b).

No association between periodontal disease and serum CRP has been reported in patients with acute myocardial infarction at the time of admission (Persson *et al* 2005), patients with acute coronary syndrome (Czerniuk *et al* 2006), type 2 diabetic patients (Takeda *et al* 2006), geriatric patients admitted to hospital (Meurman *et al* 1997), smokers (Fredriksson *et al* 1999), and obese subjects (Slade *et al* 2003). This may be because inflammatory disease, smoking and obesity may have so great an effect on serum CRP as to mask any effect of periodontal disease.

1.15.2.2 Insufficient Adjustment for Confounders

In papers without sufficient adjustment for known confounding factors, a positive association between elevated CRP and poor periodontal status was still found. However, since these papers do not adequately describe the systemic disease or smoking status (Wakai *et al* 1999, Furuichi *et al* 2003, Saito *et al* 2003, Takami *et al* 2003, Nakajima *et al* 2010) of the populations under investigation they cannot be used as valid evidence for an association between CRP and periodontal disease.

1.15.2.3 Systemic Medical Conditions

Among patients with medical conditions that can increase serum CRP, those patients with moderate-severe periodontal disease have elevated CRP compared to those with mild or no disease. Such medical conditions include pregnancy (Pitiphat *et al* 2006, Horton *et al* 2008), rheumatoid arthritis (Mercado *et al* 2001, Abou-Raya *et al* 2008), myocardial

infarction (Deliargyris *et al* 2004), cardiovascular disease (Glurich *et al* 2002, Amabile *et al* 2008), diabetes (Lim *et al* 2007), coronary heart disease and type 2 diabetes (Keles *et al* 2007), continuous ambulatory peritoneal hemodialysis (Cengiz *et al* 2007), and juvenile idiopathic arthritis (Reichert *et al* 2006). Animal models suggest that concurrent obesity has a synergistic effect with periodontitis to increase serum CRP, beyond that of either condition alone (Endo *et al* 2010). As such, the presence of systemic medical conditions makes it difficult to determine the relative significance of periodontitis on serum CRP.

1.15.2.4 Non-traditional Measures of Periodontal Disease

When non-traditional measures of periodontal infection are used, significant associations between serum CRP and periodontal status persist: serum IgG antibody levels to *Porphyromonas gingivalis* (Rahmati *et al* 2002, Craig *et al* 2003a); subgingival bacterial load (Renvert *et al* 2006); and the BANA-test (Bretz *et al* 2005).

1.15.2.5 Epidemiological Studies

Cross-sectional population-based epidemiological studies consistently show a positive relationship between serum CRP and worsening periodontal status, even after adjusting for confounders (Beck *et al* 2000, Wu *et al* 2000, Beck and Offenbacher 2002, Slade *et al* 2003, Sabbah *et al* 2008). Interesting suggestions from these studies are that pocket depth and extent of bleeding may be stronger predictors of serum CRP levels than clinical attachment loss (Wu *et al* 2000, Beck and Offenbacher 2002).

This suggests that attachment levels may not be the most appropriate measure of periodontitis when investigating association with systemic conditions (Beck and Offenbacher 2002), which is logical, as it does not describe an active inflammatory process (Zaremba *et al* 2007).

Periodontal disease was also not associated with increased CRP in obese subjects (Slade *et al* 2003). The effect of obesity on CRP may have been so great as to mask any effect of periodontal disease on CRP, as suggested by other studies (Offenbacher *et al* 2009).

Although serum CRP is independently associated with periodontal measures in these multivariate models, it is possible that the statistical adjustment for confounders is insufficient, and that this is the reason for the apparent positive association with periodontal disease.

1.15.2.6 Systemically Healthy Subjects

The strongest evidence for a positive association between serum CRP and periodontal disease comes from untreated patients, who do not have other confounding factors such as systemic disease, medication use, pregnancy or smoking, in studies using high sensitivity ELISA for detecting CRP and an appropriate control group.

Various measures of periodontal disease have been used in studies reporting significantly higher levels of CRP in periodontitis patients compared to periodontally healthy controls, including:

- ≥6 sites with clinical attachment loss (CAL) ≥5 mm (Fredriksson *et al* 1998)
- ≥8 teeth with radiographic bone loss extending into the middle one-third of the root length or beyond (Loos *et al* 2000)
- Mean attachment loss and the presence of periodontal pathogens *Porphyromonas gingivalis*, *Prevotella intermedia*, *Campylobacter rectus*, and *Bacteroides forsythus* in subgingival samples (Noack *et al* 2001)
- ≥7 sites with ≥6 mm clinical attachment loss (Buhlin *et al* 2003)
- Interproximal attachment loss on ≥ three permanent teeth other than first molars and incisors, and ≥ 10 of the sites showing bleeding/suppuration on probing (Havemose-Poulsen *et al* 2006)
- Generalised pattern of severe periodontal destruction with CAL ≥5 mm on ≥8 teeth, at least three of which are not first molars and incisors, and with demonstrated age of onset under 35 years, and localized pattern of clinical attachment loss limited to first molar or incisor teeth and up to two additional teeth with demonstrated age of onset under 30 years (Salzberg *et al* 2006)
- At least 6 teeth with PD >5 mm and loss of attachment >3 mm in 3 aspects of each tooth (Amar *et al* 2003)
- CEJ-alveolar bone crest on bitewing radiographs ≤3 mm (Bizzarro *et al* 2007).

The variations in disease definitions make it difficult to compare results between studies.

A recent systematic review and meta-analysis found a weighted mean difference in serum CRP between periodontitis patients and controls of 1.56 mg/L (95% CI: 1.21-1.90) on the basis of ten cross-sectional studies (P<0.00001) (Paraskevas *et al* 2008). This meta-analysis used papers with a clear definition of both periodontitis and control subjects, where subjects had no systemic disorders, and using high-sensitivity ELISA to measure CRP.

The following studies all reported significantly higher serum CRP in association with periodontitis in systemically healthy subjects, but were excluded from the meta-analysis due to: no control group (D'Aiuto *et al* 2004e); no high-sensitivity CRP measurement (Shklair *et al* 1968); inadequate detection limit of CRP ELISA (Slade *et al* 2000); mean and SD reported graphically only (Leivadaros *et al* 2005); and excluded following controlling for heterogeneity (Craig *et al* 2003b, Joshipura *et al* 2004).

Despite the wide variety of measures used for periodontal disease, on the basis of this stringent meta-analysis there is convincing evidence that CRP is significantly associated with periodontal disease.

1.15.2.7 Edentulousness

If periodontitis is an important contributor to serum CRP levels one would expect that edentulous patients would have reduced serum CRP levels. Indeed, this is the case in several studies (Rahman *et al* 2005, Taylor *et al* 2006, Ellis *et al* 2007).

However, results from other studies undermine these findings, and therefore the hypothesis that CRP is associated with periodontal disease. For instance, edentulousness is associated with elevated serum CRP in men, after adjustment for age, social class and smoking (1.42 vs 1.00 mg/L, P=0.03) (Lowe *et al* 2003). Although an association was also found between elevated serum CRP and edentulism, the majority (91.2%) of edentulous people had at least one established risk factor for elevated CRP (P<0.05) (Slade *et al* 2000). This may be indicative of an underlying hyperinflammatory trait leading to periodontal tooth loss and elevated systemic CRP (Slade *et al* 2000).

Another study confirmed this association with significantly more edentulous subjects having elevated serum CRP (≥3 mg/L) than those subjects with at least 20 teeth (P<0.01) (Ajwani *et al* 2003). In the edentulous subjects in this study salivary microbial counts and mucosal lesions were greater than the dentate group, and were significantly associated with elevated serum CRP in multivariate analysis. These may be important causes of elevated serum CRP in edentulous populations (Ajwani *et al* 2003).

1.15.3 Serum CRP and Periodontal Therapy Longitudinal Studies

1.15.3.1 Time Course of Changes in Serum CRP Following Periodontal Therapy

Intervention studies have studied the effect of periodontal treatment on markers of inflammation. In the two hours following commencement of non-surgical periodontal

treatment of patients with at least five teeth with PD ≥5 mm and at least two quadrants with radiographic alveolar bone loss, no change in serum CRP is detectable (Ide *et al* 2004). Treatment of severe chronic periodontitis (at least 50% of teeth presenting with PD >6 mm and marginal alveolar bone loss >30%) by full mouth debridement completed within 6 hours in patients without systemic disease induces an acute systemic inflammatory response, with a 10-fold increase in serum CRP within 24 hours (from 1.61±2.11 mg/L to 12.49±2.63 mg/L) (P<0.001) and significantly raised serum CRP concentrations for one week (P<0.01) before returning to baseline after one month (D'Aiuto *et al* 2004a). Similar results from the same research group indicate that serum CRP levels peak 24 hours after intensive periodontal therapy and return to baseline within one month (D'Aiuto *et al* 2005b, D'Aiuto and Tonetti 2005). The mean increase in serum CRP within 24 hours following intensive periodontal therapy is 15.02 mg/L (95% CI: 11.90-18.15 mg/L) (D'Aiuto and Tonetti 2005). This is a result of the intense transient bacteremia and soft tissue damage resulting from periodontal instrumentation (Forner *et al* 2006).

Similarly, serum CRP levels were significantly increased immediately following non-surgical periodontal therapy compared to baseline (from 0.8 to 1.6 mg/L, P<0.049), but returned to baseline levels by 8 weeks (Pischon *et al* 2007), and by 1 month in patients given intensive treatment (full mouth scaling and root planing and local minocycline) (Tonetti *et al* 2007).

This also implies that assessing the changes in serum CRP following periodontal therapy needs to be delayed for at least one to two months, if not longer. This would explain why treatment of acute periodontal abscess with debridement/extraction/antibiotics as indicated did not reduce serum CRP levels at one week (baseline mean 13.3 mg/L, P=0.317) (Ren and Malmstrom 2007). This also correlates with the generally accepted minimum time at which periodontal therapy should be reassessed (Caton *et al* 1982, Cerek *et al* 1983, Badersten *et al* 1984).

1.15.3.2 Non-surgical Therapy

The effect of non-surgical therapy on serum CRP seems to be influenced by the response to periodontal therapy.

Subjects responding best to periodontal therapy (as measured by reduction in BOP and PD ≥5 mm) show significant reductions in serum CRP levels (D'Aiuto *et al* 2004b). By contrast, those with a persistent periodontal infection after therapy do not show significant reductions in serum CRP levels (Ide *et al* 2003). Other factors, such as smoking

(D'Aiuto *et al* 2005b, Marcaccini *et al* 2009), baseline serum CRP levels (Mattila *et al* 2002), and obesity (Offenbacher *et al* 2009), also appear to limit the effect of periodontal therapy on serum CRP levels. This may be due to the persistence of these and other pro-inflammatory conditions that may continue to elevate serum CRP levels to the extent that the effect of periodontal therapy is masked. However, patients with both periodontitis and cardiovascular disease do exhibit significant reductions in serum CRP levels following non-surgical periodontal therapy (Montebugnoli *et al* 2005). As such, the effect of other inflammatory conditions is likely to be complex.

One author has suggested that periodontal therapy does not appear to lower serum CRP in subjects with CRP <3 mg/L at baseline, but may prevent elevations above 3 mg/L in patients with levels of 1-3 mg/L at baseline, and lower levels in those with levels initially >3 mg/L (Offenbacher *et al* 2009).

1.15.3.3 Surgical and Non-surgical Therapy

The combination of surgical and non-surgical therapy on serum CRP levels has only been assessed in three studies, with reports of both a non-significant trend towards reduction in serum CRP at 1 (Elter *et al* 2006) and 3 months (Persson *et al* 2005) and a significant reduction at 3 months post therapy (Nakajima *et al* 2010). More interesting is the finding that serum CRP was reduced after therapy only in the subgroup of patients who displayed the highest quartile of CRP at baseline (Nakajima *et al* 2010), which is consistent with previous reports (Mattila *et al* 2002, Offenbacher *et al* 2009).

1.15.3.4 Adjunctive Local Antibiotics

In studies using non-surgical therapy in conjunction with locally-delivered antibiotics, both an additional reduction (Iwamoto *et al* 2003, D'Aiuto *et al* 2005b, D'Aiuto *et al* 2006) and no additional reduction (Tonetti *et al* 2007) in serum CRP levels have been reported compared to non surgical therapy alone..

Non-surgical periodontal treatment in conjunction with minocycline ointment resulted in lowered serum CRP and HbA1c in a subset of non-smoking, type 2 diabetic patients with HbA1c 6.5-10%, with no other systemic disease at 6 months (Katagiri *et al* 2009). The only observable difference between the patients who exhibited reduction in CRP compared to those who did not was that the former groups had significantly higher BOP at baseline, indicating active inflammation was more prominent in this group (46.6% vs 26.4%, $P<0.05$) (Katagiri *et al* 2009).

With regards to lowering CRP, adjunctive local antibiotics may be beneficial for some but not all patients with periodontitis. This suggests that a particular subset of patients may benefit from such adjunctive treatment.

1.15.3.5 Adjunctive Systemic Antibiotics

The use of systemic antibiotics (metronidazole ± amoxicillin) in conjunction with non-surgical periodontal therapy has been shown to significantly reduce serum CRP levels compared to baseline levels (Seinost *et al* 2005, Blum *et al* 2007a, Higashi *et al* 2008), but did not lower levels to that of the healthy control group without periodontitis (Blum *et al* 2007b).

The effect of systemic antibiotics in conjunction with non-surgical therapy on reducing serum CRP levels, compared to non-surgical therapy alone, remains unknown as no studies have compared these two treatment arms.

1.15.3.6 Host Modulation

Host modulating agents may theoretically reduce serum CRP levels in two ways; by improving local periodontal inflammation, and by a systemic effect on inflammation. The use of adjunctive sub-antimicrobial dose doxycycline compared to placebo and non-surgical therapy for periodontitis does not result in additional reduction in serum CRP levels following therapy beyond that achieved by non-surgical therapy alone (Tuter *et al* 2007b). However, the addition of a non-steroidal anti-inflammatory drug to non-surgical therapy significantly reduced serum CRP levels beyond that of non-surgical therapy alone (Ebersole *et al* 1997).

No studies have used a host-modulating agent-only control arm. Therefore at present the adjunctive effect of host modulation drugs to non-surgical periodontal therapy on serum CRP levels is unclear, but the choice of agent may be important.

1.15.3.7 Dental Clearance

Further evidence for the effect of periodontitis on serum CRP comes from studies where the periodontal infection is completely eradicated by full dental clearance.

Treatment of end-stage periodontitis by dental clearance resulted in significantly decreased serum CRP at 3 months after completion of treatment compared to baseline (2.5

vs 1.8 mg/L, P=0.04), however when analysed according to smoking status, the difference was significant for non-smokers only (P=0.02) (Taylor *et al* 2006).

Similarly, dental clearance for end-stage periodontitis significantly reduced serum CRP levels in several other studies (Rahman *et al* 2005, Ellis *et al* 2007). The earliest time at which a significant decrease in serum CRP could be detected post-operatively was at 6 months (P=0.01), with no significant differences between 6 and 9 months, and 9 and 12 months (Rahman *et al* 2005). When patients were categorised according to baseline CRP levels, 61% of the subjects with CRP>3.0 mg/L at baseline reduced their CRP to <3.0 mg/L (Ellis *et al* 2007).

The significant reduction in serum CRP by complete eradication of periodontal infection lends support to a contributory effect of periodontitis to elevated levels of serum CRP. In this category of intervention studies, baseline CRP and other inflammatory conditions (smoking) appear to play a role on the degree of serum CRP reduction achieved by therapy, as previously discussed.

1.15.3.8 Summary

A recent meta-analysis of studies up to 2007 found only modest evidence that periodontal therapy lowered serum CRP levels with a weighted mean difference of 0.50 mg/L, P<0.00001 (95% CI: 0.08-0.93), based on four studies (D'Aiuto *et al* 2005c, Seinost *et al* 2005, D'Aiuto *et al* 2006, Tonetti *et al* 2007), and although all studies showed a favourable effect from treatment, it was concluded that there was no conclusive evidence due to the scarcity of studies (Paraskevas *et al* 2008).

Overall, serum CRP exhibits a tendency to be lowered in periodontitis patients responding best to treatment, those with highest CRP at baseline, and those without other systemic conditions that can increase systemic CRP. Furthermore, it may take up to 6 months for periodontal treatment to have a significant effect on systemic CRP.

There is a possible dose-response relationship between the extent of resolution of periodontal infection and the level of reduction in systemic inflammatory markers (D'Aiuto *et al* 2005b). However, the relationship between periodontal disease and CRP is complex, with no correlation between the severity of periodontal disease and CRP at baseline (Mattila *et al* 2002, Iwamoto *et al* 2003). Other factors seem to play a significant role in the overall level of CRP, such as the underlying genotype of the patient. The magnitude of serum CRP increase following non-surgical periodontal treatment of systemically healthy subjects with severe periodontitis was significantly influenced by the presence of

homozygosity for the +1444T allele of the CRP gene (CRP (+1444C>T) polymorphism) on day 1 (21.10±4.81 mg/L vs 12.37 ± 1.61 mg/L, P = 0.02) and day 7 (4.89 ± 0.74 mg/L vs 3.08 ± 2.00 mg/L, P < 0.01), even after adjusting for cardiovascular risk factors and baseline and peak IL-6 concentrations (D'Aiuto *et al* 2005a).

Importantly, the actual magnitude of contribution of periodontitis to systemic CRP in any individual is likely to be variable, influenced by the degree of periodontal inflammation, the concurrence of other systemic conditions and the underlying genetic predisposition to systemic inflammation. Indeed, previous studies have shown that a CRP genotype significantly influences serum CRP at baseline in systemically healthy patients and significantly influences stimulated CRP level in both systemically healthy patients and those with cardiovascular disease, even after accounting for age, sex, BMI, smoking, diabetes, and IL-6 levels (Brull *et al* 2003).

Several studies have indicated that periodontal therapy is successful in reducing serum CRP in only a subset of patients, usually those with the highest serum CRP (Mattila *et al* 2002, Nakajima *et al* 2010) or inflammatory burden as measured by periodontal parameters (D'Aiuto *et al* 2004b, Katagiri *et al* 2009) at baseline, or those responding best to periodontal treatment (Higashi *et al* 2008). Furthermore, improvements are more likely to be seen in patients without other conditions known to increase CRP, such as smoking (D'Aiuto *et al* 2005b, Taylor *et al* 2006), and obesity (Offenbacher *et al* 2009). It may be that periodontitis contributes to increased serum CRP only in some, but not all, patients (Nakajima *et al* 2010), and that periodontal treatment will therefore only be effective in reducing serum CRP in this subset. Similarly the magnitude of increased serum CRP as a result of periodontitis is likely to be variable between individuals.

Patients with elevated serum CRP levels are considered at high risk of future cardiovascular events (Pearson *et al* 2003). However, despite the improvements in serum CRP levels achieved by periodontal therapy there is currently no evidence that periodontal treatment will reduce the incidence of cardiovascular events. If CRP is a valid therapeutic target, and periodontal therapy an important modifier of serum CRP levels, then one would expect a reduction in serum CRP to reduce the risk of cardiovascular events. The actual importance of periodontal therapy in any given individual is likely to be substantially modified by concurrent inflammatory conditions, smoking, baseline serum CRP levels, and the effectiveness of periodontal therapy.

1.15.4 Longitudinal Studies of Serum CRP and Periodontal Disease Activity

During periods of active tissue destruction, locally produced cytokines gaining access to the systemic circulation could stimulate increased synthesis of CRP by the liver, resulting in elevated serum CRP levels. As with other inflammatory diseases, serum CRP may provide useful information about the course of periodontitis. Further, as CRP is shown to have many functions during inflammation, CRP may also contribute directly to the disease process.

Indeed, animal studies using ligature-induced periodontitis show that CRP significantly increased as a result of active periodontitis compared to baseline (Ebersole *et al* 2002, Hasturk *et al* 2007), and was increased compared to experimental gingivitis (Ebersole *et al* 2002).

Human longitudinal studies also support this hypothesis, at least in part. Patients with the greatest number of sites exhibiting attachment loss ≥2 mm over 6 months exhibited baseline serum CRP levels that were 17 times greater than normal (approximately 34 mg/L) and were significantly greater than periodontally healthy groups (Ebersole *et al* 1997). Similarly, multiple sites (≥2%) of disease progression (>2 mm loss of attachment 2 months post baseline) resulted in an odds ratio of 14.1 of having baseline serum CRP >2.08 mg/L according to a multiple regression model (Craig *et al* 2003b). Another study found a similar association between attachment loss and the odds of an elevated serum CRP level, but only in patients without major systemic disease (Swoboda *et al* 2008). Without measuring disease activity, merely the presence of PD ≥4 mm at baseline predicted elevated serum CRP 1 year later, in an untreated population of 10,376 Japanese men with normal baseline serum CRP (Yoshii *et al* 2009).

However, a negative effect of elevated serum CRP levels on periodontitis is unsubstantiated. Elevated serum CRP levels are not significantly associated with the presence of periodontitis compared to periodontal health (Craig *et al* 2003b). Further, serum CRP at baseline does not predict periodontal disease 1 year later in an untreated, periodontally healthy population of 4,977 Japanese men (Yoshii *et al* 2009), and pharmacological reduction of elevated serum CRP has no effect on periodontal parameters in untreated periodontitis subjects (Renvert *et al* 2009). Currently there is no evidence of a causal effect of CRP on periodontal disease.

If serum CRP is influenced by periodontal disease activity, then this has important implications for the interpretation of cross sectional and intervention studies. Treatment of periodontal disease would best lower CRP in those individuals actually undergoing

periodontal breakdown, which is likely to be a subset of the population under study at any one time, similarly, in systemically healthy subjects, CRP would only be elevated in a subset of the population. With this in mind, measurement of serum acute phase proteins may be one of the ways to identify subjects at a higher risk of destructive disease or undergoing active periodontal breakdown (Johnson *et al* 1988). This may be a real possibility in the future as CRP can be assessed by finger-prick blood sample and chair side diagnostic test within 2 minutes (Ren and Malmstrom 2007).

1.15.5 Biological Plausibility

There seem to be three main theories behind the apparent association between periodontal disease and CRP:

1. Periodontal diseases increases CRP via systemic inflammation

Periodontal bacteria can act via six different mechanisms to cause systemic effects: invasion of adjacent tissue spaces, aspiration, bacterial seeding via the circulation and colonization, endotoxaemia, systemic dissemination of other bacterial virulence factors, and induction of systemic host response through cytokine production (Kamer *et al* 2008).

In periodontal health, bacteria and the host response maintain a balance. In gingivitis, the bacteria elicit an innate immune response from the host, which is able to contain the bacterial-induced tissue damage. In periodontitis, the host response does not contain the bacteria, resulting in a systemic immune response and production of proinflammatory cytokines (Kamer *et al* 2008).

Theoretically, elevated CRP may be a response to systemically disseminated periodontal bacteria and LPS, which stimulates release of IL-6 and TNF-α, which in turn act on the liver to stimulate production of acute phase proteins including CRP (Williams and Offenbacher 2000). Indeed, *in vivo* rabbit models show that *P. gingivalis*-induced periodontitis over 6 weeks induces elevation of CRP compared to baseline ($P<0.05$) (Hasturk *et al* 2007).

Although many other stimuli, including infections, inflammatory conditions or trauma, may account for a mild increase in CRP these do not appear to negate the effects of periodontitis on increasing CRP in cross-sectional population studies (Slade *et al* 2000).

2. Hyperinflammatory trait

It has been proposed there exists a common genetic background that predisposes the individual to inflammatory conditions due to a hyperinflammatory phenotype (Moutsopoulos and Madianos 2006).

Among certain individuals there may be an underlying hyperinflammatory trait that is characterized by excessive production of pro-inflammatory cytokines by monocytes and other cell types (Beck *et al* 2000). This hyperinflammatory trait may be induced by genetic, behavioural and environmental exposure and may serve as a common precursor to both cardiovascular and periodontal disease, and other inflammatory conditions (Beck *et al* 2000). In the presence of a pathogenic oral flora, and the inevitable bacteremia which results (Geerts *et al* 2002, Forner *et al* 2006) and induces systemic inflammation, there is an added burden on the host, increasing the prevalence and severity of cardiovascular disease (Beck *et al* 2000).

Therefore, possibly only a subset of susceptible patients produce an elevated systemic CRP response to periodontitis, and this may not always correlate to those with the most severe bone loss (Mattila *et al* 2002). Treating these susceptible patients may indeed lower CRP levels, but this may not occur for all patients with periodontal disease (Mattila *et al* 2002).

Recent *in vivo* research confirms the effect of a hyperinflammatory trait on periodontitis (Trombone *et al* 2009). Genetically hyperinflammatory mice experience increased bone loss in experimental periodontitis following infection with *Aggregatibacter actinomycetemcomitans* (*A. a*) but have no difference in bacterial burden compared to hypoinflammatory mice (i.e. the hyperactive immune system is ineffective in killing bacteria) (Trombone *et al* 2009). Proinflammatory cytokines measured in the hyperinflammatory mice were significantly elevated (IL-1β, TNF-α, IL-17) in response to experimental periodontitis (Trombone *et al* 2009). Correspondingly in humans, there is clear evidence that the inflammatory response to periodontal infection is not uniform (Trombelli *et al* 2008, Behle *et al* 2009).

The periodontal burden (infection and inflammation) amplifies the individual systemic inflammatory response in a susceptible host who is predisposed to inflammation (D'Aiuto *et al* 2004c). Such characteristics of a susceptible host may be smoking, BMI, age, and polymorphisms of IL-6 and IL-1α (D'Aiuto *et al* 2004c). Indeed, the systemic inflammatory response to severe periodontitis is higher, as measured by serum CRP and IL-6, in patients with polymorphisms of IL-1α or IL-6 ($P<0.05$), even after correcting for age, BMI, gender, ethnicity and smoking (D'Aiuto *et al* 2004d).

Further evidence for a hyperinflammatory trait is that neutrophils from treated periodontitis patients appear to be hyper-reactive compared to periodontally healthy controls, where all patients were systemically healthy and non-smokers (Fredriksson *et al* 2003). In this study there were no significant differences in serum CRP between treated

periodontitis patients and controls, even though numerically serum CRP was higher in periodontitis patients. However, these patients were treated for periodontal disease before blood sampling and this may have reduced any differences in CRP levels between the two groups (Fredriksson *et al* 2003).

Importantly, an amplified inflammatory response would not be able to initiate disease on its own, but would modify the onset, severity and rate of progression if the subject is exposed to other causative factors of the disease and is susceptible to the disease (Kornman 2006).

3. Periodontal disease may simply be a marker for an underlying or unknown risk factor or exposure leading to systemic disease (Haynes and Stanford 2003, Offenbacher and Beck 2005).

In this hypothesis, periodontal disease does not contribute to systemic disease. For example, both cardiovascular disease and periodontitis share common risk factors such as smoking and diabetes. Although these factors are often adjusted for in statistical analyses of association data, it is possible that residual confounding persists and results in an apparent association between periodontitis and cardiovascular disease (Haynes and Stanford 2003). The apparent association may also be due to an as yet unidentified common risk factor, which is therefore impossible to adjust for in statistical analyses.

1.16 Conclusion

While it seems clear that there is indeed an association between both periodontal disease and systemic disease with CRP, the nature of this relationship is unclear. Nonetheless it is an important one, as highlighted by recent consensus recommendations from the editors of the *American Journal of Cardiology* and *Journal of Periodontology* (Friedewald *et al* 2009). Among these recommendations are that patients with moderate to severe periodontitis should be informed there may be an increased risk for atherosclerotic CVD associated with periodontitis, and that medical evaluation of patients with periodontitis should include measurement of systemic CRP.

CRP has a wide variety of functions that make it a possible contributor to disease and therefore a therapeutic target. Periodontal therapy may be one aspect of CRP reduction in periodontitis patients at risk of CVD, but as yet there is no way to identify which patients are likely to benefit most from intervention. Further, there is no evidence that periodontal therapy can actually reduce cardiovascular events.

As part of the understanding of the relationship between periodontitis, systemic disease and CRP it is necessary to determine the origin of CRP in the GCF. CRP in the GCF may be evidence of systemic inflammation, either in response to periodontal disease, or as a result of an underlying inflammatory condition. CRP in the GCF may also be present if CRP is produced locally during inflammation in the periodontal tissues.

The aim of the study described in Chapter 2 is to assess the likely source of the CRP in the GCF at sites of health and periodontal disease, in order to further understand the possible relationship between periodontal disease and CRP, and also CRP and systemic disease. The outcomes of this research will assist in determining the possibility of monitoring systemic or acute periodontal inflammation using less invasive tests, such as GCF analysis.

1.17 References

1. Abou-Raya, S., Abou-Raya, A., Naim, A., *et al*. (2008) Rheumatoid arthritis, periodontal disease and coronary artery disease. *Clinical Rheumatology* **27,** 421-427.
2. Agrawal, A., Shrive, A. K., Greenhough, T. J., *et al*. (2001) Topology and structure of the C1q-binding site on C-reactive protein. *Journal of Immunology* **166,** 3998-4004.
3. Agrawal, A., Simpson, M. J., Black, S., *et al*. (2002) A C-reactive protein mutant that does not bind to phosphocholine and pneumococcal C-polysaccharide. *Journal of Immunology* **169,** 3217-3222.
4. Ajwani, S., Mattila, K. J., Narhi, T. O., *et al*. (2003) Oral health status, C-reactive protein and mortality--a 10 year follow-up study. *Gerodontology* **20,** 32-40.
5. Albert, M. A., Danielson, E., Rifai, N., *et al*. (2001) Effect of statin therapy on C-reactive protein levels: The pravastatin inflammation/CRP evaluation (PRINCE): A randomized trial and cohort study. *Journal of the American Medical Association* **286,** 64-70.
6. Albert, M. A., Glynn, R. J., Buring, J., *et al*. (2004) C-reactive protein levels among women of various ethnic groups living in the United States (from the Women's Health Study). *American Journal of Cardiology* **93,** 1238-1242.
7. Albert, M. A., Glynn, R. J., Buring, J., *et al*. (2006) Impact of traditional and novel risk factors on the relationship between socioeconomic status and incident cardiovascular events. *Circulation* **114,** 2619-2626.
8. Aletaha, D., Funovits, J., Keystone, E. C., *et al*. (2007) Disease activity early in the course of treatment predicts response to therapy after one year in rheumatoid arthritis patients. *Arthritis and Rheumatism* **56,** 3226-3235.
9. Amabile, N., Susini, G., Pettenati-Soubayroux, I., *et al*. (2008) Severity of periodontal disease correlates to inflammatory systemic status and independently predicts the presence and angiographic extent of stable coronary artery disease. *Journal of internal medicine* **263,** 644-652.
10. Amar, S., Gokce, N., Morgan, S., *et al*. (2003) Periodontal disease is associated with brachial artery endothelial dysfunction and systemic inflammation. *Arteriosclerosis, Thrombosis, and Vascular biology* **23,** 1245-1249.
11. Arima, H., Kubo, M., Yonemoto, K., *et al*. (2008) High-sensitivity c-reactive protein and coronary heart disease in a general population of Japanese: The Hisayama study. *Arteriosclerosis, Thrombosis, and Vascular Biology* **28,** 1385-1391.

12. Armitage, G. C. (2004). Analysis of gingival crevice fluid and risk of progression of periodontitis. *Periodontology 2000* 34, 109-119.
13. Badersten, A., Nilveus, R. & Egelberg, J. (1984) Effect of nonsurgical periodontal therapy. III. Single versus repeated instrumentation. *Journal of Clinical Periodontology* **11,** 114-124.
14. Bahekar, A. A., Singh, S., Saha, S., *et al*. (2007) The prevalence and incidence of coronary heart disease is significantly increased in periodontitis: A meta-analysis. *American Heart Journal* **154,** 830-837.
15. Bajpai, A., Goyal, A. & Sperling, L. (2010) Should we measure C-reactive protein on earth or just on JUPITER? *Clinical Cardiology* **33,** 190-198.
16. Ballantyne, C. M., Hoogeveen, R. C., Bang, H., *et al*. (2004) Lipoprotein-associated Phospholipase A2, high-sensitivity C-reactive protein, and risk for incident coronary heart disease in middle-aged men and women in the Atherosclerosis Risk in Communities (ARIC) Study. *Circulation* **109,** 837-842.
17. Baumann, H. & Gauldie, J. (1994) The acute phase response. *Immunology Today* **15,** 74-80.
18. Beck, J. D., Couper, D. J., Falkner, K. L., *et al*. (2008) The Periodontitis and Vascular Events (PAVE) pilot study: adverse events. *Journal of Periodontology* **79,** 90-96.
19. Beck, J. D., Eke, P., Lin, D., *et al*. (2005) Associations between IgG antibody to oral organisms and carotid intima-medial thickness in community-dwelling adults. *Atherosclerosis* **183,** 342-348.
20. Beck, J. D., Elter, J. R., Heiss, G., *et al*. (2001) Relationship of periodontal disease to carotid artery intima-media wall thickness: the atherosclerosis risk in communities (ARIC) study. *Arteriosclerosis, thrombosis, and vascular biology* **21,** 1816-1822.
21. Beck, J. D. & Offenbacher, S. (2002) Relationships among clinical measures of periodontal disease and their associations with systemic markers. *Annals of Periodontology* **7,** 79-89.
22. Beck, J. D., Slade, G. & Offenbacher, S. (2000) Oral disease, cardiovascular disease and systemic inflammation. *Periodontology 2000* **23,** 110-120.
23. Behle, J. H., Sedaghatfar, M. H., Demmer, R. T., *et al*. (2009) Heterogeneity of systemic inflammatory responses to periodontal therapy. *Journal of Clinical Periodontology* **36,** 287-294.

24. Best, L. G., Zhang, Y., Lee, E. T., *et al*. (2005) C-reactive protein as a predictor of cardiovascular risk in a population with a high prevalence of diabetes: The Strong Heart Study. *Circulation* **112,** 1289-1295.
25. Bharadwaj, D., Stein, M. P., Volzer, M., *et al*. (1999) The major receptor for C-reactive protein on leukocytes is Fcgamma receptor II. *Journal of Experimental Medicine* **190,** 585-590.
26. Bizzarro, S., van der Velden, U., ten Heggeler, J. M., *et al*. (2007) Periodontitis is characterized by elevated PAI-1 activity. *Journal of Clinical Periodontology* **34,** 574-580.
27. Black, S., Agrawal, A. & Samols, D. (2003) The phosphocholine and the polycation-binding sites on rabbit C-reactive protein are structurally and functionally distinct. *Molecular Immunology* **39,** 1045-1054.
28. Black, S., Kushner, I. & Samols, D. (2004) C-reactive Protein. *Journal of Biological Chemistry* **279,** 48487-48490.
29. Blake, G. J. & Ridker, P. M. (2001) High sensitivity C-reactive protein for predicting cardiovascular disease: an inflammatory hypothesis. *European Heart Journal* **22,** 349-352.
30. Blake, G. J., Rifai, N., Buring, J. E., *et al*. (2003) Blood pressure, C-reactive protein, and risk of future cardiovascular events. *Circulation* **108,** 2993-2999.
31. Blum, A., Front, E. & Peleg, A. (2007a) Periodontal care may improve systemic inflammation. *Clinical and Investigative Medicine* **30,** E114-117.
32. Blum, A., Front, E. & Peleg, A. (2007b) Periodontal care may improve systemic inflammation. *Clinical and Investigative Medicine.* **30,** E114-117.
33. Boekholdt, S. M., Hack, C. E., Sandhu, M. S., *et al*. (2006) C-reactive protein levels and coronary artery disease incidence and mortality in apparently healthy men and women: The EPIC-Norfolk prospective population study 1993-2003. *Atherosclerosis* **187,** 415-422.
34. Boucher, N. E., Jr., Hanrahan, J. J. & Kihara, F. Y. (1967) Occurrence of C-reactive protein in oral disease. *Journal of Dental Research* **46,** 624.
35. Bray, R. A., Samberg, N. L., Gewurz, H., *et al*. (1988) C-reactive protein antigenicity on the surface of human peripheral blood lymphocytes. Characterization of lymphocytes reactive with anti-neo-CRP. *Journal of Immunology* **140,** 4271-4278.
36. Brennan, F. M., Chantry, D., Jackson, A., *et al*. (1989) Inhibitory effect of TNF alpha antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. *Lancet* **2,** 244-247.

37. Bretz, W. A., Weyant, R. J., Corby, P. M., *et al*. (2005) Systemic inflammatory markers, periodontal diseases, and periodontal infections in an elderly population. *Journal of the American Geriatrics Society* **53,** 1532-1537.
38. Brodala, N., Merricks, E. P., Bellinger, D. A., *et al*. (2005) Porphyromonas gingivalis bacteremia induces coronary and aortic atherosclerosis in normocholesterolemic and hypercholesterolemic pigs. *Arteriosclerosis, Thrombosis, and Vascular Biology* **25,** 1446-1451.
39. Brull, D. J., Serrano, N., Zito, F., *et al*. (2003) Human CRP gene polymorphism influences CRP Levels: Implications for the prediction and pathogenesis of coronary heart disease. *Arteriosclerosis, Thrombosis, and Vascular Biology* **23,** 2063-2069.
40. Bruns, A. H. W., Oosterheert, J. J., Hak, E., *et al*. (2008) Usefulness of consecutive C-reactive protein measurements in follow-up of severe community-acquired pneumonia. *European Respiratory Journal* **32,** 726-732.
41. Buhlin, K., Gustafsson, A., Pockley, A. G., *et al*. (2003) Risk factors for cardiovascular disease in patients with periodontitis. *European Heart Journal* **24,** 2099-2107.
42. Burke, G. L., Evans, G. W., Riley, W. A., *et al*. (1995) Arterial wall thickness is associated with prevalent cardiovascular disease in middle-aged adults. The Atherosclerosis Risk in Communities (ARIC) Study. *Stroke* **26,** 386-391.
43. Cairo, F., Castellani, S., Gori, A. M., *et al*. (2008) Severe periodontitis in young adults is associated with sub-clinical atherosclerosis. *Journal of Clinical Periodontology* **35,** 465-472.
44. Califano, J. V., Gunsolley, J. C., Schenkein, H. A., *et al*. (1997) A Comparison of IgG antibody reactive with *Bacteroides forsythus* and *Porphyromonas gingivalis* in adult and early-onset periodontitis. *Journal of Periodontology* **68,** 734-738.
45. Casas, J. P., Shah, T., Cooper, J., *et al*. (2006) Insight into the nature of the CRP-coronary event association using Mendelian randomization. *International Journal of Epidemiology* **35,** 922-931.
46. Caton, J. G., Proye, M. & Polson, A. M. (1982) Maintenance of healed periodontal pockets after a single episode of root planing. *Journal of Periodontology* **53,** 420-424.
47. Cengiz, M. I., Bal, S., Gokcay, S., *et al*. (2007) Does periodontal disease reflect atherosclerosis in continuous ambulatory peritoneal dialysis patients? *Journal of Periodontology* **78,** 1926-1934.

48. Cerek, J. F., Kiger, R. D., Garrett, S., *et al*. (1983) Relative effects of plaque control and instrumentation on the clinical parameters of human periodontal disease. *Journal of Clinical Periodontology* **10,** 46-56.

49. Chalmers, J. D., Singanayagam, A. & Hill, A. T. (2008) C-reactive protein is an independent predictor of severity in community-acquired pneumonia. *American Journal of Medicine* **121,** 219-225.

50. Chang, M. K., Binder, C. J., Torzewski, M., *et al*. (2002) C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: Phosphorylcholine of oxidized phospholipids. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 13043-13048.

51. Chi, M., Tridandapani, S., Zhong, W., *et al*. (2002) C-reactive protein induces signaling through FcgammaRIIa on HL-60 granulocytes. *Journal of Immunology* **168,** 1413-1418.

52. Chiu, B. (1999) Multiple infections in carotid atherosclerotic plaques. *American Heart Journal* **138,** S534-536.

53. Christodoulides, N., Mohanty, S., Miller, C. S., *et al*. (2005) Application of microchip assay system for the measurement of C-reactive protein in human saliva. *Lab on a Chip - Miniaturisation for Chemistry and Biology* **5,** 261-269.

54. Chrysohoou, C., Panagiotakos, D. B., Pitsavos, C., *et al*. (2004) Adherence to the Mediterranean diet attenuates inflammation and coagulation process in healthy adults: The ATTICA study. *Journal of the American College of Cardiology* **44,** 152-158.

55. Cicarelli, L. M., Perroni, A. G., Zugaib, M., *et al*. (2005) Maternal and cord blood levels of serum amyloid A, C-reactive protein, tumor necrosis factor-alpha, interleukin-1beta, and interleukin-8 during and after delivery. *Mediators of Inflammation* **2005,** 96-100.

56. Cid, M. C., Grant, D. S., Hoffman, G. S., *et al*. (1993) Identification of haptoglobin as an angiogenic factor in sera from patients with systemic vasculitis. *Journal of Clinical Investigation* **91,** 977-985.

57. Claus, D. R., Osmand, A. P. & Gewurz, H. (1976) Radioimmunoassay of human C-reactive protein and levels in normal sera. *The Journal of Laboratory and Clinical Medicine* **87,** 120-128.

58. Craig, R. G., Yip, J., So, M. K., *et al*. (2003a) Relationship of destructive periodontal disease to the acute-phase response. *Journal of Periodontology* **74,** 1007-1016.

59. Craig, R. G., Yip, J. K., So, M. K., *et al.* (2003b) Relationship of destructive periodontal disease to the acute-phase response. *Journal of Periodontology* **74,** 1007-1016.
60. Craig, S. R., Leaver, H. A., Yap, P. L., *et al.* (2001) Acute phase responses following minimal access and conventional thoracic surgery. *European Journal of Cardio-Thoracic Surgery* **20,** 455-463.
61. Cray, C., Zaias, J. & Altman, N. H. (2009) Acute phase response in animals: A review. *Comparative Medicine* **59,** 517-526.
62. Culley, F. J., Bodman-Smith, K. B., Ferguson, M. A. J., *et al.* (2000) C-reactive protein binds to phosphorylated carbohydrates. *Glycobiology* **10,** 59-65.
63. Curb, J. D., Abbott, R. D., Rodriguez, B. L., *et al.* (2003) C-reactive protein and the future risk of thromboembolic stroke in healthy men. *Circulation* **107,** 2016-2020.
64. Cushman, M., Arnold, A. M., Psaty, B. M., *et al.* (2005) C-reactive protein and the 10-year incidence of coronary heart disease in older men and women: The cardiovascular health study. *Circulation* **112,** 25-31.
65. Cushman, M., Legault, C., Barrett-Connor, E., *et al.* (1999) Effect of postmenopausal hormones on inflammation-sensitive proteins: The Postmenopausal Estrogen/Progestin Interventions (PEPI) study. *Circulation* **100,** 717-722.
66. Czerniuk, M. R., Gorska, R., Filipiak, K. J., *et al.* (2006) C-reactive protein in patients with coexistent periodontal disease and acute coronary syndromes. *Journal of Clinical Periodontology* **33,** 415-420.
67. D'Aiuto, F., Casas, J. P., Shah, T., *et al.* (2005a) C-reactive protein (+1444C>T) polymorphism influences CRP response following a moderate inflammatory stimulus. *Atherosclerosis* **179,** 413-417.
68. D'Aiuto, F., Nibali, L., Mohamed-Ali, V., *et al.* (2004a) Periodontal therapy: a novel non-drug-induced experimental model to study human inflammation. *Journal of Periodontal Research* **39,** 294-299.
69. D'Aiuto, F., Nibali, L., Parkar, M., *et al.* (2005b) Short-term effects of intensive periodontal therapy on serum inflammatory markers and cholesterol. *Journal of Dental Research* **84,** 269-273.
70. D'Aiuto, F., Parkar, M., Andreou, G., *et al.* (2004b) Periodontitis and atherogenesis: causal association or simple coincidence? *Journal of Clinical Periodontology* **31,** 402-411.

71. D'Aiuto, F., Parkar, M., Andreou, G., *et al*. (2004c) Periodontitis and systemic inflammation: control of the local infection is associated with a reduction in serum inflammatory markers. *Journal of Dental Research* **83,** 156-160.
72. D'Aiuto, F., Parkar, M., Brett, P. M., *et al*. (2004d) Gene polymorphisms in pro-inflammatory cytokines are associated with systemic inflammation in patients with severe periodontal infections. *Cytokine* **28,** 29-34.
73. D'Aiuto, F., Parkar, M., Nibali, L., *et al*. (2006) Periodontal infections cause changes in traditional and novel cardiovascular risk factors: results from a randomized controlled clinical trial. *American Heart Journal* **151,** 977-984.
74. D'Aiuto, F., Parkar, M. & Tonetti, M. S. (2005c) Periodontal therapy: A novel acute inflammatory model. *Inflammation Research* **54,** 412-414.
75. D'Aiuto, F., Ready, D. & Tonetti, M. S. (2004e) Periodontal disease and C-reactive protein-associated cardiovascular risk. *Journal of Periodontal Research* **39,** 236-241.
76. D'Aiuto, F. & Tonetti, M. S. (2005) Contribution of periodontal therapy on individual cardiovascular risk assessment [1]. *Archives of Internal Medicine* **165,** 1920-1921.
77. Danenberg, H. D., Grad, E., Swaminathan, R. V., *et al*. (2008) Neointimal formation is reduced after arterial injury in human crp transgenic mice. *Atherosclerosis* **201,** 85-91.
78. Danenberg, H. D., Szalai, A. J., Swaminathan, R. V., *et al*. (2003) Increased thrombosis after arterial injury in human C-reactive protein-transgenic mice. *Circulation* **108,** 512-515.
79. Danesh, J., Collins, R., Appleby, P., *et al*. (1998) Association of fibrinogen, C-reactive protein, albumin or leukocyte count with coronary heart disease. Meta-analyses of prospective studies. *Journal of the American Medical Association* **279,** 1477-1482.
80. Danesh, J., Erqou, S., Walker, M., *et al*. (2007) The Emerging Risk Factors Collaboration: Analysis of individual data on lipid, inflammatory and other markers in over 1.1 million participants in 104 prospective studies of cardiovascular diseases. *European Journal of Epidemiology* **22,** 839-869.
81. Danesh, J., Hingorani, A., Wensley, F., *et al*. (2008) Collaborative pooled analysis of data on C-reactive protein gene variants and coronary disease: Judging causality by Mendelian randomisation. *European Journal of Epidemiology* **23,** 531-540.

82. Danesh, J., Muir, J., Wong, Y. K., *et al*. (1999) Risk factors for coronary heart disease and acute-phase proteins. A population-based study. *European Heart Journal* **20,** 954-959.
83. Danesh, J., Wheeler, J. G., Hirschfield, G. M., *et al*. (2004) C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *New England Journal of Medicine* **350,** 1387-1397.
84. Danesh, J., Whincup, P., Walker, M., *et al*. (2000) Low grade inflammation and coronary heart disease: Prospective study and updated meta-analyses. *British Medical Journal* **321,** 199-204.
85. Dasu, M. R., Devaraj, S., Du Clos, T. W., *et al*. (2007) The biological effects of CRP are not attributable to endotoxin contamination: Evidence from TLR4 knockdown human aortic endothelial cells. *Journal of Lipid Research* **48,** 509-512.
86. Dave, S. & Van Dyke, T. (2008) The link between periodontal disease and cardiovascular disease is probably inflammation. *Oral Diseases* **14,** 95-101.
87. Dehghan, A., Kardys, I., de Maat, M. P., *et al*. (2007) Genetic variation, C-reactive protein levels, and incidence of diabetes. *Diabetes* **56,** 872-878.
88. Deliargyris, E. N., Madianos, P. N., Kadoma, W., *et al*. (2004) Periodontal disease in patients with acute myocardial infarction: prevalence and contribution to elevated C-reactive protein levels. *American Heart Journal* **147,** 1005-1009.
89. Desvarieux, M., Demmer, R. T., Rundek, T., *et al*. (2005a) Periodontal microbiota and carotid intima-media thickness: the Oral Infections and Vascular Disease Epidemiology Study (INVEST). *Circulation* **111,** 576-582.
90. Desvarieux, M., Demmer, R. T., Rundek, T., *et al*. (2005b) Periodontal microbiota and carotid intima-media thickness: The Oral Infections and Vascular Disease Epidemiology Study (INVEST). *Circulation* **111,** 576-582.
91. Devaraj, S., Cheung, A. T., Jialal, I., *et al*. (2007) Evidence of increased inflammation and microcirculatory abnormalities in patients with type 1 diabetes and their role in microvascular complications. *Diabetes* **56,** 2790-2796.
92. Di Napoli, M., Schwaninger, M., Cappelli, R., *et al*. (2005) Evaluation of C-reactive protein measurement for assessing the risk and prognosis in ischemic stroke: A statement for health care professionals from the CRP pooling project members. *Stroke* **36,** 1316-1329.
93. DiCamelli, R., Potempa, L. A., Siegel, J., *et al*. (1980) Binding reactivity of C-reactive protein for polycations. *Journal of Immunology* **125,** 1933-1938.

94. Diehl, E. E., Haines Iii, G. K., Radosevich, J. A., *et al.* (2000) Immunohistochemical localization of modified C-reactive protein antigen in normal vascular tissue. *American Journal of the Medical Sciences* **319,** 79-83.
95. Dietrich, T., Jimenez, M., Krall Kaye, E. A., *et al.* (2008) Age-dependent associations between chronic periodontitis/edentulism and risk of coronary heart disease. *Circulation* **117,** 1668-1674.
96. Du Clos, T. W. (1989) C-reactive protein reacts with the U1 small nuclear ribonucleoprotein. *Journal of Immunology* **143,** 2553-2559.
97. Du Clos, T. W. (1996) The interaction of C-reactive protein and serum amyloid P component with nuclear antigens. *Molecular Biology Reports* **23,** 253-260.
98. Du Clos, T. W., Zlock, L. T. & Rubin, R. L. (1988) Analysis of the binding of c-reactive protein to histones and chromatin. *Journal of Immunology* **141,** 4266-4270.
99. Ebersole, J. L. & Cappelli, D. (2000) Acute-phase reactants in infections and inflammatory diseases. *Periodontology 2000* **23,** 19-49.
100. Ebersole, J. L., Cappelli, D., Mathys, E. C., *et al.* (2002) Periodontitis in humans and non-human primates: oral-systemic linkage inducing acute phase proteins. *Annals of Periodontology* **7,** 102-111.
101. Ebersole, J. L., Machen, R. L., Steffen, M. J., *et al.* (1997) Systemic acute-phase reactants, C-reactive protein and haptoglobin, in adult periodontitis. *Clinical and Experimental Immunology* **107,** 347-352.
102. Ebersole, J. L., Taubman, M. A., Smith, D. J., *et al.* (1987) Human serum antibody responses to oral microorganisms. IV. Correlation with homologous infection. *Oral Microbiology and Immunology* **2,** 53-59.
103. Ebersole, J. L., Taubman, M. A., Smith, D. J., *et al.* (1982) Human immune responses to oral micro-organisms. I. Association of localized juvenile periodontitis (LJP) with serum antibody responses to Actinobacillus actinomycetemcomitans. *Clinical Experimental Immunology* **47,** 43-52.
104. Ebrahim, S. & Davey Smith, G. (2008) Mendelian randomization: Can genetic epidemiology help redress the failures of observational epidemiology? *Human Genetics* **123,** 15-33.
105. Egenhofer, C., Alsdorff, K., Fehsel, K., *et al.* (1993) Membrane-associated C-reactive protein on rat liver macrophages is synthesized within the macrophages, expressed as neo-C-reactive protein and bound through a C-reactive protein-specific membrane receptor. *Hepatology* **18,** 1216-1223.

106. Ellis, J. S., Averley, P. A., Preshaw, P. M., *et al*. (2007) Change in cardiovascular risk status after dental clearance. *British Dental Journal* **202,** 543-544.

107. Elter, J. R., Hinderliter, A. L., Offenbacher, S., *et al*. (2006) The effects of periodontal therapy on vascular endothelial function: a pilot trial. *American Heart Journal* **151,** 47.

108. Emery, P., Gabay, C., Kraan, M., *et al*. (2007) Evidence-based review of biologic markers as indicators of disease progression and remission in rheumatoid arthritis. *Rheumatology International* **27,** 793-806.

109. Endo, Y., Tomofuji, T., Ekuni, D., *et al*. (2010) Experimental periodontitis induces gene expression of proinflammatory cytokines in liver and white adipose tissues in obesity. *Journal of Periodontology* **81,** 520-526.

110. Engebretson, S. P., Lamster, I. B., Elkind, M. S., *et al*. (2005) Radiographic measures of chronic periodontitis and carotid artery plaque. *Stroke* **36,** 561-566.

111. Epstein, S. E. (2002) The multiple mechanisms by which infection may contribute to atherosclerosis development and course. *Circulation research* **90,** 2-4.

112. Esposito, K., Marfella, R., Ciotola, M., *et al*. (2004) Effect of a Mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: A randomized trial. *Journal of the American Medical Association* **292,** 1440-1446.

113. Fehsel, K., Plewe, D. & Kolb-Bachofen, V. (1997) Nitric oxide-induced expression of C-reactive protein in islet cells as a very early marker for islet stress in the rat pancreas. *Nitric Oxide - Biology and Chemistry* **1,** 254-262.

114. Fitzsimmons, T. R., Sanders, A. E., Bartold, P. M., *et al*. (2010) Local and systemic biomarkers in gingival crevicular fluid increase odds of periodontitis. *Journal of Clinical Periodontology* **37,** 30-36.

115. Fitzsimmons, T. R., Sanders, A. E., Slade, G. D., *et al*. (2009) Biomarkers of periodontal inflammation in the Australian adult population. *Australian Dental Journal* **54,** 115-122.

116. Floriano, P. N., Christodoulides, N., Miller, C. S., *et al*. (2009) Use of saliva-based nano-biochip tests for acute myocardial infarction at the point of care: A feasibility study. *Clinical Chemistry* **55,** 1530-1538.

117. Ford, E. S., Giles, W. H., Mokdad, A. H., *et al*. (2004) Distribution and correlates of C-reactive protein concentrations among adult US women. *Clinical Chemistry* **50,** 574-581.

118. Ford, E. S., Giles, W. H., Myers, G. L., *et al.* (2003) Population distribution of high-sensitivity C-reactive protein among US men: Findings from National Health and Nutrition Examination Survey 1999-2000. *Clinical Chemistry* **49,** 686-690.
119. Forner, L., Larsen, T., Kilian, M., *et al.* (2006) Incidence of bacteremia after chewing, tooth brushing and scaling in individuals with periodontal inflammation. *Journal of Clinical Periodontology* **33,** 401-407.
120. Fredriksson, M., Bergstrom, K. & Asman, B. (2002) IL-8 and TNF-alpha from peripheral neutrophils and acute-phase proteins in periodontitis. *Journal of Clinical Periodontology* **29,** 123-128.
121. Fredriksson, M., Gustafsson, A., Asman, B., *et al.* (1998) Hyper-reactive peripheral neutrophils in adult periodontitis: Generation of chemiluminescence and intracellular hydrogen peroxide after in vitro priming and FcgammaR-stimulation. *Journal of Clinical Periodontology* **25,** 394-398.
122. Fredriksson, M. I., Figueredo, C. M., Gustafsson, A., *et al.* (1999) Effect of periodontitis and smoking on blood leukocytes and acute-phase proteins. *Journal of Periodontology* **70,** 1355-1360.
123. Fredriksson, M. I., Gustafsson, A. K., Bergstrom, K. G., *et al.* (2003) Constitutionally hyperreactive neutrophils in periodontitis. *Journal of Periodontology* **74,** 219-224.
124. Friedewald, V. E., Kornman, K. S., Beck, J. D., *et al.* (2009) The American Journal of Cardiology and Journal of Periodontology Editors' Consensus: Periodontitis and Atherosclerotic Cardiovascular Disease. *American Journal of Cardiology* **104,** 59-68.
125. Furuichi, Y., Shimotsu, A., Ito, H., *et al.* (2003) Associations of periodontal status with general health conditions and serum antibody titers for *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. *Journal of Periodontology* **74,** 1491-1497.
126. Gabay, C. & Kushner, I. (1999) Acute-phase proteins and other systemic responses to inflammation. *New England Journal of Medicine* **340,** 448-454.
127. Gaboriaud, C., Juanhuix, J., Gruez, A., *et al.* (2003) The crystal structure of the globular head of complement protein c1q provides a basis for its versatile recognition properties. *Journal of Biological Chemistry* **278,** 46974-46982.
128. Ganter, U., Arcone, R., Toniatti, C., *et al.* (1989) Dual control of C-reactive protein gene expression by interleukin-1 and interleukin-6. *EMBO Journal* **8,** 3773-3779.

129. Geerts, S. O., Nys, M., De, M. P., *et al*. (2002) Systemic release of endotoxins induced by gentle mastication: association with periodontitis severity. *Journal of Periodontology* **73,** 73-78.

130. Genctoy, G., Ozbek, M., Avcu, N., *et al*. (2007) Gingival health status in renal transplant recipients: Relationship between systemic inflammation and atherosclerosis. *International Journal of Clinical Practice* **61,** 577-582.

131. Giffen, P. S., Turton, J., Andrews, C. M., *et al*. (2003) Markers of experimental acute inflammation in the Wistar Han rat with particular reference to haptoglobin and C-reactive protein. *Archives of Toxicology* **77,** 392-402.

132. Glurich, I., Grossi, S., Albini, B., *et al*. (2002) Systemic inflammation in cardiovascular and periodontal disease: comparative study. *Clinical and Diagnostic Laboratory Immunology* **9,** 425-432.

133. Gokce, N., Keaney Jr, J. F., Hunter, L. M., *et al*. (2003) Predictive value of noninvasively determined endothelial dysfunction for long-term cardiovascular events in patients with peripheral vascular disease. *Journal of the American College of Cardiology* **41,** 1769-1775.

134. Goodson, J. M. (2003). Gingival crevice fluid flow. *Periodontology 2000* 31, 43-54.

135. Gotschlich, E. C. & Edelman, G. M. (1967) Binding properties and specificity of C-reactive protein. *Proceedings of the National Academy of Sciences of the United States of America* **57,** 706-712.

136. Gould, J. M. & Weiser, J. N. (2001) Expression of C-reactive protein in the human respiratory tract. *Infection and Immunity* **69,** 1747-1754.

137. Greenland, P., Knoll, M. D., Stamler, J., *et al*. (2003) Major risk factors as antecedents of fatal and nonfatal coronary heart disease events. *Journal of the American Medical Association* **290,** 891-897.

138. Griffiths, G. S. (2003). Formation, collection and significance of gingival crevice fluid. *Periodontology 2000* 31, 32-42.

139. Hack, C. E., Wolbink, G. J., Schalkwijk, C., *et al*. (1997) A role for secretory phospholipase A2 and C-reactive protein in the removal of injured cells. *Immunology Today* **18,** 111-115.

140. Haffajee, A. D., Socransky, S. S., Taubman, M. A., *et al*. (1995) Patterns of antibody response in subjects with periodontitis. *Oral Microbiology and Immunology* **10,** 129-137.

141. Hansson, G. K. (2005) Mechanisms of disease: Inflammation, atherosclerosis, and coronary artery disease. *New England Journal of Medicine* **352**.

142. Haraszthy, V. I., Zambon, J. J., Trevisan, M., *et al.* (2000) Identification of periodontal pathogens in atheromatous plaques. *Journal of Periodontology* **71,** 1554-1560.

143. Hasturk, H., Kantarci, A., Goguet-Surmenian, E., *et al.* (2007) Resolvin E1 regulates inflammation at the cellular and tissue level and restores tissue homeostasis in vivo. *Journal of immunology (Baltimore, Md. : 1950)* **179,** 7021-7029.

144. Havemose-Poulsen, A., Westergaard, J., Stoltze, K., *et al.* (2006) Periodontal and hematological characteristics associated with aggressive periodontitis, juvenile idiopathic arthritis, and rheumatoid arthritis. *Journal of Periodontology* **77,** 280-288.

145. Haverkate, F., Thompson, S. G., Pyke, S. D. M., *et al.* (1997) Production of C-reactive protein and risk of coronary events in stable and unstable angina. *Lancet* **349,** 462-466.

146. Haynes, W. G. & Stanford, C. (2003) Periodontal disease and atherosclerosis: from dental to arterial plaque. *Arteriosclerosis, Thrombosis, and Vascular Biology* **23,** 1309-1311.

147. Heidari, B., Heidari, P. & Tayebi, M. E. (2007) The value of changes in CRP and ESR for predicting treatment response in rheumatoid arthritis. *APLAR Journal of Rheumatology* **10,** 23-28.

148. Heinrich, P. C., Castell, J. V. & Andus, T. (1990) Interleukin-6 and the acute phase response. *Biochemical Journal* **265,** 621-636.

149. Heuertz, R. M., Schneider, G. P., Potempa, L. A., *et al.* (2005) Native and modified C-reactive protein bind different receptors on human neutrophils. *International Journal of Biochemistry & Cell Biology* **37,** 320-335.

150. Higashi, Y., Goto, C., Jitsuiki, D., *et al.* (2008) Periodontal infection is associated with endothelial dysfunction in healthy subjects and hypertensive patients. *Hypertension* **51,** 446-453.

151. Hirasaki, S., Yamazaki, T. & Shiba, K. (2005) Changes in salivary components by drug administration in patients with heart diseases. *Journal of Medical and Dental Sciences* **52,** 183-188.

152. Hirschfield, G. M., Gallimore, J. R., Kahan, M. C., *et al.* (2005) Transgenic human C-reactive protein is not proatherogenic in apolipoprotein E-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* **102,** 8309-8314.

153. Ho, K. J., Owens, C. D., Longo, T., *et al.* (2008) C-reactive protein and vein graft disease: Evidence for a direct effect on smooth muscle cell phenotype via modulation

of PDGF receptor-beta. *American Journal of Physiology - Heart and Circulatory Physiology* **295,** H1132-H1140.

154. Horton, A. L., Boggess, K. A., Moss, K. L., *et al*. (2008) Periodontal disease early in pregnancy is associated with maternal systemic inflammation among African American women. *Journal of Periodontology* **79,** 1127-1132.
155. Hujoel, P. P., White, B. A., Garcia, R. I., *et al*. (2001) The dentogingival epithelial surface area revisited. *Journal of Periodontal Research* **36,** 48-55.
156. Hundt, M., Zielinska-Skowronek, M. & Schmidt, R. E. (2001) Lack of specific receptor for C-reactive protein on white blood cells. *European Journal of Immunology* **31,** 3475-3483.
157. Hurlimann, J., Thorbecke, G. J. & Hochwald, G. M. (1966) The liver as the site of C-reactive protein formation. *Journal of Experimental Medicine* **123,** 365-378.
158. Ide, M., Jagdev, D., Coward, P. Y., *et al*. (2004) The short-term effects of treatment of chronic periodontitis on circulating levels of endotoxin, C-reactive protein, tumor necrosis factor-alpha, and interleukin-6. *Journal of Periodontology* **75,** 420-428.
159. Ide, M., McPartlin, D., Coward, P. Y., *et al*. (2003) Effect of treatment of chronic periodontitis on levels of serum markers of acute-phase inflammatory and vascular responses. *Journal of Clinical Periodontology* **30,** 334-340.
160. Ikejima, T., Okusawa, S., Ghezzi, P., *et al*. (1990) Interleukin-1 induces tumor necrosis factor (TNF) in human peripheral blood mononuclear cells in vitro and a circulating TNF-like activity in rabbits. *Journal of Infectious Diseases* **162,** 215-223.
161. Ikonomidis, I., Andreotti, F., Economou, E., *et al*. (1999) Increased proinflammatory cytokines in patients with chronic stable angina and their reduction by aspirin. *Circulation* **100,** 793-798.
162. Iwamoto, Y., Nishimura, F., Soga, Y., *et al*. (2003) Antimicrobial periodontal treatment decreases serum C-reactive protein, tumor necrosis factor-alpha, but not adiponectin levels in patients with chronic periodontitis. *Journal of Periodontology* **74,** 1231-1236.
163. Jabs, W. J., Busse, M., Kruger, S., *et al*. (2005) Expression of C-reactive protein by renal cell carcinoma and unaffected surrounding renal tissue. *Kidney International* **68,** 2103-2110.
164. Jabs, W. J., Logering, B. A., Gerke, P., *et al*. (2003a) The kidney as a second site of human C-reactive protein formation in vivo. *European Journal of Immunology* **33,** 152-161.

165. Jabs, W. J., Theissing, E., Nitschke, M., *et al.* (2003b) Local generation of C-reactive protein in diseased coronary artery venous bypass grafts and normal vascular tissue. Circulation 108, 1428-1431.

166. Jain, A., Batista Jr, E. L., Serhan, C., *et al.* (2003) Role for periodontitis in the progression of lipid deposition in an animal model. *Infection and Immunity* **71,** 6012-6018.

167. Janket, S.-J., Baird, A. E., Chuang, S.-K., *et al.* (2003) Meta-analysis of periodontal disease and risk of coronary heart disease and stroke. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **95,** 559-569.

168. Jialal, I., Devaraj, S. & Venugopal, S. K. (2004) C-reactive protein: Risk marker or mediator in atherothrombosis? *Hypertension* **44,** 6-11.

169. Johnson, N. W., Griffiths, G. S., Wilton, J. M., *et al.* (1988) Detection of high-risk groups and individuals for periodontal diseases. Evidence for the existence of high-risk groups and individuals and approaches to their detection. *Journal of Clinical Periodontology* **15,** 276-282.

170. Joshipura, K. J., Wand, H. C., Merchant, A. T., *et al.* (2004) Periodontal disease and biomarkers related to cardiovascular disease. *Journal of Dental Research* **83,** 151-155.

171. Jousilahti, P., Salomaa, V., Rasi, V., *et al.* (2003) Association of markers of systemic inflammation, C reactive protein, serum amyloid A, and fibrinogen, with socioeconomic status. *Journal of Epidemiology and Community Health* **57,** 730-733.

172. Kamer, A. R., Craig, R. G., Dasanayake, A. P., *et al.* (2008) Inflammation and Alzheimer's disease: Possible role of periodontal diseases. *Alzheimer's and Dementia* **4,** 242-250.

173. Katagiri, S., Nitta, H., Nagasawa, T., *et al.* (2009) Multi-center intervention study on glycohemoglobin (HbA1c) and serum, high-sensitivity CRP (hs-CRP) after local anti-infectious periodontal treatment in type 2 diabetic patients with periodontal disease. *Diabetes Research and Clinical Practice*.

174. Keles, G. C., Cetinkaya, B. O., Simsek, S. B., *et al.* (2007) The role of periodontal disease on acute phase proteins in patients with coronary heart disease and diabetes. *Turkish Journal of Medical Sciences* **37,** 39-44.

175. Khera, A., McGuire, D. K., Murphy, S. A., *et al.* (2005) Race and gender differences in C-reactive protein levels. *Journal of the American College of Cardiology* **46,** 464-469.

176. Khot, U. N., Khot, M. B., Bajzer, C. T., *et al*. (2003) Prevalence of conventional risk factors in patients with coronary heart disease. *Journal of the American Medical Association* **290,** 898-904.
177. Kinane, D. F. & Hart, T. C. (2003) Genes and gene polymorphisms associated with periodontal disease. *Critical Reviews in Oral Biology and Medicine* **14,** 430-449.
178. Kinlay, S. (2007) Low-density lipoprotein-dependent and -independent effects of cholesterol-lowering therapies on C-reactive protein: a meta-analysis. *Journal of the American College of Cardiology* **49,** 2003-2009.
179. Koenig, W., Lowel, H., Baumert, J., *et al*. (2004) C-reactive protein modulates risk prediction based on the Framingham Score - Implications for future risk assessment: Results from a large cohort study in Southern Germany. *Circulation* **109,** 1349-1353.
180. Koenig, W., Sund, M., Frohlich, M., *et al*. (1999) C-reactive protein, a sensitive marker of inflammation, predicts future risk of coronary heart disease in initially healthy middle-aged men: results from the MONICA (Monitoring Trends and Determinants in Cardiovascular Disease) Augsburg Cohort Study, 1984 to 1992. *Circulation* **99,** 237-242.
181. Koj, A. (1989) The role of interleukin-6 as the hepatocyte stimulating factor in the network of inflammatory cytokines. *Annals of the New York Academy of Sciences* **557,** 1-8.
182. Koj, A. (1996) Initiation of the acute phase response ad the synthesis of cytokines. *Biochimica et Biophysica Acta* **1317,** 84-94.
183. Kolb-Bachofen, V., Puchta-Teudt, N. & Egenhofer, C. (1995) Expression of membrane-associated C-reactive protein by human monocytes: Indications for a selectin-like activity participating in adhesion. *Glycoconjugate Journal* **12,** 122-127.
184. Kones, R. (2009) The Jupiter study, CRP screening, and aggressive statin therapy-implications for the primary prevention of cardiovascular disease. *Therapeutic Advances in Cardiovascular Disease* **3,** 309-315.
185. Kornman, K. S. (2006) Interleukin 1 genetics, inflammatory mechanisms, and nutrigenetic opportunities to modulate diseases of aging. *American Journal of Clinical Nutrition* **83,** 475S-483S.
186. Kuo, H. K., Yen, C. J., Chang, C. H., *et al*. (2005) Relation of C-reactive protein to stroke, cognitive disorders, and depression in the general population: Systematic review and meta-analysis. *Lancet Neurology* **4,** 371-380.
187. Kushner, I. (1982) The phenomenon of the acute phase response. *Annals of the New York Academy of Sciences* **Vol. 389,** 39-48.

188. Kushner, I. & Feldmann, G. (1978) Control of the acute phase response. Demonstration of C-reactive protein synthesis and secretion by hepatocytes during acute inflammation in the rabbit. *Journal of Experimental Medicine* **148,** 466-477.
189. Kushner, I., Jiang, S. L., Zhang, D., *et al*. (1995) Do post-transcriptional mechanisms participate in induction of C-reactive protein and serum amyloid A by IL-6 and IL-1? *Annals of the New York Academy of Sciences* **762,** 102-107.
190. Kuta, A. E. & Baum, L. L. (1986) C-reactive protein is produced by a small number of normal human peripheral blood lymphocytes. *Journal of Experimental Medicine* **164,** 321-326.
191. Kwang, K. K., Quon, M. J., Seung, H. H., *et al*. (2004) Additive beneficial effects of losartan combined with simvastatin in the treatment of hypercholesterolemic, hypertensive patients. *Circulation* **110,** 3687-3692.
192. Laaksonen, D. E., Niskanen, L., NyyssoÃànen, K., *et al*. (2005) C-reactive protein in the prediction of cardiovascular and overall mortality in middle-aged men: A population-based cohort study. *European Heart Journal* **26,** 1783-1789.
193. Lalla, E., Lamster, I., Hofmann, M. A., *et al*. (2003) Oral infection with a periodontal pathogen accelerates early atherosclerosis in apoliporotein E-null mice. *Arteriosclerosis, Thrombosis and Vascular Biology* **23,** 1405-1411.
194. Lam, H. S. & Ng, P. C. (2008) Biochemical markers of neonatal sepsis. *Pathology* **40,** 141-148.
195. Lange, L. A., Carlson, C. S., Hindorff, L. A., *et al*. (2006) Association of polymorphisms in the CRP gene with circulating C-reactive protein levels and cardiovascular events. *Journal of the American Medical Association* **296,** 2703-2711.
196. Lavie, C. J., Milani, R. V., Verma, A., *et al*. (2009) C-reactive protein and cardiovascular diseases-Is it ready for primetime? *American Journal of the Medical Sciences* **338,** 486-492.
197. Leivadaros, E., van der Velden, U., Bizzarro, S., *et al*. (2005) A pilot study into measurements of markers of atherosclerosis in periodontitis. *Journal of Periodontology* **76,** 121-128.
198. Li, J. T., Hou, F. F., Guo, Z. J., *et al*. (2007) Advanced glycation end products upregulate C-reactive protein synthesis by human hepatocytes through stimulation of monocyte IL-6 and IL-1beta production. *Scandinavian Journal of Immunology* **66,** 555-562.

199. Li, L., Messas, E., Batista Jr, E. L., *et al*. (2002) Porphyromonas gingivalis infection accelerates the progression of atherosclerosis in a heterozygous apolipoprotein E-deficient murine model. *Circulation* **105,** 861-867.
200. Libby, P. & Aikawa, M. (2002) Stabilization of atherosclerotic plaques: New mechanisms and clinical targets. *Nature Medicine* **8,** 1257-1262.
201. Lim, L. P., Tay, F. B., Sum, C. F., *et al*. (2007) Relationship between markers of metabolic control and inflammation on severity of periodontal disease in patients with diabetes mellitus. *Journal of Clinical Periodontology* **34,** 118-123.
202. Listgarten, M. A., Lai, C. H. & Evian, C. I. (1981) Comparative antibody titers to Actinobacillus actinomycetemcomitans in juvenile periodontitis, chronic periodontitis and periodontally healthy subjects. *Journal of Clinical Periodontology* **8,** 155-164.
203. Liu, C., Wang, S., Deb, A., *et al*. (2005) Proapoptotic, antimigratory, antiproliferative, and antiangiogenic effects of commercial C-reactive protein on various human endothelial cell types in vitro: Implications of contaminating presence of sodium azide in commercial preparation. *Circulation Research* **97,** 135-143.
204. Loos, B. G. (2005) Systemic markers of inflammation in periodontits. *Journal of Periodontology* **76,** 2106-2115.
205. Loos, B. G., Craandijk, J., Hoek, F. J., *et al*. (2000) Elevation of systemic markers related to cardiovascular diseases in the peripheral blood of periodontitis patients. *Journal of Periodontology* **71,** 1528-1534.
206. Lowe, G., Woodward, M., Rumley, A., *et al*. (2003) Total tooth loss and prevalent cardiovascular disease in men and women: Possible roles of citrus fruit consumption, vitamin C, and inflammatory and thrombotic variables. *Journal of Clinical Epidemiology* **56,** 694-700.
207. Mackiewicz, A., Ganapathi, M. K., Schultz, D., *et al*. (1990) Transforming growth factor beta1 regulates production of acute-phase proteins. *Proceedings of the National Academy of Sciences of the United States of America* **87,** 1491-1495.
208. Mackiewicz, A., Speroff, T., Ganapathi, M. K., *et al*. (1991) Effects of cytokine combinations on acute phase protein production in two human hepatoma cell lines. *Journal of Immunology* **146,** 3032-3037.
209. Maes, M., Delange, J., Ranjan, R., *et al*. (1997) Acute phase proteins in schizophrenia, mania and major depression: Modulation by psychotropic drugs. *Psychiatry Research* **66,** 1-11.

210. Marcaccini, A. M., Meschiari, C. A., Sorgl, C. A., *et al*. (2009) Circulating intereukin - 6 and high - sensitivity C-reactive protein decrease after periodontal theapy in otherwise healthy subjects. *Journal of Periodontology* **80,** 594-602.
211. Mattila, K., Vesanen, M., Valtonen, V., *et al*. (2002) Effect of treating periodontitis on C-reactive protein levels: a pilot study. *BMC Infectious Diseases* **2,** 30.
212. Mattila, K. J., Nieminen, M. S., Valtonen, V. V., *et al*. (1989) Association between dental health and acute myocardial infarction. *British Medical Journal* **298,** 779-781.
213. Meier-Ewert, H. K., Ridker, P. M., Rifai, N., *et al*. (2001) Absence of diurnal variation of C-reactive protein concentrations in healthy human subjects. *Clinical Chemistry* **47,** 426-430.
214. Mendall, M. A., Patel, P., Ballam, L., *et al*. (1996) C Reactive protein and its relation to cardiovascular risk factors: A population based cross sectional study. *British Medical Journal* **312,** 1061-1065.
215. Mengel, R., Bacher, M. & Flores-De-Jacoby, L. (2002) Interactions between stress, interleukin-1beta, interleukin-6 and cortisol in periodontally diseased patients. *Journal of Clinical Periodontology* **29,** 1012-1022.
216. Mercado, F. B., Marshall, R. I., Klestov, A. C., *et al*. (2001) Relationship between rheumatoid arthritis and periodontitis. *Journal of Periodontology* **72,** 779-787.
217. Meurman, J. H., Pajukoski, H., Snellman, S., *et al*. (1997) Oral infections in home-living elderly patients admitted to an acute geriatric ward. *Journal of Dental Research* **76,** 1271-1276.
218. Meurman, J. H., Sanz, M. & Janket, S.-J. (2004) Oral health, atherosclerosis and cardiovascular disease. *Critical Reviews in Oral Biology and Medicine* **15,** 403-413.
219. Miller, D. T., Zee, R. Y. L., Danik, J. S., *et al*. (2005) Association of common CRP gene variants with CRP levels and cardiovascular events. *Annals of Human Genetics* **69,** 623-638.
220. Miller, L. L., Bly, C. G., Watson, M. L., *et al*. (1951) The dominant role of the liver in plasma protein synthesis; a direct study of the isolated perfused rat liver with the aid of lysine-epsilon-C14. *Journal of Experimental Medicine* **94,** 431-453.
221. Mochari, H., Grbic, J. T. & Mosca, L. (2008) Usefulness of self-reported periodontal disease to identify individuals with elevated inflammatory markers at risk of cardiovascular disease. *American Journal of Cardiology* **102,** 1509-1513.
222. Modena, M. G., Bonetti, L., Coppi, F., *et al*. (2002) Prognostic role of reversible endothelial dysfunction in hypertensive postmenopausal women. *Journal of the American College of Cardiology* **40,** 505-510.

223. Mold, C., Baca, R. & Du Clos, T. W. (2002) Serum amyloid P component and C-reactive protein opsonize apoptotic cells for phagocytosis through Fcgamma receptors. *Journal of Autoimmunity* **19,** 147-154.
224. Mold, C., Gresham, H. D. & Du Clos, T. W. (2001) Serum amyloid P component and C-reactive protein mediate phagocytosis through murine FcgammaRs. *Journal of Immunology* **166,** 1200-1205.
225. Montebugnoli, L., Servidio, D., Miaton, R. A., *et al.* (2005) Periodontal health improves systemic inflammatory and haemostatic status in subjects with coronary heart disease. *Journal of Clinical Periodontology* **32,** 188-192.
226. Morley, J. J. & Kushner, I. (1982) Serum C-reactive protein levels in disease. *Annals of the New York Academy of Sciences* **Vol. 389,** 406-418.
227. Motie, M., Brockmeier, S. & Potempa, L. A. (1996) Binding of model soluble immune complexes to modified C-reactive protein. *Journal of Immunology* **156,** 4435-4441.
228. Moutsopoulos, N. M. & Madianos, P. N. (2006) Low-grade inflammation in chronic infectious diseases: Paradigm of periodontal infections. *Annals of the New York Academy of Sciences* **1088,** 251-264.
229. Murphy, A. M., Daly, C. G., Mitchell, D. H., *et al.* (2006) Chewing fails to induce oral bacteraemia in patients with periodontal disease. *Journal of Clinical Periodontology* **33,** 730-736.
230. Mustapha, I. Z., Debrey, S., Oladubu, M., *et al.* (2007) Markers of systemic bacterial exposure in periodontal disease and cardiovascular disease risk: a systematic review and meta-analysis. *Journal of Periodontology* **78,** 2289-2302.
231. Myles, D. A., Rule, S. A., DeLucas, L. J., *et al.* (1990) Rotation function studies of human C-reactive protein. *Journal of Molecular Biology* **216,** 491-496.
232. Nakajima, T., Honda, T., Domon, H., *et al.* (2010) Periodontitis-associated up-regulation of systemic inflammatory mediator level may increase the risk of coronary heart disease. *Journal of Periodontal Research* **45,** 116-122.
233. Neunteufl, T., Heher, S., Katzenschlager, R., *et al.* (2000) Late prognostic value of flow-mediated dilation in the brachial artery of patients with chest pain. *American Journal of Cardiology* **86,** 207-210.
234. Nibali, L., Tonetti, M. S., Ready, D., *et al.* (2008) Interleukin-6 polymorphisms are associated with pathogenic bacteria in subjects with periodontitis. *Journal of Periodontology* **79,** 677-683.

235. Nilsson, J. (2005) CRP - Marker or maker of cardiovascular disease? *Arteriosclerosis, Thrombosis, and Vascular Biology* **25,** 1527-1528.

236. Noack, B., Genco, R. J., Trevisan, M., *et al.* (2001) Periodontal infections contribute to elevated systemic C-reactive protein level. *Journal of Periodontology* **72,** 1221-1227.

237. Norman, M. E., Baehni, P. C., Tsai, C. C., *et al.* (1979) Studies of host responses during experimental gingivitis in humans. II. Changes in acute phase reactants, serum immunoglobulins and complement during the development of gingival inflammation. *Journal of Periodontal Research* **14,** 361-369.

238. Nunomura, W. (1990) C-reactive protein in rat: In development, pregnancy and effect of sex hormones. *Comparative Biochemistry and Physiology - A Physiology* **96,** 489-493.

239. O'Brien-Simpson, N. M., Black, C. L., Bhogal, P. S., *et al.* (2000) Serum immunoglobulin G (IgG) and IgG subclass responses to the RgpA-Kgp proteinase-adhesin complex of Porphyromonas gingivalis in adult periodontitis. *Infection and Immunity* **68,** 2704-2712.

240. O'Connor, S., Taylor, C., Campbell, L. A., *et al.* (2001) Potential infectious etiologies of atherosclerosis: a multifactorial perspective. *Emerging Infectious Diseases* **7,** 780-788.

241. Offenbacher, S. & Beck, J. D. (2005) A perspective on the potential cardioprotective benefits of periodontal therapy. *American Heart Journal* **149,** 950-954.

242. Offenbacher, S., Beck, J. D., Moss, K., *et al.* (2009) Results from the Periodontitis and Vascular Events (PAVE) study: A pilot multicentered, randomized, controlled trial to study effects of periodontal therapy in a secondary prevention model of cardiovascular disease. *Journal of Periodontology* **80,** 190-201.

243. Osmand, A. P., Friedenson, B. & Gewurz, H. (1977a) Characterization of C reactive protein and the complement subcomponent C1t at homologous proteins displaying cyclic pentameric symmetry (pentraxins). *Proceedings of the National Academy of Sciences of the United States of America* **74,** 739-743.

244. Osmand, A. P., Gerwurz, H. & Friedenson, B. (1977b) Partial amino-acid sequences of human and rabbit C-reactive proteins: homology with immunoglobulins and histocompatibility antigens. *Proceedings of the National Academy of Sciences of the United States of America* **74,** 1214-1218.

245. Ouchi, N., Kihara, S., Funahashi, T., *et al*. (2003) Reciprocal association of C-reactive protein with adiponectin in blood stream and adipose tissue. *Circulation* **107,** 671-674.
246. Pai, J. K., Pischon, T., Ma, J., *et al*. (2004) Inflammatory markers and the risk of coronary heart disease in men and women. *New England Journal of Medicine* **351,** 2599-2610.
247. Pankow, J. S., Folsom, A. R., Cushman, M., *et al*. (2001) Familial and genetic determinants of systemic markers of inflammation: The NHLBI family heart study. *Atherosclerosis* **154,** 681-689.
248. Paquette, D. W. (2002) The periodontal infection-systemic disease link: a review of the truth or myth. *Journal of the International Academy of Periodontology* **4,** 101-109.
249. Paraskevas, S., Huizinga, J. D. & Loos, B. G. (2008) A systematic review and meta-analyses on C-reactive protein in relation to periodontitis. *Journal of Clinical Periodontology* **35,** 277-290.
250. Pasceri, V., Willerson, J. T. & Yeh, E. T. H. (2000) Direct proinflammatory effect of C-reactive protein on human endothelial cells. *Circulation* **102,** 2165-2168.
251. Paul, A., Ko, K. W. S., Li, L., *et al*. (2004) C-reactive protein accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. *Circulation* **109,** 647-655.
252. Pearson, T., Ballantyne, C., Sisk, C., *et al*. (2007) Comparison of effects of ezetimibe/simvastatin versus simvastatin versus atorvastatin in reducing C-reactive protein and low-density lipoprotein cholesterol levels. *American Journal of Cardiology* **99,** 1706-1713.
253. Pearson, T. A., Mensah, G. A., Alexander, R. W., *et al*. (2003) Markers of inflammation and cardiovascular disease: application to clinical and public health practice: A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. *Circulation* **107,** 499-511.
254. Pederson, E. D., Stanke, S. R., Whitener, S. J., *et al*. (1995) Salivary levels of alpha 2-macroglobulin, alpha 1-antitrypsin, C-reactive protein, cathepsin G and elastase in humans with or without destructive periodontal disease. *Archives of Oral Biology* **40,** 1151-1155.
255. Pepys, M. B. (1981) C-reactive protein fifty years on. *Lancet* **1,** 653-657.

256. Pepys, M. B., Booth, S. E., Tennent, G. A., *et al*. (1994) Binding of pentraxins to different nuclear structures: C-reactive protein binds to small nuclear ribonucleoprotein particles, serum amyloid P component binds to chromatin and nucleoli. *Clinical and Experimental Immunology* **97,** 152-157.
257. Pepys, M. B., Dash, A. C. & Fletcher, T. C. (1978) Analogues in other mammals and in fish of human plasma proteins, C-reactive protein and amyloid P component. *Nature* **273,** 168-170.
258. Pepys, M. B., Hawkins, P. N., Kahan, M. C., *et al*. (2005) Proinflammatory effects of bacterial recombinant human C-reactive protein are caused by contamination with bacterial products, not by C-reactive protein itself. *Circulation Research* **97**.
259. Pepys, M. B. & Hirschfield, G. M. (2003) C-reactive protein: A critical update. *Journal of Clinical Investigation* **111,** 1805-1812.
260. Pepys, M. B., Hirschfield, G. M., Tennent, G. A., *et al*. (2006) Targeting C-reactive protein for the treatment of cardiovascular disease. *Nature* **440,** 1217-1221.
261. Persson, G. R. & Persson, R. E. (2008) Cardiovascular disease and periodontitis: an update on the associations and risk. *Journal of Clinical Periodontology* **35,** 362-379.
262. Persson, G. R., Pettersson, T., Ohlsson, O., *et al*. (2005) High-sensitivity serum C-reactive protein levels in subjects with or without myocardial infarction or periodontitis. *Journal of Clinical Periodontology* **32,** 219-224.
263. Piconi, S., Trabattoni, D., Luraghi, C., *et al*. (2009) Treatment of periodontal disease results in improvements in endothelial dysfunction and reduction of the carotid intima-media thickness. *FASEB Journal* **23,** 1196-1204.
264. Pischon, N., Hagewald, S., Kunze, M., *et al*. (2007) Influence of periodontal therapy on the regulation of soluble cell adhesion molecule expression in aggressive periodontitis patients. *Journal of Periodontology* **78,** 683-690.
265. Pitiphat, W., Joshipura, K. J., Rich-Edwards, J. W., *et al*. (2006) Periodontitis and plasma C-reactive protein during pregnancy. *Journal of Periodontology* **77,** 821-825.
266. Pollitt, R. A., Kaufman, J. S., Rose, K. M., *et al*. (2007) Early-life and adult socioeconomic status and inflammatory risk markers in adulthood. *European Journal of Epidemiology* **22,** 55-66.
267. Potempa, L. A., Maldonado, B. A. & Laurent, P. (1983) Antigenic, electrophoretic and binding alterations of human C-reactive protein modified selectively in the absence of calcium. *Molecular Immunology* **20,** 1165-1175.

268. Potempa, L. A., Siegel, J. N. & Fiedel, B. A. (1987) Expression, detection and assay of a neoantigen (Neo-CRP) associated with a free, human C-reactive protein subunit. *Molecular Immunology* **24,** 531-541.
269. Potempa, L. A., Siegel, J. N. & Gewurz, H. (1981) Binding reactivity of C-reactive protein for polycations. II. Medulatory effects of calcium and phosphocholine. *Journal of Immunology* **127,** 1509-1514.
270. Pradhan, A. D., Manson, J. E., Rifai, N., *et al.* (2001) C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *Journal of the American Medical Association* **286,** 327-334.
271. Rahman, A. U., Rashid, S., Noon, R., *et al.* (2005) Prospective evaluation of the systemic inflammatory marker C-reactive protein in patients with end-stage periodontitis getting teeth replaced with dental implants: a pilot investigation. *Clinical Oral Implants Research* **16,** 128-131.
272. Rahmati, M. A., Craig, R. G., Homel, P., *et al.* (2002) Serum markers of periodontal disease status and inflammation in hemodialysis patients. *American Journal of Kidney Diseases* **40,** 983-989.
273. Rau, B. M. (2007) Predicting severity of acute pancreatitis. *Current Gastroenterology Reports* **9,** 107-115.
274. Rees, R. F., Gewurz, H., Siegel, J. N., *et al.* (1988) Expression of a C-reactive protein neoantigen (neo-CRP) in inflamed rabbit liver and muscle. *Clinical Immunology and Immunopathology* **48,** 95-107.
275. Reichert, S., Machulla, H. K., Fuchs, C., *et al.* (2006) Is there a relationship between juvenile idiopathic arthritis and periodontitis? *Journal of Clinical Periodontology* **33,** 317-323.
276. Ren, Y. F. & Malmstrom, H. S. (2007) Rapid quantitative determination of C-reactive protein at chair side in dental emergency patients. *Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics* **104,** 49-55.
277. Renvert, S., Lindahl, C., Roos-Jansaker, A. M., *et al.* (2009) Short-term effects of an anti- inflammatory treatment on clinical parameters and serum levels of C-reactive protein and proinflammatory cytokines in subjects with periodontitis. *Journal of Periodontology* **80,** 892-900.
278. Renvert, S., Pettersson, T., Ohlsson, O., *et al.* (2006) Bacterial profile and burden of periodontal infection in subjects with a diagnosis of acute coronary syndrome. *Journal of Periodontology* **77,** 1110-1119.

279. Ridker, P. M., Buring, J. E., Cook, N. R., *et al*. (2003) C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events: an 8-year follow-up of 14 719 initially healthy American women. *Circulation* **107,** 391-397.
280. Ridker, P. M., Buring, J. E., Rifai, N., *et al*. (2007) Development and validation of improved algorithms for the assessment of global cardiovascular risk in women: The Reynolds Risk Score. *Journal of the American Medical Association* **297,** 611-619.
281. Ridker, P. M., Cannon, C. P., Morrow, D., *et al*. (2005) C-reactive protein levels and outcomes after statin therapy. *New England Journal of Medicine* **352,** 20-28.
282. Ridker, P. M. & Cook, N. (2004) Clinical Usefulness of Very High and Very Low Levels of C-reactive Protein Across the Full Range of Framingham Risk Scores. *Circulation* **109,** 1955-1959.
283. Ridker, P. M., Cushman, M., Stampfer, M. J., *et al*. (1997) Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *New England Journal of Medicine* **336,** 973-979.
284. Ridker, P. M., Danielson, E., Fonseca, F. A. H., *et al*. (2008) Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *New England Journal of Medicine* **359,** 2195-2207.
285. Ridker, P. M., Hennekens, C. H., Buring, J. E., *et al*. (2000) C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *New England Journal of Medicine* **342,** 836-843.
286. Ridker, P. M., Rifai, N., Clearfield, M., *et al*. (2001) Measurement of C-reactive protein for the targeting of statin therapy in the primary prevention of acute coronary events. *New England Journal of Medicine* **344,** 1959-1965.
287. Ridker, P. M., Rifai, N., Rose, L., *et al*. (2002) Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *New England Journal of Medicine* **347,** 1557-1565.
288. Ridker, P. M. & Silvertown, J. D. (2008) Inflammation, C-reactive protein, and atherothrombosis. *Journal of Periodontology* **79,** 1544-1551.
289. Rifai, N., Tracy, R. P. & Ridker, P. M. (1999) Clinical efficacy of an automated high-sensitivity C-reactive protein assay. *Clinical Chemistry* **45,** 2136-2141.
290. Robey, F. A., Jones, K. D., Tanaka, T., *et al*. (1984) Binding of C-reactive protein to chromatin and nucleosome core particles. A possible physiological role of C-reactive protein. *Journal of Biological Chemistry* **259,** 7311-7316.
291. Robey, F. A. & Liu, T. Y. (1981) Limulin: A C-reactive protein from *Limulus polyphemus*. *Journal of Biological Chemistry* **256,** 969-975.

292. Rogus, J., Beck, J. D., Offenbacher, S., *et al*. (2008) IL1B gene promoter haplotype pairs predict clinical levels of interleukin-1beta and C-reactive protein. *Human Genetics* **123,** 387-398.
293. Sabbah, W., Watt, R. G., Sheiham, A., *et al*. (2008) Effects of allostatic load on the social gradient in ischaemic heart disease and periodontal disease: evidence from the Third National Health and Nutrition Examination Survey. *Journal of Epidemiology and Community Health* **62,** 415-420.
294. Saeland, E., Van Royen, A., Hendriksen, K., *et al*. (2001) Human C-reactive protein does not bind to FcgammaRIIa on phagocytic cells. *Journal of Clinical Investigation* **107,** 641-643.
295. Sahingur, S. E., Sharma, A., Genco, R. J., *et al*. (2003) Association of increased levels of fibrinogen and the -455G/A fibrinogen gene polymorphism with chronic periodontitis. *Journal of Periodontology* **74,** 329-337.
296. Saito, T., Murakami, M., Shimazaki, Y., *et al*. (2003) Association between alveolar bone loss and elevated serum C-reactive protein in Japanese men. *Journal of Periodontology* **74,** 1741-1746.
297. Salonen, E. M., Vartio, T., Hedman, K., *et al*. (1984) Binding of fibronectin by the acute phase reactant C-reactive protein. *Journal of Biological Chemistry* **259,** 1496-1501.
298. Salzberg, T. N., Overstreet, B. T., Rogers, J. D., *et al*. (2006) C-reactive protein levels in patients with aggressive periodontitis. *Journal of Periodontology* **77,** 933-939.
299. Samberg, N. L., Bray, R. A., Gewurz, H., *et al*. (1988) Preferential expression of neo-CRP epitopes on the surface of human peripheral blood lymphocytes. *Cellular Immunology* **116,** 86-98.
300. Schillinger, T., Kluger, W., Exner, M., *et al*. (2006) Dental and periodontal status and risk for progression of carotid atherosclerosis: the inflammation and carotid artery risk for atherosclerosis study dental substudy. *Stroke* **37,** 2271-2276.
301. Schutte, K. & Malfertheiner, P. (2008) Markers for predicting severity and progression of acute pancreatitis. *Best Practice and Research in Clinical Gastroenterology* **22,** 75-90.
302. Scirica, B. M., Morrow, D. A., Verma, S., *et al*. (2006) Is C-reactive protein an innocent bystander or proatherogenic culprit? The verdict is still out. *Circulation* **113,** 2128-2134.

303. Seinost, G., Wimmer, G., Skerget, M., *et al*. (2005) Periodontal treatment improves endothelial dysfunction in patients with severe periodontitis. *American Heart Journal* **149,** 1050-1054.

304. Shen, J. & Ordovas, J. M. (2009) Impact of genetic and environmental factors on hsCRP Concentrations and response to therapeutic agents. *Clinical Chemistry* **55,** 256-264.

305. Shields, M. J. (1993) A hypothesis resolving the apparently disparate activities of native and altered forms of human C-reactive protein. *Immunologic Research* **12,** 37-47.

306. Shklair, I. L., Loving, R. H., Leberman, O. F., *et al*. (1968) C-reactive protein and periodontal disease. *Journal of Periodontology* **39,** 93-95.

307. Shrive, A. K., Cheetham, G. M. T., Holden, D., *et al*. (1996) Three dimensional structure of human C-reactive protein. *Nature Structural Biology* **3,** 346-354.

308. Sibraa, P. D., Reinhardt, R. A., Dyer, J. K., *et al*. (1991) Acute-phase protein detection and quantification in gingival crevicular fluid by direct and indirect immunodot. *Journal of Clinical Periodontology* **18,** 101-106.

309. Siegel, J., Osmand, A. P., Wilson, M. F., *et al*. (1975) Interactions of C reactive protein with the complement system. II. C reactive protein mediated consumption of complement by poly L lysine polymers and other polycations. *Journal of Experimental Medicine* **142,** 709-721.

310. Singh, U., Devaraj, S., Dasu, M. R., *et al*. (2006) C-reactive protein decreases interleukin-10 secretion in activated human monocyte-derived macrophages via inhibition of cyclic AMP production. *Arteriosclerosis, Thrombosis, and Vascular Biology* **26,** 2469-2475.

311. Singh, U., Devaraj, S., Vasquez-Vivar, J., *et al*. (2007) C-reactive protein decreases endothelial nitric oxide synthase activity via uncoupling. *Journal of Molecular and Cellular Cardiology* **43,** 780-791.

312. Slade, G. D., Ghezzi, E. M., Heiss, G., *et al*. (2003) Relationship between periodontal disease and C-reactive protein among adults in the atherosclerosis risk in communities study. *Archives of Internal Medicine* **163,** 1172-1179.

313. Slade, G. D., Offenbacher, S., Beck, J. D., *et al*. (2000) Acute-phase inflammatory response to periodontal disease in the US population. *Journal of Dental Research* **79,** 49-57.

314. Smith, J. W., 2nd, Urba, W. J., Curti, B. D., *et al*. (1992) The toxic and hematologic effects of interleukin-1 alpha administered in a phase I trial to patients with advanced malignancies. *Journal of Clinical Oncology* **10,** 1141-1152.
315. Spector, T. D., Hart, D. J., Nandra, D., *et al*. (1997) Low-level increases in serum C-reactive protein are present in early osteoarthritis of the knee and predict progressive disease. *Arthritis and Rheumatism* **40,** 723-727.
316. Steel, D. & Whitehead, A. S. (1994) The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. *Immunology Today* **15,** 81-88.
317. Stein, M. P., Edberg, J. C., Kimberly, R. P., *et al*. (2000) C-reactive protein binding to FcgammaRIIa on human monocytes and neutrophils is allele-specific. *Journal of Clinical Investigation* **105,** 369-376.
318. Suk Danik, J., Chasman, D. I., Cannon, C. P., *et al*. (2006) Influence of genetic variation in the C-reactive protein gene on the inflammatory response during and after acute coronary Ischemia. *Annals of Human Genetics* **70,** 705-716.
319. Sun, H., Koike, T., Ichikawa, T., *et al*. (2005) C-reactive protein in atherosclerotic lesions: Its origin and pathophysiological significance. *American Journal of Pathology* **167,** 1139-1148.
320. Swanson, S. J., McPeek, M. M. & Mortensen, R. F. (1989) Characteristics of the binding of human C-reactive protein (CRP) to laminin. *Journal of Cellular Biochemistry* **40,** 121-132.
321. Swoboda, J. R., Kiyak, H. A., Darveau, R., *et al*. (2008) Correlates of periodontal decline and biologic markers in older adults. *Journal of Periodontology* **79,** 1920-1926.
322. Tabeta, K., Yamazaki, K., Hotokezaka, H., *et al*. (2000) Elevated humoral immune response to heat shock protein 60 (hsp60) family in periodontitis patients. *Clinical and Experimental Immunology* **120,** 285-293.
323. Takami, Y., Nakagaki, H., Morita, I., *et al*. (2003) Blood test values and community periodontal index scores in medical checkup recipients. *Journal of Periodontology* **74,** 1778-1784.
324. Takeda, M., Ojima, M., Yoshioka, H., *et al*. (2006) Relationship of serum advanced glycation end products with deterioration of periodontitis in type 2 diabetes patients. *Journal of Periodontology* **77,** 15-20.
325. Tanke, H. J. (2007) Genomics and proteomics: The potential role of oral diagnostics. *Annals of the New York Academy of Sciences* **1098,** 330-334.

326. Taylor, B. A., Tofler, G. H., Carey, H. M., *et al.* (2006) Full-mouth tooth extraction lowers systemic inflammatory and thrombotic markers of cardiovascular risk. *Journal of Dental Research* **85,** 74-78.
327. Taylor, K. E., Giddings, J. C. & Van Den Berg, C. W. (2005) C-reactive protein-induced in vitro endothelial cell activation is an artefact caused by azide and lipopolysaccharide. *Arteriosclerosis, Thrombosis, and Vascular Biology* **25,** 1225-1230.
328. Thompson, D., Pepys, M. B. & Wood, S. P. (1999) The physiological structure of human C-reactive protein and its complex with phosphocholine. *Structure* **7,** 169-177.
329. Thorand, B., Lowel, H., Schneider, A., *et al.* (2003) C-reactive protein as a predictor for incident diabetes mellitus among middle-aged men: Results from the MONICA Augsburg Cohort Study, 1984-1998. *Archives of Internal Medicine* **163,** 93-99.
330. Tillet, W. S. & Francis, T. (1930) Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. *Journal of Experimental Medicine* **52,** 561-571.
331. Tonetti, M. S., D'Aiuto, F., Nibali, L., *et al.* (2007) Treatment of periodontitis and endothelial function. *New England Journal of Medicine* **356,** 911-920.
332. Torzewski, J. (2005) C-reactive protein and atherogenesis: New insights from established animal models. *American Journal of Pathology* **167,** 923-925.
333. Torzewski, J., Torzewski, M., Bowyer, D. E., *et al.* (1998) C-reactive protein frequently colocalizes with the terminal complement complex in the intima of early atherosclerotic lesions of human coronary arteries. *Arteriosclerosis, Thrombosis, and Vascular Biology* **18,** 1386-1392.
334. Torzewski, M., Rist, C., Mortensen, R. F., *et al.* (2000) C-reactive protein in the arterial intima: Role of C-reactive protein receptor-dependent monocyte recruitment in atherogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology* **20,** 2094-2099.
335. Tracy, R. P., Lemaitre, R. N., Psaty, B. M., *et al.* (1997a) Relationship of C-reactive protein to risk of cardiovascular disease in the elderly: Results from the Cardiovascular Health Study and the Rural Health Promotion Project. *Arteriosclerosis, Thrombosis, and Vascular Biology* **17,** 1121-1127.
336. Tracy, R. P., Psaty, B. M., Macy, E., *et al.* (1997b) Lifetime smoking exposure affects the association of C-reactive protein with cardiovascular disease risk factors and subclinical disease in healthy elderly subjects. *Arteriosclerosis, Thrombosis, and Vascular Biology* **17,** 2167-2176.

337. Trion, A., De Maat, M. P. M., Jukema, J. W., *et al.* (2005) No effect of C-reactive protein on early atherosclerosis development in apolipoprotein E*3-Leiden/human C-reactive protein transgenic mice. *Arteriosclerosis, Thrombosis, and Vascular Biology* **25,** 1635-1640.

338. Trombelli, L., Farina, R., Minenna, L., *et al.* (2008) Experimental gingivitis: Reproducibility of plaque accumulation and gingival inflammation parameters in selected populations during a repeat trial. *Journal of Clinical Periodontology* **35,** 955-960.

339. Trombone, A. P. F., Ferreira Jr, S. B., Raimundo, F. M., *et al.* (2009) Experimental periodontitis in mice selected for maximal or minimal inflammatory reactions: Increased inflammatory immune responsiveness drives increased alveolar bone loss without enhancing the control of periodontal infection. *Journal of Periodontal Research* **44,** 443-451.

340. Tsai, C. C., McArthur, W. P., Baehni, P. C., *et al.* (1981) Serum neutralizing activity against Actinobacillus actinomycetemcomitans leukotoxin in juvenile periodontitis. *Journal of Clinical Periodontology* **8,** 338-348.

341. Turu, M. M., Slevin, M., Matou, S., *et al.* (2008) C-reactive protein exerts angiogenic effects on vascular endothelial cells and modulates associated signalling pathways and gene expression. *BMC Cell Biology* **9**.

342. Tuter, G., Kurtis, B. & Serdar, M. (2007a) Evaluation of gingival crevicular fluid and serum levels of high-sensitivity C-reactive protein in chronic periodontitis patients with or without coronary artery disease. *Journal of Periodontology* **78,** 2319-2324.

343. Tuter, G., Kurtis, B., Serdar, M., *et al.* (2007b) Effects of scaling and root planing and sub-antimicrobial dose doxycycline on oral and systemic biomarkers of disease in patients with both chronic periodontitis and coronary artery disease. *Journal of clinical periodontology* **34,** 673-681.

344. U.S. Food and Drug Administration (2005) Guidance for industry and FDA staff. review criteria for assessment of C-reactive protein (CRP), HIGH-SENSITIVITY C-reactive protein (hsCRP) and cardiac C-reactive protein (cCRP) assays. (eds.) U.S. Department of Health and Human Services, Center for Devices and Radiological Health, Office of In Vitro Diagnostic Device Evaluation and Safety, *et al.* Rockville, MD: U.S. Food and Drug Administration,.

345. Vainas, T., Lubbers, T., Stassen, F. R., *et al.* (2003) Serum C-reactive protein level is associated with abdominal aortic aneurysm size and may be produced by aneurysmal tissue. *Circulation* **107,** 1103-1105.

346. van Leeuwen, M. A., va Rijswijk, M. H., Sluiter, W. J., *et al*. (1997) Individual relationship between progression of radiological damage and the acute phase response in early rheumatoid arthritis. Towards development of a decision support system. *Journal of Rheumatology* **24,** 20-27.

347. Van Leeuwen, M. A., Van der Heijde, D. M. F. M., Van Rijswijk, M. H., *et al*. (1994) Interrelationship of outcome measures and process variables in early rheumatoid arthritis. A comparison of radiologic damage, physical disability, joint counts, and acute phase reactants. *Journal of Rheumatology* **21,** 425-429.

348. Vermeire, S., Van Assche, G. & Rutgeerts, P. (2005) The role of C-reactive protein as an inflammatory marker in gastrointestinal diseases. *Nature Clinical Practice. Gastroenterology & Hepatology* **2,** 580-586.

349. Verzilli, C., Shah, T., Casas, J. P., *et al*. (2008) Bayesian Meta-Analysis of Genetic Association Studies with Different Sets of Markers. *American Journal of Human Genetics* **82,** 859-872.

350. Visser, M., Bouter, L. M., McQuillan, G. M., *et al*. (1999) Elevated C-reactive protein levels in overweight and obese adults. *Journal of the American Medical Association* **282,** 2131-2135.

351. Volanakis, J. E. & Kaplan, M. H. (1971) Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide. *Proceedings of the Society for Experimental Biology and Medicine* **136,** 612-614.

352. Volanakis, J. E. & Wirtz, K. W. A. (1979) Interaction of C-reactive protein with artificial phosphatidylcholine bilayers. *Nature* **281,** 155-157.

353. Wakai, K., Kawamura, T., Umemura, O., *et al*. (1999) Associations of medical status and physical fitness with periodontal disease. *Journal of Clinical Periodontology* **26,** 664-672.

354. Wang, H. W. & Sui, S. F. (2001) Dissociation and subunit rearrangement of membrane-bound human C-reactive proteins. *Biochemical and Biophysical Research Communications* **288,** 75-79.

355. Wang, H. W., Wu, Y., Chen, Y., *et al*. (2002) Polymorphism of structural forms of C-reactive protein. *International Journal of Molecular Medicine* **9,** 665-671.

356. Wang, T. J., Gona, P., Larson, M. G., *et al*. (2006) Multiple biomarkers for the prediction of first major cardiovascular events and death. *New England Journal of Medicine* **355,** 2631-2639.

357. Weinhold, B. & Ruther, U. (1997) Interleukin-6-dependent and -independent regulation of the human C-reactive protein gene. *Biochemical Journal* **327 (Pt 2),** 425-429.
358. Whitehead, A. S., Bruns, G. A. P. & Markham, A. F. (1983) Isolation of human C-reactive protein complementary DNA and localization of the gene to chromosome 1. *Science* **221,** 69-71.
359. Wigmore, S. J., Fearon, K. C. H., Maingay, J. P., *et al*. (1997) Interleukin-8 can mediate acute-phase protein production by isolated human hepatocytes. *American Journal of Physiology - Endocrinology and Metabolism* **273**.
360. Williams, R. C. & Offenbacher, S. (2000) Periodontal medicine: the emergence of a new branch of periodontology. *Periodontology 2000* **23,** 9-12.
361. Wu, T., Trevisan, M., Genco, R. J., *et al*. (2000) Examination of the relation between peridontal health status and cardiovascular risk factors: serum total and high density lipoprotein cholesterol, C-reactive protein and plasma fibrinogen. *American Journal of Epidemiology* **151,** 273-282.
362. Xing, Z., Gauldie, J., Cox, G., *et al*. (1998) IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *Journal of Clinical Investigation* **101,** 311-320.
363. Yamazaki, K., Honda, T., Domon, H., *et al*. (2007) Relationship of periodontal infection to serum antibody levels to periodontopathic bacteria and inflammatory markers in periodontitis patients with coronary heart disease. *Clinical and Experimental Immunology* **149,** 445-452.
364. Yasojima, K., Schwab, C., McGeer, E. G., *et al*. (2000) Human neurons generate C-reactive protein and amyloid P: upregulation in Alzheimer's disease. *Brain Research* **887,** 80-89.
365. Yasojima, K., Schwab, C., McGeer, E. G., *et al*. (2001) Generation of C-reactive protein and complement components in atherosclerotic plaques. *American Journal of Pathology* **158,** 1039-1051.
366. Ying, S. C., Gewurz, H., Kinoshita, C. M., *et al*. (1989) Identification and partial characterization of multiple native and neoantigenic epitopes of human C-reactive protein by using monoclonal antibodies. *Journal of Immunology* **143,** 221-228.
367. Yoshii, S., Tsuboi, S., Morita, I., *et al*. (2009) Temporal association of elevated c-reactive protein and periodontal disease in men. *Journal of Periodontology* **80,** 734-739.

368. Zaremba, M., Gorska, R., Suwalski, P., *et al*. (2007) Evaluation of the incidence of periodontitis-associated bacteria in the atherosclerotic plaque of coronary blood vessels. *Journal of Periodontology* **78,** 322-327.

369. Zhang, D., Jiang, S. L., Rzewnicki, D., *et al*. (1995) The effect of interleukin-1 on C-reactive protein expression in Hep3B cells is exerted at the transcriptional level. *Biochemical Journal* **310,** 143-148.

370. Zhang, Y. X., Cliff, W. J., Schoefl, G. I., *et al*. (1999) Coronary C-reactive protein distribution: Its relation to development of atherosclerosis. *Atherosclerosis* **145,** 375-379.

371. Zouki, C., Haas, B., Chan, J. S. D., *et al*. (2001) Loss of pentameric symmetry of C-reactive protein is associated with promotion of neutrophil-endothelial cell adhesion. *Journal of Immunology* **167,** 5355-5361.

Chapter Two. C-reactive Protein in Gingival Crevicular Fluid may be Indicative of Systemic Inflammation

Emma Megson[1,2], Tracy Fitzsimmons[1,2], Kencana Dharmapatni[3], P. Mark Bartold[1,2]

[1]School of Dentistry, Faculty of Health Sciences, University of Adelaide,
[2]Colgate Australian Clinical Dental Research Centre, University of Adelaide,
[3]Discipline of Pathology, School of Medical Sciences, University of Adelaide

Conflict of Interest and Source of Funding Statement

The authors declare that there are no conflicts of interest in this study.

This study was supported by a grant from the National Health and Medical Research Council of Australia (Project Grant # 565341).

2.1 Introduction

Periodontal disease has been associated with a number of other inflammatory diseases, including diabetes mellitus, rheumatoid arthritis and cardiovascular disease (Lagervall *et al* 2003). The status of many of these systemic inflammatory diseases can be measured using serum markers of inflammation. One such molecule that has been widely investigated is the acute-phase protein, C-reactive protein (CRP). Serum CRP concentrations are normally in the order of ng/ml, but are capable of rising rapidly 100-1000-fold in response to inflammation or tissue necrosis (Claus *et al* 1976). As CRP concentrations closely follow the course of inflammation (Gabay and Kushner 1999), they are used as a measure for many disease processes (Ebersole and Cappelli 2000). Even mildly elevated serum levels of CRP are regarded as evidence of an active tissue-damaging process (Pepys 1981). Aside from being used as a marker of inflammation, CRP is now believed to itself have potent pro-inflammatory properties (Ho *et al* 2008, Turu *et al* 2008, Dasu *et al* 2007).

Not surprisingly, CRP has also been detected in the serum of periodontitis patients, and levels are significantly higher than those of non-periodontitis subjects (Paraskevas *et al* 2008, Cairo *et al* 2008, Higashi *et al* 2008). Further, there is mounting evidence that effective periodontal therapy can lower serum CRP (Paraskevas *et al* 2008; Offenbacher *et al* 2009; Marcaccini *et al* 2009). CRP has also been detected in the saliva and gingival crevicular fluid (GCF) of periodontitis patients (Sibraa *et al* 1991, Pederson *et al* 1995, Hirasaki *et al* 2005, Christodoulides *et al* 2005, Tuter *et al* 2007, Fitzsimmons *et al* 2009, Sanders *et al* 2009, Fitzsimmons *et al* 2010). GCF is a transudate of serum and, as such, contains both serum components and locally produced molecules.

In general CRP is primarily produced by the liver (Steel and Whitehead 1994, Miller *et al* 1951), but several papers have reported extra-hepatic production of CRP, such as lymphocytes (Kuta and Baum 1986), neurons of patients with Alzheimer's disease (Yasojima *et al* 2000), nasal polyp epithelium (Gould and Weiser 2001), smooth muscle cells and macrophages of atherosclerotic plaques (Yasojima *et al* 2001), aortic aneurysmal tissue (Vainas *et al* 2003), renal epithelium of acute rejection renal allografts (Jabs *et al* 2003a), adipose tissue from patients with coronary artery disease (Ouchi *et al* 2003), smooth muscle cells of diseased coronary artery venous bypass grafts (Jabs *et al* 2003b), and renal cell carcinoma epithelium (Jabs *et al* 2005). As such, the presence of CRP in GCF of periodontitis patients may be due, in part, to local production of CRP within the

periodontal tissues. Local production of CRP would mean that CRP levels detected in the GCF could not be used to make inferences about systemic inflammation.

To date there have been no published investigations of local production of CRP in the periodontal tissues. Therefore, the aim of this study was to determine if CRP detected in the GCF is the result of local production of CRP within the gingival tissues.

2.2 Methods

2.2.1 Study Population

2.2.1.1 Patients

The University of Adelaide Human Ethics Research Committee and South Australian Dental Service approved the study, in accordance with the guidelines of the National Health and Medical Research Council of Australia. Patients attending the Adelaide Dental Hospital Periodontology Department for periodontal surgery were invited to participate in the study following written and informed consent. Periodontitis affected tissue was collected during periodontal flap surgery carried out for the treatment of persistent periodontal pocketing (PD ≥5 mm) following initial non-surgical therapy carried out at least 3 months previously. Tissue samples from non-periodontitis sites included gingival hyperplasia without periodontal attachment loss, gingivae resected during crown lengthening surgery, or from soft tissue biopsy prior to tooth extraction. A thorough medical history and smoking history was taken for each patient, as well as questioning whether anti-inflammatory medication, antibiotics or steroids had been taken within the last six months. Patients were not excluded on the basis of their medical history or smoking status. Disease classification was in accordance with the American Academy of Periodontology classification (Armitage 1999), and periodontitis sites exhibited radiographic alveolar bone loss, deepened probing depths and attachment loss to a degree consistent with the rest of the dentition.

2.2.2 Sample Collection, Processing and Analysis

2.2.2.1 Gingival Crevicular Fluid (GCF) Collection and Analysis

GCF samples were collected from the deepest periodontal pocket in the area of periodontal surgery prior to any clinical measurements (Offenbacher *et al* 1984). All clinically detectable supragingival plaque was removed without touching the gingiva, to minimize contamination of the paper strips by plaque. GCF samples were taken after air-

drying of the site, using isolation with cotton rolls and a saliva ejector isolation if necessary, to prevent salivary contamination. One paper strip was used for each collection site. A Periopaper™ strip (OraFlow, Smithtown, NY, USA) was introduced into the gingival crevice 1 mm, with care being taken not to traumatise the tissues. The strip was left in place for 30 seconds and then transferred for volume determination to a chairside Periotron 8000 unit (OraFlow, Smithtown, NY, USA). The unit was calibrated regularly and standard curves generated to determine the volume in each strip. The Periopaper™ strip containing the sampled GCF was wrapped in foil and stored at -20°C until further processing.

GCF was subsequently eluted from the paper strips by placing the strips into the wells of a sterile 96-well plate (Flow Laboratories, McLean, VA, USA), and adding 230µl sterile phosphate buffered saline (PBS, pH 7.2) into each well. The plate was then sealed and placed on a shaker for 30 minutes. The amount of CRP in GCF was determined as previously described (Fitzsimmons *et al* 2009). Each sample was assayed in duplicate using commercially available ELISA kits (human C-reactive protein Instant ELISA, Bender MedSystems, Vienna, Austria) in accordance with the manufacturers' instructions. Colour development was monitored using a BioTek Powerwave microplate reader (BioTek Instruments, Winooski, VT, USA) until optimum optical density was reached, stop solution added, and the optical density read at 450 nm. Standard curves were then generated using KC4 software (BioTek Instruments) and used to determine the CRP concentration in each sample. The lower limit of detection for the CRP assay was 3 pg/ml. The concentration of CRP in GCF was determined by dividing the total amount by the volume of GCF collected, as previously described (Tang *et al* 2009).

2.2.2.2 Immunohistochemistry for C-reactive protein

Gingival tissue samples and human liver tissue (positive control) for immunohistochemical analysis were fixed in 10% buffered formalin for 24 hours prior to paraffin embedding. The first section cut (5 µm) from each block was stained with hematoxylin and eosin for standard histological assessment. For immunohistochemical detection, sections were dewaxed in 2 changes of xylene and 2 changes of alcohol. Endogenous peroxidase activity was inhibited using 0.3% hydrogen peroxide in PBS/0.1% sodium azide. Following rinsing in PBS the sections were incubated with the primary antibody (monoclonal antibody mouse anti human CRP, clone CRP-8, Sigma, St Louis, MO, USA) at concentration 9.1 µg/mL (1 in 1000 dilution) in PBS with 1%

(weight/volume) bovine serum albumin (BSA) and incubated overnight in a wet chamber. For control sections the primary antibodies were omitted or a purified mouse IgG1 isotype control was substituted for the primary antibody (BD Biosciences Pharmingen, Franklin Lakes, NJ, USA). The primary antibody was detected by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (DAKO, Botany, NSW, Australia), added for 30 minutes, followed by incubation with HRP-conjugated swine anti-goat IgG antibody (Biosource, Carslbad, CA, USA) for another 30 minutes. The HRP-conjugated antibodies were diluted in 1% PBS-BSA with 10% (volume/volume) normal human serum as blocking serum. All incubations were carried out at room temperature. HRP activity was detected using hydrogen peroxide as a substrate and aminoethylcarbazole (AEC) dye (DAKO). Slides were counterstained with Mayer's hematoxylin, and after washing with distilled water, mounted in Aquatex (Merck, Whitehouse Station, NJ, USA).

After immunohistochemical staining, sections stained with antibodies for CRP were analyzed under a microscope (Nikon Microphot FXA Photomicroscope, Nikon Instruments Inc, Melville, NY, USA).

2.2.2.3 Real-Time Polymerase Chain Reaction for CRP gene detection

Gingival tissue samples for real time-PCR analysis of CRP mRNA were placed in RNAlater solution (RNA Stabilization Solution, Applied Biosystems, Austin, TX, USA) at 4°C overnight. The following day the supernatant was removed before storing at -80°C until isolation of total RNA.

Total RNA was extracted from homogenized tissue specimens using TriReagent Solution according to the manufacturer's protocol (Ambion, Austin, TX, USA). Total RNA was then quantified in triplicate by ultra-violet spectroscopy (Nanodrop 1000, Thermo Scientific, Wilmington, DE, USA) and the quality assessed using the ratio of absorbance at 260 nm and 280 nm.

One microgram of total RNA was then converted to cDNA by reverse transcription using Superscript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The resulting cDNA was stored at -20°C and later assessed for CRP gene expression.

The single copy gene 36B4 (acidic ribosomal phosphoprotein PO) was used as a housekeeping gene. Previously published primer sequences specific for CRP (Jabs *et al* 2003a) and 36B4 mRNA (Cawthon 2002) were used to assess CRP and 36B4 gene expression, respectively. The primer sequences for CRP were 5'GAA CTT TCA GCC

GAA TAC ATC TTT T3' (forward) and 5'CCT TCC TCG ACA TGT CTG TCT3' (reverse). 36B4 primers were 5'CAG CAA GTG GGA AGG TGT AAT CC3' and 5'CCCA TTC TAT CAT CAA CGG GTA CAA3', forward and reverse, respectively, all purchased from Geneworks (Hindmarsh, SA, Australia). The levels of CRP gene expression were analyzed using a standard curve of known amounts of reverse transcribed human liver RNA (Stratagene, La Jolla, CA, USA) which served as a positive control.

The final reaction mix consisted of 1x EXPRESS SYBR Green ER qPCR Supermix Universal (Invitrogen), with final concentrations of 200 nM CRP forward and reverse primers, or 250 nM and 200 nM 36B4 forward and reverse primers respectively, and 1µL cDNA in a final volume of 20µL. Each cDNA sample was measured in triplicate. This mix was then amplified at 95°C for 20 seconds followed by 40 cycles of 95°C for 3 seconds, 62°C for 30 seconds (or 58°C for 36B4) and 72°C for 30 seconds, followed by melt curve analysis on a Corbett Research Rotor Gene 3000 real-time thermal cycler (Corbett Life Sciences, Sydney, NSW, Australia).

Amplification efficiency of both CRP and 36B4 PCR runs were assessed using the Corbett Rotor-Gene 6.1 software (Corbett Life Sciences), with efficiency between 90-110% and $R^2 \geq 0.99$.

Verification of PCR products was conducted using gene sequencing (Molecular Pathology Unit, Institute of Medical and Veterinary Sciences, Adelaide, SA, Australia).

2.2.3 Statistical Analysis

Data were entered into a computer database and corrected for implausible data. For the basis of analysis, patients with high blood pressure (including those taking anti-hypertensive medication), cardiovascular disease, high cholesterol, diabetes, arthritis, Crohn's disease or recent antibiotic, anti-inflammatory medication or steroid use were categorised as "systemically unhealthy", as these conditions may influence systemic levels of CRP. Patients without these conditions were categorised as "systemically healthy". Statistical analysis was performed using GraphPad Prism computer software (GraphPad Software, Inc., La Jolla, CA, USA). When comparing the numeric characteristics between periodontitis and non-periodontitis groups, non-parametric Mann-Whitney test statistics were calculated to test the hypothesis of no difference between the groups. When comparing non-numerical characteristics, non-parametric Fisher's exact test statistics were calculated to test the hypothesis of no difference between the groups. Two-sided tests were performed, with statistical significance set at $p \leq 0.05$.

2.3 Results

2.3.1 Subject demographics and sample collection

A total of 64 samples were taken from 59 individual patients (Table 1). Of the 64 samples, 50 were collected from 46 patients with periodontitis, and the remaining 14 were collected from 13 patients who were defined as non-periodontitis, but may have exhibited gingival hyperplasia or gingivitis without periodontal bone loss/attachment loss. The pocket depth for the periodontitis sample sites ranged from 5-10 mm with a mean of 7.2±1.3 mm (± SD) and median of 7.0 mm (6.0-8.0) (25%-75% percentiles). This was significantly deeper than that measured for the non-periodontitis samples with a mean of 3.6±1.7 mm and median of 3.0 mm (2.0-5.3) (Mann-Whitney test, $p<0.0001$). Similarly, the percentage of sites that bled upon probing (measured following GCF collection) was significantly higher for the periodontitis sites, compared to the non-periodontitis sites (82.0% vs 28.6%, Fisher's exact test, $p=0.0003$). The mean GCF volume for the periodontitis samples was 0.98±0.69 μL and the median was 0.85 μL (range 0.28-1.70), which was also significantly different from the non-periodontitis samples at mean 0.52±0.55 μL and median 0.30 μL (range 0.12-0.78) (Mann-Whitney test, $p=0.0159$).

Among the periodontitis samples, 39.1% were from systemically healthy patients without any known medical conditions and not taking any medications, while among the non-periodontitis samples, 46.2% were systemically healthy, and this difference was not significantly different (Fisher's exact test). The two most common conditions experienced amongst the sample population were current prescription of antihypertensive medication or anti-inflammatory medication (including aspirin).

2.3.2 Detection of CRP in GCF samples

The subject and sample demographics and clinical parameters for samples with detectable CRP in GCF, in comparison to the total study population are described in Table 2. Approximately 41% of the 64 GCF samples tested by ELISA had detectable CRP. Numerically, a higher percentage of non-periodontitis GCF samples had detectable CRP compared to the periodontitis GCF samples, but this difference was not statistically significant (50% vs 38%, Fisher's exact test). Bleeding on probing did not appear to have a positive effect on CRP detection in the GCF, with only 35.6% of bleeding on probing positive (BOP+) sites subsequently found to be positive for CRP, compared to 52.6% of

BOP- sites, which was not a significant difference (Fisher's exact test). In addition, there were no significant differences in the proportions of BOP+ sites with detectable CRP in the GCF between the periodontitis compared to the non-periodontitis groups (34.1% vs. 50.0%, Fisher's exact test). Also there was no significant difference in the volume of GCF for those with detectable CRP compared to the total study population (Mann-Whitney test, data not shown). Systemic health status also did not appear to have a significant influence on CRP detection in the GCF with similar proportions of detection in both systemically healthy and unhealthy groups. Overall, 10 of the 24 (41.6%) systemically healthy patients and 16 of the 35 (45.7%) patients who were not systemically healthy had detectable CRP in GCF, and this was not statistically significant (Fisher's exact test). The 10 systemically healthy patients included 2 current smokers, and 2 of the systemically unhealthy patients were also current smokers, so that overall 4 of the 11 (36%) current smokers had detectable CRP in the GCF. Only three of the patients with detectable CRP in the GCF were former smokers, and 19 were never smokers. Due to the small number of current smokers in the study statistical significance of smoking on CRP detection was not determined in the current study. However, the proportion of current smokers with detectable CRP was not significantly different from the proportion of former or never smokers with detectable CRP (36.3% vs. 45.8%, Fisher's exact test).

Table 3 describes the subject and sample demographics and clinical parameters for only the samples with detectable CRP in GCF. Of the 26 samples with CRP detectable in the GCF, 73.1% were periodontitis samples, in keeping with the higher proportion of periodontitis samples in the total sample collection. Seventy-four percent of the CRP positive periodontitis GCF samples and 28.6% of the non-periodontitis GCF samples were taken from sites that subsequently bled upon probing, this difference approached statistical significance at p=0.0687 (Fisher's exact test), and overall 61.5% of the sites with detectable CRP subsequently bled upon probing. These figures reflect the proportions of BOP+ sites in the periodontitis and non-periodontitis samples seen in the total study population. There were no significant differences in the volume of GCF between periodontitis and non-periodontitis samples (Mann Whitney test). Interestingly, a numerically higher proportion of non-periodontitis samples with detectable CRP in the GCF were also systemically healthy, compared to the proportion of periodontitis samples that were systemically healthy, but this difference was not statistically significantly (57.1% vs. 31.6%, p= 0.3692, Fisher's exact test). Three of the seven patients with both non-periodontitis tissue and CRP detectable in the GCF had other medical conditions that could affect CRP (Type II diabetes, HBP, Crohn's disease), two were current smokers, and the

remaining two had no known systemic condition that could affect CRP. The reason for detection of CRP in these otherwise medically healthy and non-periodontitis patients is unknown. There were no significant differences in the amounts of CRP between periodontitis and non-periodontitis samples, nor between systemically healthy and systemically unhealthy samples (Mann Whitney test, data not shown).

2.3.3 Immunohistochemical staining for CRP in gingival tissue

Immunohistochemical staining of gingival tissue samples was carried out to determine the distribution of CRP in the periodontal tissue (Figure 2). Positive staining for CRP was observed around blood vessels within the connective tissue and diffusely within the connective tissues, and no cell associated staining was seen. Liver tissue was used as a positive control and clearly showed cell associated CRP staining in the hepatocytes. No staining was observed in any of the negative controls included in this study.

2.3.4 Real-time CRP analysis of gingival tissue for CRP mRNA gene expression

Real-time PCR was used to assess whether cells located in gingival tissue were expressing mRNA for CRP. Of the 64 gingival tissue samples that had paired GCF samples, none had detectable levels of CRP mRNA An additional 22 gingival tissue samples without paired GCF samples were also analysed by PCR for CRP mRNA, but none of these had detectable CRP mRNA. All samples were positive for expression of the housekeeping gene 36B4 (data not shown). All human liver samples (positive control) had detectable levels of CRP.

2.4 Discussion

In this study, as in previous studies, CRP has been detected in the gingival crevicular fluid and periodontal tissues of both non-periodontitis and periodontitis sites and subjects. However, the origin of this protein in GCF has not been investigated previously. Based on the absence of CRP mRNA in the periodontal tissues, it can be deduced that the origin of CRP in GCF is not from the local periodontal tissues. Further support for the absence of local production of CRP is that within the periodontal tissues the distribution of CRP was diffuse throughout the connective tissue and was not cell-associated. Therefore the presence of CRP in the GCF and periodontal tissues appears to be of systemic origin.

The main source of CRP is acknowledged to be the liver (Hurlimann *et al* 1966), and although several other tissues have recently been shown to produce CRP (Yasojima *et al* 2000, Yasojima *et al* 2001, Jabs *et al* 2003a, Jabs *et al* 2003b, Gould and Weiser 2001), these are regarded as having minimal contribution to serum levels of CRP (Black *et al* 2004). Systemic CRP detected in GCF and periodontal tissue may be a result of systemic inflammation resulting from disease elsewhere in the body (Blake and Ridker 2001, Blake *et al* 2003, Bruns *et al* 2008, Schutte and Malfertheiner 2008, Aletaha *et al* 2007), as well as systemic inflammation induced by periodontitis (Paraskevas *et al* 2008). Indeed, in this study the majority of patients with detectable CRP had either periodontitis or systemic inflammatory disease, and it is very possible that the remaining patients had an undiagnosed systemic inflammatory disease or a recent infection, which may explain the detection of CRP, given that it is not of local origin.

In the current study, serum levels of CRP were not analysed. While the collection of serum samples would have enabled correlation between GCF and serum levels of CRP, the aim was primarily to determine if CRP was locally produced in the periodontal tissues and not to correlate CRP in the GCF with CRP in the serum. Collecting serum samples was not necessary to determine if CRP was produced locally, and GCF samples were primarily included to assess whether CRP was present or absent in the periodontal tissues. As GCF is a transudate of serum, and our results establish that CRP is not produced locally in the periodontal tissues, CRP in the GCF must be derived from serum CRP, and be indicative of systemic inflammation. Nonetheless, future studies may wish to address the correlation between GCF and serum levels of CRP. If the levels of CRP in GCF mirror those in serum, then the simple collection of GCF could be a non-invasive means of screening for CRP serum levels to determine the severity of systemic inflammation. Indeed, chairside kits for CRP measurement using oral fluids are currently under development (Christodoulides *et al* 2005, Floriano *et al* 2009) and could provide a novel means to screen patients for systemic inflammation.

We detected CRP in some, but not all, of the GCF and periodontal tissues of both non-periodontitis and periodontitis patients. This is consistent with previous studies (Sibraa *et al* 1991, Tuter *et al* 2007, Fitzsimmons *et al* 2009, Sanders *et al* 2009, Fitzsimmons *et al* 2010). Such findings are not surprising if CRP is not produced in the periodontal tissues, as the systemic inflammatory response between individuals is not uniform. Genetic susceptibility to inflammation may modify CRP levels, in response to periodontal destruction or systemic disease, such that patients with a hyperinflammatory phenotype exhibit higher levels of CRP (Beck *et al* 2000). As such, it is likely only a subset of

patients produce a systemic CRP response to periodontitis, due to their genetic predisposition (Mattila *et al* 2002). Indeed, an equivalent hypothesis has been confirmed in genetically hyperinflammatory mice, showing elevated systemic inflammatory markers as a result of experimental periodontitis, compared to hypoinflammatory mice (Trombone *et al* 2009). Such patients may be at higher risk of systemic inflammatory diseases due to their hyperinflammatory trait.

The incidence of detection of CRP in GCF in this study was consistent with that of recently published studies (Sanders *et al* 2009, Fitzsimmons *et al* 2010, Fitzsimmons *et al* 2009, Tuter *et al* 2007). In our study CRP was not detected in the GCF of all patients. An explanation for this is that the levels of CRP may have been below the sensitivity of the ELISA assay used, although this was the same as Fitzsimmons *et al* (2009), Sanders *et al* (2009) and Fitzsimmons *et al* (2010) and greater than that of Tuter *et al* (2007). Our study differed from these others in several aspects. The samples described in Fitzsimmons *et al* (2009), Sanders *et al* (2009), and Fitzsimmons *et al* (2010) were taken from a population study (Slade *et al* 2007), and no history was given of prior periodontal treatment, medical conditions or medication use, all of which are associated with systemic CRP levels (Paraskevas *et al* 2008, Pearson *et al* 2007, Kinlay 2007, Dehghan *et al* 2007, Pradhan *et al* 2001). The paper by Tuter *et al* (2007) also used untreated periodontitis patients, but excluded those with medical conditions or medications that could increase or decrease CRP levels. In our study, the periodontitis patients had either completed a hygienic phase of periodontal therapy while the non-periodontitis patients had not received any such initial therapy. The participants in this study were not excluded on the basis of medical conditions or smoking status. The prior periodontal treatment may have reduced the influence of periodontal inflammation on systemic CRP, as periodontal treatment has been shown to have a moderate effect on reducing systemic CRP levels (Paraskevas *et al* 2008). Also, it is acknowledged that many medical conditions do influence systemic CRP, and that many patients in our study had these conditions. However, the exact effect on systemic CRP levels may be difficult to determine. This is because the majority of patients in our study with a medical condition that may affect systemic CRP were also taking medication to counteract that condition and this could further modify systemic CRP. As CRP is not produced locally, the inclusion of patients with medical conditions that may affect CRP does not change the interpretation of this main result.

Since the detection of CRP in both serum and GCF of patients with periodontitis is not uniform, those individuals with detectable CRP may be more susceptible to developing other systemic inflammatory conditions, indeed perhaps due to a hyperinflammatory

phenotype (Beck *et al* 2000). Those subjects with a hyperinflammatory phenotype may be more susceptible to periodontal bone loss, as demonstrated by recent animal studies (Trombone *et al* 2009). It may therefore be desirable to be able to identify these individuals, as they may be at greater risk of periodontal disease progression. Additionally, identifying hyperinflammatory trait individuals may highlight to the clinician a need for a more aggressive management of periodontal and cardiovascular disease risk factors. In our study, a small proportion of non-periodontitis patients had no known medical conditions that could affect CRP, and the reason for detection of CRP in the GCF of these patients is unknown. These patients could have an undiagnosed inflammatory condition.

In conclusion the findings of this study indicate that CRP detected in the GCF and periodontal tissue is not of local origin. This implies that elevated serum CRP in periodontitis patients is not due to local production but is indicative of systemic inflammation, either as a result of periodontal infection or systemic disease. As GCF is a serum transudate, it may be considered as a substitute source with which to assess systemic inflammation as measured by CRP. Further studies need to correlate levels of CRP in the GCF with that of serum. GCF may therefore be suitable as a source for the non-invasive assessment of the degree of systemic inflammation in both periodontitis and non-periodontitis patients.

2.5 References

1. Aletaha, D., Funovits, J., Keystone, E. C. & Smolen, J. S. (2007) Disease activity early in the course of treatment predicts response to therapy after one year in rheumatoid arthritis patients. *Arthritis and Rheumatism* 56, 3226-3235.
2. Armitage, G. C. (1999) Development of a classification system for periodontal diseases and conditions. *Annals of Periodontology* 4, 1-6.
3. Beck, J. D., Slade, G. & Offenbacher, S. (2000) Oral disease, cardiovascular disease and systemic inflammation. *Periodontology 2000* 23, 110-120.
4. Black, S., Kushner, I. & Samols, D. (2004) C-reactive protein. *The Journal of Biological Chemistry* 279, 48487-48490.
5. Blake, G. J. & Ridker, P. M. (2001) High sensitivity C-reactive protein for predicting cardiovascular disease: an inflammatory hypothesis. *European Heart Journal* 22, 349-352.
6. Blake, G. J., Rifai, N., Buring, J. E. & Ridker, P. M. (2003) Blood pressure, C-reactive protein, and risk of future cardiovascular events. *Circulation* 108, 2993-2999.
7. Bruns, A. H. W., Oosterheert, J. J., Hak, E. & Hoepelman, A. I. M. (2008) Usefulness of consecutive C-reactive protein measurements in follow-up of severe community-acquired pneumonia. *European Respiratory Journal* 32, 726-732.
8. Cairo, F., Castellani, S., Gori, A. M., Nieri, M., Baldelli, G., Abbate, R. & Pini-Prato, G. P. (2008) Severe periodontitis in young adults is associated with sub-clinical atherosclerosis. *Journal of Clinical Periodontology* 35, 465-472.
9. Cawthon, R. M. (2002) Telomere measurement by quantitative PCR. *Nucleic Acids Research* 30, e47.
10. Christodoulides, N., Mohanty, S., Miller, C. S., Langub, M. C., Floriano, P. N., Dharshan, P., Ali, M. F., Bernard, B., Romanovicz, D., Anslyn, E., Fox, P. C. & McDevitt, J. T. (2005) Application of microchip assay system for the measurement of C-reactive protein in human saliva. *Lab on a Chip - Miniaturisation for Chemistry and Biology* 5, 261-269.
11. Claus, D. R., Osmand, A. P. & Gewurz, H. (1976) Radioimmunoassay of human C-reactive protein and levels in normal sera. *The Journal of Laboratory and Clinical Medicin*e 87, 120-128.
12. Dasu, M. R., Devaraj, S., Du Clos, T. W. & Jialal, I. (2007) The biological effects of CRP are not attributable to endotoxin contamination: Evidence from TLR4 knockdown human aortic endothelial cells. *Journal of Lipid Research* 48, 509-512.

13. Dehghan, A., Kardys, I., de Maat, M. P., Uitterlinden, A. G., Sijbrands, E. J., Bootsma, A. H., Stijnen, T., Hofman, A., Schram, M. T. & Witteman, J. C. (2007) Genetic variation, C-reactive protein levels, and incidence of diabetes. *Diabetes* 56, 872-878.
14. Ebersole, J. L. & Cappelli, D. (2000) Acute-phase reactants in infections and inflammatory diseases. *Periodontology 2000* 23, 19-49.
15. Fitzsimmons, T. R., Sanders, A. E., Bartold, P. M. & Slade, G. D. (2010) Local and systemic biomarkers in gingival crevicular fluid increase odds of periodontitis. *Journal of Clinical Periodontology* 37, 30-36.
16. Fitzsimmons, T. R., Sanders, A. E., Slade, G. D. & Bartold, P. M. (2009) Biomarkers of periodontal inflammation in the Australian adult population. *Australian Dental Journal* 54, 115-122.
17. Floriano, P. N., Christodoulides, N., Miller, C. S., Ebersole, J. L., Spertus, J., Rose, B. G., Kinane, D. F., Novak, M. J., Steinhubl, S., Acosta, S., Mohanty, S., Dharshan, P., Yeh, C. K., Redding, S., Furmaga, W. & McDevitt, J. T. (2009) Use of saliva-based nano-biochip tests for acute myocardial infarction at the point of care: A feasibility study. *Clinical Chemistry* 55, 1530-1538.
18. Gabay, C. & Kushner, I. (1999) Acute-phase proteins and other systemic responses to inflammation. *New England Journal of Medicine* 340, 448-454.
19. Gould, J. M. & Weiser, J. N. (2001) Expression of C-reactive protein in the human respiratory tract. *Infection and Immunity* 69, 1747-1754.
20. Higashi, Y., Goto, C., Jitsuiki, D., Umemura, T., Nishioka, K., Hidaka, T., Takemoto, H., Nakamura, S., Soga, J., Chayama, K., Yoshizumi, M. & Taguchi, A. (2008) Periodontal infection is associated with endothelial dysfunction in healthy subjects and hypertensive patients. *Hypertension* 51, 446-453.
21. Hirasaki, S., Yamazaki, T. & Shiba, K. (2005) Changes in salivary components by drug administration in patients with heart diseases. *Journal of Medical and Dental Sciences* 52, 183-188.
22. Ho, K. J., Owens, C. D., Longo, T., Sui, X. X., Ifantides, C. & Conte, M. S. (2008) C-reactive protein and vein graft disease: Evidence for a direct effect on smooth muscle cell phenotype via modulation of PDGF receptor-beta. *American Journal of Physiology - Heart and Circulatory Physiology* 295, H1132-H1140.
23. Hurlimann, J., Thorbecke, G. J. & Hochwald, G. M. (1966) The liver as the site of C-reactive protein formation. *Journal of Experimental Medicine* 123, 365-378.

24. Jabs, W. J., Busse, M., Kruger, S., Jocham, D., Steinhoff, J. & Doehn, C. (2005) Expression of C-reactive protein by renal cell carcinoma and unaffected surrounding renal tissue. *Kidney International* 68, 2103-2110.
25. Jabs, W. J., Logering, B. A., Gerke, P., Kreft, B., Wolber, E.-M., Kliger, M. H. F., Fricke, L. & Steinhoff, J. (2003a) The kidney as a second site of human C-reactive protein formation in vivo. *European Journal of Immunology* 33, 152-161.
26. Jabs, W. J., Theissing, E., Nitschke, M., Bechtel, M. J. F., Duchrow, M., Mohamed, S., Jahrbeck, B., Sievers, H.-H., Steinhoff, J. & Bartels, C. (2003b) Local generation of C-reactive protein in diseased coronary artery venous bypass grafts and normal vascular tissue. *Circulation* 108, 1428-1431.
27. Kinlay, S. (2007) Low-density lipoprotein-dependent and -independent effects of cholesterol-lowering therapies on C-reactive protein: a meta-analysis. *Journal of the American College of Cardiology* 49, 2003-2009.
28. Kuta, A. E. & Baum, L. L. (1986) C-reactive protein is produced by a small number of normal human peripheral blood lymphocytes. *Journal of Experimental Medicine* 164, 321-326.
29. Lagervall M, Jansson L, Bergström J. (2003) Systemic disorders in patients with periodontal disease. *Journal of Clinical Periodontology* 30, 293-299.
30. Marcaccini, A. M., Meschiari, C. A., Sorgl, C. A., Saraiva, M. C. P., De Souza, A. M., Faccioli, L. H., Tanus-Santos, J. E., Novaes, A. B. & Gerlach, R. F. (2009) Circulating intereukin - 6 and high - sensitivity C-reactive protein decrease after periodontal theapy in otherwise healthy subjects. *Journal of Periodontology* 80, 594-602.
31. Mattila, K., Vesanen, M., Valtonen, V., Nieminen, M., Palosuo, T., Rasi, V. & Asikainen, S. (2002) Effect of treating periodontitis on C-reactive protein levels: a pilot study. *BMC Infectious Diseases* 2, 30.
32. Miller, L. L., Bly, C. G., Watson, M. L. & Bale, W. F. (1951) The dominant role of the liver in plasma protein synthesis; a direct study of the isolated perfused rat liver with the aid of lysine-epsilon-C14. *The Journal of Experimental Medicine* 94, 431-453.
33. Moutsopoulos, N. M. & Madianos, P. N. (2006) Low-grade inflammation in chronic infectious diseases: Paradigm of periodontal infections. *Annals of the New York Academy of Sciences* 1088, 251-264.
34. Offenbacher, S., Beck, J. D., Moss, K., Mendoza, L., Paquette, D. W., Barrow, D. A., Couper, D. J., Stewart, D. D., Falkner, K. L., Graham, S. P., Grossi, S.,

Gunsolley, J. C., Madden, T., Maupome, G., Trevisan, M., Van Dyke, T. E. & Genco, R. J. (2009) Results From the Periodontitis and Vascular Events (PAVE) Study: A pilot multicentered, randomized, controlled trial to study effects of periodontal therapy in a secondary prevention model of cardiovascular disease. *Journal of Periodontology* 80, 190-201.

35. Offenbacher, S., Odle, B. M., Gray, R. C. & Van Dyke, T. E. (1984) Crevicular fluid prostaglandin E levels as a measure of the periodontal disease status of adult and juvenile periodontitis patients. *Journal of Periodontal Research* 19, 1-13.
36. Ouchi, N., Kihara, S., Funahashi, T., Nakamura, T., Nishida, M., Kumada, M., Okamoto, Y., Nagaretani, H., Kishida, K., Nishizawa, H., Maeda, N., Kobayashi, H., Hiraoka, H. & Matsuzawa, Y. (2003) Reciprocal association of C-reactive protein with adiponectin in blood stream and adipose tissue. *Circulation* 107, 671-674.
37. Paraskevas, S., Huizinga, J. D. & Loos, B. G. (2008) A systematic review and meta-analyses on C-reactive protein in relation to periodontitis. *Journal of Clinical Periodontology* 35, 277-290.
38. Pearson, T., Ballantyne, C., Sisk, C., Shah, A., Veltri, E. & Maccubbin, D. (2007) Comparison of effects of ezetimibe/simvastatin versus simvastatin versus atorvastatin in reducing C-reactive protein and low-density lipoprotein cholesterol levels. *The American Journal of Cardiology* 99, 1706-1713.
39. Pederson, E. D., Stanke, S. R., Whitener, S. J., Sebastiani, P. T., Lamberts, B. L. & Turner, D. W. (1995) Salivary levels of alpha 2-macroglobulin, alpha 1-antitrypsin, C-reactive protein, cathepsin G and elastase in humans with or without destructive periodontal disease. *Archives of Oral Biology* 40, 1151-1155.
40. Pepys, M. B. (1981) C-reactive protein fifty years on. *Lancet* 1, 653-657.
41. Pradhan, A. D., Manson, J. E., Rifai, N., Buring, J. E. & Ridker, P. M. (2001) C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *Journal of the American Medical Association* 286, 327-334.
42. Sanders, A. E., Slade, G. D., Fitzsimmons, T. R. & Bartold, P. M. (2009) Physical activity, inflammatory biomarkers in gingival crevicular fluid and periodontitis. *Journal of Clinical Periodontology* 36, 388-395.
43. Schutte, K. & Malfertheiner, P. (2008) Markers for predicting severity and progression of acute pancreatitis. *Best Practice and Research in Clinical Gastroenterology* 22, 75-90.

44. Sibraa, P. D., Reinhardt, R. A., Dyer, J. K. & DuBois, L. M. (1991) Acute-phase protein detection and quantification in gingival crevicular fluid by direct and indirect *immunodot. Journal of Clinical Periodontology* 18, 101-106.
45. Slade, G. D., Spencer, A. J.,Roberts-Thomson, K. F (2007) Australia's Dental Generations: the National Survey of Adult Oral Health 2004–06, Australian Institute of Health and Welfare. http://www.arcpoh.adelaide.edu.au/project/distribution/nsaoh_pdf%20files/nsaoh_report.pdf Canberra: Dental Statistics and Research Series [accessed on 20 February 2010]
46. Steel, D. & Whitehead, A. S. (1994) The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. *Immunology Today* 15, 81-88.
47. Tang, T. H., Fitzsimmons, T. R. & Bartold, P. M. (2009) Effect of smoking on concentrations of receptor activator of nuclear factor Œ∫ B ligand and osteoprotegerin in human gingival crevicular fluid. *Journal of Clinical Periodontology* 36, 713-718.
48. Trombone, A. P. F., Ferreira Jr, S. B., Raimundo, F. M., De Moura, K. C. R., Avila-Campos, M. J., Silva, J. S., Campanelli, A. P., De Franco, M. & Garlet, G. P. (2009) Experimental periodontitis in mice selected for maximal or minimal inflammatory reactions: Increased inflammatory immune responsiveness drives increased alveolar bone loss without enhancing the control of periodontal infection. *Journal of Periodontal Research* 44, 443-451.
49. Turu, M. M., Slevin, M., Matou, S., West, D., Rodriguez, C., Luque, A., Grau-Olivares, M., Badimon, L., Martinez-Gonzalez, J. & Krupinski, J. (2008) C-reactive protein exerts angiogenic effects on vascular endothelial cells and modulates associated signalling pathways and gene expression. *BMC Cell Biology* 9. Article 47
50. Tuter, G., Kurtis, B. & Serdar, M. (2007) Evaluation of gingival crevicular fluid and serum levels of high-sensitivity C-reactive protein in chronic periodontitis patients with or without coronary artery disease. *Journal of Periodontology* 78, 2319-2324.
51. Vainas, T., Lubbers, T., Stassen, F. R., Herngreen, S. B., van Dieijen-Visser, M. P., Bruggeman, C. A., Kitslaar, P. J. & Schurink, G. W. (2003) Serum C-reactive protein level is associated with abdominal aortic aneurysm size and may be produced by aneurysmal tissue. *Circulation* 107, 1103-1105.

52. Yasojima, K., Schwab, C., McGeer, E. G. & McGeer, P. L. (2000) Human neurons generate C-reactive protein and amyloid P: upregulation in Alzheimer's disease. *Brain Research* 887, 80-89.

53. Yasojima, K., Schwab, C., McGeer, E. G. & McGeer, P. L. (2001) Generation of C-reactive protein and complement components in atherosclerotic plaques. *American Journal of Pathology* 158, 1039-1051.

Tables and Figures

	Total	Periodontitis	Non-periodontitis
Number of samples	64	50	14
Number of subjects	59	46	13
Age* (years)	53.6±15.2	55.5±12.1	46.9 ±18.5
Female	36/59=61%	29/46=63%	7/13=54%
Male	23/59=39%	17/46=37%	6/13=46%
PD* (mm)	6.4±2.0	7.2±1.3†	3.6±1.7
Recession* (mm)	1.3±1.6	1.4±1.5 ^{NS}	0.9±2.0
BOP+	45/64=70%	41/50=82%‡	4/14=29%
GCF volume* (μL)	0.88±0.68	0.98±0.69†	0.52±0.55
Current smoker	11/59=19%	8/46=17% ^{NS}	3/13=23%
Systemically healthy	24/59=41%	18/46=39% ^{NS}	6/13=46%
Systemically unhealthy	35/59=59%	28/46=61%	7/13=54%
- Cardiovascular disease	3	1	2
- Diabetes	9	8	1
- Arthritis	8	7	1
- Crohn's disease	1	0	1
- Antihypertensive medication	16	13	3
- Cholesterol lowering medication	12	10	2
- Anti-inflammatory medication (including aspirin)	17	14	3
- Antibiotics	2	1	1
Samples with CRP detectable in GCF	26/64=41%	19/50=38% ^{NS}	7/14=50%

*Numbers represent mean ± SD

†*p*<0.01, Mann Whitney test, compared to non-periodontitis samples

‡*p*<0.01, Fisher's exact test, compared to non-periodontitis samples

^{NS} No statistically significant differences between periodontitis and non-periodontitis groups

Cardiovascular disease included a history of myocardial infarction, angina, or placement of a cardiac stent

Table 1. Subject and sample demographics and clinical parameters in the study population

	Total	Periodontitis	Non-periodontitis
Number of samples	26/64=41%	19/50=38% $^{\mathrm{NS}}$	7/14=50%
BOP+	16/45=36% $^{NS}_{1}$	14/41=34% $^{\mathrm{NS}}$	2/4=50%
BOP-	10/19=53%	5/9=56%	5/10=50%
GCF volume* (µL)	0.79±0.68 ^{NS}	0.90±0.70 $^{\mathrm{NS}}$	0.51±0.56
Systemically healthy	10/24=42% $^{NS}_{2}$	6/18=33% $^{\mathrm{NS}}$	4/6=67%
Systemically unhealthy	16/35=46%	13/28=46%	3/7=43%
Current smoker	4/11=36% $^{NS}_{3}$	2/8=25%	2/3=67%
Former or never smoker	22/48=46%	17/38=45%	5/10=50%

$^{\mathrm{NS}}$ No statistically significant differences between periodontitis and non-periodontitis groups

^{NS} No statistically significant differences between those with detectable CRP in GCF compared to total sample population

$^{NS}_{1}$ No statistically significant differences in proportion of those with detectable CRP in GCF in BOP+ sites compared to BOP- sites

$^{NS}_{2}$ No statistically significant differences in proportion of those with detectable CRP in GCF who were systemically healthy compared to those who were not

$^{NS}_{3}$ No statistically significant differences in proportion of those with detectable CRP in GCF who were current smokers compared to those who were former or never smokers

Table 2. Proportion of samples with detectable CRP in GCF compared to total sample population

	Total	Periodontitis	Non-periodontitis
Number of subjects	26	19	7
Number of samples	26	19/26=73%	7/26=27%
BOP+	16/26=62%	14/19=74% $^{\mathrm{NS}}$	2/7=29%
GCF volume* (µL)	0.79±0.68	0.90±0.70 $^{\mathrm{NS}}$	0.51±0.56
Systemically healthy	10/26=38%	6/19=32% $^{\mathrm{NS}}$	4/7=57%
CRP amount (pg)*	0.12±0.18	0.13±0.21 $^{\mathrm{NS}}$	0.08±0.09

*Numbers represent mean ± SD

$^{\mathrm{NS}}$ No statistically significant differences between periodontitis and non-periodontitis groups

Table 3. Subject and sample demographics and clinical parameters in the samples with detectable CRP in GCF

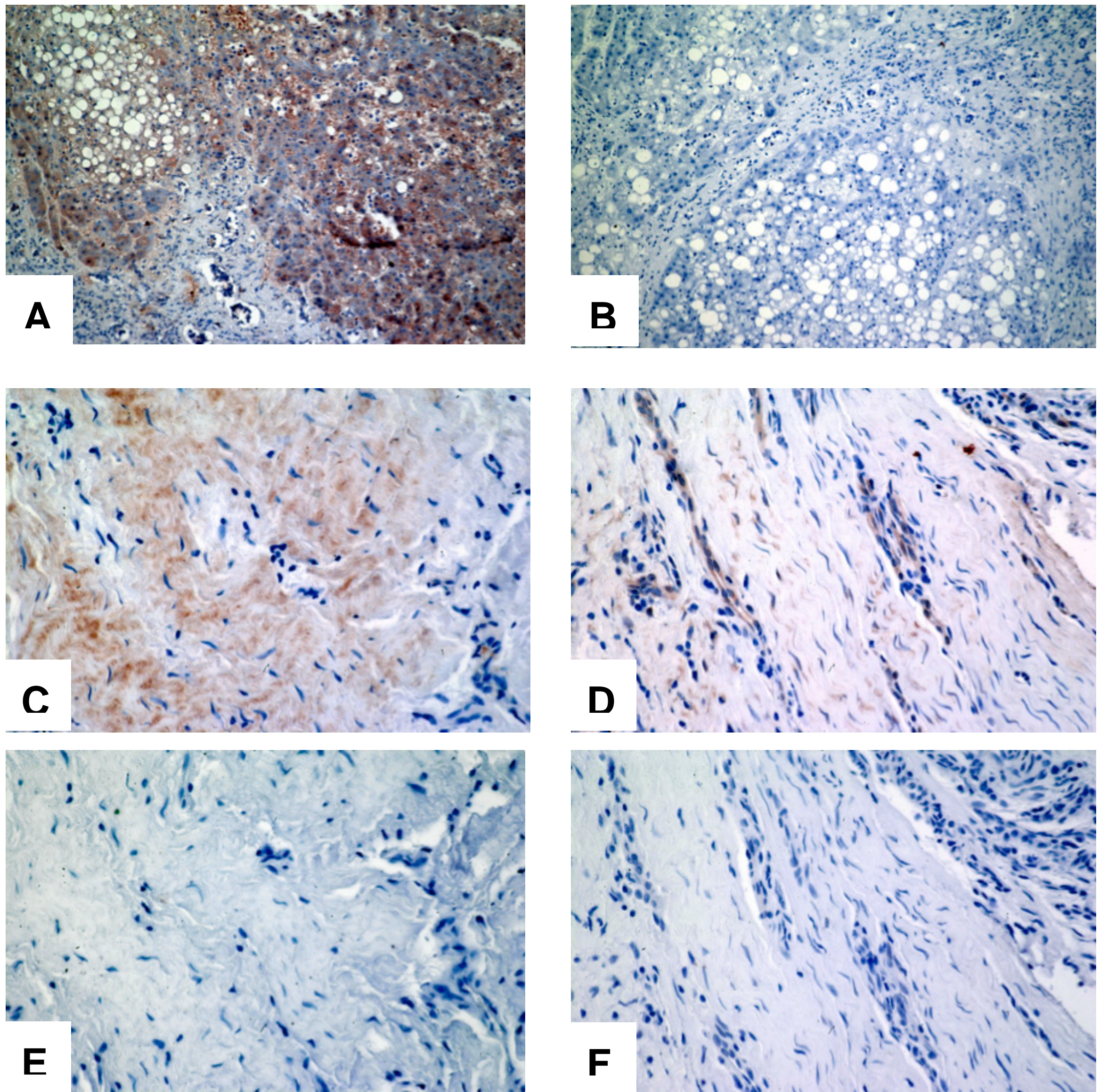

Figure 2. Immunohistochemical staining of gingival tissue for CRP

A. Positive control tissue (liver) immunostaining demonstrates positivity for CRP antibody in hepatocytes (red), and counterstaining in blue (hematoxylin), magnification x50.

B. Negative Control (liver) IgG1 isotype staining in liver tissue. Hematoxylin counterstaining, magnification x50.

C. CRP immunostaining in gingival tissue demonstrates positivity within connective tissue (red). Hematoxylin counterstaining, magnification x100.

D. CRP immunostaining in gingival tissue (red) demonstrates positivity around vessels (looks like endothelial cells). Hematoxylin counterstaining, magnification x100.

E. Negative control (omission of antibody) in gingival tissue. Hematoxylin counterstaining, magnification x100.

F. Negative control (IgG1 isotype antibody staining) in gingival tissue. Hematoxylin counterstaining, magnification x100.

Chapter Three. Addendum

3.1 Addendum

Following completion of our research and acceptance for publication in the *Journal of Clinical Periodontology*, a similar paper has been published, also in the *Journal of Clinical Periodontology* (Lu and Jin 2010).

This paper presents findings in direct contrast to our own, and the reasons for this are unknown. Several aspects of the materials and methods are different our research and this may in part explain the conflicting findings.

Immunohistochemistry was performed using the same antibody as in our study (monoclonal anti- body mouse anti human CRP, clone CRP-8 (Sigma, St. Louis, MO, USA)) but at a 1:400 dilution, whereas we used 1:1000 dilution. The staining technique used was also different. While we used a three-step immunoperoxidase method, they used a commercial kit for ABC technique (Santa Cruz Biotechnology, Santa Cruz, CA, USA). They did not describe a positive control, and used CRP antibody pre-incubated in an excess of CRP blocking peptide (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) as a negative control. We used liver as a positive control and both omission of the primary antibody and substitution of the primary antibody with an IgG1 isotype as negative controls.

RT-PCR was performed using different primers to our study. Lu and Jin (2010) used primers as described in (Wilson *et al* 2007), who also found 100% of samples exhibiting mRNA expression in coronary artery plaques, non-atherosclerotic artery and atrium tissue. This is in contrast to several other papers reporting extra-hepatic production of CRP in which only a subset of samples exhibit CRP mRNA (Jabs *et al* 2003a, Jabs *et al* 2003b, Vainas *et al* 2003, Jabs *et al* 2005).

Jabs *et al* (2003) noted that "there are some difficulties in designing CRP exon-junction spanning primer, in particular because the 3'-end of the only intron has a very high homology to the 3'-end of the first exon (Lei *et al* 1985)". They specifically confirmed that genomic DNA could not be amplified by the selected primer/probe combination.

It is not mentioned whether the samples for PCR were run in duplicate/triplicate to exclude the possibility of false positives, nor is the use of a positive control. Similarly quality control tests such as RNA quality assessment and amplification efficiency are not mentioned. While they verified their PCR product via gel electrophoresis we used gene sequencing.

Another possible explanation for the different findings is the differing ethnicity of the study populations. Our samples were collected from patients with a variety of ethnic backgrounds, but the majority were Caucasian. In contrast, Lu and Jin (2010) used Chinese subjects. Variation in CRP expression between subjects of differing ethnicities has been documented (Albert & Ridker, (2006)). However, subjects of Chinese ancestry appear to have lower levels of CRP compared to Caucasians, and as such ethnicity is unlikely to explain the conflicting findings between the two papers.

As such, clear differences in methodology exist between the two papers. It is disappointing that they are in conflict and that further research is now needed to clarify the issue.

3.2 References

1. Albert, M. A. & Ridker, P. M. (2006) C-reactive protein as a risk predictor: Do race/ethnicity and gender make a difference? *Circulation* 114 (5)**,** 67-74
2. Jabs, W. J., Busse, M., Kruger, S., *et al*. (2005) Expression of C-reactive protein by renal cell carcinoma and unaffected surrounding renal tissue. *Kidney International* 68, 2103-2110.
3. Jabs, W. J., Logering, B. A., Gerke, P., *et al*. (2003a). The kidney as a second site of human C-reactive protein formation in vivo. *European Journal of Immunology* 33, 152-161.
4. Jabs, W. J., Theissing, E., Nitschke, M., *et al*. (2003b) Local generation of C-reactive protein in diseased coronary artery venous bypass grafts and normal vascular tissue. *Circulation* 108, 1428-1431.
5. Lei, K. J., Liu, T. & Zon, G. (1985) Genomic DNA sequence for human C-reactive protein. *Journal of Biological Chemistry* 260, 13377-13383.
6. Lu, Q. & Jin, L. (2010) Human gingiva is another site of C-reactive protein formation. *Journal of Clinical Periodontology* 37, 789-873.
7. Vainas, T., Lubbers, T., Stassen, F. R., *et al*. (2003) Serum C-reactive protein level is associated with abdominal aortic aneurysm size and may be produced by aneurysmal tissue. *Circulation* 107, 1103-1105.
8. Wilson, A. M., Swan, J. D., Ding, H., *et al*. (2007) Widespread vascular production of C-reactive protein (CRP) and a relationship between serum CRP, plaque CRP and intimal hypertrophy. *Atherosclerosis* 191, 175-181.