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An investigation on the effects of non surgical periodontal treatment of chronic periodontitis on the levels of C reactive protein, IL-6 and IL-10 as measured in blood, saliva and gingival crevicular fluid.

A thesis submitted to the University of Dublin in partial fulfillment of the degree of
Doctorate in Dental Surgery D.Ch.Dent. (Periodontology)

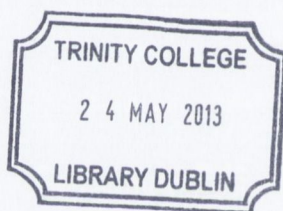
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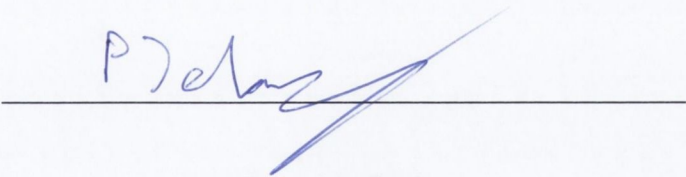


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September 2012

Summary

Cardiovascular disease is one of the main causes of morbidity in the western world. As well as common risk factors for developing this disease, several other potential contributors have been suggested. One of these is periodontal disease, which has been shown to be significantly associated with cardiovascular disease in epidemiological studies.

In this study 20 patients with moderate or severe chronic periodontitis were recruited and treated with non-surgical therapy performed in a sequential side by side basis. On each of the two treatment visits and at the re-evaluation visit blood CRP was assessed using the Quikread machine and saliva and GCF samples were taken. These samples were analysed for CRP, IL-6 and IL-10 using commercial ELISA kits.

The results indicate that the therapy caused a short sharp increase in both local and systemic inflammation, as assessed from changes in the inflammatory markers. At the completion of the study, systemic CRP was decreased to below baseline but the change was not significant. Local CRP and IL-6 however showed an initial marked increase just after therapy followed by a sharp decrease at 3 months. The local markers at re-evaluation were significantly less than baseline values suggesting a positive effect of therapy on the inflammatory status of the periodontium. A relationship was observed between systemic and local CRP values. This was most pronounced on day 2, just after initial therapy, which indicated that these values were strongly dependent on each other. The relationship between CRP in blood and saliva showed the closest correlation.

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This study also indicates that not only can periodontal therapy reduce systemic inflammation, but that it can have a pronounced effect on local inflammatory markers. Many markers in question were strongly affected by the treatment and this was reflected by changes in their concentrations at the study time points. Strong relationships were observed between changes in systemic and local CRP values. It would seem that alterations in local inflammation can have a causal effect systemically and perhaps, vice versa.

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1. Introduction and Literature Review

1.1 Introduction

Periodontitis is a chronic inflammatory disease caused by gram negative bacteria and it is the leading cause of tooth loss worldwide today. It is in fact, one of the most common infectious diseases in the world. Plaque is a soft amorphous layer of bacteria that quickly builds up on the surface teeth soon after brushing. It displays all the characteristics of a biofilm. A period of inadequate oral hygiene or general poor oral hygiene habits can lead to plaque deposits building up on teeth. This can lead to gingivitis and in some cases can progress to periodontitis, affecting the surrounding structures of the teeth, such as the periodontal ligament and bone. This happens when the plaque biofilm forms beneath the gingival margin and causes an inflammatory reaction locally, by means of direct bacterial effects and the host mediated response. The destruction of the periodontal tissue can lead to reduced tissue support for the teeth and this can lead to them becoming loose over time. It is this outcome from which the phrase “long in the tooth” is derived. The ultimate result of periodontal disease is tooth loss.

Cardiovascular disease represents the main cause of death worldwide, particularly in western society. Atherosclerosis of the coronary and cerebral arteries is caused mainly by lipid accumulation to form plaques which build up within a vessel and eventually occlude the artery. Furthermore, these plaques may rupture to cause occlusion at a distant site. These diseases are known to be modulated by inflammatory processes, which form an integral part of the pathophysiology of atherosclerosis. Initial damage to the endothelial lining of the vessel allow for the attachment of leukocytes, such as monocytes and

macrophages, via adhesion molecules. The atheroma now accumulates more lipids and grows. Chemokines released promote the recruitment of other immune cells.

Smoking, age, gender, diabetes, high blood pressure and high blood lipids are traditional risk factors for cardiovascular disease (CVD). Over the last number of years there has been interest in the possible role of chronic infections in the atherosclerosis pathway. It has been noted that biomarkers of inflammation are raised in patients with known CVD and these can be increased in the years leading up to a cardiovascular (CV) event. One such biomarker is C Reactive Protein (CRP). This protein is thought to be highly predictive of CVD. Much research has been ongoing as to its role in CVD pathogenesis and it has been shown to be associated with an increased risk of myocardial infarction (MI).

Over the last couple of decades there has been increasing evidence of an association between periodontal disease and cardiovascular disease. Although no causal relationship has been discovered as yet, the evidence of a positive correlation mounts. Although the mechanism underlying the association isn't known, it is thought to be due to a generalised hyper-inflammatory state induced by chronic periodontitis. This is supported by the fact that periodontal disease causes elevated serum markers of inflammation such as CRP and various cytokines such as interleukin-6 (IL-6). These are the same markers seen to be increased in CVD. Chronic infections such as periodontal disease possibly cause indirect damage by releasing inflammatory mediators in the blood which elicit an immune response and contribute to an increased systemic inflammatory state.

It is for this reason the much research has been done and is going into the link between periodontal disease and cardiovascular disease, with much of the focus on CRP and other common biomarkers of both diseases.

1.2 Periodontal Diseases

Periodontal disease is a general term used to describe a chronic condition that affects the gingiva and causes damage to the supporting structures of the teeth such as the ligament and bone (Williams, 1990). The overall prevalence of periodontitis worldwide is in the order of 50% for gingivitis and 10% - 15% for the more severe forms of periodontitis (Burt, 2005). The prevalence of severe periodontitis for those over 50 years of age is approximately 30% (Oliver et al., 1998). Periodontal disease is caused by specific bacteria within plaque, which is a biofilm. Evidence for the role of bacteria in the pathogenesis of periodontitis was first demonstrated in the 1960's with the experimental induction of gingivitis in subjects by the restriction of oral hygiene (Loe et al., 1965). Once the plaque biofilm forms and migrates subgingivally a complex array of pathways lead to tissue destruction either directly by bacteria and their products or indirectly by the host immune response. Putative periodontal pathogens include *Actinobacillus actinomycetecomitans*, *Porphyromonas gingivalis*, *Bacteriodes forsythus* and *Tannerella* species. Analysis of subgingival plaque samples have shown that certain bacterial species occur together in 'complexes' (Socransky et al., 1998). The authors accorded each group or complex a

different colour, with the lower ones representing health and the orange/red complexes being most associated with periodontitis. Plaque from patients without periodontitis tends to have a lower proportion of orange and red complex species than patients with periodontitis.

The accumulation of plaque bacteria in the gingival pocket results in the release of microbial substances such as lipopolysaccharide which enter the connective tissues. An inflammatory response is produced in the tissues, which results in local vessel vasodilatation and chemotaxis of immune cells to the area. Initial cells recruited to the area are mainly neutrophils (PMN's) which can phagocytose and kill bacteria (Preshaw et al., 2005). Later, bacterial products in the circulation cause the recruitment of B lymphocytes which are transformed to plasma cells, which produce antibodies to bacterial antigens. The PMNs involved in the immune reaction release large quantities of enzymes and inflammatory mediators such as matrix metalloproteases (MMP's). These MMP's break down collagen in the periodontal tissues. T cells become evident in the periodontal tissues and these produce cytokines such as IL2, IL-6, IL-10 and tumor necrosis factor (TNF). Other cells such as macrophages produce prostaglandins such as PGE2 and interleukins such as IL-1 and IL-6. Prostaglandins and the interleukins stimulate osteoclasts to resorb bone. IL-6 causes the fusion of monocytes to form multinucleated cells that also resorb bone.

With the bacterial challenge the periodontal tissues become increasingly populated by these immune cell lines such as B lymphocytes and macrophages. These cells can produce

cytokines that initiate and regulate the inflammatory response which results in a net loss of connective tissue and bone. In this scenario the tissue destructive phase is dominant and this slowly and insidiously results in attachment loss around the affected teeth, which over the years will manifest itself as clinical periodontal disease.

1.3 Periodontal and Cardiovascular disease interface

The literature suggests that there is an association between periodontal disease (PD) and cardiovascular disease (CVD). The first study providing evidence of an association between dental health and cardiovascular health was done by Mattila et al. (1989b). In a case control study, subjects with a recent history of acute myocardial infarction had significantly more dental problems, as described by a dental index for severity of dental disease. This association was found to be independent of other risk factors for cardiovascular disease such as age, smoking, serum lipids and diabetes after adjustment with logistic regression analysis. The study assessed dental health in terms of periodontal disease and other parameters such as dental caries and apical infections. Subsequently, numerous studies have investigated this relationship.

Mattila et al. (1995) later carried out a prospective cohort study to assess the link between oral disease as assessed by the Total Dental Index (TDI) and CVD. With a study population of 214 subjects, they found a significant association between the two after adjustment for other CVD risk factors. An increased relative risk ratio (RR) of 1.21 (CI 1.08

– 1.36) was observed for developing CVD in individuals with a high TDI score when compared with those who had a lower TDI score.

In another study with a follow-up period of 14 years, the relationship between periodontal disease and coronary heart disease (CHD) was explored (DeStefano et al., 1993). A total of 9,760 subjects were included from the National Health and Nutritional Epidemiologic Survey (NHANES I). They observed a 25% increased risk for CHD in subjects who had periodontal disease after controlling for confounders (RR 1.25, CI 1.06-1.48). Briggs et al. (2006) did a case control study to investigate whether an association could be demonstrated between CHD and chronic periodontitis. All cases had angiographically confirmed CHD in at least one coronary vessel. A periodontal examination carried out on all patients confirmed that the cases had poorer periodontal status compared to the controls, with fewer teeth and a higher percentage of deep sites. The CHD patients also had a significantly higher level of serum CRP, a known CVD risk indicator. It was also observed that 58% of patients within the CVD group who had a poor periodontal status had a high CRP level ($>2\text{mg/l}$) compared to 33% of those with good periodontal health. It was seen that poor periodontal status and smoking were strongly linked with CHD with an odds ratio for the association between poor periodontal status and CHD being 3.06 even after adjustment for confounding effects, which was seen to be significant ($p = 0.002$).

Evidence to support the link between PD and CVD continues to accumulate. A systematic review and meta analysis of 5 cohort studies, 5 case control studies and 5 cross sectional studies found that the risk of developing CHD was significantly raised in patients who had

periodontal disease when compared to controls (Bahekar et al., 2007). After adjusting for other risk factors for CVD such as smoking, diabetes, alcohol, obesity and blood pressure the five prospective cohort studies with a total of 86,092 patients found that subjects with PD had a 1.14 times higher risk of developing CHD than controls (RR 1.14, 95% CI 1.074-1.213, $p<0.001$). The case control studies involving 1,423 patients showed an even higher risk of developing CHD (OR 2.22, 95% CI 1.59-3.117, $p<0.001$) for those with periodontitis. The prevalence of CVD in the cross sectional studies were also significantly increased among patients with PD (OR 1.59, 95% CI 1.329-1.907, $p<0.001$). Also further analysis of the cohort studies revealed that patients with fewer than 10 teeth had an increased risk of developing CHD to the order of 1.24. The authors noted that 3 of the cohort studies were good quality and the criteria to define CVD were within accepted limits. They concluded that both the prevalence and incidence of CHD were significantly increased in patients with periodontal disease, which may have a role in CVD.

Further evidence of the link between periodontitis and atherosclerosis is the finding of periodontal pathogens such as *Porphyromonas gingivalis* and *Streptococcus sanguis* in arterial plaques from carotid endarterectomies (Haraszthy et al., 2000). A more recent study explored these findings by analysing biopsy samples from coronary arteries and internal mammary arteries for the presence of putative periodontal pathogens (Pucar et al., 2007). In this study a total of 15 patients who had a diagnosis of coronary artery disease and were scheduled for coronary bypass graft surgery were recruited. During surgery the atherosclerotic area of the coronary artery was removed and a specimen of

the internal mammary artery was taken also. *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia* and *Tannerella forsythensis* were detected by polymerase chain reaction analysis. Bacterial DNA for the designated pathogens were found in 60% of coronary artery samples, with 5 samples (33%) having more than one type of bacteria. Interestingly, no periodontal bacteria were detected in the internal mammary artery samples. It is known that these internal mammary arteries are rarely affected by atherosclerosis. Therefore, the fact that these pathogens, most commonly associated with periodontal disease, were found in coronary artery atherosclerotic plaque samples further supports the concept that periodontitis and its organisms may play a role in the development and progression of atherosclerosis. However, the authors noted that the lack of any atherosclerotic plaque in the internal mammary arteries may be a reason why no periodontal pathogens were detected there.

One large prospective study – the Atherosclerosis Risk in Communities study (ARIC) has looked at the relationship between the prevalence of coronary heart disease and the signs of clinical periodontal disease and IgG antibodies in up to 17 oral bacteria (Beck et al., 2005). In contrast to other studies, this study showed that clinical periodontitis was not significantly associated with CHD. However, when analyzing antibodies to the various oral bacteria amongst the study population it was seen that systemic antibody response was associated with CHD. Significant unadjusted associations were seen between high antibody levels of most organisms and heart disease. Furthermore after adjusting for confounders in a logistic regression analysis the association between antibody levels and

CHD remained for several bacteria, but appeared to differ between smokers and never smokers. In ever smokers (former or current), high antibody levels for certain oral bacteria were significantly associated with increased odds of having CHD. High antibody levels to *Treponema denticola* and were significantly associated with CHD (OR 1.7). Amongst the subjects who had never smoked high antibody levels to *Prevotella nigrescens* and *Actinobacillus actinomycetemcomitans* were significantly associated with CHD with OR of 1.7 for both. The systemic response to known oral and periodontal bacteria seemed to be associated with heart disease

In the same study it was noted that exposure to more than one oral bacteria further increased the risk of CHD. For example, ever smoker subjects with high antibody levels of both *Treponema denticola* and *Veillonella parvula* had a prevalence for coronary heart disease of 11%. This compared with a prevalence of 5.3% for individuals with low antibodies to both. Systemic exposure to more than one organism in never smokers resulted in a bigger difference in prevalence for CHD compared to those with low antibody levels for the same organisms (OR2.3). Overall these findings suggest that it is the hosts' response to various oral pathogens that may be the most accurate measurement of the systemic burden of infection and subsequently of the risk for cardiovascular disease.

The Oral Infections and Vascular Disease Epidemiology Study (INVEST) looked at the relationship between periodontal disease, tooth loss and carotid atherosclerosis (Desvarieux et al., 2003). In a study population of 711 patients with no history of stroke or

MI they found that the presence or prevalence of carotid plaques was not related to parameters of current or past periodontal disease such as pocket depth or clinical attachment level. However, it was seen that the greater the severity of the periodontal disease the greater the number of missing teeth. Amongst those with missing teeth, there was a significant association between tooth loss levels and the presence of carotid artery plaques. Among those with >10 teeth missing, the prevalence of carotid plaque was of the order of 60% ($p<0.05$). Other studies have found a direct link between the severity of periodontal disease and atherosclerotic extent (Amabile et al., 2008). It was seen that those with angiographically determined lesions with at least 50% stenosis of at least one coronary artery had more severe periodontitis compared to the healthy controls. This was determined according to the periodontal pocket depth which was significantly higher in the case patients. Amongst the cases subgroup the American Heart Association derived angiographic score was correlated to pocket depth ($r=0.68$, $p<0.001$). After adjusting for inflammatory markers the relationship retained its significance. Furthermore, in logistic regression analysis periodontal disease was identified as being a predictor of coronary artery disease amongst the whole study population with an OR of 2.38, after adjustment for confounding factors. More recently, it has been seen that in patients with periodontitis the common carotid artery intima media area values are higher than in controls (Yakob et al., 2010). A total of 93 patients with periodontitis and 41 controls were recruited and the intima media area of the common carotid artery was determined by carotid β - mode ultrasound. The patients with periodontitis had higher cIMA values than controls with periodontitis associated with increased cIMA (OR 3.82; CI 1.19-12.26).

1.4 CRP and Cardiovascular disease

In many ways, atherosclerosis is a chronic inflammatory disease. Along with traditional risk factors such as smoking, obesity, diabetes, hypertension, recent data demonstrates that chronic infection plays a role in the initiation, progression and destabilization of atherosclerotic plaques (Libby, 2000). Several markers of inflammation have been shown to be elevated, often years before a cardiovascular event. In a large prospective study of 15,000 men levels of interleukin 6 (IL-6) were shown to be significantly elevated amongst the men who subsequently experienced a myocardial infarction when compared to controls (Ridker et al., 2000). It was recently reported that levels of IL-6 were significantly raised in patients with coronary artery diseases when compared to controls (Jha et al., 2008).

However, the inflammatory marker most studied and associated with CAD is high sensitivity CRP (hsCRP). It is an acute phase protein which is raised in response to injury, infection and other sources of inflammation. CRP has been shown to be a strong predictor of future cardiovascular events. In a 10 year cohort study of nearly 28,000 women CRP was shown to be a stronger predictor of cardiovascular events and death than low density lipoprotein (LDL) (Ridker et al, 2002).

A more recent prospective case control study was performed as part of the European Prospective Investigation into Cancer (EPIC – Norfolk) population study 1993-2003 (Boekholdt et al., 2006). The study included 26,553 healthy men and women between the

ages of 45 and 79. Participants were recruited as part of the 10 country collaboration EPIC study which was designed to investigate cancer determinants. Additional data collected as part of this study was obtained and eligible to be used into investigations of other diseases. Participants were understood to have coronary artery disease if they were admitted to hospital for a CAD cause or died from a CAD event. Samples of serum were taken from all participants at the beginning of the study and analysed for CRP using an ELISA assay. Controls included all participants in the study who didn't develop any form of CAD during the study follow-up period. A total of 1,108 individuals developed CAD and these were matched to 2,164 controls. CRP levels were significantly higher in cases than controls ($p < 0.0001$). Taking into account matching for gender, age, and adjusting for traditional cardiovascular risk factors the odds ratio for developing CAD was 1.66 (CI 1.31-2.12) for those in the highest quartile verses those in the lowest quartile of CRP values. Interestingly the OR for future CAD were significantly higher for people in the highest CRP quartile for fatal CAD (OR=2.92; 95% CI 1.83-4.67) than for non fatal CAD (OR=1.25; 95% CI 0.93-1.66). In fact when only fatal CAD was considered, the predictive value of CRP was substantially higher than for other risk factors such as diabetes, smoking, LDL and BMI. It would seem that CRP values are strongly predictive of future CAD events.

This study was included in a recent systematic review and meta analysis of CRP as a risk factor for coronary heart disease (Buckley et al., 2009). This review and analysis was undertaken to help the U.S. Preventive Services Task Force (USPSTF) determine whether CRP level should be incorporated into guidelines for coronary and cardiovascular risk

assessment in primary care. Prospective cohort, case-control and nested case-control studies were included and reviewed by the authors. The quality of each study was evaluated. A total of 22 studies in 23 cohorts were included. The meta analysis revealed an increased risk ratio for coronary heart disease (RR 1.6; 95% CI 1.43-1.78) for those with high values of CRP (>3mg/l) verses those with low values (<1mg/l). Analysis found that including CRP values in risk models such as the Framingham risk score allowed for improved risk classification among participants, especially for those with intermediate risk. Overall the authors concluded that there is strong evidence that CRP is associated with CHD.

CRP has not only been shown to be a marker of systemic inflammation, linked to cardiovascular disease, but it is thought the CRP may play an active role in the inflammatory process of atherosclerosis (Zakynthinos and Nikolitsa, 2009). It is thought CRP is produced by an atherosclerotic plaque, which has been demonstrated via the existence of a CRP gradient in coronary arterial blood. This CRP gradient in the coronary blood increased up to 48 hours after stenting procedures. Circulating levels of CRP have been shown to be correlated to various well known cardiovascular risk factors such as smoking, diabetes, obesity and hypertension (Cook et al., 2000).

It has also recently been shown that reduction of circulating CRP levels can lead to a reduction of cardiovascular events (Ridker et al., 2008). In this intervention trial evaluating Rosuvastatin (JUPITER), it was shown that daily use of a statin can lower an elevated CRP level (Ridker et al., 2008). JUPITER was a randomized, double-blind, placebo-controlled,

multicenter trial conducted at 1,315 sites in 26 countries. There were 17,802 study participants. Men were included over 50 years of age and women at 60 and above. No participant had a history of CVD and was otherwise healthy. They had to have a low level of LDL and a CRP level of at least 2mg/l to be included. They were given 20mg rosuvastatin or a placebo. The primary outcome was a first cardiovascular event, defined as nonfatal myocardial infarction, nonfatal stroke, hospitalization for unstable angina, an arterial revascularization procedure, or confirmed death from cardiovascular causes. This trial ran for up to 5 years. The mean CRP level at baseline in rosuvastatin and placebo groups was 4.2 and 4.3 respectively. At the 12 month stage of the study the median CRP level for the rosuvastatin group was 2.2mg/l versus 3.5mg/l for the placebo group. At this stage, the rosuvastatin group, as compared with the placebo group, had a 50% lower median LDL cholesterol level. These effects persisted throughout the study period. At the end of the study, 142 cardiovascular events had occurred in the rosuvastatin group compared to 251 in the placebo. The rates of the primary end points were 0.77 and 1.36 per 100 person years of follow up for rosuvastatin and placebo groups respectively. There was also a reduction in individual end points of the study, such as myocardial infarction, with an end point rate 0.17 and 0.37 per 100 person years of follow up for the drug and placebo respectively. The corresponding event rates for stroke were 0.18 and 0.34; 0.41 and 0.77 for arterial revascularization or unstable angina. The authors concluded that in this study of apparently healthy men and women with elevated CRP levels but with lipid levels within the normal range, the use of statins significantly reduced the incidence of major cardiovascular events. This suggests that statins can have effects on non lipid parameters.

Also as we have seen from the above studies, elevated CRP levels are correlated to cardiovascular events and have been shown to alter traditional cardiovascular risk evaluation of patients. The lowering of CRP levels in subjects who are deemed to have elevated levels but are not eligible for statin treatment or be considered high risk for cardiovascular disease under normal risk evaluation may benefit from statin therapy. Indeed the centre for disease control and prevention and the American Heart Association previously issued a set of guidelines for CRP levels as far back as 2003. The guidelines issued suggested that a CRP level of <1mg/l be considered low, a level between 1 and 3mg/l be considered moderate and a level above 3mg/l be considered high when considering CRP levels and risk prediction for vascular disease in the future (Pearson et al., 2003).

1.4.1 CRP

CRP is an acute phase protein produced in the hepatocyte cells of the liver. It is a pentamer of 23 kDa subunits. It is normally at a low concentration in the blood but can increase several hundred fold with acute systemic inflammation, infection or injury (Black et al., 2004). While it has been thought that CRP is an inflammatory marker which reflects the inflammatory load of the body, more recently it has been suggested that it may have a pro-inflammatory role (Yeh, 2004). CRP is stimulated by the pro-inflammatory cytokine interleukin 6 (IL-6). Once produced it can bind to various natural ligands on damaged cell

membranes and it can function as an opsonin binding to various pathogenic pathogens which can facilitate their phagocytosis (Du Clos, 2000). CRP can also activate the complement system by binding C1q, the first component of the complement cascade. Unlike other activators of the complement cascade, complement activation by CRP seems to be limited to the early part of the cascade. It has been shown that C4b-binding protein (C4BP), which can inhibit the complement pathway, can bind directly to CRP (Sjöberg et al., 2006). This is thought to limit excessive complement activation locally during the acute phase response, would limit the damage to host cells and tissue by the complement system itself.

CRP levels have been investigated and shown to increase after trauma and fractures (Yoon et al., 1993, Ellitsgaard et al., 1991), after fracture reduction and fixation (Iizuka and Lindqvist, 1991), with infections (Spapen et al., 2006), after plastic surgery and neurosurgery procedures (Al-Jabi and El-Shawarby, 2010, Wright and Khan, 2010). CRP increases have been shown to increase after the event, often to reach a peak after two days and subsequently decrease. Secondary CRP increases or persistently elevated levels have been shown to be associated with postoperative infections (Wright and Khan, 2010). CRP levels have been shown to differ according to the degree and type of surgical intervention, with the most surgically traumatic procedure causing the highest post-operative CRP levels (Al-Jabi and El-Shawarby, 2010, Neumaier et al., 2006). CRP levels after such trauma can be indicative of the degree of tissue damage and the invasiveness of the procedure and reflect the level of peri-operative stress on a patient.

1.4.2 Interleukin 6

Interleukin 6 (IL-6) is a pro-inflammatory cytokine produced by a range of immune cells such as T-cells, B-cells, macrophages, endothelial cells and fibroblasts. It binds to the IL-6 receptor (IL-6R) and this complex binds to gp130, a membrane bound protein (Fonseca et al., 2009). This in turn activates various intracellular pathways which can initiate the various functions of IL-6. IL-6 induces fever, angiogenesis, leukocytosis and can stimulate the production of acute phase proteins such as CRP by hepatocytes (Castell et al., 1988) . It has also been shown that IL-6 can stimulate osteoclast formation and can lead to bone destruction (Tamura et al., 1993).

1.4.3 Interleukin 10

Interleukin 10 (IL-10) is an anti-inflammatory cytokine mainly produced by Treg cells and also other T cell subsets, such as Th1, Th2, Th17 and CD8 T cells (Seymour and Gemmell, 2001) . IL-10 inhibits the synthesis of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF (de Waal Malefyt et al., 1993). It has been shown to be present in healthy as well as inflamed periodontal tissues, with a lack of IL-10 leading to increased alveolar bone loss (Al-Rasheed et al., 2004). It inhibits the activities of Th1 cells, Th2 cells and macrophages by inhibiting the stimulatory molecules and other inflammatory cytokines that signal their activation (Couper et al., 2008). It would seem that the strength of the IL-10 response

depends on the inflammatory response which triggers it and that different and increased cell lines produce IL-10 depending on the strength and virulence of the infection

1.5 Periodontitis and inflammatory Markers

1.5.1 Systemic CRP and Periodontitis

While there is emerging evidence that periodontitis is associated with cardiovascular disease, cardiovascular events and the underlying pathogenesis, atherosclerosis, the exact causal nature has not been established. One school of thought is that inflammation produced by periodontal disease causes systemic markers of inflammation to rise and thus can contribute to the cardiovascular disease process indirectly. It is also well established from the above literature that several inflammatory markers and agents are increased in cardiovascular disease patients, sometimes for years in advance of any cardiovascular event. The markers mainly associated with CVD are CRP, as well as IL-6, fibrinogen and other pro-inflammatory mediators. The question that arose was – is CRP elevated in periodontal patients? Indeed research on this started appearing in the periodontal literature relatively recently. Several early studies positively showed increased systemic levels of CRP in periodontitis patients (Loos et al., 2000, Loos, 2005). In the first study, Loos et al did a case control study involving 107 patients with localized and generalised periodontitis compared to 43 healthy controls. They found that the subjects with periodontitis had higher and median levels of CRP than controls (1.45mg/l and

1.3mg/l respectively versus 0.9mg/l, $p=0.03$). In this study they also looked at serum IL-6 levels and found that the IL-6 levels were significantly higher amongst periodontitis patients and that the levels correlated to CRP levels in generalized periodontitis patients ($r=0.47$). D'Aiuto *et al* showed that serum concentrations of CRP were significantly correlated to the extent and severity of periodontal disease (D'Aiuto et al., 2004). When the authors looked at the study population and categorized them according to the AHA/CDC guidelines they found that those patients with more widespread periodontitis (as expressed by a greater number of pockets ≥ 5 mm) had an increased odds of 5.6 (95% CI 1.2-27.4, $p<0.04$) and 3.5 (95% CI 1.2-10.1) of being in the highest CRP group (>3 mg/l) compared to the lowest (<1 mg/l) and medium (1-3mg/l) CRP groups, respectively.

A more recent systematic review and meta-analysis which included some of the above studies found that there was evidence from the studies analysed that systemic CRP is elevated in periodontitis patients compared to controls (Paraskevas et al., 2008). The authors selected cross-sectional case control and longitudinal studies which used high-sensitivity CRP measurements. In total 18 studies were included and evaluated. For those studies that gave median values of CRP, five out of six studies reported higher median values of CRP for periodontal patients compared to controls. However, only two showed these differences to be statistically significant. Of the studies that gave mean CRP results, of which there were ten totaling 702 patients and 902 controls, the majority showed significant differences in mean CRP values between subjects and controls. A meta-analysis of these cross-sectional studies revealed a weighted mean difference (WMD) of 1.56mg/l

(95% CI 1.21-1.90) in CRP levels between periodontitis patients and controls ($p=0.00001$). Consistently, all studies showed higher CRP values for patients with values always seen to be $>2.1\text{mg/l}$. They concluded that plasma CRP was elevated compared to controls as deduced from these studies. It would also seem that the degree of elevation of CRP levels can have an effect on the severity of periodontitis (Linden et al., 2008). These study subjects were part of a cohort study of men in Northern Ireland who had CVD. These subjects were screened ten years apart and at the second screening, a periodontal exam was undertaken. Only subjects with a high ($>3\text{mg/l}$) or a low ($<3\text{mg/l}$) value at both time points were included. It was observed that a significant portion of those within the higher CRP group had advanced periodontitis compared to those within the low CRP group (18% v 6%, $p=0.0001$). The OR for advanced periodontitis being associated with the higher CRP values was 3.62 ($p=0.0003$). After adjustment for confounders such as smoking, diabetes, BMI, SES and age the association remained with OR of 2.49 ($p=0.02$).

A more recently study confirmed previous findings on this topic that CRP levels in serum are elevated in periodontitis patients compared to controls (Nakajima et al., 2010). The authors were assessing the levels of inflammatory mediator levels in relation to periodontitis and its treatment. They found, as previous authors have, that the serum CRP levels were elevated in the subjects compared to controls at the baseline examination. The periodontal treatment did decrease the levels of CRP, most notably for the group with the highest initial concentration of CRP.

While it has been shown that periodontitis patients have elevated levels of systemic CRP, its overall effect on systemic health and CVD particularly has been difficult to explore. If periodontitis is associated with higher CRP levels, does it influence systemic health? A recent study has shown periodontal disease to be associated with increased CRP in acute myocardial infarction (AMI) patients (Kodovazenitis et al., 2011). This study looked at periodontal parameters amongst 47 patients admitted for AMI and compared them to angiographically confirmed non-obstructive coronary disease. The AMI group had significantly higher frequency of periodontal disease compared to the coronary disease group (38% versus 17.5%, $p=0.03$). As expected the AMI had a much higher level of CRP compared to the other group but, interestingly, those people with periodontitis in both groups had higher mean levels of CRP, which was in the order of 1.5 times greater than the non-periodontitis subgroup. This difference reached significance in the AMI group (52.5mg/l vs. 36.1mg/l, $p=0.04$). Multivariable linear regression analysis for CRP values in AMI patients showed significant association with mean PPD, mean CAL and overall periodontitis in terms of percent of sites with CAL ≥ 3 mm and pocket depth ≥ 4 mm. This contribution of periodontal disease to CRP was strong and independent of other risk factors in a population who had never smoked and weren't diabetics, which are too major confounders for periodontitis and heart disease.

1.5.2 Gingival crevicular fluid

Gingival crevicular fluid (GCF) is a transudate of plasma which crosses the epithelial barrier and flows through the periodontal pocket into the mouth. It has long been known that host inflammatory products can be found in the GCF and that the host inflammatory products in question can be categorized as follows: (1) enzymes released by inflammatory cells; (2) products of cellular/tissue breakdown; and (3) inflammatory cytokines, mediators, complement and other products released by activated immunology cells, such as antibodies (Offenbacher et al., 1993). It has been hoped that these products would allow for easy diagnostic tests to detect disease activity and predict future activity in diseases such as periodontitis. GCF is easily obtained in a painless non invasive manner, therefore a potentially valuable diagnosis tool.

The amount of GCF volume tends to increase in patients with diseased or unhealthy periodontium (Smith et al., 1992). In this same study, the amount of GCF collected tended to increase in posterior sites irrespective of health status. It was also noted that the GCF constituents such as myeloperoxidase and lactoferrin also increased at more posterior sampling sites in the diseased patients. This finding was also seen when looking at the volume and composition of GCF in healthy subjects from different sites over time (Smith and Geegan, 1990). Repeated samples were taken from the same 6 sites in 11 patients six times over a six week period. There was a difference between GCF volumes among subjects as expected. However, the authors observed a change in GCF volume by site and by visit when calculated for each site for the mean of the 11 subjects at each of the 6

visits. These findings illustrate that differences exist among patients, among sites within patients and between different sampling times at these same sites. This has implications for evaluating and measuring GCF markers.

1.5.3 GCF CRP and Periodontitis

There are limited and sometimes conflicting studies on GCF inflammatory markers relating to periodontitis. The most studied inflammatory marker in GCF is CRP. In a recent study comparing the CRP levels in serum and GCF of patients with periodontitis with and without coronary artery disease and controls, it was observed that GCF levels of CRP were not statistically elevated between groups (Tuter et al., 2007). However, the serum CRP values were significantly higher in periodontitis patients with and without CAD than in the control group. There was also a correlation between all clinical parameters and serum CRP levels which were significant. Furthermore, there was no correlation between the clinical parameters and the GCF CRP levels. Indeed there was also no correlation between serum and GCF CRP levels. Although many studies have previously shown serum CRP levels to be increased in patients with periodontitis, this study failed to show an increase in CRP at a local level in periodontally involved patients. In contrast it has recently been shown that having higher amounts of IL-1 β and CRP increased the odds of having periodontitis (Fitzsimmons et al., 2010). In this case-control study, GCF samples were collected from 939 subjects selected from the 2004-2006 Australian National Survey of Adult Oral Healthy. There were 430 cases with periodontitis and 509 healthy controls. ELISA analysis was used to detect for IL-1 β and CRP in the GCF of subjects. It was found that subjects

with detectable CRP were almost twice as likely to have periodontitis as those with undetectable levels (OR 1.9, 95% CI = 1.5-2.5). Detectable levels of CRP were found in significantly higher numbers of older subjects and those with medical complications. After adjustment for covariates, detectable CRP levels retained its significance for periodontitis risk. The odds of severe periodontitis was shown to be even more likely with CRP having a stronger effect (OR 3.3, 95% CI = 2.0-5.5). It seems that patients with evidence of local or systemic inflammation in GCF have a higher risk of periodontal disease.

The question remains as to the source of inflammatory markers, such as CRP, in GCF. If it is systemic, it may be that the CRP in GCF is from plasma, as GCF is a transudate of this. However if produced locally it may lead to increased systemic levels of CRP and account for the increased serum CRP levels seen in periodontitis patients. Although mainly synthesized in the liver, it has recently been shown for the first time that the gingival tissues can produce CRP (Lu and Jin, 2010). In all 94 biopsies were taken from 44 patients with chronic periodontitis and 18 healthy controls. CRP was detected by immunohistochemistry and western blotting. The samples were also used to detect for CRP and IL-6 mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time PCR. CRP was detected in gingival epithelium and connective tissue, with no significant difference between groups. CRP and IL-6 mRNAs were detected in all categories of gingival tissue sampled with RT-PCR. The CRP mRNA was strongly correlated with IL-6 mRNA ($R=0.694$, $p<0.001$), suggesting that IL-6 was associated with CRP expression. The authors concluded that human gingiva is able to produce CRP in situ and

that it may be associated with IL-6 activity. However, in contrast, another study also looked at whether CRP in GCF is locally produced and found conflicting results (Megson et al., 2010). Gingival samples and GCF were taken for periodontitis patients and healthy patients and analysed for CRP and CRP mRNA. While they found CRP in both gingivae and GCF, no detectable levels of CRP mRNA were found in gingivae using real-time PCR. Both periodontitis patients and non periodontitis had detectable CRP levels in GCF and vice versa. While CRP was seen in gingival biopsy samples, the fact that no gene expression for CRP was found lead the authors to conclude that CRP was not produced locally and may be indicative of systemic inflammation, perhaps due to the periodontal infection itself or another systemic source. Serum CRP values were not determined in this study, which could have been correlated to GCF levels and would have been of use.

1.5.4 GCF IL-6 and Periodontitis

Several authors have found that serum IL-6 levels are significantly higher in patients with periodontal disease compared to controls in cross sectional studies (Marcaccini et al., 2009, Sun et al., 2009). There have been fewer studies on the levels of IL-6 and other inflammatory cytokines present in oral fluids such as gingival crevicular fluid or saliva. It is thought that these markers of inflammation are produced locally and play a role in the disease response and tissue destruction that occurs with periodontal disease. It is thought that they can be used as possible markers of active disease. An early study found that IL-6

levels are higher in the GCF of periodontitis patients (Mogi et al., 1999). Patients were stratified as having mild or severe periodontitis and GCF samples were taken from sites with Periopaper strips and the samples analysed with a sandwich ELISA kit. They found that the concentration of IL-6 for the group with severe periodontitis was three times that of the control group and that the difference was significant ($p < 0.05$). Although there was a difference between the mild periodontitis group and the controls in terms of IL-6 levels, the difference was not significant, which suggests that those with more severe disease have more inflammation at the local site which may be indicative of increased tissue destruction and bone resorption. This was confirmed by a more recent study which looked at well matched groups of patients with chronic periodontitis, aggressive periodontitis, gingivitis and a healthy periodontium (Becerik et al., 2012). The periodontitis groups had significantly increased volumes of GCF and higher levels of IL-6 compared to the other groups. On the contrary, the IL-6 plasma levels within these patients didn't show any difference between the groups. Also there was no correlation between GCF IL-6 levels and plasma IL-6 levels amongst any patient group so it seems the systemic cytokine level may be independent of inflammatory agents produced locally in the periodontal tissues.

In the previous study the IL-6 levels and those of other cytokines assessed were not predictably correlated to clinical periodontal parameters. However, it has been shown that inflammatory cytokines are not only higher in periodontal patients but are correlated to pocket depths (Giannopoulou et al., 2003). Of the four cytokines evaluated in the GCF

of periodontally involved patients and healthy controls, IL-1, IL-6 and IL-8 were seen in higher amounts in patients compared to healthy subjects. These are known pro-inflammatory cytokines and pocket depths were significantly associated with the amounts of all cytokines. IL-6 levels were also significantly correlated to smoking ($p=0.024$) and stress ($p=0.001$). While stress is known to have an effect on several systemic diseases its influence is hard to quantify. Smoking on the other hand is a known risk factor for cardiovascular disease and several other systemic diseases as well as periodontitis, so the enhanced production of inflammatory cytokines in smokers may have clinical significance. It also seems that while production of IL-6 may vary due to inflammation, stress and smoking, it may also be the case that different bacterial species can affect the production of inflammatory cytokines, including IL-6 (Stathopoulou et al., 2010). When epithelial cells from periodontally healthy donors were challenged with four common oral bacterial species associated with periodontitis, which were live or heat killed, *F. nucleatum* elicited the highest response of IL-6 and other tested cytokines. Live *A. Actinomycetemcomitans* also elicited a high IL-6 response which was statistically significant compared to that produced by unchallenged cells ($p<0.001$). It was also observed that IL-10, the secretion of which was also screened for, was not detected for any of the bacteria involved under any of the conditions.

1.5.5 GCF IL-10 and Periodontitis

IL-10 is a traditional “stop signal” for inflammation as it plays a role in suppressing immune and inflammatory responses. It inhibits the production of proinflammatory cytokines such as IL-1, IL-6 and IL-8 it was thought that IL-10 may be important in regulating and limiting the extent of inflammation in periodontitis. It has been shown that IL-10 gene polymorphisms at position -597 is associated with severe generalized periodontitis (Sumer et al., 2007). Other polymorphisms of IL-10 have been associated with severe periodontitis in populations and these polymorphisms have been shown to be associated with different expression of IL-10 in localized periodontal lesions (Donati et al., 2008). While IL-10 levels and polymorphisms have been associated with periodontitis, little is known of its role in periodontitis and what happens at a local level. Goutoudi *et al* found that the concentration of IL-10 in GCF was significantly higher in non diseased sites than diseased sites of patients with chronic periodontal disease (Goutoudi et al., 2004). Also the total amount of IL-10 was significantly higher in non smokers than smokers ($p<0.01$). These findings would suggest a role for IL-10 in maintaining health in periodontal disease or at least acting to reduce inflammation. Conversely, in other studies looking at IL-10 levels in the GCF of periodontitis patients, it has been difficult to detect the cytokine and to differentiate between disease states (Gamonal et al., 2000). The authors detected IL-10 in only 43% of sites in periodontal patients and none were detected in healthy controls. Furthermore, although not significant, IL-10 levels were seen to be higher in periodontally active sites. Detection rates in another study looking at IL-10

levels in the GCF of periodontitis patients and controls ranged from 26% to 57% depending on the pocket depth (Toker et al., 2008). There were no significant differences between detection rates at diseased sites and control sites and no differences within test subjects regarding pocket depth. Also, the actual levels of IL-10 in sites where it was detected did not differ significantly across various pocket depths compared to healthy controls.

1.5.6 Salivary CRP and Periodontitis

While much research has focused on levels of various cytokines and inflammatory mediators in the serum and GCF of periodontitis patients, there has been less work on their presence in saliva. Many publications have focused on the potential diagnostic properties of saliva which could not only lead to the diagnosis of oral diseases and their activity, but also be used to diagnose systemic conditions. It is hoped that saliva could be used as a non invasive tool to measure biomarkers to allow rapid and cost effective diagnosis of various diseases in the body. In much the same way, it is hoped that markers of periodontal disease released into saliva could be used to diagnose the disease and especially periods of activity and progression. It is believed that most of the cytokines present in saliva are from the gingival fluid (Ruhl et al., 2004). The authors measured the levels of several common cytokines, including IL-6, in whole saliva and in the salivary secretion from the parotid and submandibular glands. They found that the cytokine levels

were significantly higher in whole saliva compared to the glandular secretions, which would suggest that the GCF was the source of these cytokines and accounted for the difference. As we have seen from the earlier studies, levels of various cytokines have been found to be higher in the GCF of periodontally diseased sites of patients compared to controls. Whole saliva is a mixture of salivary gland secretions and GCF so it is conceivable that the cytokine levels in saliva are directly related to and derived from the GCF as demonstrated by Ruhl *et al.*

CRP has been detected in the saliva of periodontitis patients (Pederson *et al.*, 1995, Christodoulides *et al.*, 2007). The study by Pederson *et al.* evaluated CRP values and those of other proteins in whole saliva of 45 patients distributed amongst four groups representing oral health, gingivitis, moderate periodontitis and severe periodontitis. They found that CRP values were statistically lower in the healthy group compared to the disease groups ($P = 0.0427$) and there was a significant positive trend for CRP values to steadily increase from groups I to IV ($P = 0.0238$). However, the number of participants in each group was small. Christodoulides *et al.* also looked at salivary CRP values in 28 subjects with moderate-to-severe periodontitis and compared them to 28 periodontally healthy controls using a lab-on-a-chip method (LOC) and a standard enzyme linked immunosorbent assay (ELISA). The patients were well matched and it was observed that the saliva of the periodontitis patients had CRP values 18.2 times higher than those of healthy patients as detected by the LOC method ($P < 0.0004$). In a more recent pilot study on the effects of smoking on salivary CRP levels, it was observed that active smokers had

significantly higher levels of salivary CRP compared to non/never smokers ($p=0.003$) (Azar and Richard, 2011). This study included 45 participants and is a pilot for a larger study seeking to validate salivary CRP against serum CRP. The study population was controlled for potential confounders at the recruitment phase and all were selected from a healthy student population. They did not have periodontitis, however this was self described so it can't be certain what their actual periodontal status was. Although, it is interesting that smoking status, a major contributor to periodontal disease and CVD, did influence salivary CRP levels. One study that did look at salivary CRP levels compared to serum CRP values failed to show a high correlation between the two (Dillon et al., 2010). Although CRP was detected in all saliva and serum samples from 69 healthy subjects, linear regression analysis failed to find a strong correlation. All subjects in this study were recruited from a healthy student population but no information was gathered on oral health status, particularly periodontal health, which could have acted as a confounder. Also, the authors questioned the sources of the CRP in saliva and serum and whether they were the same and if one could be used to validate the other.

1.5.7 Salivary IL-6 and IL-10 levels and Periodontitis

Although scarce, other authors have investigated the different salivary cytokine levels in subjects with periodontitis (Teles et al., 2009). In a cross sectional study 74 subjects with chronic periodontitis and 44 periodontally healthy subjects were assessed to see if the

levels of 10 different cytokines in saliva were affected by periodontal status. Interestingly, levels of IL-6 and IL-10 were not seen to be significantly different between the groups. Indeed none of the cytokines screened were seen to differ between groups. There was a weak negative correlation between IL-10 and clinical attachment level which reached significance ($r = -0.2$, $p < 0.05$). So in contrast to salivary CRP and these same cytokines in GCF it seems that, in saliva, they are not associated with active periodontal disease.

1.6 Periodontal Therapy: Clinical Outcomes

Periodontal therapy traditionally comprises initial non-surgical debridement followed by re-evaluation. At this stage the need for further therapy is determined. Non-surgical treatment consists of mechanical supra- and subgingival root debridement and oral hygiene instruction (OHI). These measures are undertaken to reduce and alter the microbial composition, in the form of a biofilm, to allow reduction in inflammation and return to relative periodontal health. It has been shown that subgingival debridement without adequate OHI results in a limited clinical healing response (Magnusson et al., 1984). Conversely OHI alone without subgingival debridement also results in limited clinical response (Cercek et al., 1983).

Badersten et al, studied the effect of combined therapy of OHI and supra- and subgingival debridement of the non molar teeth only in patients with advanced disease (Badersten et al., 1984). The mean plaque and bleeding scores were reduced to <20% irrespective of

initial pocket depth. However, the deepest pockets were seen to achieve the biggest reduction in pocket depths – in the order of 1-2mm usually. This came about by a combination of gingival recession and gain of clinical attachment levels. It seems that the amount of re-adaptation of tissues at the base of the pocket is related to the height of the soft tissue. Conversely the initially shallow sites (<3mm) were seen to lose attachment. The highest proportion of sites with attachment loss was found in sites with initial pocketing ≤ 3.5 mm. Nordland et al also looked at the healing response to non surgical therapy in all teeth (Nordland et al., 1987). They found similar results to Badersten et al, although the deep sites did not do as well, probably due to the inclusion of molar teeth. It was seen that deep non-molar and molar sites healed comparably. The deep sites at molar furcations showed impaired healing overall and a much higher frequency of sites losing attachment compared to molar/non-molar flat surfaces with similar depth – up to 21% of sites losing attachment. This is in agreement with (Loos et al., 1989) who showed up to 29% of deep molar furcation sites losing attachment over two years.

A later hypothesis that gained popularity in non-surgical therapy was the concept of 'full mouth disinfection'. This initially involved scaling and root planning all quadrants within 24 hours in combination with the application of chlorhexidine to all intraoral areas for up to 2 months afterwards at home. Compared to conventional, quadrant by quadrant approach to non-surgical treatment, the clinical and microbiological parameters were improved after debridement was completed within 24 hours and combined with simultaneous and postoperative full mouth disinfection in an early study on this concept

(Bollen et al., 1998). These results confirmed those of an earlier pilot study by the same group. The findings suggest that re-infection can occur during the healing phase from the remaining untreated sites. A later study seemed to suggest that most of the benefit was due to the scaling and root debridement within a 24 hour period and that the chlorhexidine had no adjunctive effects (Quirynen et al., 2000). They found that patients who had received root debridement within 24 hours with and without concomitant use of chlorhexidine showed significant clinical gains compared to patient who had received quadrant debridement at two week intervals. There was additional gain of attachment of 2mm and additional pocket depth reduction of 1.5mm for sites, initial ≥ 7 mm deep in both 'full mouth' groups compared to the traditional quadrant wise modality. A more recent systematic review of available studies looked at the effectiveness of full mouth debridement within 24 hours with (FMD) and without (FMS) chlorhexidine compared to quadrant wise debridement, which acted as the control (Eberhard et al., 2008). In total seven randomized controlled trials were included for analysis. Overall it was found that while all modalities lead to significant improvement in clinical parameters, the mean difference between FMD and control in terms of pocket depth reduction was 0.53 mm (95% CI, 0.28 to 0.77) in moderately deep pockets of single rooted teeth. No significant differences were seen between FMS and control for any parameters while in one study FMS lead to an improved gain of clinical attachment level of 0.73mm over FMD. The authors concluded that while FMD lead to improved clinical outcomes over quadrant debridement, these improvements were only modest in size. It seems that while full mouth disinfection does infer some clinical advantage over traditional debridement, these

differences vary and the treatment modality selected depends on the practical considerations relating to the clinical setting and patient preferences (Kinane and Papageorgakopoulos, 2008).

1.7 Periodontal therapy: Inflammatory marker outcomes

1.7.1 Periodontal therapy effect on CRP and other cytokine levels

While it has been widely observed that periodontitis is associated with cardiovascular disease and other systemic diseases and that the levels of inflammatory markers often associated with these diseases are also significantly raised in periodontitis, thus offering a possible explanatory link between the two. However, the most crucial aspect of any potential causal pathway would be to determine if the treatment of the periodontal disease could result in improved systemic inflammatory markers and thus potentially reduce the risk of developing systemic disease. There have been several such studies evaluating what happens to the levels of serum CRP, IL-6 and other inflammatory markers post treatment for periodontitis (Ide et al., 2003, Nakajima et al., 2010, D'Aiuto et al., 2005). Ide *et al* studied the effects of periodontal treatment on serum CRP, IL-6, TNF- α , fibrinogen and IL-1 β in a randomized control trial. In all 24 test subjects received periodontal treatment and were re-evaluated six weeks later. Blood samples were taken at each appointment and analysed for the above inflammatory markers. At the re-evaluation exam there was significant improvement in clinical parameters as expected. However the levels of CRP, IL-6 and other markers did not change significantly within the

treatment group and there was no difference after treatment compared to the untreated control group. This contrasts with D'Aiuto et al who showed that serum inflammation levels were reduced after periodontal treatment compared to untreated controls in an RCT. Using two treatment regimes – standard quadrant debridement (SPT) and intensive therapy done over four hours (IPT) – it was seen that CRP reduced significantly over the controls after 2 months (0.5 ± 0.2 mg/L for SPT, $p = 0.030$ and 0.8 ± 0.2 mg/L for IPT, $p = 0.001$). It was also seen that IL-6 reduced significantly.

More recently, a systematic review on the effects of periodontal treatment on CRP levels was published (Ioannidou et al., 2006). In total, the authors included three RCT and seven single cohort studies. In the cohort studies, three were eligible for meta-analysis and the overall mean reduction of CRP levels due to therapy was 0.2mg/l (95% CI -0.15 to 0.55). These changes were not significant ($p > 0.05$). Two of the RCTs identified were eligible for meta-analysis – the studies by Ide et al (2003) and D'Aiuto et al (2005). When they were combined for analysis the mean difference in CRP levels before and after treatment was -0.18mg/l (95% CI -0.7 to 0.35mg/l). Although there was an effect in favour of therapy, analysis showed it not to be significant ($p = 0.49$). The other RCT not included did show a positive effect of treatment on CRP levels but was excluded because the authors didn't report detailed pre- and post-treatment results with CRP levels per group (Ebersole et al., 1997). Additionally these authors used long term adjunctive non-steroidal anti-inflammatory drugs which would have had an impact on the level of systemic inflammation irrespective of treatment. Overall the authors of the review concluded that

there was insufficient evidence to support the hypothesis that periodontal therapy could reduce systemic CRP levels. They suggested the inclusion of repeated root debridement and even surgery in study designs to assess the outcome of periodontal therapy, which would allow a more stable endpoint of treatment.

However, subsequent research did show a positive impact on systemic inflammation resulting from non surgical periodontal therapy (Marcaccini et al., 2009, Vidal et al., 2009). Marcaccini et al, 2009 assessed the levels of circulating IL-6 and CRP before and three months after periodontal treatment in 25 patients with periodontal disease. 20 healthy patients were used as controls in the study. The patients in the test group received OHI and quadrant debridement followed by review appointments every two weeks until the re-examination appointment at the third month. The CRP was analysed using a high-sensitivity methodology in a spectrophotometre. Interestingly, the authors did not show a significant difference in CRP values between test and healthy controls at baseline. However, the criteria used to determine periodontitis level was slightly opaque and the authors didn't mentioned what severity of periodontitis patient was included, so many of them may have had mild periodontitis. It may be supposed that the amount of systemic inflammation in terms of CRP levels was lower than other studies have shown. The median values for CRP in the periodontitis group was 1.2mg/l at baseline and there was a decrease in hs-CRP value after therapy which was significant ($p=0.006$). The CRP value in the control group was unchanged. Serum IL-6 was also significantly decreased after therapy in the treatment group, illustrating that systemic inflammation was reduced

after periodontal therapy. Vidal et al, did an RCT to assess the effects of non surgical therapy on the levels of CRP and IL-6 in plasma. Twenty two patients with periodontitis were recruited and assigned to the treatment and non-treatment (control) groups. Those in the control group had their treatment delayed for 3 months until the study was completed. The test group received non-surgical therapy in the form of OHI and root debridement over 4 to 6 visits. There was no difference in CRP values between the groups at baseline. All clinical parameters were well matched between groups also. However the CRP values for the treated groups were significantly lower at the re-evaluation appointment (1.4mg/l +/- 1 vs. 0.9mg/l +/-0.8 (p=0.005). There was also a statistical difference between the IL-6 values in the treatment group before and after treatment. Interestingly, the plasma CRP and IL-6 values in the control group were significantly higher at 3 months compared to baseline (p=0.01), which suggests that the untreated disease had contributed to systemic inflammation over that time period.

Often the case with periodontal therapy is that the initial course of treatment reduces the number of deep sites and individual pocket depths, but often there remain active or non responsive sites. The usual course of treatment is to redebride residual sites or to do surgery, depending on the clinical situation. Most studies evaluating the response of CRP and other systemic inflammatory markers to periodontal therapy have had short follow up periods of 1 month to 3 months. Three months is the most common in the literature, as this is the point when periodontal re-evaluation is traditionally done. A more recent study evaluated the systemic inflammatory response to periodontal therapy over a longer

period (Nakajima et al., 2010). A total of seventy eight patients with moderate to severe periodontitis were enrolled in the study with forty healthy patients acting as controls. Serum samples were taken at baseline and at least three months after completion of active therapy. In contrast to earlier studies, patients received OHI, scaling and root planning as well as surgical intervention for the treatment of residual pockets. Surgical intervention varied from patient to patient depending on their needs and each patient received antibiotics after surgical therapy. This treatment protocol would seem to ensure a more comprehensive periodontal therapy and would allow patients to possibly be more stable periodontally after their active treatment, which would in turn affect their systemic inflammatory status. The mean pocket depth of 3.8mm in the treated patients at baseline was significantly reduced at the end (2.4, $p<0.0001$), as were the percentage of sites >6 mm or 4 to 6mm deep. The median CRP level at baseline was 0.42mg/l in the periodontitis group and 0.19mg/l in controls, a difference that was significant ($p<0.001$). After therapy, the median level of CRP in serum decreased to 0.36mg/l, a significant drop ($p<0.01$). The authors also analysed the effect of periodontal therapy of hs-CRP levels using CRP quartile groups. They found that only those in the highest quartile according to CRP values had a significant reduction in CRP values. The other quartiles did not show a significant reduction. Also, various factors did not differ amongst these quartiles such as gender and smoking. Furthermore, the clinical parameters did not differ by quartile at baseline also. The authors concluded that although the periodontal therapy did reduce the overall CRP values significantly, there was a subgroup that seemed most at risk of having an elevated CRP level associated with periodontitis. This susceptible group would

also seem more responsive to periodontal therapy in terms of reducing the CRP associated CHD risk factor.

1.7.2 Acute effects of Periodontal therapy on Inflammatory Markers

From the above research studies, it has been shown that there is evidence of altered systemic inflammatory levels after periodontal therapy. However, there is more limited evidence on what the acute effect periodontal treatment has on circulating levels of proinflammatory mediators. Some groups have attempted to study the short term effects of periodontal therapy on the host response by determining the response of various systemic inflammatory markers (Ide et al., 2004). This group looked at the fluctuations of circulating levels of lipopolysaccharide (LPS), CRP and IL-6 at various stages after root debridement in patients with chronic periodontitis. Blood samples were taken at baseline, 15, 30, 60 and 120 minutes after treatment and analysed for the markers in question. LPS was studied to determine the bacterial load in blood which was hypothesized to occur after therapy and lead to a systemic response. The findings showed that LPS levels did increase to suggest a low grade bacteremia but didn't approach significance. However systemic levels of IL-6 did increase significantly at 60 and 120 minutes after therapy. CRP levels, which were measured only at baseline and 120 minutes only, did not show significant alteration, possibly due to the fact that CRP levels tend to lag behind those of other initiating cytokines. What this study did show was that periodontal therapy can generate a low grade elevated systemic inflammatory response. This acute systemic response to therapy has been confirmed by other studies (Tonetti et al., 2007, D'Aiuto et

al., 2005). Both these groups showed significant spikes in circulating CRP levels 24 hours after periodontal therapy ($p < 0.001$ both). These levels were seen to return to baseline after one week and one month respectively. Tonetti *et al* also assessed endothelial function in their study, using flow-mediated dilatation. They showed after 1 day, that endothelial function as determined by flow-mediated dilatation was significantly lower in the treatment group compared to control. However, after 60 and 180 days, the endothelial function was seen to be increased in the treatment group compared to control ($p = 0.02$ and $p < 0.001$). This approach showed improved endothelial function due to therapy which was significant because endothelial dysfunction is thought to represent a pathway through which atherosclerosis may be triggered. More recently, research has expanded on the concept of systemic inflammation and oxidative stress in association with chronic periodontitis, as oxidative stress has been linked to both periodontitis and systemic inflammation (D'Aiuto et al., 2010). When comparing oxidative stress in the form of Diacron-reactive oxygen metabolites (D-ROM) and systemic inflammation in the form of CRP and IL-6 between 145 cases and 56 controls, it was observed that periodontitis patients had higher D-ROM and CRP than the controls. There was also a positive correlation between serum CRP and D-Rom levels amongst all participants (CRP, $r = 0.4$, $p < 0.001$), a correlation which increased in magnitude amongst cases (CRP, $r = 0.5$, $p < 0.001$). A small subset of the periodontitis patients (14) received periodontal treatment over one prolonged visit and D-Rom levels were assessed at days 1, 5, 7 and 30 after therapy. The reactive oxygen species increased sharply over the first week post therapy

($p < 0.01$) compared to baseline, which suggests that acute inflammation immediately after debridement is associated with raised systemic levels of reactive oxygen species.

Increases in systemic CRP levels are not confined to non surgical periodontal therapy and have been shown to peak 24 hours after surgical periodontal therapy (Graziani et al., 2010). This prospective cohort study included 14 patients who received non surgical periodontal therapy initially followed by at least two surgical interventions six months later. CRP levels were assessed 24 hours after full mouth debridement and the surgeries. Interestingly the 24 hour CRP level increased more so after the non surgical therapy ($985 \pm 392\%$, $p < 0.01$) than after the surgeries ($237 \pm 67\%$, $p < 0.05$). This would suggest that CRP and other systemic inflammatory marker perturbations after periodontal therapy are not just due to mechanical trauma of the periodontium, as surgical intervention would involve a trauma of greater magnitude. It would seem non-surgical debridement in an untreated periodontitis patients would lead to greater bacteraemia than surgery due to the greater quantity and quality of pathogens in the initially untreated pockets, that can contribute to a systemic inflammatory reaction.

1.7.3 Effects of Periodontal therapy on Localised Inflammation

1.7.3.1 Interleukin-6

Although various research is available on systemic inflammation and markers after periodontal therapy there is relatively scant information on the local inflammatory response in the periodontal tissues, and by extension the GCF. Some authors have attempted to address this by analysing levels of pro-inflammatory markers in oral fluids and their response to treatment, including IL-6 and IL-10. Periodontal therapy has been shown to decrease IL-6 levels in the GCF of patients with chronic periodontitis (Goutoudi et al., 2012). The study was designed as a randomized, longitudinal, split mouth study with one quadrant in each patient receiving scaling and root planning with another quadrant receiving non surgical therapy followed by surgical periodontal treatment. The authors took GCF samples from 72 diseased and 24 non diseased sites in the experimental quadrants of 12 patients before as well as 6, 12 and 32 weeks after periodontal therapy. A site was designated diseased if the pocket depth (PD) was $\geq 5\text{mm}$ and non diseased if the PD was $\leq 3\text{mm}$. IL-6 was detected in 87.5% of diseased sites and 95.83% of non-diseased sites. The concentration of IL-6 was seen to be higher in non-diseased sites than diseased sites both at baseline ($p < 0.01$) and following therapy ($p < 0.05$). Periodontal treatment resulted in significant increase in IL-6 concentration in diseased sites at 6 weeks ($p < 0.01$) and this remained increased over the course of the study. The treatment modality did not have affect on the IL-6 concentration. It was also seen that IL-6 concentration was negatively correlated to PD and CAL ($p < 0.05$). These findings were interesting because as

IL-6 is a proinflammatory cytokine, it would have been expected that there would have been a difference in concentration between diseased/non-diseased sites and that the IL-6 concentration would have decreased after therapy. This finding was replicated in another earlier study comparing diabetics and non-diabetic patients with chronic periodontitis (Kardesler et al., 2011). This group measured IL-6 levels in the GCF of 20 type 2 diabetics and 22 non diabetics at baseline, 1 month and 3 months after therapy. While it was observed that total amounts of IL-6 were higher in diabetics before treatment and lower after treatment, the change in IL-6 amounts amongst diabetic patients did not reach significance. Similarly, IL-6 concentrations actually increased significantly amongst diabetics after therapy. These findings may reflect poorer healing and increased inflammation in diabetic patients. IL-6 concentrations or total amounts did not significantly decrease after treatment.

1.7.3.2 Interleukin-10

Levels of IL-10 in GCF before and after treatment have also been analysed with varying outcomes (Goutoudi et al., 2004, Toker et al., 2008). In the former study GCF samples were taken from diseased and non-diseased sites using Periopaper strips at baseline, 6, 16 and 32 weeks. The study design and outlay was similar as to the study by Goutoudi et al, 2004 on IL-6 levels in GCF before and after periodontal therapy. In this study, the authors found that the total amount of IL-10 per sample was similar in diseased and non diseased sites before and after treatment. In contrast, IL-10 concentration was significantly higher

in non-diseased sites ($p < 0.01$) and increased significantly after therapy. IL-10 is cytokine with a supposed anti-inflammatory regulatory function, so the finding of increased concentrations in healthier sites adds credence to this theory. Toker *et al*, 2008 examined the effects of periodontal therapy on IL-10 levels in the GCF of patients with aggressive periodontitis and compared them to samples taken from healthy controls. 15 patients with aggressive periodontitis were included and four samples were taken per patient from sites with different depth categories (shallow, moderate and deep sites) and found that the amount of IL-10 per sample was similar for all pocket depth categories and did not alter after therapy. This was in contrast to the previous study. The detection rate of IL-10 in samples in the study by Toker *et al*, 2008 was typically much lower than that for Goutoudi *et al*, 2004 (26.7%-57.5% versus 79-92%) and IL-10 was most often detected in moderate and deep sites than in shallow and control sites.

As to our knowledge, there are no studies evaluating CRP levels in the GCF or saliva of periodontitis patients in response to therapy.

1.8 Study Aims

The aims of this study were as follows:

1. To assess the levels of CRP in blood, GCF and saliva and to assess the levels of IL-6 and IL-10 in the oral fluids at three time points in patients undergoing non-surgical periodontal treatment.
2. To ascertain if there is a relationship between what is occurring to the levels of systemic and local inflammatory markers at the various times of the study.

2. Materials and Methods

2.1 Study Design

This study design was a prospective single cohort study.

2.2 Ethical Approval

Ethical approval was sought for health related research studies which was not an interventional clinical trial of medicinal products from the St James's Hospital Joint Ethical committee and was received in July 2010.

2.3 Subjects

22 subjects were recruited consecutively from the Dublin Dental University Hospital general waiting list for periodontal therapy. The recruitment period was from September 2010 to June 2011.

The participant age range was 21 – 75. Both male and female patients were recruited after a diagnosis of moderate or severe generalised chronic periodontitis was made according to the classification by Armitage 1999.

The patients were selected according to the following criteria

2.3.1 Inclusion Criteria

1. Ten or more teeth present.
2. Diagnosis of moderate to severe periodontitis.
3. Presence of more than 2 teeth with pocket depths $\geq 5\text{mm}$ and clinical attachment levels $\geq 6\text{mm}$
4. Presence of proximal attachment loss of $\geq 5\text{mm}$ in $\geq 30\%$ of teeth present.
5. No periodontal treatment in the previous 12 months.

2.3.2 Exclusion Criteria

1. Diabetes.
2. Psychiatric illness
3. Pregnancy or Lactation.
4. Recent bacterial, viral or fungal infections.
5. Chronic Inflammatory or Immunological Disease.
6. Taking of Statins, anti-biotics, anti – inflammatory drugs and any other medication known to affect inflammatory processes and CRP levels within the last six months

7. Cardiovascular disease or history of cardiovascular disease

2.4 Consent

The patients who were deemed suitable for the study were asked to participate in a gated manner by a designated gatekeeper. On the assessment visit they were informed of the study and its nature. An information leaflet explaining what the study entailed and how enrollment would affect them was given to each patient (*Appendix 2*). A consent form was also given to the patient on that day (*Appendix 3*). The information leaflet contained all the relevant information for the patients in relation to this study. A contact number was provided on the leaflet of the main researcher in case they had any additional questions after the assessment visit. Patients were informed that their participation was totally voluntary and that their treatment outcome would not be affected should they decline to participate. One week after the assessment visit the patients were contacted by phone and asked if they wanted to participate in the study.

2.5 Clinical Parameters

At the initial assessment and 3 month re-evaluation visits the patients were examined clinically and radiographically. Six sites were examined around each tooth: mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual. One trained

examiner (PD) took all measurements and recorded the results. The following clinical parameters were measured.

1. Pocket depths (mm)
2. Attachment level (mm)
3. Bleeding scores (0/1)
4. Suppuration sites (0/1)
5. Plaque Score (0/1) – (O'Leary et al., 1967)
6. Tooth mobility (Grade 1, 2 or 3) - (Miller, 1950)
7. Furcation involvement (Grade 1, 2 or 3)

The patients were also asked about their smoking history and a full medical history was taken, including medications being used and any known allergies.

2.6 Periodontal Treatment.

Each patient received oral hygiene instruction (OHI) at the initial visit and this was reinforced at the following treatment appointments. Depending on individual needs, this usually included brushing techniques and advice on the use of interproximal cleaning aids such as interdental brushes and/or floss. At the treatment appointments the patients underwent scaling and root debridement under local anesthesia (2% lidocaine with 1:80000 adrenaline, Septodont, Austria) on a side by side basis i.e. the right side was

scaled and root debrided on the first treatment visit and the left side was done on the second visit. Scaling and root debridement was performed using ultrasonic scalers (EMS, Nyon, Switzerland) and Gracey curettes (Hu Friedy, Chicago, USA). No medicaments were used during scaling or afterwards.

2.7 Measurement of Whole Blood CRP

At each treatment visit and at the 3 month re-evaluation the whole blood CRP level was measured through standard clinical analysis using the Quikread CRP machine (Orion Diagnostica, Espoo, Finland). This device is widely used in medical practice to determine CRP values at chairside and has been shown to correlate closely with laboratory assays (Esposito et al., 2005, Papaevangelou et al., 2006). The supplied card was read and validated. One ml of the buffer solution was pipetted into the cuvettes. Next 20µl of blood was taken from the patient's finger using the supplied capillary tubes. This blood sample was added to the buffer and the solution was gently mixed until it appeared to clear up as a result of haemolysis. The CRP reagent cap was placed onto the open end of the cuvette and the cuvette was then placed into the measuring well of the QuikRead machine. The blank measurement was read and on the prompting of the machine the reagent was added to the solution by pressing on the inner part of the cap. The cuvette was then shaken vigorously to dissolve the reagent and put back into the measurement well. The CRP result was then displayed within 2 minutes. The CRP range for the Quikread CRP machine was 5-160mg/l.

2.8 Sample Collection

At each of the treatment visits and at the 3 month re-evaluation visit saliva and gingival crevicular fluid (GCF) samples were taken. The saliva samples were collected by getting the patient to expectorate approximately 2mls of whole saliva into a container. This container was placed in an ice box until processing at the end of the clinic.

GCF samples were also taken at the two treatment visits and 3 months after periodontal therapy. Sampling sites were selected randomly and had a baseline pocket depth of $\geq 5\text{mm}$. One GCF sampling site was chosen per quadrant.

Prior to GCF sampling the area was isolated from saliva using cotton wool rolls. The site was cleared of supragingival plaque using curettes. Filter paper strips (Periopaper, Oroflow Inc., Smithtown, NY, USA) were placed in the orifice of the gingival sulcus to a depth of at least 2mm for 30 seconds. Three periopaper strip samples were taken from each site sequentially, corresponding to the 3 cytokines to be tested. Each paper strip was placed in a separate polypropylene eppendorf tube and they were placed in an ice box until processing after the clinic. Any Periopaper strip seen to be contaminated with blood was discarded and a new sample taken

2.9 Treatment Flow Chart

Day 0 – clinical examination and patients recruited to study.

Oral hygiene instruction.

Day 1 - First treatment visit comprising of scaling and root debridement on the right hand side of mouth – upper and lower quadrants were treated.

The whole blood CRP was measured and saliva and gingival crevicular fluid samples were taken.

Day 2 - Second treatment visit the following day comprising of scaling and root debridement on the left hand side of mouth. Upper and lower quadrants were treated.

The whole blood CRP was measured.

Saliva and gingival crevicular fluid samples were taken.

Day 90 - Re-evaluation visit (3months) when all clinical parameters were measured again.

Whole blood CRP, saliva samples and GCF samples were taken again.

2.10 Sample Storage

The saliva sample collected on each day was spun down at the end of clinic to remove any debris. The saliva sample was allocated to 4 separate Eppendorf tubes with approximately 0.5ml in each tube.

All saliva and GCF filter paper samples were numbered sequentially and stored at -80C until laboratory analysis.

2.11 Laboratory Analysis.

The levels of CRP, IL-6 and IL-10 in saliva and GCF were determined using commercially available ELISA kits [Biolegend® (IL-6 and IL-10) and R&D Systems (CRP)]. All manufacturers' guidelines were followed.

The filter paper strips were removed from the freezer prior to the ELISA test and the GCF was eluted from the individual strips by adding 250µl Phosphate Buffer Saline* (PBS) into the Eppendorf tubes. The tubes were then placed on an ELISA plate rocker for approximately 75 minutes prior to the appropriate ELISA being performed.

The saliva samples were removed from the freezer and allowed to come to room temperature for approximately 15 minutes prior to being analysed. A description is given below.

- Phosphate Buffer saline (PBS) was made up prior to the analysis as follows:- 8.0g NaCl, 1.16g Na_2HPO_4 , 0.2g KH_2PO_4 , 0.2g KCL were added to approximately 800mls of deionised water. Once the agents were added and dissolved, the solution was brought up to 1 litre. The pH was checked using a calibrated pH meter and titrated to 7.4 exactly by adding the appropriate amount of acid [hydrochloric acid (HCL)] or base [sodium hydroxide (NaOH)].

2.11.1 Interleukin-6 Analysis.

1. 100 μl Capture antibody was added to each well the night before the assay.
2. On the day of the assay, all standards and reagents were prepared as per manufacturers guidelines.
3. The capture antibody was washed out of the well using wash buffer* and dried. This process was repeated 4 times.
4. 200 μl Assay diluent was added to each well and left for 1 hour.
5. The plate was washed again 4 times.
5. 100 μl of standards and samples were added to the wells. The standards and samples were assayed in duplicate.
6. The plate was left on the rocker plate for 2 hours at room temperature.

7. The plate was washed 4 times.
 8. 100µl IL-6 detection antibody was added to the wells and left for 1 hour.
 9. The plate was washed 4 times.
 10. 100µl Avidin-HRP solution was added to each well and left for 30 minutes.
 11. The plate was washed 5 times.
 12. 100µl TMB substrate solution was added to the wells and left at room temperature for 30 minutes in the dark.
 13. 100µl stop solution was added to the wells.
 14. The absorbance was read by a plate reader at 450nm within 30 minutes.
- * Wash buffer solution was made by adding 0.05% Tween-20 to 1 litre pre-prepared PBS

2.11.2 Intereukin-10 Analysis

The procedure outlined above was repeated except IL-10 detection antibody was added at step 8.

2.11.3 CRP analysis.

1. All samples and reagents were prepared as per manufacturers guidelines.

2. 100µl assay diluent was added to wells.
3. 50µl Standard and samples were added to each well and incubated for 2 hours at room temperature.
4. The plate was washed 4 times.
5. 200µl CRP conjugate was added to each well and allowed to incubate for 2 hours at room temperature.
6. The plate was washed 4 times.
7. 200µ substrate solution was added to each well and incubated for 30minutes in darkness.
8. 50µl stop solution was added to each well and the absorbance was read in a spectometre at 450nm within 30 minutes.

2.12 Biomarker Concentrations

All standards and sample concentrations were determined from standard curves generated with GraphPad Prism software (GraphPad software, Inc., California, USA) using linear regression analysis.

2.13 Statistical Analysis

All data collected was entered in an Excel database and proofed for any errors. The data was analysed using a commercial statistical package (JMP). Frequency distributions were determined for all variables at all time points. Data were compared between baseline and subsequent sequential time points (Day 2 and 3 months). This was done using a series of paired *t*-tests or Wilcoxon signed rank matched pairs test for normally and not normally distributed variables respectively. The clinical parameters such as probing pocket depth, bleeding and plaque score was normally distributed. The biomarker concentrations were for the most part seen to be not normally distributed with the results being skewed.

The CRP results from the three fluids (Blood, saliva and GCF) were assessed for a correlation relationship using Spearman Rank coefficient correlation between each pair of results, at each of the three time points in question (Day 1, Day2 and Day 90). A Bland-Altman plot was also used to assess the agreement between these different parameters at each time point. A Bland-Altman plot is a visual tool for measuring two different methods of measuring the same parameter, the purpose of which is to determine if a new method of measurement is in agreement with an established one (Bland and Altman, 1986). The Bland-Altman plot is a visual representation of agreement or lack of agreement and evidence of bias.

Finally, a Fishers exact test was performed between the number of patients where blood CRP level was $<5\text{mg/l}$ at each time point and those patients where blood CRP level was $\geq 5\text{mg/l}$ at each time point to assess the proportions of patient within each group.

3. Results

3.1 Study Population Characteristics

A total of 22 patients were recruited to the study and all patients returned for the 3 month re-evaluation and completed the study. The patient demographics and parametres are demonstrated below.

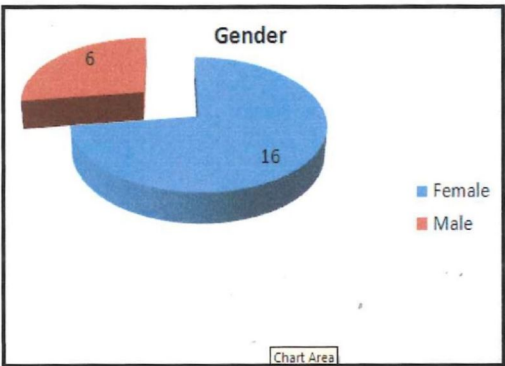


Figure 3.1.1: Gender distribution

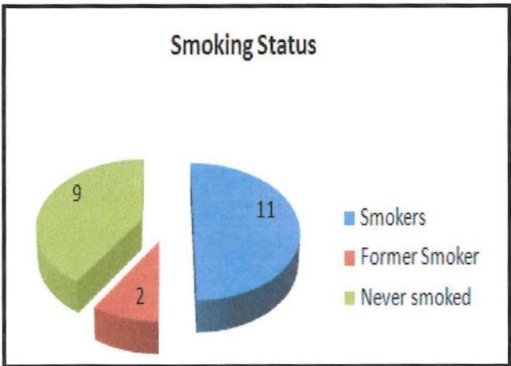


Figure 3.1.2: Smoking status distribution

	18-35	35-50	50+
Age Range	2	12	8

Table 3.1: Age distribution

3.2 Clinical Results and Frequency Distributions

3.2.1 Pocket Depth Distribution

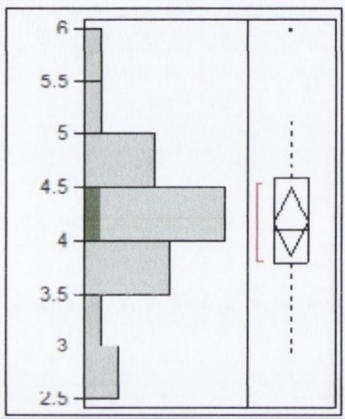


Figure 3.2.1.1: Basline pocket distribution, Mean – 4.17mm

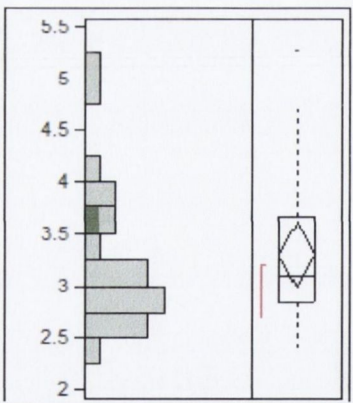


Figure 3.2.1.2 Re-evaluation pocket depth distribution, Mean – 3.304mm

3.2.2 Bleeding score distribution

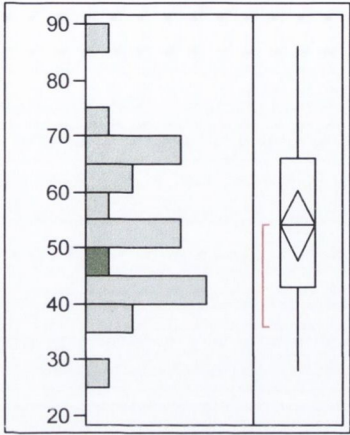


Figure 3.2.2.1 Baseline bleeding score distribution, Mean – 54% ± 14.3

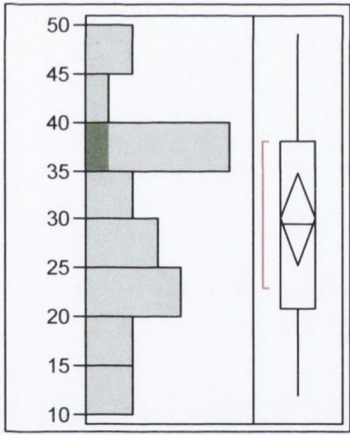


Figure 3.2.2.2 Re-evaluation bleeding score distribution, Mean – 30% ± 10.57

3.2.3 Plaque score distribution

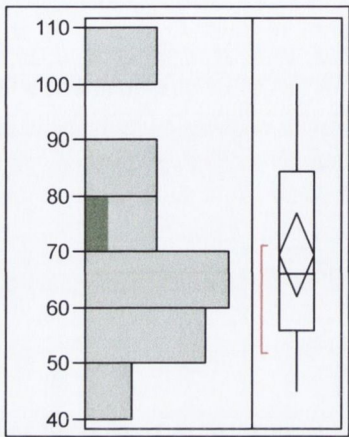


Figure 3.2.3.1 Baseline plaque score distribution, mean – 69.4% ± 16.96

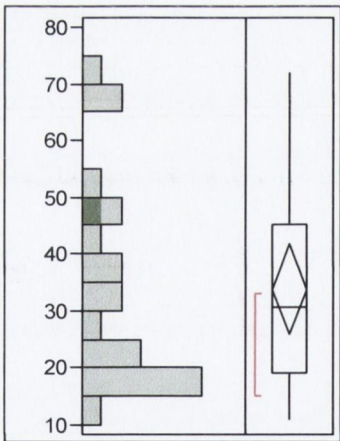


Figure 3.2.3.2 Re-evaluation plaque score distribution, mean – 33.77% ± 17.9

3.3 Cytokine Frequency Distribution

3.3.1 Blood CRP Frequency Distribution

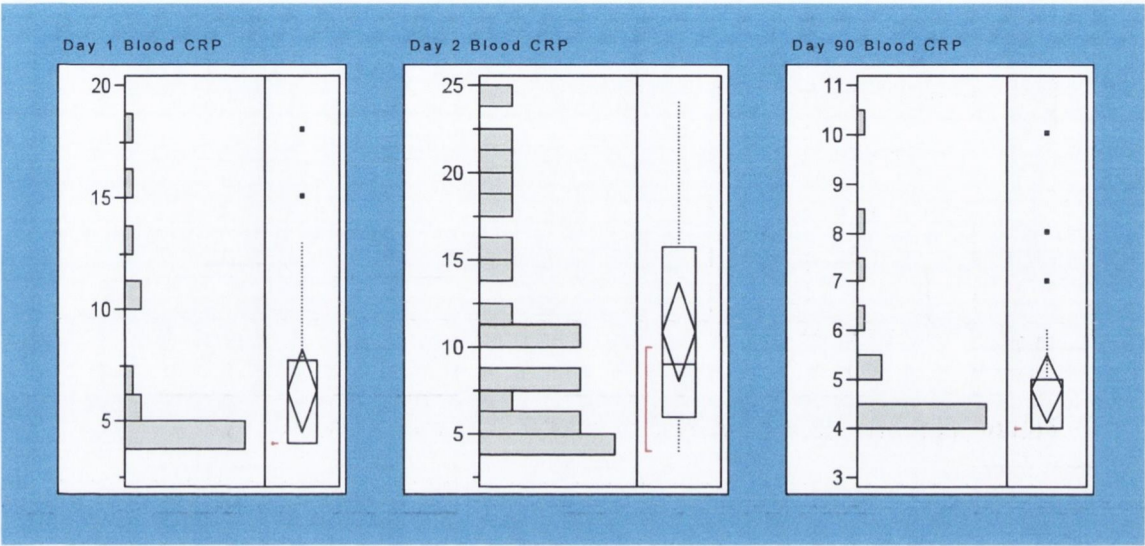


Figure 3.3: Blood CRP frequency distributions (mg/l)

Mg/l	Day 1	Day 2	Day 90
Mean	6.4	10.86	4.8
Median	4	9	4
Interquartile Range	4-7.75	6-15.75	4-5

Table 3.3: CRP means, medians and ranges

3.3.2 GCF CRP Frequency

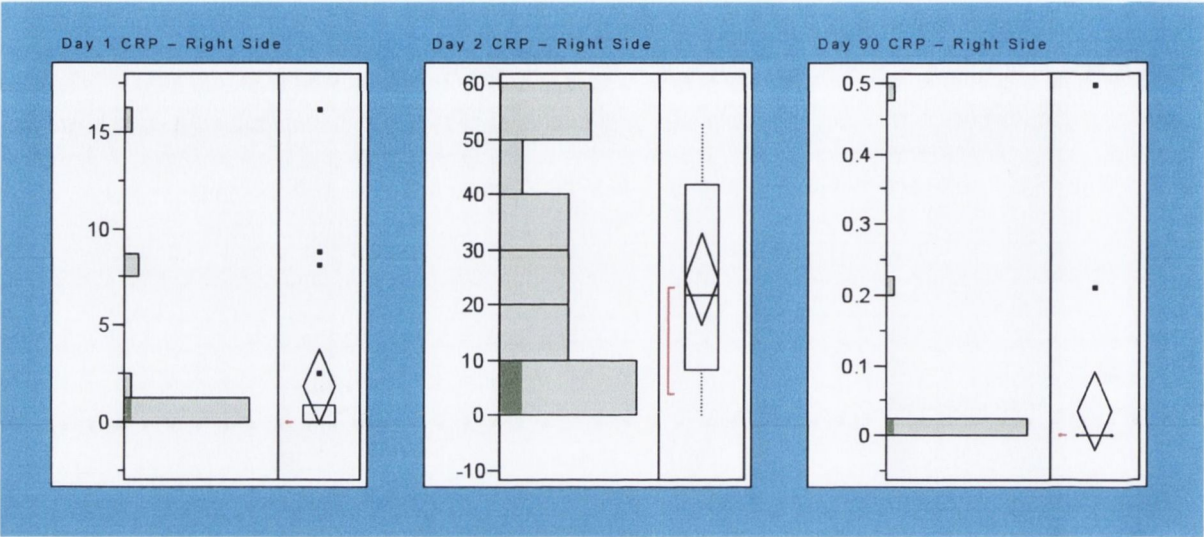


Figure 3.3.2 GCF CRP frequency distributions (ng/ml)

ng/ml	Day 1	Day 2	Day 90
Mean	1.8	24.66	0.03
Median	0	21.73	0
Interquartile Range	0-0.83	8.4-41.6	0-0

Table 3.3.2 GCF CRP means, medians and ranges

3.3.3 Saliva CRP Frequency

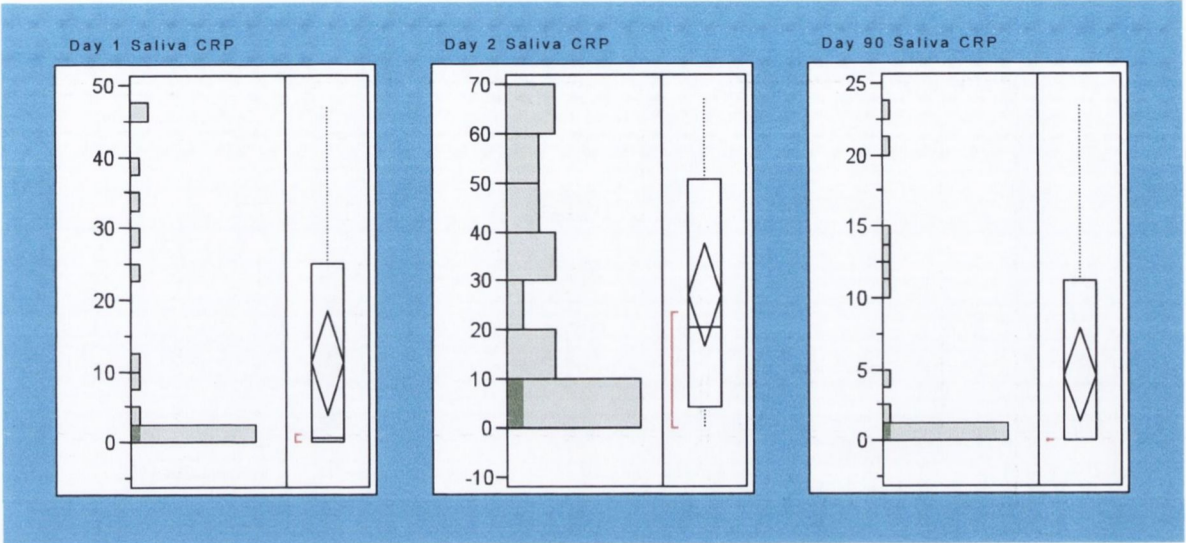


Figure 3.3.3: Saliva CRP frequency distribution (ng/ml)

ng/ml	Day 1	Day 2	Day 90
Mean	6.4	10.86	4.8
Median	0.54	20.39	0
Interquartile Range	0-25	4.2-50.7	0-11.16

Table 3.3.3: Saliva CRP means, medians and ranges

3.3.4 GCF IL-6 Frequency Distribution and Analysis

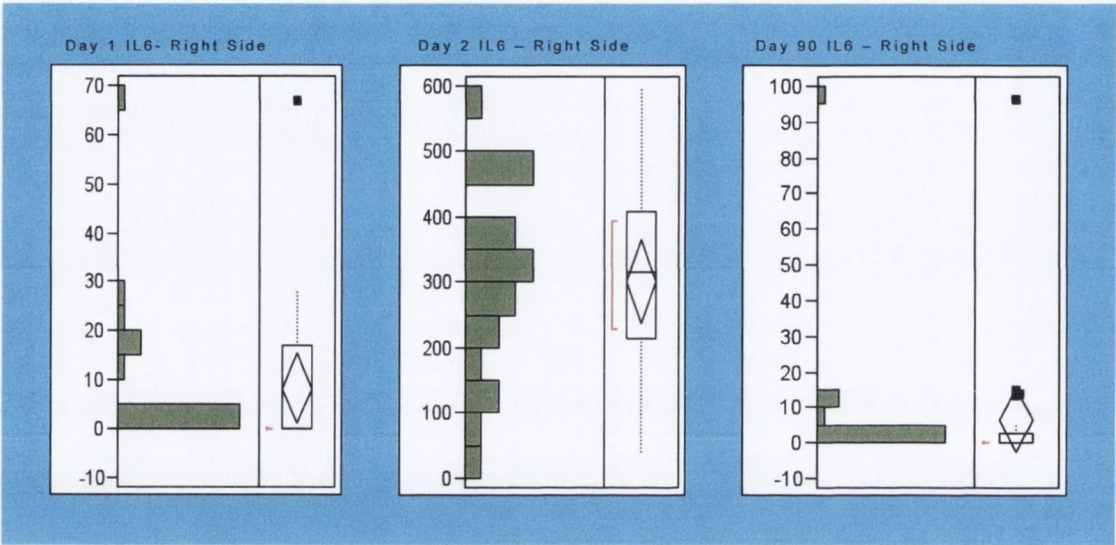


Figure 3.3.4: GCF IL-6 frequency distribution (pg/ml)

Wilcoxon signed ranks tests between the time points showed a statistical difference between Day 1 and Day 2 ($p=0.0001$); between Day 2 and Day 90 ($p=0.0001$). The difference between Day 1 and Day 90 was not seen to be significant ($p=0.32$)

Pg/ml	Day 1	Day 2	Day 90
Mean	8.3	301.8	6.63
Median	0	314.8	0
Interquartile Range	0-16.8	213.7-409.6	0-2.9

Table 3.3.4: GCF IL-6 means, medians and ranges (pg/ml)

3.3.5 Salivary IL-6 Frequency Distribution and Analysis

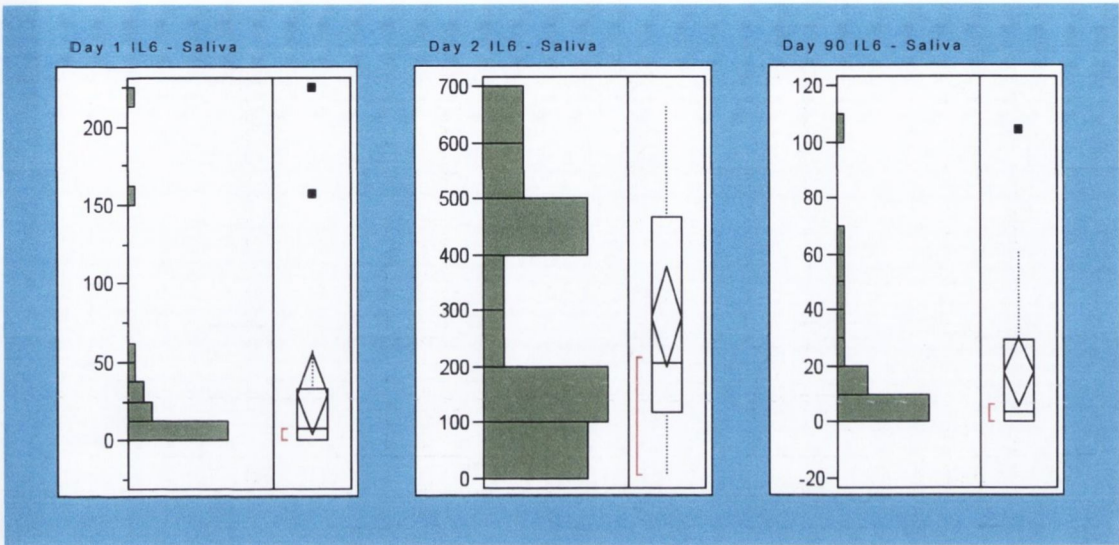


Figure 3.3.5: Salivary IL-6 frequency distribution (pg/ml)

Wilcoxon signed ranks tests between the time points showed a statistical difference between Day 1 and Day 2 ($p=0.001$); between Day 2 and Day 90 ($p=0.0001$). The differences between Day 1 and Day 90 were not significant ($p=0.26$).

	Day 1	Day 2	Day 90
Mean	29.5	288.4	17.9
Median	7.89	205.82	3.86
Interquartile Range	0-32.6	117.4-466.5	0-29

Table 3.3.5: Salivary IL-6 means, medians and ranges (pg/ml)

3.3.6 GCF IL-10 Frequency Distribution and Analysis

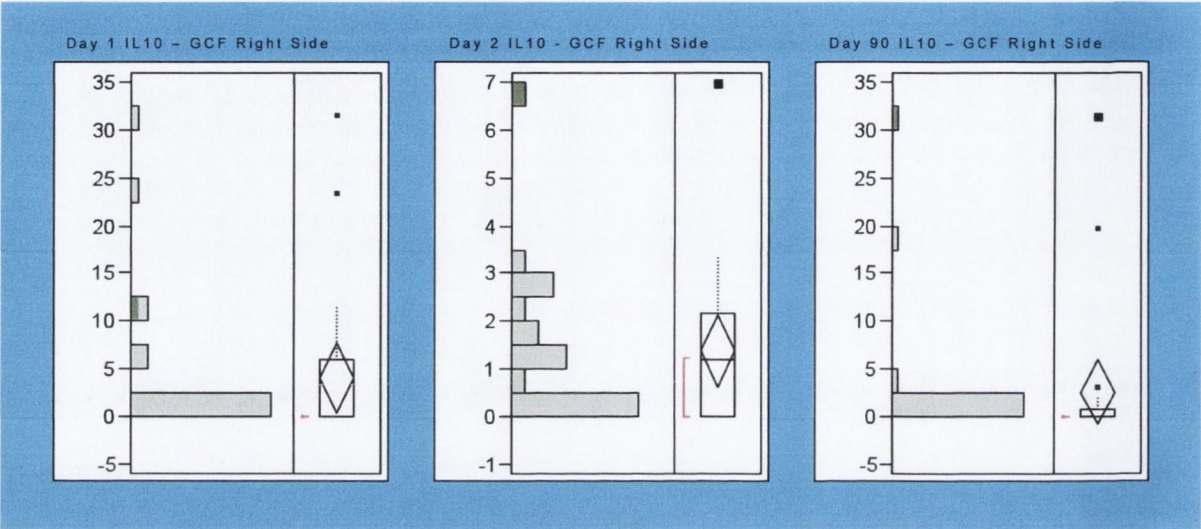


Figure 3.3.6: GCF IL-10 frequency distribution (pg/ml)

Wilcoxon signed ranks tests between the time points showed no statistical difference between Day 1 and Day 2 ($p=0.42$); no difference between Day 2 and Day 90 ($p=0.38$). The difference between Day 1 and Day 90 were also not significant ($p=0.36$)

	Day 1	Day 2	Day 90
Mean	8.19	2.75	5.2
Median	0	2.4	0
Interquartile Range	0-11.9	0-4.4	0-1.6

Table 3.3.6: GCF IL-10 means, medians and ranges (pg/ml)

3.3.7 Salivary IL-10 Frequency Distribution and Analysis

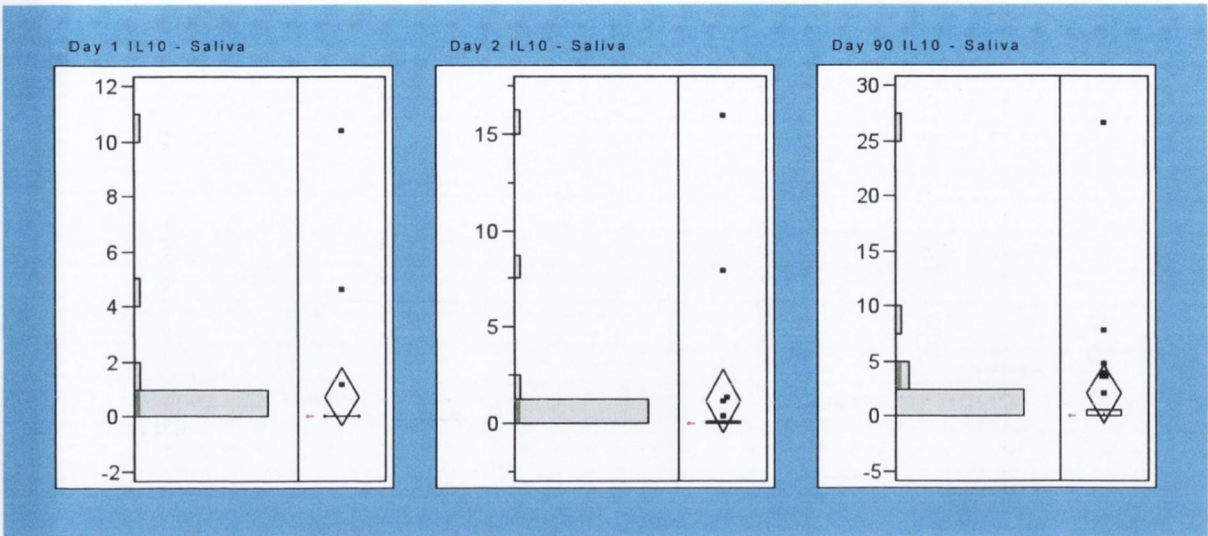


Figure 3.3.7: Salivary IL-10 frequency distribution (pg/ml)

Wilcoxon signed ranks tests between the timepoints showed no statistical difference between Day 1 and Day 2 ($p=0.62$); no difference between Day 2 and Day 90 ($p=0.94$). The difference between Day 1 and Day 90 were also not significant ($p=0.31$)

	Day 1	Day 2	Day 90
Mean	0.72	1.19	2
Median	0	0	0
Interquartile Range	0-0	0-0	0-0.5

Table 3.3.7: Salivary IL-10, means medians and ranges (pg/ml)

3.4 CRP Analysis

3.4.1 Blood CRP

Boxplot diagrams for blood CRP levels over the three time points. Wilcoxon signed ranks tests between the time points showed a statistical difference between Day 1 and Day 2 ($p=0.0003$); between Day 2 and Day 90 ($p=0.0002$). The difference between Day 1 and Day 90 were not significant, although it was approaching significance ($p=0.073$)

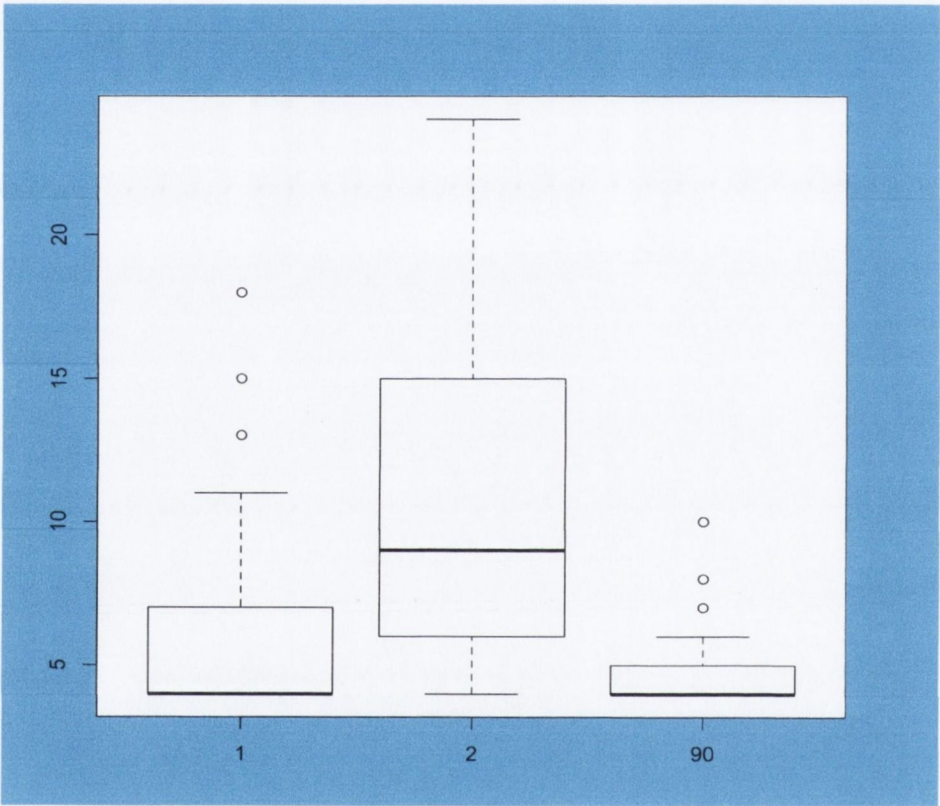


Figure 3.4.1: Boxplot of blood CRP levels days 1, 2 and 90 (mg/l)

Day 1	Day 2	Day 90
4	9	4

Table 3.4.1: Median values blood CRP (mg/l)

3.4.2 Salivary CRP

Boxplot diagrams for saliva CRP levels over the three time points. Wilcoxon signed ranks tests between the time points showed a statistical differences between Day 1 and Day 2 ($p<0.0001$); between Day 2 and Day 90 ($p<0.0001$). The difference between Day 1 and Day 90 was also significant ($p=0.006$)

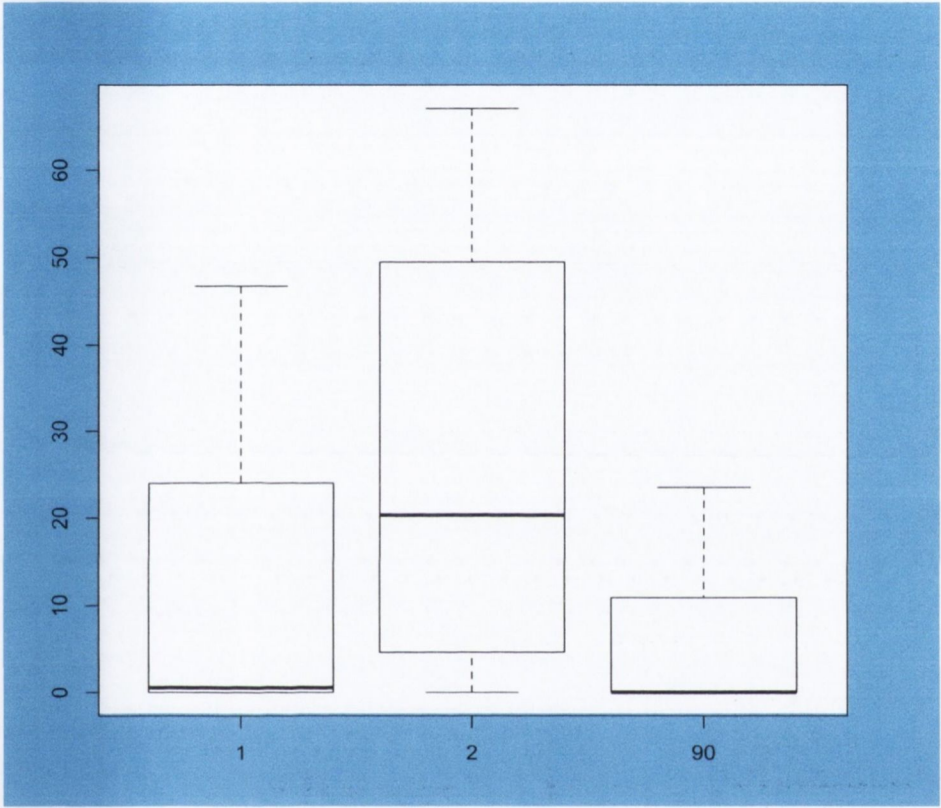


Figure 3.4.2: Boxplot salivary CRP day 1, 2 and 90 (ng/ml)

Day 1	Day 2	Day 90
0.541	20.396	0

Table 3.4.2: Median salivary CRP values (ng/ml)

3.4.3 GCF CRP

Boxplot diagrams for GCF CRP levels over the three time points. Wilcoxon signed ranks tests between the time points showed a statistical differences between Day 1 and Day 2 ($p=0.0001$); between Day 2 and Day 90 ($p=0.0001$). The difference between Day 1 and Day 90 was also significant ($p=0.02$)

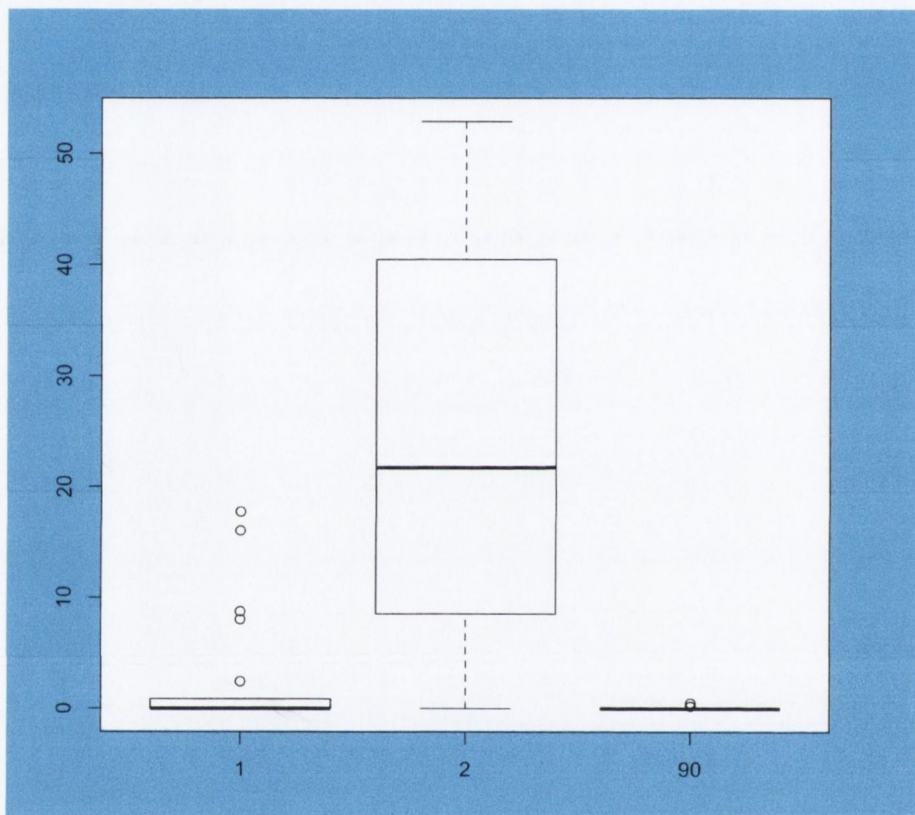


Figure 3.4.3: Boxplot of GCF CRP day 1, 2 and 90 (ng/ml)

Day 1	Day 2	Day 90
0	21.73	0

Table 3.4.3: Median GCF CRP values (ng/ml)

3.5 Clincial Results

3.5.1 Pocket Depth

Boxplot diagram for the probing pocket depth at baseline and at re-evaluation (Day 90).

The median value at baseline was 4.11mm and at re-evaluation was 3.09mm. Wilcoxon signed rank test showed a significant difference between the time points ($p<0.0001$)

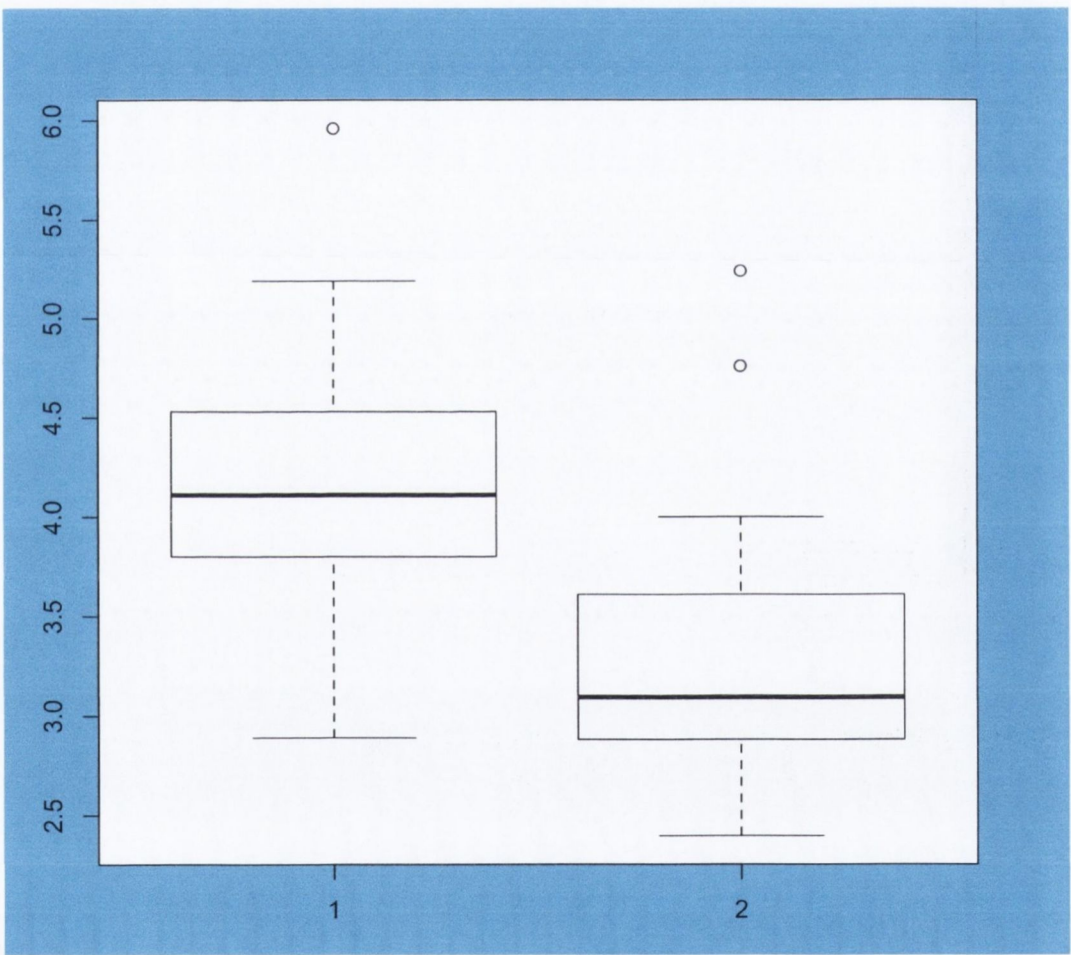


Figure 3.5.1: Boxplot of mean pocket dept at baseline and re-evaluation

3.5.2 Bleeding Scores

Boxplot Diagram for the bleeding scores at baseline and at re-evaluation (day 90). The median value at baseline was 54% and at re-evaluation was 29%. Wilcoxon signed rank test showed a significant difference between the timepoints ($p < 0.0001$)

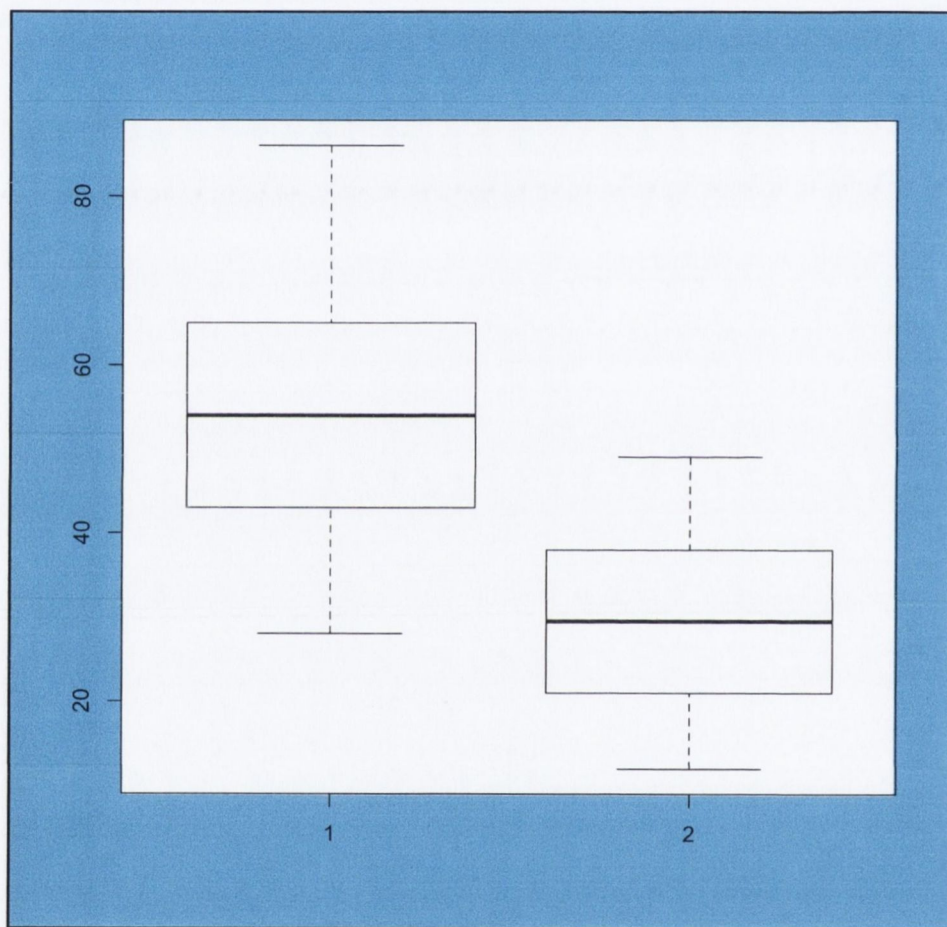


Figure 3.5.2: Boxplot of mean bleeding score at baseline and re-evaluation

3.6 Frequency of detection

GCF detection rates

	Day 1	Day 2	3 months
CRP	8/22 samples	21/22 samples	2/22 samples
IL-6	23/88 samples	56/88 samples	10/88 samples
IL-10	27/88 samples	29/88 samples	22/88 samples

Table 3.6.1: GCF detection rates

Saliva detection rates

	Day 1	Day 2	3 months
CRP	11/22 samples	21/22 samples	10/22 samples
IL-6	15/22 samples	22/22 samples	9/22 samples
IL-10	3/22 samples	5/22 samples	5/22 samples

Table 3.6.2: Saliva detection rates

3.7 CRP Correlations

3.7.1 Salivary/GCF CRP Day 1

The results were plotted against one another, as below. A Spearman rank correlation was done which showed a coefficient correlation of $r = 0.75$ ($p < 0.0001$). A Bland – Altman difference plot as undertaken to assess agreement.

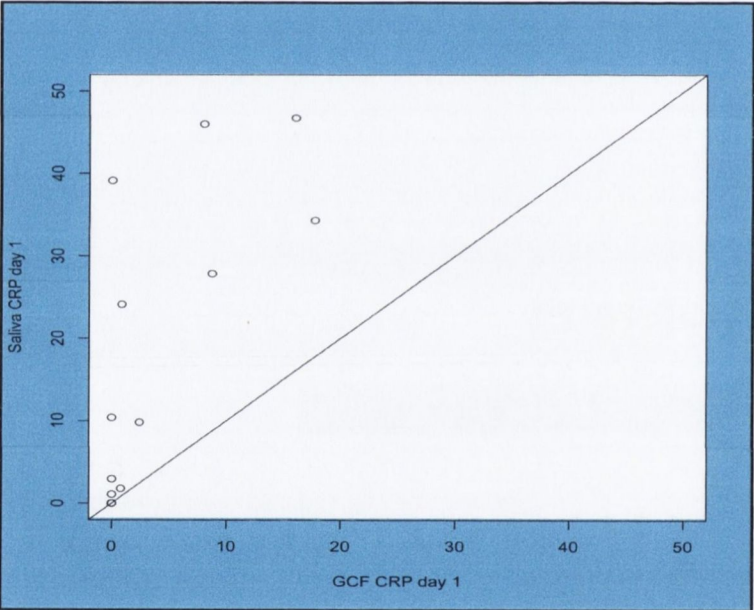


Figure 3.7.1.1: Plot of salivary CRP to GCF CRP on day 1

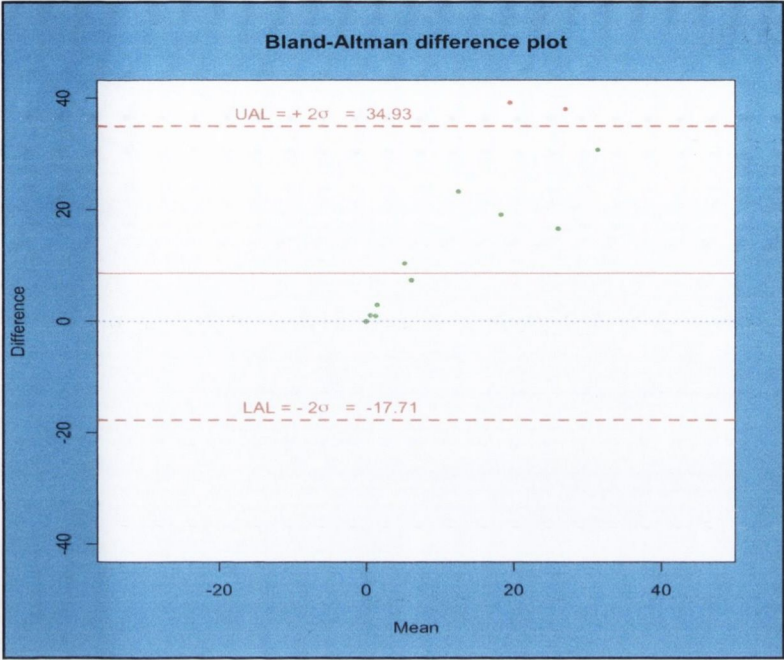


Figure 3.7.1.2: Bland Altman plot of salivary CRP to GCF CRP on day 1

Using the Bland Altman Difference plot the mean difference was 8.613 and the SD difference was 13.1. The wide spread of data about the line suggests agreement was relatively weak.

3.7.2 Salivary/GCF CRP Day 2

The results were plotted against one another, as below. A Spearman rank correlation was done which showed a coefficient correlation of $r = 0.79$ ($p < 0.0001$). A Bland – Altman difference plot as undertaken to assess agreement.

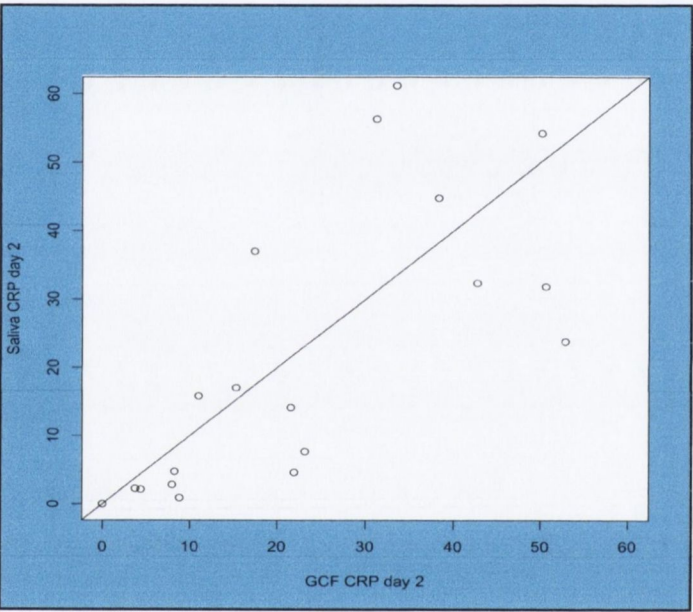


Figure 3.7.2.1: Plot of salivary CRP to GCF CRP on day 2

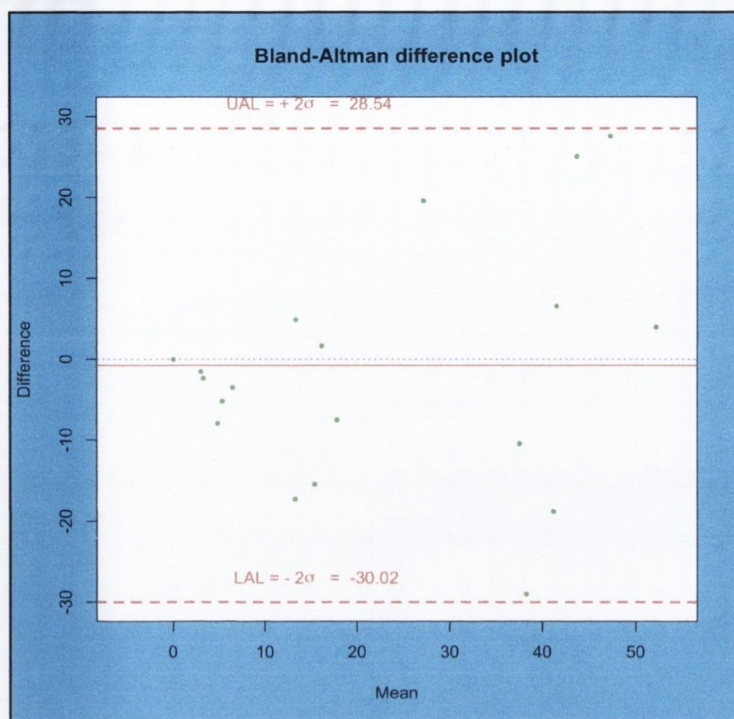


Figure 3.7.2.2: Bland Altman plot of salivary CRP to GCF CRP on day 2

Using the Bland Altman Difference plot the mean difference was 0.744 and the SD difference was 14.6. While the mean difference was small, the even distribution of data about this line suggests better agreement than Day 1, but with the wide spread of data about the line suggests agreement was relatively weak.

3.7.3 Salivary/GCF CRP Day 90

The results were plotted against one another, as below. A Spearman rank correlation was done which showed a coefficient correlation of $r = 0.38$ ($p = 0.07$). A Bland – Altman difference plot as undertaken to assess agreement.

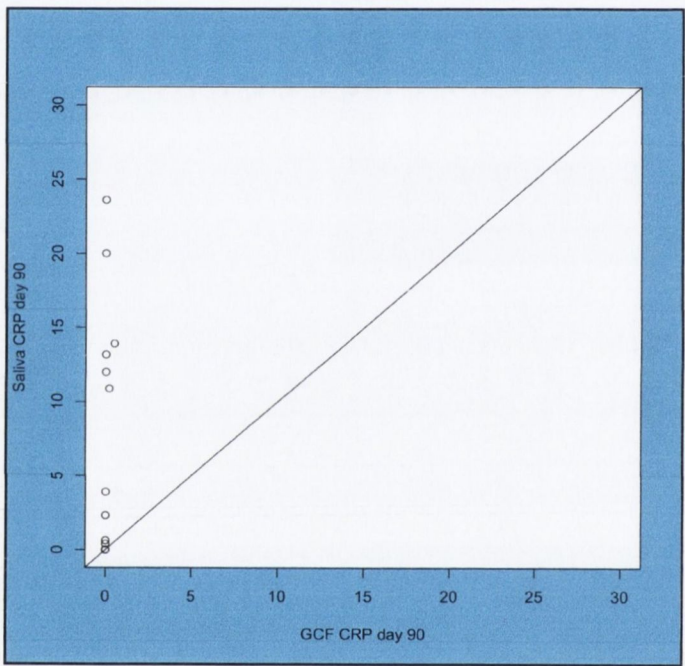


Figure 3.7.3.1: Plot of salivary CRP to GCF CRP on day 90

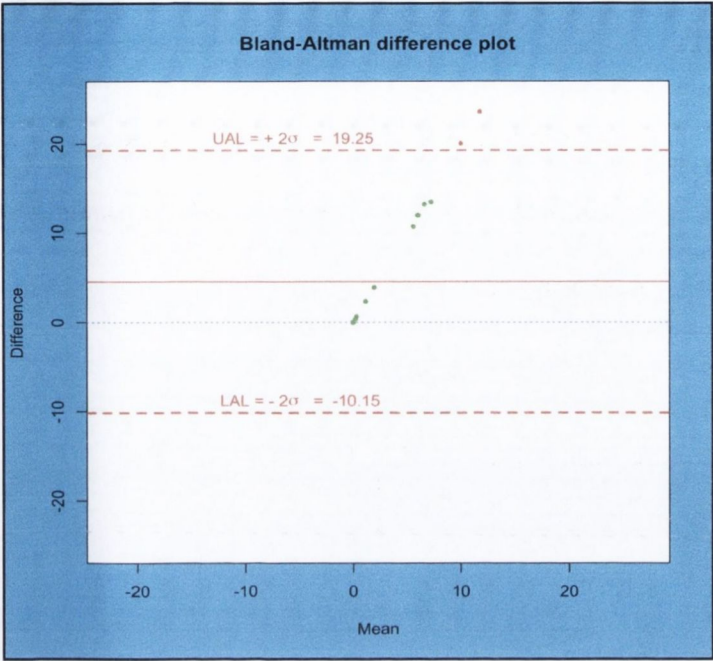


Figure 3.7.3.2: Bland Altman plot of salivary CRP to GCF CRP on day 90

Mean difference = 4.549 SD Difference = 7.3

Using the Bland Altman Difference plot the mean difference was 4.549 and the SD difference was 7.3. The mean difference between the measurements was small and the wide spread of data about the line suggests agreement was relatively weak.

3.7.4 Blood / GCF CRP Day 1

The results were plotted against one another, as below. A Pearson's correlation was done which showed a coefficient correlation of $r = 0.5$ ($p = 0.018$). A Bland – Altman difference plot was undertaken to assess agreement.

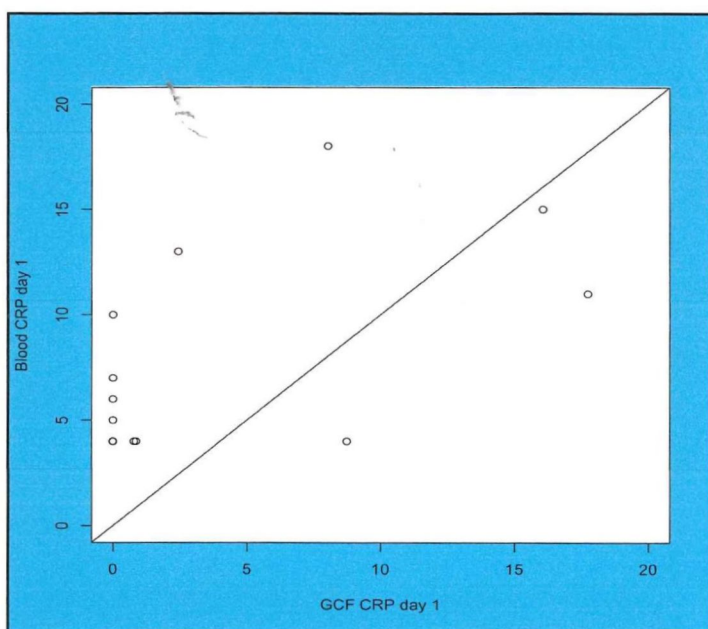


Figure 3.7.4.1 Plot of blood/GCF CRP to Day 1

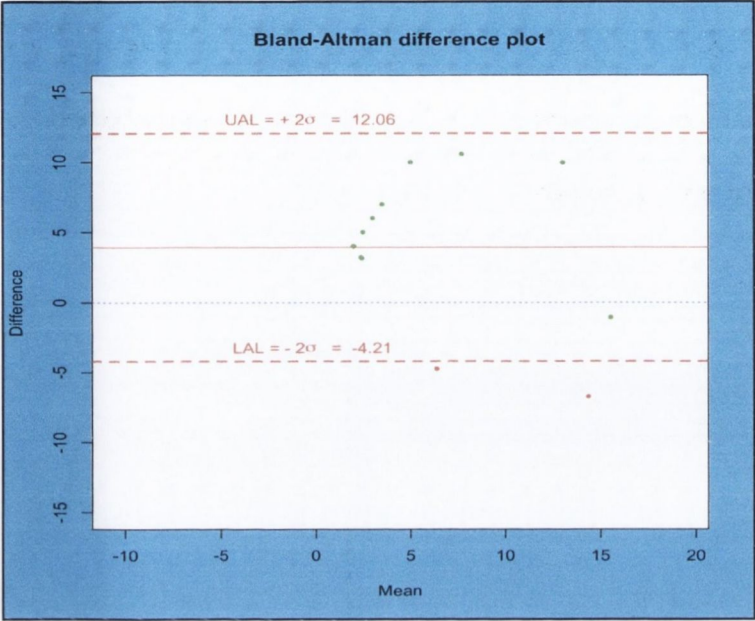


Figure 3.7.4.1: Bland Altman plot of blood/GCF CRP to Day 1

Mean difference = 3.925 SD Difference = 4.0

Using the Bland Altman Difference plot the mean difference was 3.925 and the SD difference was 4.1. The wide spread of data about the line suggests agreement was relatively weak.

3.7.5 Blood/GCF CRP Day 2

The results were plotted against one another, as below. A Spearman rank correlation was done which showed a coefficient correlation of $r = 0.71$ ($p = 0.0003$). A Bland – Altman difference plot was undertaken to assess agreement.

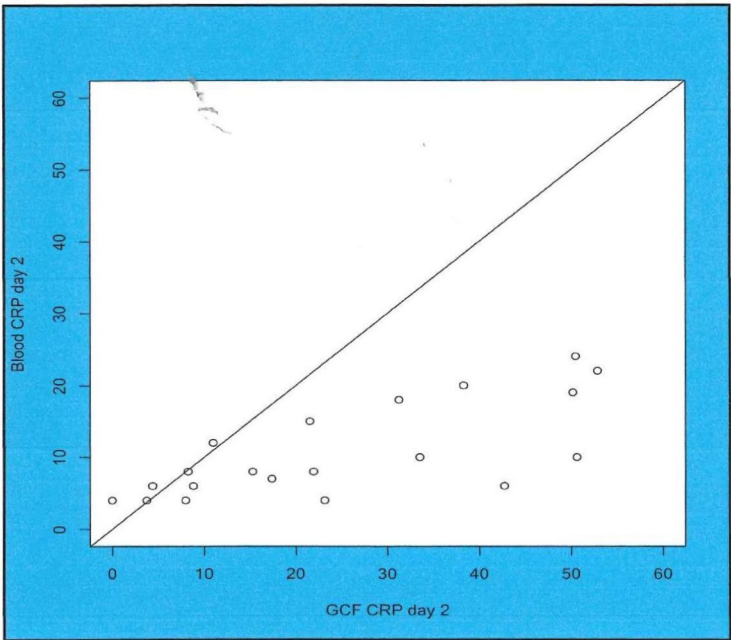


Figure 3.7.5.1: Plot of blood/GCF CRP to Day 2

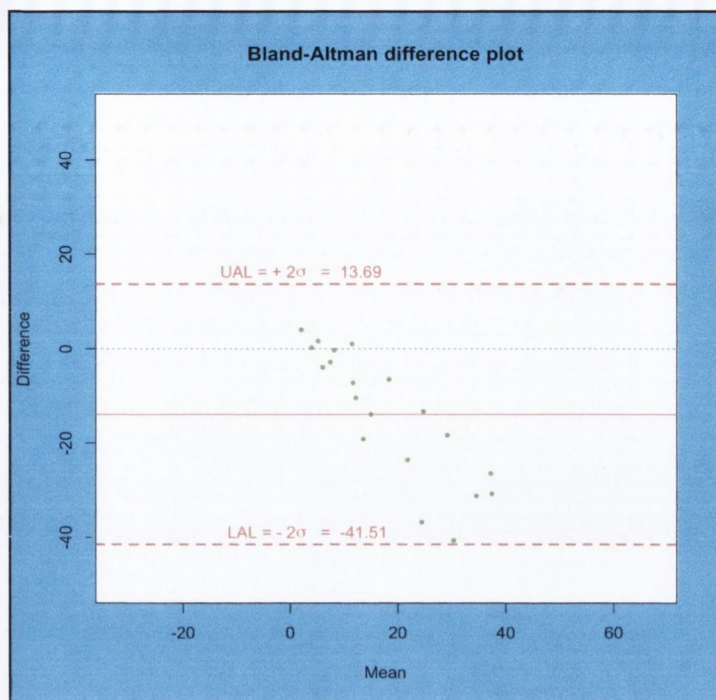


Figure 3.7.5.2: Bland Altman plot of blood/GCF CRP to Day 2

Mean difference = 13.911 SD Difference = 13.7

Using the Bland Altman Difference plot the mean difference was 13.911 and the SD difference was 13.7. The large mean difference and wide spread of data about the line suggests agreement was relatively weak.

3.7.6 Blood/GCF CRP Day 90

The results were plotted against one another, as below. A Spearman rank correlation was done which showed a coefficient correlation of $r = 0.54$ ($p = 0.008$). A Bland – Altman difference plot as undertaken to assess agreement.

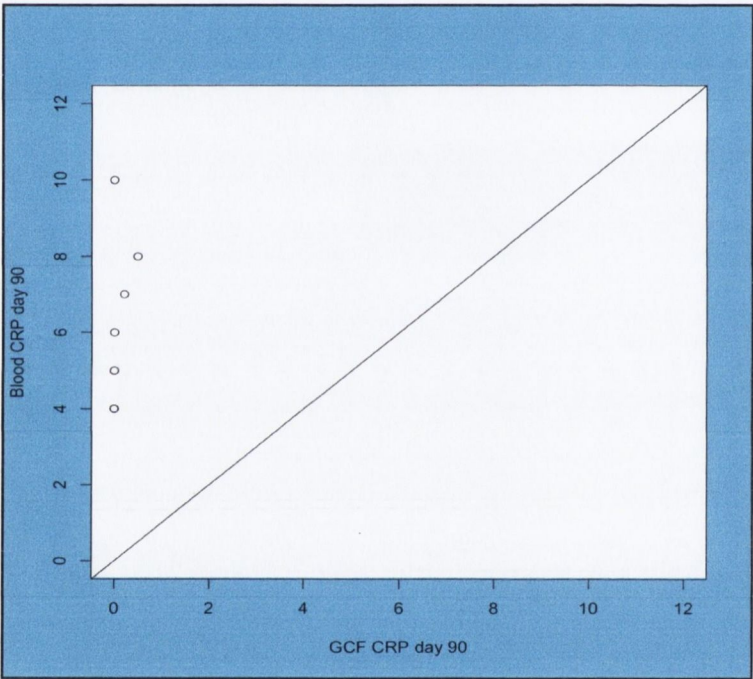


Figure 3.7.6.1: Plot of blood/GCF CRP to Day 90

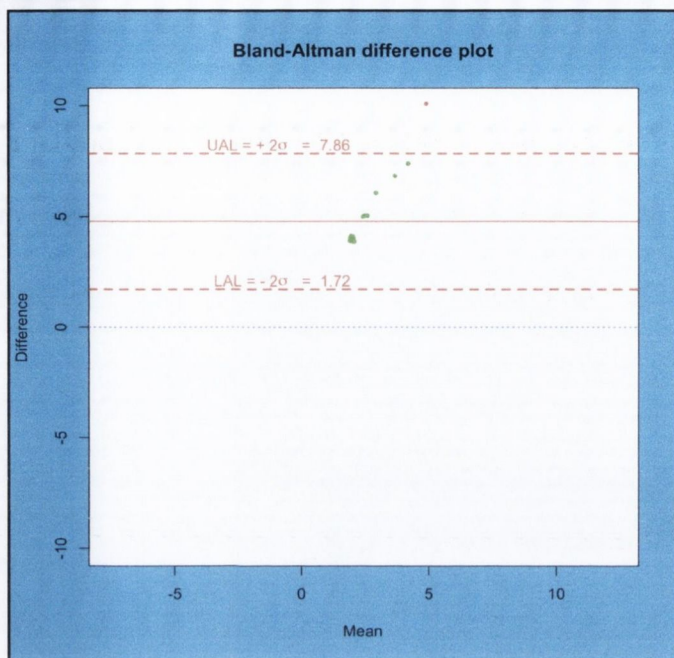


Figure 3.7.6.2: Bland Altman plot of blood/GCF CRP to Day 90

Mean difference = 4.786 SD Difference = 1.5

Using the Bland Altman Difference plot the mean difference was 4.786 and the SD difference was 1.5. The relatively large difference suggests bias but the skewed spread of data about the mean line suggests agreement was modest at this time point.

3.7.7 Blood/Salivary CRP Day 1

The results were plotted against one another, as below. A Spearman rank correlation was done which showed a coefficient correlation of $r = 0.68$ ($p = 0.0004$). A Bland – Altman difference plot as undertaken to assess agreement.

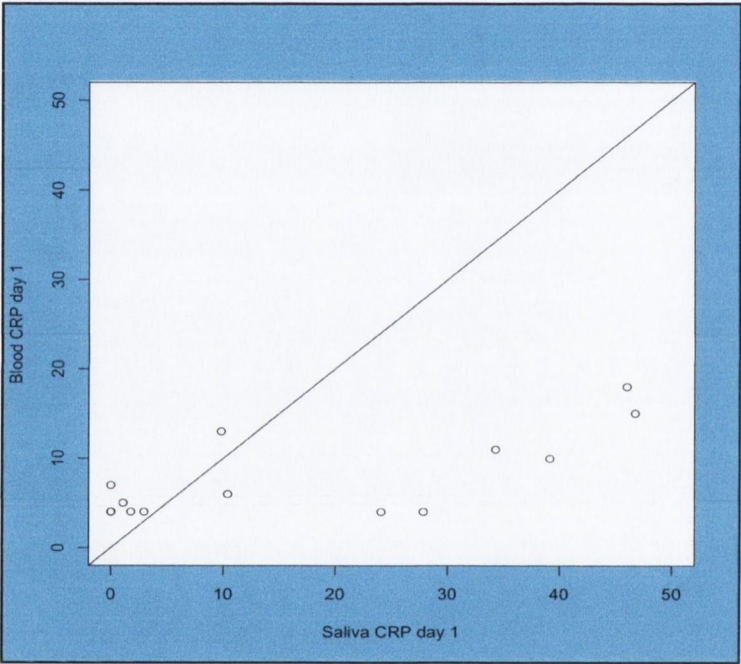


Figure 3.7.7.1: Plot of blood/salivary CRP Day 1

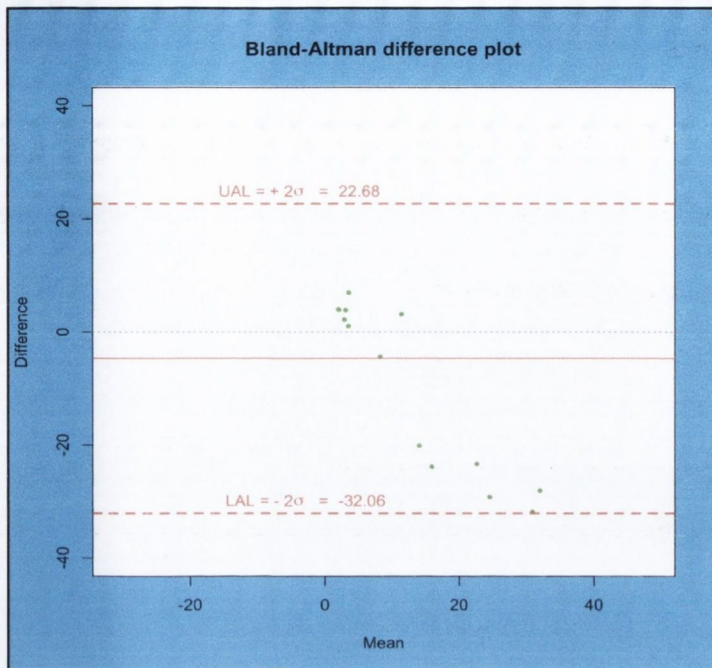


Figure 3.7.8.2: Bland Altman plot of blood/salivary CRP Day 1

Mean difference = 4.688 SD Difference = 13.6

Using the Bland Altman Difference plot the mean difference was 4.688 and the SD difference was 13.6. The small mean difference and even spread of data about the line suggests agreement was relatively good, although the spread was wide.

3.7.8 Blood/Salivary CRP Day 2

The results were plotted against one another, as below. A Spearman rank correlation was done which showed a coefficient correlation of $r = 0.70$ ($p = 0.0002$). A Bland – Altman difference plot as undertaken to assess agreement.

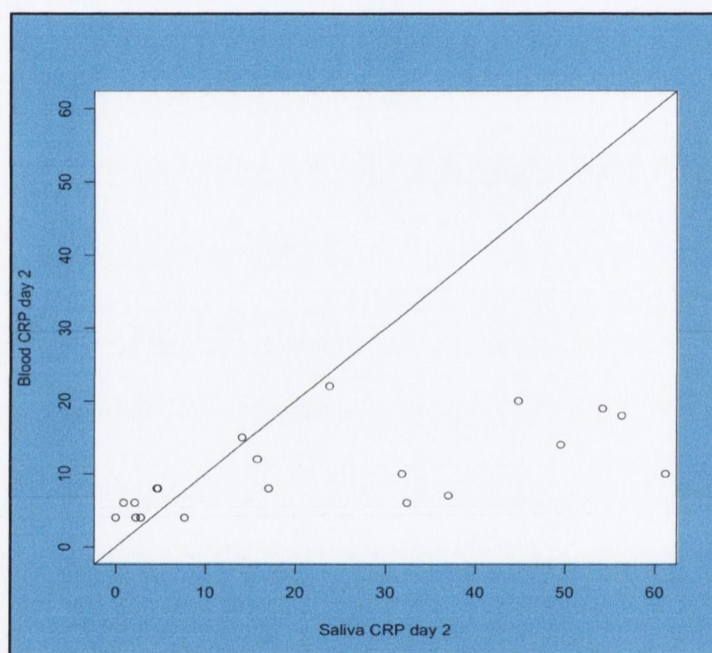


Figure 3.7.8.1: Plot of blood/GCF CRP Day 2

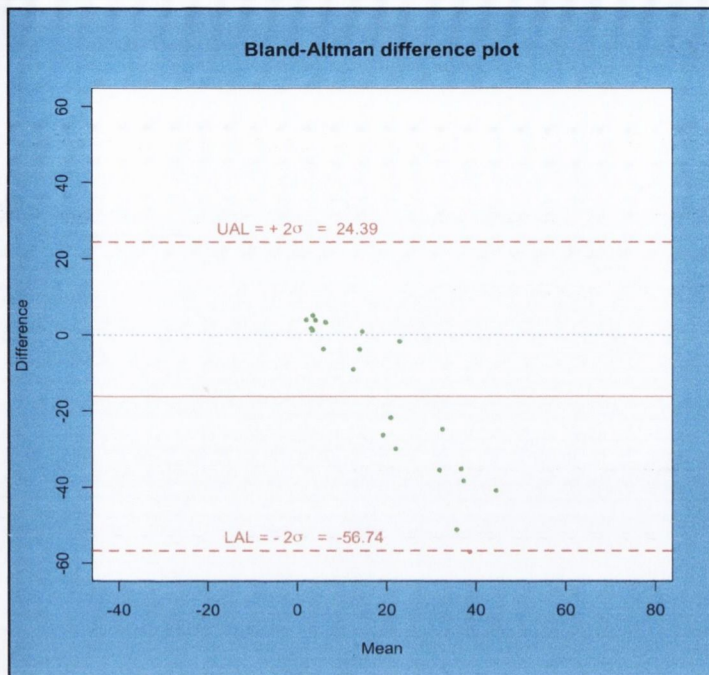


Figure 3.7.8.2: Bland Altman plot of blood/salivary CRP Day 2

Mean difference = 16.176 SD Difference = 20.2

Using the Bland Altman Difference plot the mean difference was 16.176 and the SD difference was 20.2. The large mean difference and wide spread of data about the line suggests agreement was relatively weak.

3.7.9 Blood/Salivary CRP Day 90

The results were plotted against one another, as below. A Spearman rank correlation was done which showed a coefficient correlation of $r = 0.52$ ($p = 0.01$). A Bland – Altman difference plot as undertaken to assess agreement.

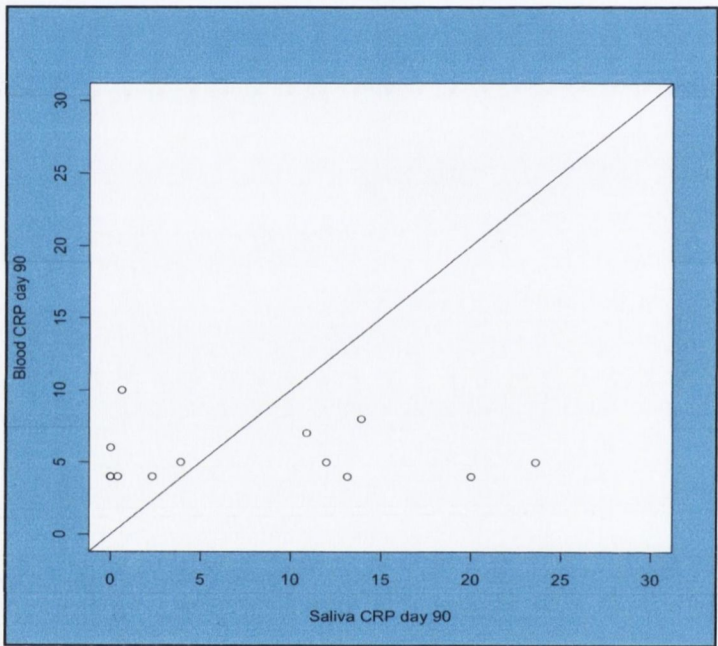


Figure 3.7.9.1: Plot of blood/GCF CRP to Day 90

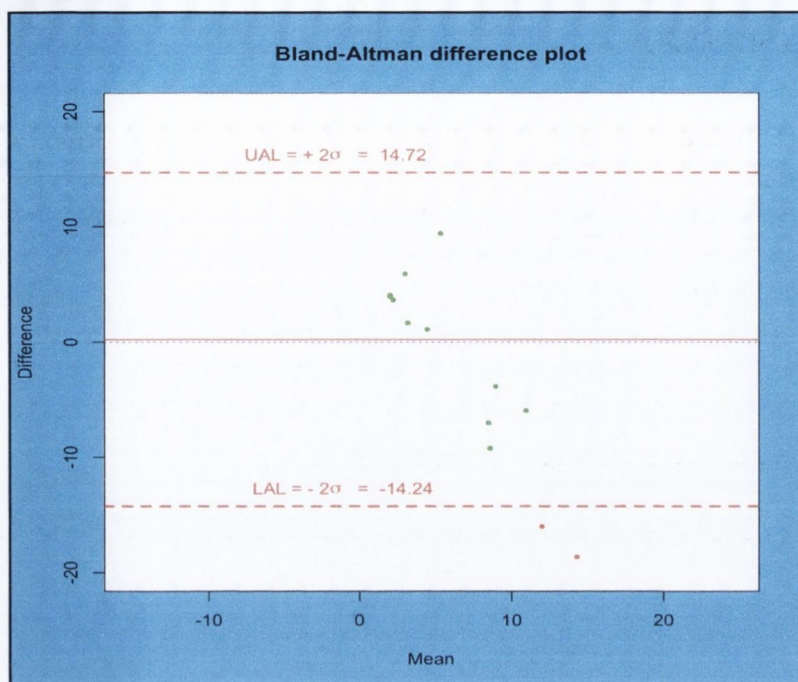


Figure 3.7.9.2: Bland Altman plot of blood/salivary to CRP Day 90

Mean difference = 0.236 SD Difference = 7.2

Using the Bland Altman Difference plot the mean difference was 0.236 and the SD difference was 7.2. The mean difference between measurements was very small with a wide but even distrubtion of data which suggest relatively good agreement.

3.8 Fishers Exact Tests Blood CRP

Fisher Exact tests were done to compare the number of patients in which blood CRP was detectable and was not detectable between the three time points of the study

	<5mg/l	≥5mg/l	Total
Patients Day 1	14	8	22
Patients Day 2	4	18	22

Table 3.8.1: Fishers exact test Day 1-2; $p= 0.0051$

	<5mg/l	≥5mg/l	Total
Patients Day 2	4	18	22
Patients Day 90	15	7	22

Table 3.8.2: Fishers exact test Day 2-90; $p= 0.001$

	<5mg/l	≥5mg/l	Total
Patients Day 1	14	8	22
Patients Day 90	15	7	22

Table 3.8.3 Fishers exact test Day 1-90; $p= 1$

4. Discussion

The aim of this study was to assess the changes in CRP levels in blood, saliva and GCF in response to initial therapy for chronic periodontitis and to monitor the alterations of the levels of IL-6 and IL-10 in the saliva and GCF of these patients. The concentration of these markers and cytokines have been shown to alter following periodontal therapy in previous studies, but few, if any studies have looked at their concentrations locally in the oral fluids and systemically as measured in the blood and sought to investigate a possible relationship between them.

In this study the clinical situation was significantly improved in terms of improvement in pocket depth (PD), bleeding scores (BS) and plaque scores (PS). Patient's means were used to quantify the PDs in patients before treatment and at re-evaluation. The differences between the examinations in terms of pocketing was seen to be significant and closely matched the results seen in other studies employing mean results in studies evaluating non surgical therapy (Serino et al., 2001). Significant improvements were also seen in terms of BS and PS, although the level of bleeding and plaque at re-evaluation was seen to be higher than that reported in some previous studies (Badersten et al., 1984, Nordland et al., 1987). These findings can perhaps be attributed to the fact that in these and other such studies, patients often received repeated oral hygiene instruction and /or tooth polishing at repeated intervals during the study. In our study the patients received OHI at their initial assessment visit and this was reinforced at the two debridement visits. Also, the duration of the current study was only three months to allow observation of the corresponding changes in cytokines levels in response to therapy. It is often the case that

initial therapy is followed by re-debridement of unresponsive sites or indeed surgical therapy to gain access to deeper sites or facilitate corrective therapy. As a result, in this group of patients, further therapy was often provided as necessary but did not fall within the timelines of the study.

Blood CRP was measured at each time point using the Quikread CRP machine. While the number of patients with detectable CRP (5mg/l and above) values on Day 1 was only seven, on Day 2 this number went up to 18. Fishers exact test showed a statistically significant difference between the proportions of patients within each group on the first two days ($p=0.001$). The mean CRP value in blood on day 1 was 6.4mg/l and this increased to 10.86mg/l on day two, after the first session of debridement. This is in agreement with previous findings by other groups that periodontal therapy results in an acute systemic inflammatory response and in particular, that the systemic levels of CRP are increased twenty four hours after this therapy (Tonetti et al., 2007). At the three month re-evaluation (Day 90), the mean blood CRP level was seen to have decreased to 4.8mg/l. Although statistical analysis did not show a significant difference between the baseline and re-evaluation CRP levels, there was a marked difference in the CRP values following the periodontal treatment. There have been conflicting findings amongst studies assessing the CRP response to periodontal therapy with some authors showing a significant difference and others not demonstrating such a relationship (Vidal et al., 2009, Ide et al., 2003). What is evident is that in most of these studies, high sensitivity (hs) assays were used to assess levels of hs-CRP. The method we used has been shown to be

as reliable as the laboratory assays but the lower sensitivity limit of 5mg/l did restrict the lower limit of detection in our study group. This was done for ease of procedure and patient comfort, as it was felt repeated blood sampling may deter patients from returning and completing the study. This was perhaps a contributing factor in the 100% completion rate in the current study. However, had hs-CRP assays been undertaken, the more sensitive detection of the CRP at lower minimum levels may have lead to an increased number of blood samples in which this analyte was positively determined. This in turn may have shown that the blood CRP values decreased significantly following periodontal therapy, as the difference was approaching significance in our study results.

CRP values analysed in saliva and GCF showed that there were significant increases in values at Day 2 and that this spike in CRP was seen to decrease significantly at three months. Additionally, there was a significant decrease in locally measured CRP values in both saliva and GCF between baseline and at the end of the study. Therefore the periodontal therapy seemed to reduce the local CRP levels as measured in the oral fluids.

IL-6 and IL-10 were also measured in saliva and GCF at the three time points of the study. Both these cytokines play an active role in the inflammatory process, with IL-6 seen to have a pro-inflammatory role and IL-10 an anti-inflammatory one. IL-6 in the GCF was observed to increase significantly twenty four hours after the first debridement session and then to decrease to around baseline level at three months. The difference between the baseline levels and three month levels were not significantly different. A similar pattern was seen with IL-6 as measured in saliva at the three time points. Initial

debridement resulted in a large, significant increase in salivary IL-6 when measured twenty four hours later. At the re-evaluation stage, the cytokine level had reduced to a level similar to what was seen at baseline.

The detection rate ranges of CRP and IL-6 in both GCF and saliva was 9-95% and 41-100% respectively over the course of the study. This was somewhat in agreement with previous studies looking at these cytokines in GCF (Tuter et al., 2007, Goutoudi et al., 2012). In the present study the lowest rate of detection for CRP was 9% in GCF at the three month examination – this may be due to the fact that the improved periodontal condition and reduced local inflammation caused a reduction in the amount of CRP. It may also be that there was less GCF in these more healthy sites and thus there may not have been enough GCF eluted at the analysis stage to allow detection of the analytes in question. Although most studies assessing levels of cytokines in periodontal patients use ELISA techniques, adequate amounts of aliquots are necessary to allow detection. Three separate periopaper samples were taken from each site at each time point give adequate GCF volume to allow individual analysis for each cytokine but it may be the amount of GCF volume and cytokine within the GCF was too low and below the lower detection range of the ELISA assay used. Other techniques such as multiplexed bead immunoassay (Luminex multi-analyte profiling) can be used to detect multiple cytokines in a single sample and require less sample volume to do so. However, multiple cytokines are often detected on a single plate, many of which were not of consideration in this study. Also, when a large

number of samples need to be analysed, as in the current study, the extra costs involved with multiplex assays can be excessive.

With IL-10 the detection rate of 25-31% in GCF and only 3-6% in saliva did not allow meaningful analysis. It was observed that there were no significant differences between IL-10 in GCF or saliva at any of the time points investigated. This is actually in agreement with earlier studies which measured IL10 levels in GCF before and after periodontal treatment (Toker et al., 2008, Goutoudi et al., 2004). These studies failed to show a positive effect of treatment on IL-10 concentrations. The detection rates in these studies varied from 26-92%. One possible explanation for the disagreement in the frequency in detection of IL10 in GCF between these studies and our study could be attributed to the sensitivity and specificity of the immunoassays used. Also, Goutoudi *et al* noted that IL-10 concentrations were significantly higher in non diseased sites. This would fit its profile as a cytokine with an anti-inflammatory function. All of the GCF samples were taken from sites $\geq 5\text{mm}$ and many were seen to be bleeding at the assessment visit, indicating active inflammation and disease in these sites. Taking into consideration that all of the study participants had moderate or severe generalised chronic periodontitis, when combined with high initial baseline bleeding scores indicating disease, it can deduced that the majority of sites in our study population were diseased and so this may have contributed to the very low detection frequency of IL-10 in GCF as well as in saliva.

The relationships between CRP values in the three different fluids of blood, saliva and GCF was investigated and explored. Spearman rank correlations were first applied to establish the relationships between CRP in blood, GCF and saliva at the three time points in question. The relationship between saliva and GCF was seen to be strongest on Days 1 and 2 with coefficient correlations of 0.75 and 0.71 respectively. The Bland –Altman analysis is primarily used to assess agreement between two differing parameters and is a visual representation of the agreement between two continuous measurements. The purpose of a Bland Altman plot is to try and determine whether a new method of measurement is in agreement with an established one. Ideally, when there is good agreement between both measurements, the points will be randomly distributed around the zero line on the plot - a tight distribution about this line would suggest excellent agreement and no bias. For Day 1 saliva/GCF the agreement would seem modest at best with some bias, but this improves on Day 2, when both measurements of CRP values increased significantly. Day 90 saliva/GCF relationship was not as close with a coefficient of correlation of 0.38 and relatively poor agreement as observed on the Bland –Altman plot.

In the relationship between blood and GCF, the relationship was most pronounced on the second day again, with a correlation coefficient of 0.71 for the spearman rank test. On the other days (1 and 90) the correlation was in the order of 0.5. The agreement as deduced from the Bland-Altman was seen to be poor between blood and GCF for all times assessed, with day 2 being the most closely in agreement. This would suggest that the

large spike in GCF CRP following debridement may have an influence on CRP blood levels, but at times of normal activity the relationship is not as linear.

The relationship between blood CRP and saliva CRP at the three time points of the study appeared to have better agreement. Spearman rank coefficient correlations of 0.7, 0.68 and 0.5 were seen between the CRP measures in these two fluids at day 1, 2 and 90 respectively. All correlations were significant, with those correlations on days 1 and 2 being bigger, suggesting a closer relationship. The Bland-Altman plots suggest improved agreement between the measures of CRP in blood and saliva in comparison to the agreement that was seen between other CRP measures. Although it would appear that the differences between the measurements from the respective fluids are relatively widely scattered, with wide confidence intervals, the points are evenly distributed about the bias line. Interestingly, it would appear from visual inspection that the agreement as deduced from the Bland –Altman plots is most pronounced on Days 1 and 90, which is prior to any therapy and at re-evaluation after therapy at 3 months. On Day 2, after active debridement, samples measured for CRP in both saliva and blood showed sharp increases in individuals and average CRP values as discussed earlier. Although the correlation on this day was strongest between the two measures (0.7), the agreement was the poorest, suggesting that although CRP values changed that day, the values did not seem to alter in a uniform manner within both fluids and within individual patients. However, as seen from the correlations and agreement plots between saliva CRP and blood CRP on days 1 and 90, it would appear that there is a close relationship between the two measures. It

hasn't been previously shown in any studies that blood CRP and saliva CRP can be measured individually and show a corresponding relationship. These findings would suggest a tentative relationship between the two, at least when measured without any active periodontal treatment or ongoing known systemic inflammatory source.

It has being widely shown that periodontal disease is associated with increased systemic inflammation, in the form of cytokines such as IL-6 and acute phase proteins such as CRP (Loos et al., 2000) . Many of these same inflammatory markers have been shown to be associated with and be markers for, cardiovascular disease (Ridker and Silvertown, 2008). Following on from this, several clinical interventional trials have sought to explore and characterise this relationship between periodontal disease and systemic inflammation and inflammatory markers. It has been shown that non surgical periodontal therapy can significantly reduce both serum CRP and IL-6 levels (D'Aiuto et al., 2005). Patients treated with non surgical therapy also showed a significant increase in systemic CRP and IL-6 levels shortly after intervention possibly caused by the massive bacterial invasion of the blood stream or localised trauma releasing inflammatory mediators (Ide et al., 2004, D'Aiuto et al., 2005). IL-6 levels increased within a couple of hours of therapy whereas CRP values tending to peak after 24 hours or so. Our results also showed a sharp increase in systemic CRP and local CRP and IL-6 levels approximately 24 hours after treatment, which were seen to fall back to baseline level at 3 months, or indeed below such levels. In our study, the levels of blood CRP at 3 months were below the baseline values, but this was not significant. Results from elsewhere have shown conflicting results as regards whether

periodontal therapy can reliably reduce CRP levels, although a recent systematic review found modest evidence of a treatment induced reduction in systemic CRP in the order of 0.5mg/l (Paraskevas et al., 2008). The mean change observed within the present study was in the order of 1.4mg/l.

A limitation of this study was possibly the use of the Quikread CRP machine. While this allowed quick and easy access to patient blood samples which could be analysed at chairside, the lower sensitivity level of 5mg/l did not allow us to accurately quantify all blood CRP values. If high sensitivity CRP analysis had been used, we could have assessed the changes occurring as a result of therapy in a more accurate fashion. As it is, the fluctuations in CRP values in the short term due to debridement were amply demonstrated, with a subsequent decline to below baseline values after 3 months. If the suggested guidelines of the AHA are considered, the Quikread device may indeed be very useful in accurately identifying patients with increased levels of systemic CRP due to periodontal disease who thus may be at risk of CVD. Person *et al* suggested that CRP levels in blood >3mg/l be considered in the highest risk category for CVD development (Pearson et al., 2003) In total, one third of patients had detectable levels of CRP at baseline, levels which were $\geq 5\text{mg/l}$. These levels are clinically much higher than the concentration suggested by the AHA as putting patients into the highest risk category for future CVD. Chair side analysis can be used to accurately assess a patients systemic CRP levels. This simple test, when taken in conjunction with a full medical and dental history

can be reliable enough indicate the need for further assessment by the patient's own medical practitioner, especially in the absence of other potential inflammatory sources.

While there are studies that have shown improvements in inflammatory markers and other surrogate markers of CVD such as endothelial dysfunction after periodontal therapy (Tonetti et al., 2007), virtually no studies have looked at hard clinical endpoints in terms of cardiovascular disease. One study which tried to do so and focused on the secondary prevention of cardiac events was the Periodontitis and Vascular Events (PAVE) study (Offenbacher et al., 2009). Not only were clinical periodontal results similar for those in treatment and preventative groups, but adverse cardiovascular events were of similar frequency for both groups, suggesting that the treatment did not have a positive impact on cardiac events. A recent scientific statement from the American Heart Association called into question the validity of much of the research suggesting a direct link between periodontal disease and cardiovascular disease (Lockhart et al., 2012). The authors did an exhaustive review of the available literature to ascertain the association between CVD and PD. While the association is evident between the two diseases, as outlined in our earlier literature review, no causal relationship has been fully elicited. While the association is independent of known confounders, it may be that adjustment for these confounders was inadequate, as these factors such as smoking play such a huge role in each disease. While the authors of the statement also acknowledged that many interventional studies have shown an improvement in systemic markers and other CVD risk factors, there is a paucity of studies that have been published which adequately show a

definitive positive effect of periodontal therapy on CVD in terms of long term outcomes and hard clinical endpoints, such as a reduction in MI, stroke or fatal coronary heart disease. More research is required in this area, with well designed and adequately powered studies to properly evaluate this possible relationship.

In conclusion, the current study indicates that periodontal therapy causes an initial sharp, acute increase in systemic inflammation which decreases to below baseline levels at the re-evaluation stage. These systemic changes are also mirrored at local level by an acute increase in inflammatory markers in saliva and GCF just after therapy, followed by a significant reduction in their levels over the course of the study. Significant relationships were observed between systemic and local CRP values at various times. This was most pronounced between CRP in blood and saliva and on the second day of the study which would suggest that debridement is eliciting an acute reaction locally which in turn is having an effect on the systemic level. The systemic CRP values observed in this current study were often found to be above suggested thresholds for being a risk for CVD events. The authors suggest that such chair side screening can be incorporated into the full periodontal assessment in order evaluate the response to periodontal therapy but also to isolate patients who could benefit from further investigation.

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6.Appendix

Appendix 1

THIS NOTEPAPER MUST NOT BE USED FOR
PRESCRIPTIONS OR INVOICING PURPOSES



**THE ADELAIDE & MEATH
HOSPITAL, DUBLIN**
INCORPORATING
THE NATIONAL CHILDREN'S HOSPITAL

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Dr. Paddy Delaney
Department of Periodontology
Dublin Dental School & Hospital
Lincoln Place
Dublin 2

June 28th 2010

REC reference: 2010/06/01

(Please quote REC reference and EudraCT number on all correspondence)

**Re: An Investigation of the Effects of Periodontal Treatment on Inflammatory
Markers in Blood, Saliva and Gingival Crevicular Fluid in Patients with Chronic
Periodontitis.**

Dear Dr. Delaney,

The SJH / AMNCH Research Ethics Committee, having reviewed the above
application at its meeting held on June 23rd 2010, decided to give ethical approval to
this proposed study.

The Committee however notes that you have directed any study participant with
questions concerning their rights in connection with this study to contact the
SJH/AMNCH Research Ethics Committee. The Committee asks that this statement be
removed from the Consent Form.

Yours sincerely,

Dr. Ray McDermott,
Chairman,
SJH/AMNCH Research Ethics Committee

Appendix 2

Patient information:

Study: An investigation of the effects of periodontal treatment on inflammatory markers in blood, saliva and gingival crevicular fluid in patients with chronic periodontitis.

Patient Name: _____

Patient Number: _____

Staff conducting research: Dr Paddy Delaney
Prof N Claffey
Dr Ioannas Polyzois

You are being asked to participate in a research study. In order to make an informed judgement on whether or not you want to be part of this research study, you should understand its potential risks and benefits. This process is called informed consent. This consent form gives you information about the research study which will be discussed with you. Once you understand the study, you will be asked to sign if you wish to participate.

Nature and duration of study:

This study looks at the levels of a certain protein in the blood of patients with periodontal (gum) disease and some other proteins in patients' saliva and gum fluid. The protein is called C – reactive protein (CRP) and is present in everyone in the population at various points. Ordinarily, this protein is not detectable in most people unless they have an infection of some sort. Nowadays there is increasing evidence that the CRP protein is present in a very slightly raised state in people who have periodontal disease and that it may be raised chronically or in the long term. While not causing any adverse affects, a raised CRP level has, in recent years, been linked to increased thickening of the walls of some arteries, which in susceptible patients can lead to coronary artery

disease. The other proteins to be studied are linked to this CRP and we want to see how they are also affected by gum treatment.

This study is to find out if patients with periodontal disease have raised CRP levels and other inflammation proteins associated with this CRP and if these are lowered after treatment of the periodontal disease. We know from other studies that the CRP is slightly increased in those with periodontal disease but as yet we are not sure how much treatment of the gum disease affects this protein.

What we propose to do if you decide to partake is to measure your CRP level before your periodontal treatment begins. We will do this using a drop of blood from your finger. We also will take a sample of fluid from the pocket around your tooth and a saliva sample. Then, the treatment of the gum disease takes the usual route of intense cleaning of the teeth under local anaesthesia, usually in 2 or 3 visits. Immediately after your treatment, we will measure the CRP level again and take the saliva and fluid samples again. Then at your 3 month recall to assess how well the gums have healed, we will again measure the CRP level and take oral fluid samples again. From the beginning you will be asked to maintain excellent oral hygiene as part of your treatment, in order to get the best results for your gums. All the treatment you receive is no different to what a patient who is not included in the study would get. The CRP sample is taken at the chairside using a special machine. It involves a small prick to your index finger to get a drop of blood to analyse. The fluids from your mouth are collected by using a small piece of paper and a saliva collector cup. There are no routine blood samples needed and you don't have to go to your doctor. Once we assess your gums at the recall visit and have our results, you will exit the study. If, at that point, it is decided you need some more treatment, you will receive the appropriate follow up care irrespective of your participation in the study.

Potential Risks:

Those who volunteer for this study have the same treatment as patients who do not, so there is no extra risks attached to the study.

Possible alternatives:

You may choose not to participate in this study. Your decision not to partake will have no impact on your treatment in the Dublin Dental School and Hospital.

Contact Details:

Dr Paddy Delaney,
Periodontology Postgraduate,
Trinity DDSH,
Lincoln place,
Dublin 2.

0879229539

Appendix 3

AGREEMENT TO CONSENT

The research project and the procedures associated with it have been explained to me. All experimental procedures have been identified and no guarantee has been given about the possible results. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that my participation is voluntary and that I may withdraw my consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to health care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner.

I understand that the sponsors and investigators have such insurance as is required by law in the event of injury resulting from the research.

I, the undersigned, hereby consent to participate in the above described project conducted at the Dublin Dental School and Hospital. I have received a copy of this consent form for my records. I understand that if I have any questions regarding this research, I can contact any of the doctors listed above. If I have questions concerning my rights in connection with this research, I can contact the involved clinician(Dr P. Delaney).

After reading this entire consent form, if you have no further questions about giving consent, please sign where indicated.

Doctor _____

Signature of subject _____

Witness _____

Date _____