

**PROTEASE INHIBITORS AND IRON-BINDING PROTEINS  
IN GINGIVAL CREVICULAR FLUID RELATED TO  
PERIODONTAL DISEASE STATUS**

**By**

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**To Dimitris**

## ΙΘΑΚΗ

Σαν βγεις στον πηγαιμο για την Ιθακη,  
να ευχεσαι ναυα μακρυσ ο δρομος,  
γεματος περιπετειες, γεματος γνωσεις.  
Τους Λαστρυγονας και τους Κυκλωπας,  
τον θυμωμενο Ποσειδωνα μη φοβασαι,  
τετοια στον δρομο σου ποτε σου δεν θα βρεις,  
αν μεν' η σκεψη σου υψηλη, αν εκλεκτη  
συγκινησις το πνευμα και το σωμα σου αγγιζει.  
Τους Λαστρυγονας και τους Κυκλωπας,  
τον αγριο Ποσειδωνα δεν θα συναντησεις,  
αν δεν τους κουβανεις μες στην ψυχη σου,  
αν η ψυχη σου δεν τους στηνει εμπρος σου.

Να ευχεσαι ναυα μακρυσ ο δρομος.  
Πολλα τα καλοκαιρινα πρωια να εινα  
που με τι ευχαριστησι, με τι χαρα  
θα μπανεις σε λιμενας πρωτοειδωμενους  
να σταματησεις σ' εμπορεια Φοινικικα,  
και τες καλες πραγματειες ν' αποκτησεις,  
σεντεφια και κοραλλια, κεχριμπαρια κ' εβενους,  
και ηδονικα μυρωδικα καθε λογης,  
οσο μπορείς πιο αφθονα ηδονικα μυρωδικα  
σε πολεις Αιγυπτιακες πολλες να πας,  
να μαθεις και να μαθεις απ'τους σπουδασμενους.

Παντα στο νου σου ναχεις την Ιθακη.  
Το φθασιμον εκει ειν' ο προορισμος σου.  
Αλλα μη βιαζεις το ταξειδι διολου.  
Καλλιτερα χρονια πολλα να διαρκεσει  
και γερος πια ν' αραξεις στο νησι,  
πλουσιος με οσα κερδισες στον δρομο,  
μη προσδοκωντας πλουτη να σε δωσει η Ιθακη.

Η Ιθακη σ' εδωσε τ' ωραιο ταξειδι.  
Χωρις αυτην δεν θαβγαινες στον δρομο.  
Αλλα δεν εχει να σε δωσει πια.

Κι αν πτωχικη την βρεις, η Ιθακη δεν σε γελασε.  
Ετσι σοφος που εγινες,  
με τοση πειρα,  
ηδη θα το καταλαβες η Ιθακες τι σημαινουν.

## ΙΘΑΚΑ

When you start on your journey to Ithaca,  
then pray that the road is long,  
full of adventure, full of knowledge.  
Do not fear the Lestrygonians  
and the Cyclopes and the angry Poseidon.  
You will never meet such as these on your path,  
if your thoughts remain lofty, if a fine  
emotion touches your body and your spirit.  
You will never meet the Lestrygonians,  
the Cyclopes and the fierce Poseidon,  
if you do not carry them within your soul,  
if your soul does not raise them up before you.

Then pray that the road is long.  
That the summer mornings are many,  
that you will enter ports seen for the first time  
with such pleasure, with such joy!  
Stop at Phoenician markets,  
and purchase fine merchandise,  
mother-of-pearl and corals, amber and ebony,  
and pleasurable perfumes of all kinds,  
buy as many pleasurable perfumes as you can;  
visit hosts of Egyptian cities,  
to learn and learn from those who have knowledge.

Always keep Ithaca fixed in your mind.  
To arrive there is your ultimate goal.  
But do not hurry the voyage at all.  
It is better to let it last for long years;  
and even to anchor at the isle when you are old,  
rich with all that you have gained on the way,  
not expecting that Ithaca will offer you riches.

Ithaca has given you the beautiful voyage.  
Without her you would never have taken the road.  
But she has nothing more to give you.

And if you find it poor, Ithaca has not defrauded you.  
With all the great wisdom you have gained,  
with so much experience,  
you must surely have understood by then what Ithacas mean.

Κ. Καβαφης

C. Cavafys \*

\* Rae Dawen. The complete poems of Cavafy. The Hoggarth Press, 1971.

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## ABBREVIATIONS

<b><math>\alpha</math>1-AT:</b>	$\alpha$ 2-macroglobulin
<b><math>\alpha</math>2-M:</b>	$\alpha$ 1-antitrypsin
<b>AL:</b>	attachment level
<b>Alb:</b>	albumin
<b>ANOVA:</b>	analysis of variance
<b>APD:</b>	advanced periodontal disease
<b>BOP:</b>	bleeding on probing
<b>BSA:</b>	bovine serum albumin
<b>CB:</b>	coating buffer
<b>CEJ:</b>	cementoenamel junction
<b>CPITN:</b>	community periodontal index of treatment needs
<b>CV:</b>	coefficient of variation
<b>ELAM-1:</b>	endothelial leucocyte adhesion molecule 1
<b>ELISA:</b>	enzyme linked immunosorbent assay
<b>GCF:</b>	gingival crevicular fluid
<b>GCW:</b>	gingival crevicular washing
<b>GM-CSF:</b>	granulocyte/macrophage colony stimulating factor
<b>HRP:</b>	horseradish peroxidase
<b>IB:</b>	incubation buffer
<b>IB/M:</b>	incubation buffer plus 5% w/v MARVEL
<b>ICAM-1:</b>	intercellular adhesion molecule 1
<b>IL-1:</b>	interleukin-1
<b>LF:</b>	lactoferrin
<b>LPS:</b>	lipopolysaccharide
<b>MANOVA:</b>	multivariate analysis of variance

**MGI:** modified gingival index  
**OD:** optical density  
**OPD:** ortho-phenylene diamine  
**PBS:** phosphate buffered saline  
**PBST:** phosphate buffered saline plus 0.05 % Tween 20  
**PD:** probing/pocket depth  
**PG:** prostaglandin  
**PI:** plaque index  
**PMN:** polymorphonuclear leucocyte  
**S:** suppuration  
**SB:** substrate buffer  
**SD:** standard deviation  
**SE:** standard error  
**TF:** transferrin  
**TIMP:** tissue inhibitor of metalloproteinases  
**TNF- $\alpha$ :** tumor necrosis factor  $\alpha$   
**WMGI:** whole mouth modified gingival index  
**WPI:** whole mouth plaque index  
**HETE:** hydroperoxyeicosatetraenoic acid

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## **Declaration**

This thesis is the original work of the author.

Evagelia Adonogianaki

## SUMMARY

Little research has been conducted into the role of protease inhibitors and iron-binding proteins in periodontal diseases, despite their possible implication in the modulation of the local inflammatory process. This thesis aimed to investigate the major protease inhibitors  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M) and  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT) and the iron-binding proteins transferrin (TF) and lactoferrin (LF) in gingival crevicular fluid (GCF), in relation to periodontal disease status.

Preliminary experiments were performed to establish new or modify existing enzyme linked immunosorbent assays (ELISA) for the assessment of these proteins in GCF. The simultaneous quantification of  $\alpha$ 2-M,  $\alpha$ 1-AT, TF and LF in the same GCF sample was the ultimate objective. Initial attempts resulted in the establishment of two competitive ELISAs for the assessment of  $\alpha$ 2-M and TF in GCF. In an effort to improve detectability limits, the establishment of sandwich ELISAs for the detection of these proteins and, in addition, of  $\alpha$ 1-AT, LF and albumin (Alb) was achieved. Application of the sandwich ELISA methodology resulted in an approximately 10 fold decrease in the detectability limits compared to the competitive ELISAs. Consequently, the assessment of all five proteins in the same GCF sample was possible in later studies.

The clinical studies conducted in this thesis include cross-sectional studies on gingival crevicular washings (GCWs) and GCF and longitudinal studies on GCF. GCF samples were obtained using paper strips for 30s and GCF volume was assessed using the Periotron 6000.

LF is contained specifically and in abundance in neutrophils (PMNs) but not in other leucocytes and only in trace amounts in serum. PMNs are widely implicated in the pathogenesis of destructive periodontal diseases. The aim of the cross-sectional study on GCWs was, therefore, to examine whether LF in the crevice could act as a marker of crevicular polymorphonuclear leucocytes (PMN). A positive and significant association was obtained indicating that LF has potential as a PMN marker in the gingival crevice.

The aim of the cross-sectional studies on GCF was to test the ability of GCF protease inhibitors ( $\alpha$ 2-M,  $\alpha$ 1-AT) and iron-binding proteins (TF, LF) to distinguish between healthy, gingivitis and periodontitis sites. The absolute amounts (ng/30s) of each protein were significantly higher at diseased (gingivitis and periodontitis) when compared to healthy sites. However, gingivitis could not be differentiated from periodontitis sites. When results were expressed as concentrations (ng/ $\mu$ l GCF), the predominately serum derived  $\alpha$ 2-M,  $\alpha$ 1-AT and TF demonstrated an increase in diseased (gingivitis and periodontitis sites) compared to healthy sites whereas the PMN derived LF demonstrated a

decrease in its concentration in diseased sites. However, only the changes in GCF  $\alpha$ 2-M concentration were statistically significant depicting the higher degree of variability that was observed when results were expressed as concentrations.

The aim of the experimental gingivitis study was to establish the dynamics of GCF protease inhibitors and iron-binding proteins during experimentally induced gingivitis. This would provide information on the effect of gingival inflammation on the levels of these proteins in the crevice.  $\alpha$ 2-M,  $\alpha$ 1-AT and TF revealed a very similar pattern during the experimental gingivitis trial, reflecting their common origin in GCF. Their levels increased significantly from baseline. This was attributed to increased permeability of the subepithelial vasculature and is supported by the simultaneous rise in GCF volume. In addition, local production of these proteins by resident cells of the periodontium could also be implicated. Vascular permeability was maintained for at least one week after the end of the experimental gingivitis period and this contributed to increased levels of these proteins in the crevice one week after the reinstatement of oral hygiene. The PMN derived LF demonstrated a different overall picture rising significantly during the experimental gingivitis trial but dropping rapidly to almost baseline levels after the reinstatement of oral

hygiene measures and when chemotactic stimuli for PMNs were removed.

The final clinical study involved a three month duration longitudinal trial of periodontitis patients on maintenance care. The aim of this study was to investigate whether incipient periodontal disease breakdown could be associated with changes in GCF protease inhibitor or iron-binding protein levels. In addition, the potential of clinical indices to act as predictors/indicators of significant attachment level change was investigated. Duplicate attachment level (AL) measurements were taken using the Florida Probe and a custom fabricated stent from a total of 384 sites. The lower variability of duplicate AL measurements using the Florida probe, resulted in earlier detection of small changes in attachment level. When the tolerance method was used to detect significant AL change, 3.9% of the sites lost attachment. When a less stringent criterion of AL change of  $\geq 1\text{mm}$  was used 9.9% of the sites lost attachment during the three-month observation period. With the exception of subject average probing depth, baseline clinical parameters failed to predict attachment level change. Fourteen active periodontitis sites that demonstrated significant attachment loss during the three month observation period were paired to stable periodontitis sites within the same patient according to baseline scores of gingival inflammation and probing depth. The levels of protease inhibitors and iron-binding proteins

(ng/30s and ng/ $\mu$ g Alb) in GCF failed to differentiate between active and stable periodontitis sites at baseline and three months.

In conclusion, assessment of different GCF constituents is possible, although existing methodology may have to be modified to help overcome problems associated with the low volumes of GCF obtained when non-invasive sampling procedures are used. Constituents of different origin in GCF may respond differently according to periodontal disease status. This was demonstrated in these studies for the mainly serum derived  $\alpha$ 2-M,  $\alpha$ 1-AT and TF when compared to the PMN derived LF. The degree of gingival inflammation of the tissues adjacent to the crevice/pocket seems to influence the levels of protease inhibitors and iron-binding proteins in gingival crevicular fluid to a greater extent than probing attachment loss.

# **CHAPTER 1**

## **Introduction**

## 1.1 General Introduction

During the past decade research on periodontal disease has evolved enormously. Carefully designed clinical studies and appropriate statistical analysis of epidemiological data, have led to the development of new concepts regarding the epidemiology and progression of periodontal disease. Instruments have been developed, using current technology, to help the clinician overcome problems associated with clinical measurements of the periodontium. In addition, classical concepts of the pathophysiology of periodontal disease have been transformed by advances in basic science. Substantial research has been directed towards understanding basic mechanisms of the pathogenesis of periodontal diseases. The ultimate goal is that the diagnosis, treatment and prevention of periodontal disease is based upon aetiology rather than experience.

The literature review that follows aims to address basic issues of clinical and experimental periodontology that apply today. Moreover, it aims to provide background information which led to the investigations pursued in this thesis.

Initially, this literature review deals with aspects of clinical periodontology. The objective is to aid the understanding of the clinical entity under investigation, the material to be used in these studies (gingival

crevicular fluid), and the methods used for the clinical assessment of the periodontium. Finally, the inadequacy of clinical criteria alone, to diagnose or predict active periodontal disease, is reviewed.

The second part deals with the pathogenesis of periodontal disease. Current concepts are summarised and some mechanisms of interest are presented in detail. Background information on protease inhibitors and iron-binding proteins is provided, together with their relevance to periodontal diseases.

## **1.2 Definitions**

### **1.2.1 The 'normal' periodontium: Clinical and histological appearance**

Supporting structures of the teeth are known collectively as the periodontium, from the Greek words 'peri' meaning around, and 'odontos' of the tooth. They comprise the alveolar bone, the periodontal ligament, the root cementum and the gingivae which together form a functional unit the 'attachment apparatus' (Lindhe, 1990).

The normal periodontium is characterised, clinically, by a gingival unit which is pale pink in colour, has a scalloped outline, is firm in texture, fills the entire space between adjacent teeth and does not bleed on gentle probing

(Wennström, 1988). In health, a gingival sulcus rarely exists. Histologically, no inflammatory infiltrate is present and only a few polymorphonuclear leucocytes can be seen traversing the connective tissue and junctional epithelium. The alveolar bone is located 1mm apical to the cemento-enamel junction. This classical picture of the 'normal' periodontium has recently been an issue of debate, as investigators have found that an inflammatory infiltrate, comprising of both polymorphonuclear leucocytes and small round mononuclear cells is always present in gingival biopsies from clinically healthy gingiva (Seymour, Powell & Aitken, 1983a; Seymour et al., 1983b; Brex et al., 1987). Therefore, the term 'clinically healthy' compared to 'normal' periodontium is preferable (Wennström, 1988).

### **1.2.2 Periodontal disease: Clinical features and epidemiology**

The term periodontal disease, if used in the general sense, should denote all diseases which could affect the periodontium. As the periodontium comprises tissues of different origin (mesenchymal and ectodermal), an array of systemic conditions with oral/periodontal manifestations could be included (Kinane & Davies, 1990). However, in this text, the term periodontal disease will only be used to describe gingivitis and periodontitis, which are inflammatory processes of the periodontium associated with

the presence of dental plaque (plaque-associated periodontal disease).

In gingivitis, pathological changes are confined to the superficial periodontal tissues i.e. the gingivae, and are clinically manifested by a change in colour (redness) and appearance (swelling) of the gingivae, together with an increased tendency to bleeding on gentle probing. Periodontitis affects the deeper structures of the periodontium (bone, periodontal ligament and cementum), resulting in loss of periodontal support, and is frequently associated with the presence of periodontal pockets. Radiographically bone loss is apparent. At least four different forms of periodontitis have been defined (Page & Schroeder, 1982). It is generally accepted that forms of periodontitis affecting children and young adults are rare and differ from those commonly seen in older individuals. These are termed as prepubertal and juvenile periodontitis respectively. Whether these forms of periodontitis present as typical plaque associated disorders is debatable, especially as far as prepubertal periodontitis is concerned where most, if not all, of the cases have genetic basis. Periodontitis affecting the adult population is defined as rapidly progressive and adult periodontitis depending mainly on the severity and age of onset of the disease.

Early epidemiological studies indicated that gingivitis is widespread among children and young adolescents (James,

1963). Similarly, Marshall-Day, Stephen and Quigley (1955) reported that the prevalence of periodontitis, as indicated by radiographic bone loss, reached approximately 100% by the age of 40, while the prevalence of gingivitis without involvement of the deeper structures of the periodontium reduced with age. Finally, Russell (1963) reported that age and oral hygiene could explain most periodontal disease variance in several ethnologically different populations. Thus the concepts arose that: a) periodontitis is a global disease affecting high proportions of the population; b) the disease starts as gingivitis at an early age which if left untreated will develop to periodontitis; and c) most of the variance in periodontal disease is associated with deficient oral hygiene and age. These concepts dominated periodontal literature until recently, when a more thorough analysis of epidemiological data was made. When the extent and severity of periodontal involvement were taken into consideration a different overall picture of the prevalence and progression of destructive periodontal disease was evident. Recent cross-sectional studies have shown that although a periodontium of reduced height becomes common with increasing age, relatively few subjects in each age group suffer from advanced periodontal destruction. These subjects account for most of the sites which are severely periodontally involved (Yoneyama *et al.*, 1988; Papapanou, Wennström & Gordhal, 1988; Jenkins & Kinane, 1989). For example, Papapanou *et al.* (1988) reported that 75% of the total sites with previous attachment loss of at least 6mm

occurred in 23% of the individuals examined. Similar findings were reported by Löe *et al.* (1986) who conducted a 15 year long study of the natural history of periodontal disease in Sri Lanka. These investigators identified a group of subjects, comprising 8% of the total sample, who showed rapid progression of periodontal disease, as defined by tooth mortality rates and interproximal attachment levels. In the same study, however, 11% of the subjects did not show progression of periodontal disease beyond gingivitis. Longitudinal studies have in addition shown, that relatively few sites undergo extensive periodontal destruction within a given observation period (Haffajee *et al.*, 1983a, b; Lindhe, Haffajee & Socransky, 1983). Lindhe *et al.* (1983) reported that during a six year follow up of 64 Swedish subjects, showing signs of previous periodontal destruction but not receiving active periodontal treatment, only 3.9% and 11.6% of their sites showed attachment loss greater than 2mm from baseline at 3 and 6 years respectively. Similar percentages (3.2%) of sites exhibiting periodontal attachment loss were reported in the same study for a group of American subjects who were followed over a 1 year period. Moreover, it seems that relatively few individuals account for the sites that show active periodontal destruction over an observation period. Thus, Lindhe *et al.* (1989) in a recent study reported that 70% of the sites that deteriorated by 3mm or more during a 2 year monitoring period, occurred in only 12% of the 265 Japanese subjects that were followed longitudinally.

The current view, therefore, is that periodontal disease is subject related, with a small subset of individuals within a given population suffering from advanced periodontal destruction, and with relatively few subjects and sites undergoing active periodontal destruction within a given period. In addition, in the absence of treatment, the mere presence of inflammatory periodontal lesions does not necessitate their progression (Papapanou, 1989).

### **1.2.3 Gingival crevicular fluid: Production, methods of collection and volume determination**

Gingival crevicular fluid (GCF) is an inflammatory exudate or preinflammatory transudate present in the gingival crevice. Its components are derived mainly from: a) microbial sources; b) interstitial fluid and locally produced factors of host origin; c) plasma; and, d) tissue degradation/turnover products (Cimasoni, 1983).

Production of GCF due to an inflammatory increase in the vascular permeability of the subepithelial vasculature was shown in early studies (Brill & Bjorn, 1959; Egelberg, 1966a). Egelberg (1966a) in an experiment in dogs, showed that chemical (topical application of histamine) or mechanical stimulation (massage of the marginal gingivae with a ball burnisher or scraping with a blunt probe) of the gingival unit will result in increased vascular permeability and increased GCF flow. Moreover, heavy

vascular labelling (by deposition of carbon particles) was observed in the stimulated areas. In an earlier study Brill and Bronnestam (1960) had already shown the presence of serum proteins in GCF from humans using immunoelectrophoresis.

Production of GCF in clinically healthy gingivae seems to be governed by different mechanisms. Alfano (1974) in his original hypothesis suggested that in clinically healthy gingivae, during initial plaque accumulation, macromolecules from gingival plaque may diffuse through the endothelial cells until they reach the barrier of the basement membrane. Accumulation of macromolecules at the basement membrane area is responsible for the creation of an osmotic gradient and induction of GCF flow. Later, Pashley (1976) developed a mathematical model which explains GCF production in both health and disease. He suggested that net GCF production can be accounted for by relative differences in: a) passage of fluid from capillaries into the tissues; b) uptake by lymphatics; and c) filtration through the junctional epithelium into the gingival crevice. These processes are in turn governed by differences in the filtration coefficients of the membranes delineating the different compartments as well as differences in hydrostatic and oncotic pressures between capillaries, lymphatics, tissue and sulcular areas. Bickel, Cimasoni and Andersen (1986) attempted to verify the above model and studied the contribution of serum in

GCF production during early (pre-inflammatory) plaque accumulation by assessing albumin concentration in GCF and its ratio to that of serum. They reported no recovery of fluid from healthy gingivae and low concentrations of albumin during pre-inflammatory plaque accumulation, supporting the osmotic gradient model of Alfano (1974). With increasing plaque accumulation, both the albumin GCF concentration and its ratio to that of serum, increased. At chronically inflamed sites GCF:serum albumin ratio of approximately 1 was obtained.

Sampling methods commonly employed for the collection of GCF vary greatly and reflect the type of analysis to be performed on the sample and the amount of fluid required. They include, the absorbent paper strips or paper points, the capillary tubes or micropipettes and the gingival washings (Cimasoni, 1983). The most commonly used method is the paper strip (Golub *et al.*, 1976; Villela *et al.*, 1976; Condacci, Cimasoni & Ahmad-Zadeh, 1982; Cimasoni & Giannopoulou, 1988; Griffiths, Curtis & Wilton, 1988; Curtis *et al.*, 1990; Giannopoulou *et al.*, 1990, 1992; Lamster, Hartley & Oshrain, 1990). Capillary tubes (Ishikawa *et al.*, 1972; Lah *et al.*, 1986; Larivee, Sodek & Ferrier, 1986) or micropipettes (Eisenhauer *et al.*, 1983) are used when a large amount of fluid is required, whereas gingival crevicular washings are employed when cellular elements of the fluid are studied (Skapski & Lehner, 1976; Baehni *et al.*, 1975; Salonen and Paunio, 1991). Choice and

standardisation of the sampling procedure is particularly important as different protocols will influence the quantity and constituents of GCF recovered. Cimasoni (1983) concluded that capillary tubes or micropipettes are difficult to handle and may cause irritation of the gingival tissues especially in the healthy crevice. Thus, the paper strip seems to be the method of choice when microamounts of fluid are to be collected and when healthy as well as diseased crevices are to be studied.

Egelberg (1966b) has shown that the insertion of a paper strip in the crevice will cause a degree of gingival irritation and induce vascular permeability. Thus, when paper strips are used for the collection of gingival crevicular fluid several factors must be taken into consideration. A number of recent reports have focused on the effect of sampling time and sampling repetition, on GCF flow rate when using paper strips and have shown that GCF volume tends to be depleted by repeated sampling (Binder, Goodson & Socransky, 1987; Curtis *et al.*, 1988; Lamster *et al.*, 1989; Persson & Page, 1990). Although in most cases repeated sampling resulted in a reduction in total amounts of GCF constituents (Persson & Page, 1990; Lamster *et al.*, 1989; Binder *et al.*, 1987), some investigators reported an increase or a steady flow rate (Binder *et al.*, 1987; Villela *et al.*, 1987). This discrepancy could have however been related to lengthy sampling periods (Villela *et al.*, 1987) or deep intracrevicular sampling techniques (Binder

et al., 1987). When repeated sampling for 30 seconds was employed, GCF constituents did not return to baseline levels even 60 minutes after the initial sampling (Lamster et al., 1989). Despite the above evidence some investigators have chosen to discard the first GCF sample and use a subsequent sample for analysis (Binder et al., 1987).

The effect of sampling time has also been investigated (Persson & Page, 1990; Cao & Smith, 1989). Persson and Page (1990) employed four sampling times (5, 10, 20 and 30 seconds) and four different sampling sequences, thus alternating the order by which samples of different time periods were taken. They suggested that 5s or 10s first time sampling of a crevice is adequate whereas lengthier sampling periods (20s or 30s) tend to dilute aspartate aminotransferase activity. However, Cao and Smith (1989) have shown that myeloperoxidase activity is higher in 30s compared to 5s samples from the same crevice when these are taken a week apart. Standardisation of the length of the strip inserted in the crevice is also an issue to be taken into consideration as deeper insertion will result in higher flow rates (Mann, 1963). In conclusion, although a general consensus concerning sampling procedures does not exist, it seems that sampling for 30s or less with a paper strip would be the method of choice as it minimises irritation of the gingival tissues and simultaneously provides enough sample for subsequent analysis.

Volume assessment of GCF collected on paper strips can be performed by assessing the wetted area of the strip, by weighing prior and after sample collection or by the use of the Periotron (Harco Electronics, Winnipeg, Canada). The area of the strip wetted can be assessed directly using a microscope fitted with a graticule (Egelberg & Attström, 1973) or after staining with ninhydrin (Egelberg, 1964). Although staining facilitates viewing and assessment of the wetted area, it renders the sample unsuitable for analysis. Weighing prior to and after GCF sample collection (Cimasoni & Giannopoulou, 1988) seems to be suitable for assessing volumes beyond the measuring range of the Periotron. The Periotron is an electronic device used to assess GCF volume. Both weighing of the strip and the use of the Periotron, offer the advantage of being compatible with subsequent biochemical analysis of the GCF sample. The Periotron 6000, the latest version of the Periotron, has been extensively used by investigators of GCF and it has proven satisfactory for the assessment of GCF volume (Cimasoni, 1983). However, assessing minute amounts of GCF by any of the above methods (volumes of 0.01-0.2 $\mu$ l) is demanding and could be subject to uncontrollable errors (Lamster *et al.*, 1988a).

### **1.3 Clinical assessment of the periodontium**

#### **1.3.1 Introduction**

Clinical assessment of the periodontium is currently performed using indices that distinguish between healthy tissues, gingival inflammation and destructive periodontal disease. Such indices are based on visual observation, palpation/percussion of teeth, the use of the periodontal probe and radiographic examination. More recently GCF flow has also been used to supplement other clinical criteria. Oral hygiene status and plaque accumulation assessment is performed on a separate basis via plaque indices. Generally accepted criteria for an ideal index include simplicity, validity, sensitivity, reproducibility and amenability to statistical analysis (Hazen, 1974).

#### **1.3.2 Assessment of oral hygiene**

Oral hygiene indices are based on the measurement of the tooth surface area covered by dental plaque and/or the thickness of the material in the area measured (Table 1.1). Among the earliest indices for the assessment of accumulation of plaque/debris are the 'simplified' Oral Hygiene Index (OHI) (Greene & Vermillion, 1964) as well as the plaque index of Quigley and Hein (1962). Since then numerous indices have been developed to assess plaque accumulation (reviewed by Fischman, 1986). One of the most

**Table 1.1** Commonly used plaque indices.

<b>Index</b>	<b>Reference</b>	<b>Score</b>	<b>Comments</b>
Oral Hygiene Index (OHI)	Greene & Vermillion, 1964	0-3	Assessment of the tooth area covered by 'soft debris'
Plaque Index	Quigley & Hein, 1962	0-5	Assessment of tooth area covered by dental plaque
Plaque Index (PI)	Silness & L�e, 1964 L�e, 1967	0-3	Assessment of the thickness of plaque at the gingival margin

widely used indices is the plaque index (PI) described by Silness and Løe (1964) which was first used to assess the association between oral hygiene and periodontal condition in pregnant women. This index was more fully described by Løe (1967) and scores plaque accumulation from 0 to 3. The Plaque Index can be used to score individual sites as single site scores, teeth as averages of four sites per tooth (buccal, lingual and interproximal) and individuals or groups of teeth, the latter two being based on site scores divided by the number of sites assessed. This index remains one of the most widely used indices although attention has been given to its subjective nature as well as its disruptive character when distinguishing between scores of 0 and 1. The use of a single examiner throughout clinical trials when using this index has been recommended (Fischman, 1986). The advantage of the plaque index is that it allows for assessment of very subtle changes in plaque accumulation (Lindhe, 1990). In addition, it has been suggested (Fischman, 1986) that the PI is suitable when plaque/ gingivitis relationships are to be considered.

### **1.3.3 Assessment of gingival inflammation**

Gingivitis indices rely upon one or more of the following criteria: gingival colour, gingival contour, gingival bleeding, extent of gingival involvement and GCF flow (Fischman, 1988). Such indices (Table 1.2) were initially developed for use in epidemiological surveys (reviewed by

**Table 1.2** Commonly used gingival indices.

<b>Index</b>	<b>Reference</b>	<b>Score</b>	<b>Comments</b>
Papillary, Marginal, Attached Index (PMA)	Schour & Massler, 1947	0-4	Visual assessment of gingival tissues
Gingival Index (GI)	Löe & Silness, 1963 Löe, 1967	0-3	Visual assessment of gingival tissues combined with bleeding on probing (scores 2-3)
Sulcus bleeding Index (SBI)	Muhlemann & Mazor, 1958 Muhlemann & Son, 1971	0-5	Visual assessment of gingival tissues combined with bleeding on probing (scores 2-5)
Papillary bleeding index (PBI)	Muhlemann, 1977	0-4	Assessment of the severity of bleeding on probing of the interdental papilla (score 1-4)
Bleeding Index (BI)	Ainamo & Bay, 1975	-	Dichotomous scoring of bleeding on probing after 10s
Modified PBI	Barnett et al., 1980	0-3	Assessment of the time elapsing for the appearance of bleeding after probing
Modified GI (MGI)	Lobene et al., 1986	0-4	Visual assessment of the severity and extent of gingival inflammation

Ciancio, 1986) and in a very early attempt Schour and Massler (1947) introduced the PMA index in order to measure the extent of gingival inflammation to the papillary (P), marginal (M) and attached (A) gingival units. The Periodontitis Index (Russell, 1956) and the Periodontal Disease Index (Ramfjord, 1959) incorporated scores for the assessment of gingival inflammation at the lower parts of their scales, reflecting the notion, dominating at the time, that gingivitis precedes periodontal destruction. More recent attempts to assess gingival inflammation include indices that are based on visual changes and/or bleeding on probing of the gingival unit, like the Gingival Index (GI) (Löe & Silness, 1963; Löe, 1967), the sulcular bleeding index (SBI) (Muhlemann & Mazar, 1958; Muhlemann & Son, 1971), the papillary bleeding index (PBI) (Muhlemann, 1977; Barnett, Ciancio & Mather, 1980) and the gingival bleeding index (Ainamo & Bay, 1975). The Gingival Index of Löe and Silness (1963), when it was first introduced, provided a conceptual change in the assessment of gingival inflammation as it scores both its severity and location and has been widely accepted and used. Consistent with the PI, the GI scores inflammation as 0-3. Scores 2 and 3 incorporate the bleeding on gentle pressure component which was later modified to bleeding upon probing (Löe, 1967). One of the disadvantages of the GI is its invasive nature which becomes important in longitudinal studies of gingivitis where trauma of the gingival tissues could lead to an altered response. Moreover a non-invasive index

facilitates other procedures, being employed during the same appointment such as GCF sampling which is influenced by the irritation of the gingival tissues. The GI has recently been modified by Lobene et al. (1986) (MGI). The bleeding upon probing component was eliminated and the lower part of the scale expanded resulting in a scoring system of 0-4. This index provides greater sensitivity to the earliest changes of gingivitis and by eliminating the bleeding upon probing element yields a completely non-invasive procedure. Moreover, it correlates well with the GI (Lobene et al., 1989) allowing comparisons with other studies where the GI has been used for the assessment of gingival inflammation and suggesting that relying only on visual assessment does not adversely affect reliability. Both the GI and MGI can be used to provide information at specific sites, teeth, groups of teeth or individuals in a fashion similar to the PI.

An advantage of indices incorporating the bleeding upon probing component is that they are useful not only in assessing gingival inflammation via the visual changes of superficial tissues, but could also contribute to the assessment of inflammation at the depth of the pocket in cases of periodontitis. The use, therefore, of two separate indices, when feasible, one for assessing the visual changes of the gingival tissues and one for assessing bleeding upon probing might be the best alternative in cases of periodontitis studies. When

bleeding upon probing is assessed pressure sensitive probes are the instrument of choice as they allow a standardised force to be applied in order to test for bleeding.

Brill (1960) was the first to suggest that GCF flow could be used for the assessment of gingival inflammation. Since then both cross-sectional (Mann, 1963; Egelberg, 1964; Loe & Holm-Pedersen, 1965; Borden, Golub & Kleinberg, 1977) and experimental gingivitis studies (Loe & Holm-Pedersen, 1965; Egelberg & Attström, 1973) have been conducted in order to assess the association between GCF flow and the degree of gingival inflammation, and positive relationships have been obtained. When the Periotron (section 1.2.3) was introduced, trials aiming to establish the relationship of Periotron units to gingival inflammation were conducted (Suppipat, Johansen & Gjermo, 1977; Garnick, Pearson & Harrell, 1979) and positive correlations with clinical signs of gingival inflammation were reported. The Periotron was initially designed for chairside use for exactly this purpose and in a rather simplistic approach the manufacturer assigned different degrees of disease severity (from health or incipient gingivitis to periodontitis) to increasing Periotron units. This indeed could be called the GCF flow index.

#### 1.3.4 Assessment of probing pocket depth and attachment level

Probing pocket depth and attachment level are at present the clinical means used to assess destruction of the attachment apparatus usually supplemented by radiographic examination. They both rely upon the identification of the most apical extent of the periodontal pocket and measure its distance from either the gingival margin (probing/pocket depths) or a fixed reference point which is usually the cemento-enamel junction (attachment level). There are several problems inherent in such measurements which include: i) variation in the probing force; ii) errors in the graduation of the probe; iii) variations in the size (diameter) of the probe; iv) errors in probe placement; v) errors in reading the probe and data recording; and vi) errors in the precision of measurement (Jeffcoat *et al.*, 1986). In addition to these physical errors, pocket probing depth assessment is complicated by biological variation or error as seen with the transient fluctuation in gingival margin location over time. When assessing attachment level, location of the cemento-enamel junction may introduce another source of error (Badersten, Nilveus & Egelberg, 1984; Clark *et al.*, 1987). Badersten *et al.* (1984) reported that, when assessing attachment level at single rooted teeth, using a conventional periodontal probe and an occlusal stent, inter and intra examiner agreement was within 1mm at approximately 90% of

the duplicate probings compared to approximately 75% to 84% when using the cemento-enamel junction as a reference point. Clark et al. (1987) pointed out that presence of calculus or the subgingival location of the cemento-enamel junction made its identification impossible in 17% of the sites used in their study. Van der Velden (1979) reported that variation of the probing force and variation in the diameter of the probe will influence the penetration of the probe through the junctional epithelium and gingival tissues. Higher forces yielded deeper probing depths, and a plateau in pocket depth measurement was reached at a probing force of approximately 1.25 N using a probing tip of 0.63mm diameter. Variation in the graduations of the probe may also be a source of error (Van der Velden, 1978). Probe penetration may also be influenced by the inflammatory status of the gingiva and deeper penetration of the probe was obtained in areas showing signs of increased inflammation (Armitage, Svanberg & Löe, 1977; Caton, Greenstein & Polson, 1981). In order to control probing force, pressure sensitive probes have been introduced (Van der Velden & de Vries, 1978; Polson et al., 1980), whereas in an effort to reduce errors related to the location/relocation of the probe or the identification of the cemento-enamel junction, occlusal stents have been used (Isidor, Karring & Attström, 1984; Badersten et al., 1984; Clark et al., 1987). Recent developments of periodontal probes tend to address different aspects of the methodological problems associated with measurement of

probing depth and attachment level and reflect the current trends and considerations of periodontal assessment. Jeffcoat *et al.* (1986) introduced a pressure sensitive probe with automated detection of the cemento-enamel junction. Gibbs *et al.* (1988) developed an electronic pressure sensitive probe, the Florida Probe, which measures probing depths to the nearest 0.2mm. The different probe handpieces allow pocket depth measurements as well as attachment level assessment from a custom-made stent or from the occlusal surface (Magnusson *et al.*, 1988a; Osborn *et al.*, 1990). An electronic pressure sensitive probe described by Birek, McGulloch and Hardy (1987) and its later modification, the Toronto probe (Karim, Birek & McGulloch, 1990), allows attachment level measurements to the nearest 0.1mm under controlled angulation.

#### **1.3.5 Detection of attachment level change**

All the indices introduced above represent measures of either the transient inflammatory status of the superficial structures of the periodontium (gingival/bleeding indices and GCF flow) or the oral hygiene status of sites/patients (plaque indices). At best, probing depth or better attachment level could provide a historical measure of the past periodontal disease experience of a site/patient. When trying to assess periodontal disease activity at a specific site, longitudinal measurements of attachment level are therefore required, spaced at appropriate time

intervals. The variable of interest is then the change in attachment level which represents the best estimator, at present, of previous/current disease activity. It became crucial, therefore, to introduce methods that would allow one to define the change in attachment level that safely exceeds measurement error. Several studies were conducted in an attempt to define measurement error when using a conventional periodontal probe to measure probing attachment level with or without an occlusal stent (Hafajee et al., 1983b; Badersten et al., 1984; Isidor et al., 1984; Clark et al., 1987; Best et al., 1990). In an attempt to reduce false positive detection of attachment level change Hafajee et al. (1983b) suggested a criterion of 3 standard deviations of the difference of replicate measurements, which in their study was assessed to be 2.46mm (SD:0.82mm), which was further rounded to 3mm. The 3mm threshold was subsequently applied in several studies, although application of a threshold derived from one population to another, without confirming that the precision of the measurement is the same has been criticised (Fleiss et al., 1991). Refinements in probing attachment level measurement techniques described above, aimed to provide a more accurate assessment of probing attachment level than manual conventional probing. This would reduce variability between replicate or sequential measurements allowing for lower thresholds of attachment level changes needed for deciding that true attachment level change has occurred.

Hafajee *et al.* (1983b) described three more sophisticated ways of detecting significant attachment level change: the regression, the running median and the tolerance methods. In the regression method the slope and y intercept of a regression line are computed from a linear least squares fit of attachment level measurements as a function of time and the slope is tested for a significant departure from 0 while a threshold of projected attachment level change per unit time has also got to be exceeded. In the running median method attachment level measurements are plotted against time and systematic variations can be differentiated from minor changes by considering the medians of 3 successive data points and setting an arbitrary threshold based on the variability of replicate measurements. Finally, the tolerance method is based on standard deviations of replicate, usually 2, measurements at each time point. For a pair of attachment level measurements to be considered different from the preceding pair, attachment level change has to exceed three different thresholds. These are derived from population, subject and site standard deviations of replicate measurements. Badersten *et al.* (1987) used the regression analysis for the identification of attachment level change and in addition developed an end point analysis method, in order to compensate for the insensitivity of the former to detect abrupt changes at the early/late phases of the study. In the end-point analysis method, the deeper of the pair of initial measurements is compared against the shallower of

the final pair of measurements and if the change exceeds a specified threshold, attachment level change is declared. Recently a fourth method has been developed based on the cumulative sum of successive measurements (cumsum) (Aeppli & Pihlstrom, 1989; Jeffcoat & Reddy, 1991). In the cumsum method, differences from baseline are computed for each observation, which are subsequently added to obtain a sequence of sums which increases or decreases with the number of positive or negative changes. If the rate of change surpasses a predetermined critical value, a significant change is declared.

The most commonly used methods in clinical trials are the regression and tolerance methods. The regression method is most suitable for assessing linear changes in attachment level over a long period of time, whereas the tolerance method requires measurements at as few as 2 points in time and is more suitable in detecting abrupt differences in short periods in time (Haffajee *et al.*, 1983b).

#### **1.3.6 Association of probing attachment loss to clinical parameters**

Several attempts have been made to correlate clinical indices with periodontal disease activity as assessed by longitudinal probing attachment level measurements (Greenstein & Caton, 1990). Plaque accumulation, due to its transient character demonstrates poor association with

destructive periodontal disease in untreated or maintained subjects (Haffajee et al., 1983a; Jenkins et al., 1988; Lindhe et al., 1989; Badersten et al., 1990; Kaldahl et al., 1990). Thus, Haffajee et al. (1983a), in one of the earliest reports on the ability of clinical parameters to act as predictors of periodontal destruction, reported a specificity and sensitivity of 0.42 and 0.71 respectively, for the presence of plaque at specific sites just prior to the attachment loss episode. Similar specificity and sensitivity values for plaque were obtained just after the attachment loss episode. Later, Badersten et al. (1990) reported that only 30% of the sites exhibiting plaque  $\geq 75\%$  of the times they were examined, during a 6 year monitoring period, actually demonstrated attachment loss. In a more recent investigation, Haffajee et al. (1991) used chi-square analysis to demonstrate positive associations between percentage of sites exhibiting plaque above certain thresholds and the number of subjects exhibiting attachment loss over 3mm during the 1 year observation period. Thus, the higher the percentages of sites demonstrating plaque within a subject the more likely the subsequent probing attachment loss. The authors however suggested that this association could have been artificial and could be due to the strong association of plaque at baseline with baseline attachment level which in turn was strongly related to subsequent attachment loss.

Bleeding upon probing has been traditionally treated as an index of high diagnostic potential and efforts have been made to correlate bleeding on probing as well as gingival inflammation to active tissue destruction in periodontal diseases. Recent evidence (Lang et al., 1990), however, suggests that although absence of bleeding on probing is a good indicator for the maintenance of periodontal stability (i.e. bleeding on probing has a high negative predictive value), it could not be used as an effective indicator/predictor of periodontal destruction. This, in addition, applies to the presence of gingival inflammation (Haffajee et al., 1983a; Lang et al., 1986; Jenkins et al., 1988; Badersten et al., 1990). However, it has been recently demonstrated that there is a positive association between percentage of sites within a patient that demonstrate bleeding on probing, or demonstrate overt gingival inflammation at baseline, and risk of future attachment loss (Haffajee et al., 1991). The authors, however, suggested that association of both gingival redness and bleeding on probing with subject baseline attachment level, as described above for plaque scores, could perhaps explain their association with future attachment loss.

Deeper probing depths at specific sites did not necessarily predict/ diagnose periodontal destruction (Haffajee et al., 1983a; Jenkins et al., 1988). Residual probing depths  $\geq 7$ mm showed 50% and 52% diagnostic predictability respectively

in a 3.5 and 5 year longitudinal study of periodontitis patients on maintenance (Badersten et al., 1987; Badersten et al., 1990). This indicates that long monitoring periods may be required to improve the diagnostic value of clinical parameters at specific sites. Mean patient probing depths and attachment level above increasing thresholds (Grbic et al., 1991; Haffajee et al., 1991) as well as percentages of sites within a patient demonstrating probing depths or attachment level >3mm (Hafajee et al., 1991) were however a risk indicator for subsequent attachment loss suggesting that previous periodontal destruction predisposes to future periodontal breakdown.

In conclusion, the studies referred to above, have shown that clinical assessment of the periodontium can not predict or diagnose active periodontal destruction. This information has bewildered the clinician who had long learnt to rely on clinical diagnostic criteria in prognostic assessments. During the past decade significant research has focused on the pathogenesis of periodontal disease which, in addition to elucidating possible mechanisms of periodontal destruction, could perhaps lead to the development of laboratory based diagnostic tests. The following sections therefore, will focus on the pathogenesis of periodontal disease and will introduce in more detail some of the possible mechanisms of interest which led to the investigations pursued in this thesis.

#### 1.4 Background information on the aetiology and pathogenesis of periodontal disease

In early times (Hunter, 1835) the concept of the periodontium had not yet evolved, and diseases affecting the periodontal tissues were thought to reside either in the gums or the alveolar bone, but not in both. Since then our understanding of periodontal disease has developed greatly, much of this progress occurring during the past three decades. Many questions however, concerning the aetiology, pathogenesis and progression of periodontal disease, still remain unanswered.

Chronic inflammation is the hallmark of periodontal disease and it is now established that accumulation of bacterial plaque is responsible for the initiation and maintenance of the inflammatory process (Löe, Theilade & Jensen, 1965; Hellden & Lindhe, 1973; Axelsson & Lindhe, 1978). Löe *et al.* (1965) introduced the experimental gingivitis model to investigate the development of gingival inflammation in response to accumulation of dental plaque. They recruited 12 individuals who, after an initial baseline examination, refrained from all oral hygiene procedures. All individuals enrolled in the study developed clinical signs of inflammation, after a period of no oral hygiene ranging from 10 to 21 days. The experimental design of this study could, however, be criticised, as subjects withdrew from oral hygiene measures for variable time periods and thus no

statistical analysis of the data could be performed. In addition, some subjects participating in the study exhibited an initial degree of gingival inflammation and therefore baseline gingival conditions were not standardised. However, these investigators were the first to unequivocally show that plaque accumulation leads to gingival inflammation and this study remains fundamental. Since then the experimental gingivitis model has been widely applied by investigators of the developing gingival inflammation.

The temporal sequence of the histopathological changes in the inflammatory lesion of gingivitis and periodontitis have been described by Page and Schroeder (1976). Four different stages have been proposed: within 2 to 4 days of plaque accumulation in the dentogingival region, the 'initial lesion' develops. This lesion is characterised by increased vascular permeability, emigration of polymorphonuclear leucocytes (PMN) from the vessels in the connective tissue and eventually in the gingival crevice, and loss of perivascular collagen. If plaque is left undisturbed within 4 to 7 days the 'early' lesion develops. At this stage, the predominant infiltrate is small and medium size lymphocytes, whereas all other changes described in the initial lesion are aggravated (PMN emigration, increased vascular permeability, loss of perivascular collagen). Moreover, the first cytopathic effects on the gingival fibroblasts together with a slight

lateral proliferation of the junctional epithelium in its coronal portion are now obvious. Two to three weeks of plaque accumulation are required, according to this model, for the shift to a plasma cell dominated lesion to occur. This is the pathognomonic feature of the 'established' lesion. As well as increased vascular permeability, the continued loss of perivascular collagen and the increased exudation, severe cytopathic effects on gingival fibroblasts are evident with concomitant increase in the lateral width of the junctional epithelium, which has now formed rete pegs extending into the subepithelial connective tissue. The 'initial' and 'early' lesions represent the acute stage of gingival inflammation whereas the 'established' lesion designates more chronic gingivitis. The established lesion may (or may not) eventually convert to the 'advanced' lesion whose additional feature is the loss of connective tissue attachment (Sharpey fibres) and alveolar bone. Ulceration of the (now) pocket epithelium may also be present and osteoclasts can be found at areas of bone resorption. The 'advanced' lesion is synonymous with periodontitis.

Although the above model has evolved mainly through early animal studies (Garant & Mulvihill, 1972; Attström, Graf-de Beerand & Schroeder, 1975; Schroeder, Graf-de Beer & Attström, 1975; Schroeder & Lindhe, 1975), it has provided a temporal framework for both scientists and clinicians and has dominated descriptions of periodontal pathology since

it was first introduced. One should not, however, forget that there is no clear cut division of the phases of an inflammatory process *in vivo*, and all the above changes could occur simultaneously, with the more chronic changes being superimposed on the more acute. Moreover, recent evidence suggests that a much longer period than two to three weeks is required for the shift to a plasma cell dominated lesion to occur. Thus, Brex *et al.* (1988a) who conducted an experimental gingivitis study of six month duration, reported that although the plasma cell fraction of the inflammatory infiltrate in the gingival connective tissue increased with time, a period of neglected oral hygiene of greater than 6 months may be required to lead to a plasma cell dominated lesion. PMNs and lymphocytes comprised the greatest part of the connective tissue infiltrate even 6 months after cessation of oral hygiene measures in their study. It has, therefore, been suggested that it is the plasma cell dominance that discriminates the advanced from the established lesion and/or active from stable periodontitis sites in humans (Seymour, 1991). Moreover, as mentioned in section 1.2.1, there is always a degree of PMN and lymphoid cell infiltration of the connective tissue even after extended periods of optimum oral hygiene leading to clinically healthy gingivae (Brex *et al.*, 1987) suggesting that this may be necessary for the maintenance of gingival health.

The initial and early lesions are associated with a primarily Gram positive, aerobic microflora in the gingival crevice. However, weakening of the junctional epithelium and its detachment from the tooth surface allows initial subgingival proliferation of the Gram positive plaque and colonisation with Gram negative anaerobes and spirochetes (Löe et al., 1965; Theilade et al., 1966). It is this type of flora that comprises the major periodontal pathogens. The 'initial', 'early' and perhaps the 'established' lesions can be induced by allowing plaque to accumulate on the teeth for a period of two to three weeks (Zachrisson, 1968; Brex et al., 1988a). However, the exact mechanisms of periodontal destruction as well as the factors that control the transition from gingivitis to periodontitis ('established' to 'advanced' lesion) are not well understood.

Bacteria may cause direct damage to the host and in a classical infection this would be initiated by the microorganisms adhering to and invading the host tissues. Bacterial invasion has been shown to occur in cases of acute ulcerative gingivitis and the consequences are disastrous to the gingival tissues (Listgarten, 1965). Some authors have suggested that active invasion of microorganisms in the gingival/pocket tissues may also occur in cases of advanced periodontitis (Frank, 1980; Saglie et al., 1982) although others have challenged these findings, and the debate as to whether true invasion or translocation

occurs, remains to be resolved (Liakoni, Barber & Newman, 1987; Newman, 1990). Even if true invasion does not occur microorganisms may be the cause of direct damage as they elaborate a variety of soluble substances which could penetrate into the gingival connective tissue and have detrimental effects to the host. Such substances include the leucotoxin produced by *Actinobacillus actinomycetemcomitans* (Baehni et al., 1979a), which can cause damage to crevicular as well as connective tissue PMNs, various enzymes, like phosphatases, aminopeptidases, glycosidases, proteinases, hyaluronidase, chondroitin sulphatase etc (Slots, 1981; Nitzan, Sperry & Wilkins, 1978; Steffen & Hentges, 1981), as well as waste products of bacterial metabolism. However, whether such substances would be able to reach effective concentrations within the tissues in order to induce damage is highly questionable.

Apart from direct, mainly enzyme related, damage that bacteria may cause to the host, indirect damage of the host tissues via upregulation of inflammatory and/or immunopathogenetic host response mechanisms by bacterial derived substances must be considered. Toxic, chemotactic, mitogenic and antigenic factors, derived from dental plaque, may initiate an inflammatory host response in the periodontium. A typical example is lipopolysaccharide (LPS) which is a common component of Gram negative bacteria and has been shown to penetrate into the gingival tissues

in dogs through intact crevicular epithelium (Schwartz, Stinson & Parker, 1972). Endotoxin, together with other bacterial derived substances, may lead to the initiation of an inflammatory reaction. A variety of endogenous mediators of inflammation, including prostaglandins and leucotrienes, lymphokines/cytokines, complement components and proteases are then released in the area inducing damage to the host tissues (reviewed by Page, 1991). Particularly important in this process are interleukin 1 (IL-1) and tumor-necrosis factor  $\alpha$  (TNF- $\alpha$ ) which possess potent proinflammatory and catabolic capacities (Bevilacqua et al., 1985; Richards & Rutherford, 1988; Stashenko et al., 1987; Meikle et al., 1989). Recent studies have shown the presence of IL-1 $\alpha$  and IL-1 $\beta$  in GCF from patients with periodontitis (Masada et al., 1990). Moreover, in a recent experimental gingivitis study IL-1 activity in GCF, measured using a bioassay, peaked prior to the development of clinically detectable inflammation (Kinane et al., 1992b). Prostaglandins seem also to be implicated (Klein & Raisz, 1970) and PGE<sub>2</sub> concentration in GCF above certain thresholds may be used as an indicator of increased patient susceptibility to future periodontal breakdown (Offenbacher et al., 1986). A key event in the pathogenesis of periodontal disease seems to be the activation and/or release within the gingival connective tissue of host-derived proteases which are the effectors of periodontal destruction as will be reviewed in section 1.6.

If the continual (or intermittent) stimulation by plaque is maintained an immune reaction of both humoral and cell-mediated type may develop which will amplify and perpetuate the inflammatory reaction. Antigen presenting cells, residing within the gingival connective tissue or epithelium, will uptake the antigen and process it, for presentation to T and B lymphocytes (B-cells may in addition recognise antigen by direct binding to their surface immunoglobulin). T-cells (CD4+) will, in turn, be activated to produce lymphokines such as IL-2, IL-4 and IL-6 and induce an immune response. The major antigen presenting cell type is the macrophage but in addition Langerhans cells are also important in cross-epithelial antigen traffic. Langerhans cells are dendritic HLA-DR+ cells found within the epithelium of the gingiva (Moughal, Adonogianaki & Kinane, 1992). In a recent experimental gingivitis study, Langerhans cell numbers in gingival epithelium rose initially, during the early stages of the development of gingival inflammation, and subsequently dropped to almost baseline levels after 21 days of plaque accumulation. Langerhans cells may thus circulate from the epithelium to the local lymph nodes where an immune response would be elicited (Moughal *et al.*, 1992a). Specific antibodies against putative periodontal pathogens can be detected in both serum and GCF (Kinane *et al.*, 1992a).

In conclusion, there is evidence that bacteria may cause damage indirectly by stimulating the host response (Shenker, 1987; Seymour, 1991; Genco, 1992). However, the exact mechanisms leading to periodontal destruction, are interwoven, extremely complex and in a constant state of reevaluation.

Depression of the host immune response during the initial stages of exposure to dental plaque is another pathway suggested to lead to periodontal destruction in a model produced by Shenker (1987). Autoimmunity, mainly to collagen components, has also been speculated as contributing to periodontal breakdown (Ftis, Singh & Dolby, 1986; Hirsch et al., 1988;) although it is more possible that autoantibodies detected in periodontal disease are derived from natural antibodies whose function is the elimination of dead cells and damaged tissue constituents (Anusaksathien & Dolby, 1991).

## **1.5 Polymorphonuclear leucocytes**

### **1.5.1 Introduction**

The PMN is a short lived cell with a characteristic appearance: it has a lobulated nucleus and numerous granules within its cytosole. PMN granules are distinguished into two main types the primary (azurophil), forming first during myelopoiesis, and the secondary

(specific) which appear later in maturation (Bainton, Ulliyot & Farquhar, 1971). PMN granule constituents are summarised in Table 1.3 (Falloon & Gallin, 1986). The primary PMN granules contain microbicidal enzymes, neutral proteases, acid hydrolases and bactericidal cationic proteins. The secondary granules are twice as numerous as the primary granules (Bainton *et al.*, 1971) and contain mainly lysozyme, collagenase and lactoferrin. A third type of granules (C particles) contain gelatinase. The two major granule types (primary and secondary) serve different functions, with the secondary granules serving the environmental modification or external secretory function, whereas the primary granules act more as lysosomes fusing with the phagosome during phagocytosis and providing the necessary digestive enzymes (Henson, 1971b; Leffel & Spitznagel, 1975; Wright & Gallin, 1979).

PMNs in the circulation are attracted to a site of host defence via chemotactic mechanisms which could be elicited from microbially derived substances. PMNs initially adhere to the vessel wall, migrate through the endothelium and are chemotactically driven towards the stimulus. PMN trafficking is regulated by adhesion molecules either stimulated or constitutively expressed on endothelial cells. These molecules include intercellular adhesion molecule 1 (ICAM-1) and endothelial leucocyte adhesion molecule 1 (ELAM-1). The ligands for these molecules on PMNs (molecules of the CD11 family) seem to be located on

**Table 1.3 Polymorphonuclear leucocyte granule contents.**

<b>Class of constituent</b>	<b>Azurophilic granules</b>	<b>Specific granules</b>
Microbicidal enzymes	Myeloperoxidase Lysozyme	Lysozyme
Neutral serine proteases	Elastase Cathepsin G Proteinase 3	
Metalloproteinases		Collagenase
Acid Hydrolases	N-acetyl-b-glucosaminidase Cathepsin B Cathepsin D $\beta$ -Glucoronidase $\beta$ -Glycerophosphatase $\alpha$ -Mannosidase	
Other	Bactericidal cationic proteins	Lactoferrin Vitamin B <sub>12</sub> -binding proteins  Cytochrome b Histaminase FMLP receptors C3bi receptors

From Falloon and Gallin (1986)

the surface of the secondary PMN granules which fuse with the external membrane during chemotaxis providing receptors and the surface membrane required (Gallin, 1984; Todd et al., 1984; Hoffstein, Freidman & Weissmann, 1982). During this process secondary granule constituents are released in the area and it has been shown that PMN secondary granule contents are released earlier and more readily than primary granules during *in vitro* PMN stimulation (Bentwood & Henson, 1980). Moreover, PMN secondary granules have been shown to be preferentially released, over primary granules, during both adherence *in vitro* and migration through epithelium *in vivo* (Wright & Gallin, 1979). When PMNs arrive at the area of defence, phagocytosis is facilitated by the opsonisation of particles either via antibody or complement. CR3, the receptor for C3bi, the C3b hydrolysis product, is also located in the secondary granules (O'Shea et al., 1985). Both primary and secondary PMN granule release may, in addition, occur during fusion of lysosomes with incompletely formed phagocytic vacuoles, during adherence of the neutrophil on non-phagocytosable surfaces or during uptake of additional particles into a preformed phagolysosome (Henson, 1971a and 1971b) and upon cell lysis/death.

### 1.5.2 Polymorphonuclear leucocytes in the pathogenesis of periodontal disease

The polymorphonuclear leucocyte (PMN) is the predominant leucocyte within the gingival crevice in both health and disease. PMNs from the circulation are attracted to the area via chemotactic stimuli elicited from dental plaque (Hellden and Lindhe, 1973) and PMNs can be seen traversing the gingival connective tissue in inflammation (Brex et al., 1988a; Seymour et al., 1983a; Moughal et al., 1992b). Thus, Attström and Egelberg (1970), in an experiment on dogs, have shown that carbon labelled peripheral blood neutrophils from the circulation migrate in the gingival crevice and their migration rate seems to be higher in inflamed crevices. PMN numbers increase in the gingival crevice with the development of experimental gingivitis (Kowashi, Jaccard & Cimasoni, 1980; Thurre et al., 1984) and more PMNs are found in periodontitis compared to gingivitis sites although their viability and function assessed by trypan blue exclusion and ability to phagocytose, were decreased in the former (Thurre et al., 1984). As in other tissues, migration of leucocytes from the vessels into the gingival connective tissue, and through the junctional epithelium into the gingival crevice, is controlled via adhesion molecules. In a recent experimental gingivitis study Moughal et al. (1992b), showed that vessels in the gingival connective tissue express ELAM-1 and ICAM-1 in health and in gingival

inflammation and PMNs were found in greater abundance in areas expressing intense ELAM-1 and ICAM-1 staining. In addition, crevicular and junctional epithelium stained strongly positive for ICAM-1, suggesting the importance of this adhesion molecule in allowing PMN migration in the gingival crevice.

PMNs in the gingival crevice form the first line of defence against periodontal pathogens. This is illustrated by the fact that qualitative and quantitative deficiencies result in gross periodontal destruction (Wilton *et al.*, 1988, Kinane & Davies, 1990). Wilton (1986) summarised the stimuli leading to PMN degranulation (Table 1.4) and concluded that these stimuli occur in the crevice and that secondary granule release is more likely. PMNs will therefore release the contents of their granules during emigration towards the gingival crevice as well as during phagocytosis or upon encounter of antigen antibody complexes. Thus, PMNs have been shown to interact *in vivo* with oral bacteria and to release their intralysosomal contents to the extracellular environment (Taichman *et al.*, 1977; Tsai *et al.*, 1978). The lysosomal release was increased by incubation with serum (heat inactivated or not) indicating that opsonization by complement components and immunoglobulins could enhance the process. Scanning electron microscopy studies (Baehni *et al.*, 1977, 1978; Taichman *et al.*, 1977) indicated that lysosomal release occurred under similar circumstances to those described by

**Table 1.4** Stimuli leading to lysosomal granule release by neutrophils.

STIMULUS	GRANULE EXOCYTOSIS	
	Azurophilic	Specific
During phagocytosis (regurgitation) during feeding	++	+++
non-specific adherence	±	++
by attachment to surface-bound immune complexes or non-opsonised bacteria	+++	+++
bacterial toxins e.g. leucocidin	+++	+++
as a response to membrane binding by adherent cells of IgG, IgA, C3b, C5a, chemotaxins and opsonised bacteria	±	++
calcium ionophore	+	+++
concanavalin A	-	+++
HETE	-	++
adherence to surfaces in the presence of Ca <sup>++</sup> and during <i>in vivo</i> and <i>in vitro</i> migration	+	+++
on cell death and subsequent lysis	+++	+++

From Wilton (1986)

Henson (1971a, b), i.e. reverse endocytosis, regurgitation during feeding and cell death. It seems therefore that although the PMN has a protective role, the interplay between microbial plaque components, complement, immune complexes and PMNs, initiates the release of PMN intracellular contents, including potent proteases, during inflammation which may cause damage to the periodontal tissues (Ishikawa *et al.*, 1982; Wilton, 1986; Deguchi *et al.*, 1990, Altman *et al.*, 1992). Thus the PMN appears to have both a protective and potentially destructive role in the pathogenesis of periodontal disease. Two models have been proposed in order to justify this somewhat contradictory statement.

The first model, proposed by Wilton (1986), involves crevicular PMN function which he suggests, can be viewed as a two phase system: fluid and surface. In the fluid phase, PMNs may phagocytose plaque bacteria while their granule content release (mainly secondary) may help this process by inhibiting plaque bacteria adherence and re-adherence. Enzymes released during this process are readily neutralised by protease inhibitors present in the area. In contrast when PMNs attach to the plaque surface or the sulcular and junctional epithelium, PMN granule enzyme release as well as the release of oxygen metabolites, takes place in a closed environment, inaccessible to naturally occurring protease inhibitors (Wright & Silverstein, 1984).

The second model which has been proposed by Page and Schroeder (1982) involves gingival connective tissue PMNs. The authors hypothesise that two compartments exist for PMN function: the gingival connective tissue and the pocket/crevice. If the epithelial barrier is disrupted (perhaps by adhesion and degranulation of crevicular PMNs as described in the previous model), then an open entrance for bacterial components and metabolites which flood the gingival connective tissue is established, and the chemotactic gradient driving PMN emigration towards the crevice/pocket is disturbed. The effect is that PMNs now move randomly within the gingival tissue encounter microbial derived substances in the connective tissue compartment, and together with other activated systems and host cells cause periodontal damage. When the epithelial barrier and chemotactic gradient is re-established the inflammatory process subsides, as PMNs are again driven towards the crevice/pocket where they encounter bacteria and bacterial substances. Although both the above models are over simplifications of the processes leading to periodontal destruction they provide paradigms of the PMN contribution to the pathogenesis of periodontal disease.

In conclusion, PMNs seem to play a central role in the pathogenesis of periodontal disease. Periodontal disease activity episodes could be associated with increased PMN emigration and activation both within the gingival connective tissue as well as in the gingival crevice and it

has been suggested that crevicular PMN numbers could be a useful indicator of acute inflammatory processes in this area (Fine & Mandel, 1986; Curtis et al., 1989)

## **1.6 Proteinases**

### **1.6.1 Introduction**

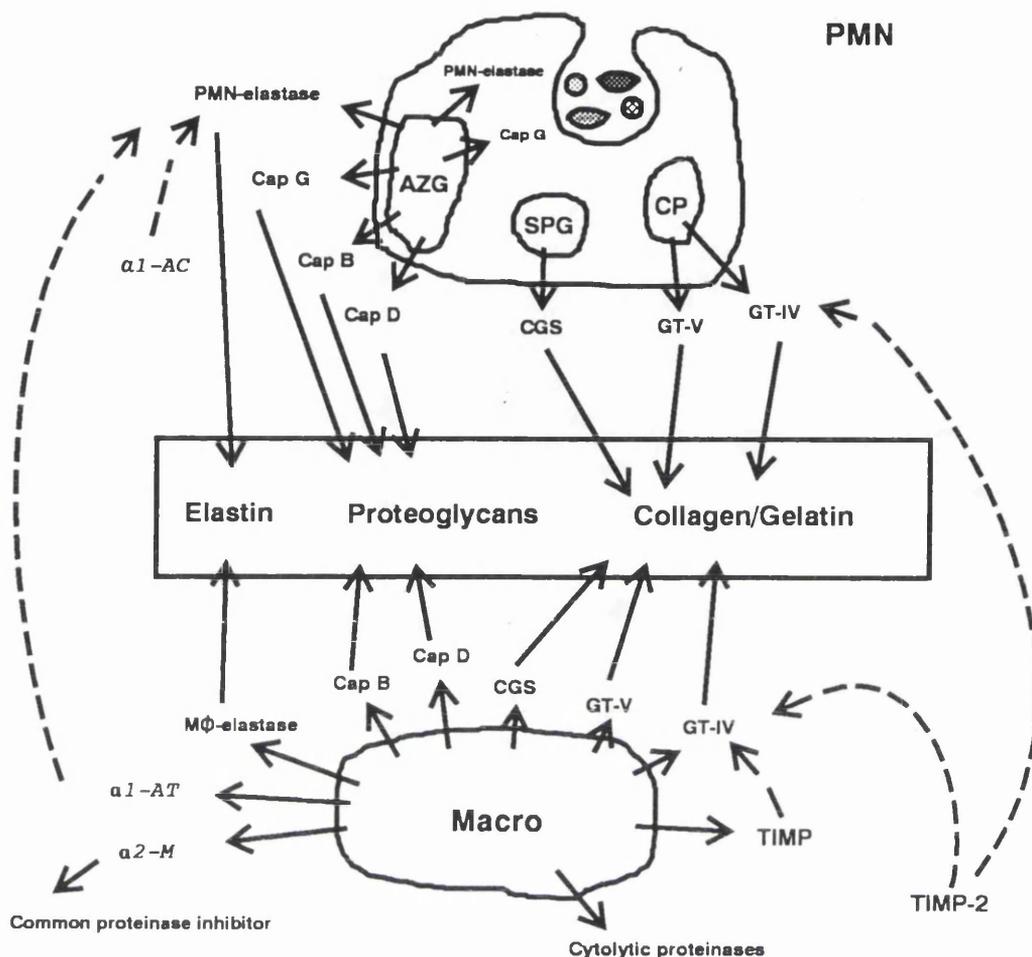
Proteolytic enzymes may be termed 'peptide bond hydrolases' (peptidases=proteases) and further classified into one of the two major classes: a) endopeptidases (proteinases) and; b) exopeptidases, depending on the location of activity of the enzyme against its substrate (Rappay, 1989). The enzymes of the first category cleave bonds in their substrate away from the end of the polypeptide chain, whereas the exopeptidases cleave their substrate only one or two residues from the end of the polypeptide chain. In the case of endopeptidases the essential catalytic group of the enzyme forms the basis for their classification into cysteine (formerly thiol), serine, aspartic (formerly carboxyl), metallo and finally proteinases of unknown catalytic class.

### **1.6.2 Proteinases in the pathogenesis of periodontal disease**

Exopeptidases have received limited attention by periodontal disease investigators. In contrast,

endopeptidases have been extensively investigated, due to their biological functions and their possible implication to the pathogenesis of periodontal disease. These potent proteinases are the effectors of periodontal damage. Their main source are the polymorphonuclear leucocytes and monocytes/macrophages but other host cells may also contribute (fibroblasts, epithelial cells) (Fig. 1.1) Mechanisms of their release have been reviewed in sections 1.5.1 and 1.5.2.

As shown in Table 1.5, over the past two decades numerous studies have been conducted in order to assess endopeptidase activity/amount in GCF. These studies include both experimental gingivitis, cross-sectional studies and longitudinal studies before and after periodontal treatment. In most cases assessment of proteinase activity has been attempted via the use of either native proteins (earlier studies) or chromogenic and fluorogenic substrates, although immunoassays have also been used for the assessment of the amount of the enzyme. An association with disease severity has been attempted in several of these studies and a positive association or a reduction of proteinase levels with treatment has in most cases been obtained (Table 1.5). Endopeptidase activity, including collagenase (Uitto, Turto & Saxen, 1978), elastase-like and trypsin-like (Uitto, 1987), as well as serine and cysteine proteinases (Eley & Cox, 1990, 1991), have also been detected in homogenates of gingival tissue.



**Figure 1.1** Proteinases are released by several cells resident at or arriving at the site of inflammation including polymorphonuclear leucocytes (PMN) and macrophages (Macro). Release of such enzymes contributes to tissue damage. The activity of such enzymes is regulated by inhibitors including  $\alpha 2$ -macroglobulin,  $\alpha 1$ -antitrypsin,  $\alpha 1$ -antichymotrypsin and tissue inhibitors of metalloproteinases (From Evans & Whicher, 1992)

Mφ:macrophage; AZG: azurophilic granules (1°); SPG: secondary granules (2°); CP: C particles; Cap: Cathepsin; CGS: collagenase; GT: gelatinase  
 $\alpha 1$ -AT:  $\alpha 1$ -antitrypsin;  $\alpha 2$ -M:  $\alpha 2$ -macroglobulin;  $\alpha 1$ -AC:  $\alpha 1$ -antichymotrypsin  
 TIMP: tissue inhibitor of metalloproteinases;  
 —→ release or degradation; -----> inhibition.

**Table 1.5 Studies on gingival crevicular fluid (GCF) endopeptidases (proteinases). Association with disease severity.**

Reference	Subjects	Endopeptidase	Comments
Ishikawa et al., 1972	AP	Cathepsin D- like activity	Total amounts correlated positively with PD and % attachment loss
Ohlsson et al., 1974	H, AP	Elastase (PMN), collagenase (PMN), collagenase & elastase activity, non-specific protease/fibrinolytic activity	Inflamed crevices yielded higher amounts of proteinases
Baehni et al., 1975	EX G	Cathepsin D-like activity	Concentration in gingival washings rose with the development of gingivitis
Golub et al., 1976	AP	Collagenase activity	Host origin; total and concentration correlated positively with GI
Tzamouranis et al., 1977	EX G	Cathepsin D-like activity	Concentration in gingival washings followed the changes of the clinical indices
Kowashi et al., 1979	EX G	Collagenase activity, neutral protease	Concentration in washings rose with the development of gingivitis
Eisenhauer et al., 1983	G, AP	Cathepsin B-like activity	Development of fluorometric assay; detection in GCF from inflamed sites
Lah et al., 1986	AP	Cathepsin D- & L- like activity	Concentration correlated positively with PD
Larivee et al., 1986	H, JP	Collagenase activity	Higher collagenase activity in diseased sites; host origin

**Table 1.5 Cont'd. Studies on GCF endopeptidases (proteinases). Association with disease severity.**

Reference	Subjects	Endopeptidase	Comments
Villela et al., 1987	H, G, AP, JP	Collagenase activity	Total amounts demonstrated positive association with disease severity at patient level: $H < G < AP$
Cox & Eley, 1989a	G, AP	Tryptase like activity	Detection in GCF
Cox & Eley, 1989b	G, AP	Cathepsin B/L, elastase, trypsin & trypsin like activities	Host origin; elastase activity the highest in GCF
Beighton & Life, 1989	G	Trypsin like activity	Total amounts correlated positively with GI PI and PD
Kunimatsu et al., 1990b	EX G, AP	Cathepsin B-, H- & L- like activity	Total amounts correlated positively with PD
Beighton et al., 1990	G, AP	Endopeptidase activity	Total amounts correlated positively with GI and BI
Gangbar et al., 1990	H, AP, JP	Collagenase & gelatinase activity	AP and JP patients demonstrated higher activities than H controls in mouthrinse samples
Sorsa et al., 1990	AP	Collagenase activity	PMN origin

**Table 1.5 Cont'd. Studies on GCF endopeptidases (proteinases). Association with disease severity.**

Reference	Subjects	Endopeptidase	Comments
Kunimatsu et al., 1990a	EX G, AP	Meddulasin	Concentration dropped with treatment in the AP group
Talonpoika et al., 1991	H, G, AP	Plasmin/fibrinolytic activity	Total amounts correlated positively with clinical parameters; levels dropped with treatment
Eley & Cox, 1992a	AP	As Cox and Eley, 1989b	Positive association of total amounts and concentrations with clinical parameters
Eley & Cox, 1992b Cox & Eley, 1992	AP	As Cox & Eley, 1989b	Reduction of total amounts or concentrations with treatment (scaling and/or periodontal surgery)
Gustafsson et al., 1992	AP	Antigenic elastase & elastase activity	Total amounts and concentration demonstrated positive association with clinical parameters
Palcanis et al., 1992	AP	Elastase activity	Higher total elastase in periodontally active sites
Suomalainen, 1992	AP	Collagenase, elastase, cathepsin G activity	Higher levels in diseased sites. Levels dropped with treatment; no association between autoactive collagenase and Cathepsin G

AP: Periodontitis patients; JP: Juvenile Periodontitis patients; H: Healthy controls; EX G: Healthy subjects during experimental gingivitis; GI: gingival index; BI: Bleeding index; PD: pocket/probing depth; PMN: polymorphonuclear leucocyte

Moreover, tissue plasminogen activator activity associated with the epithelium lining of the crevice has been recently shown (Schmidt, Cohen & Chambers, 1991).

Table 1.6 lists properties of the endopeptidases /proteinases studied in the gingival crevice and it is obvious that all of them could be implicated in the amplification of the inflammatory process. These proteinases could contribute to connective tissue damage which is critical to the development of periodontal destruction. Crude polymorphonuclear leucocyte extracts have been shown to induce ulcerations of the crevice epithelium in rabbits *in vivo* (Ishikawa et al., 1982) and purified PMN elastase can cause *in vitro* enlargement of the intracellular spaces in the oral epithelium of the gingivae and collagen loss in the underlying connective tissue (Cergneux et al., 1982).

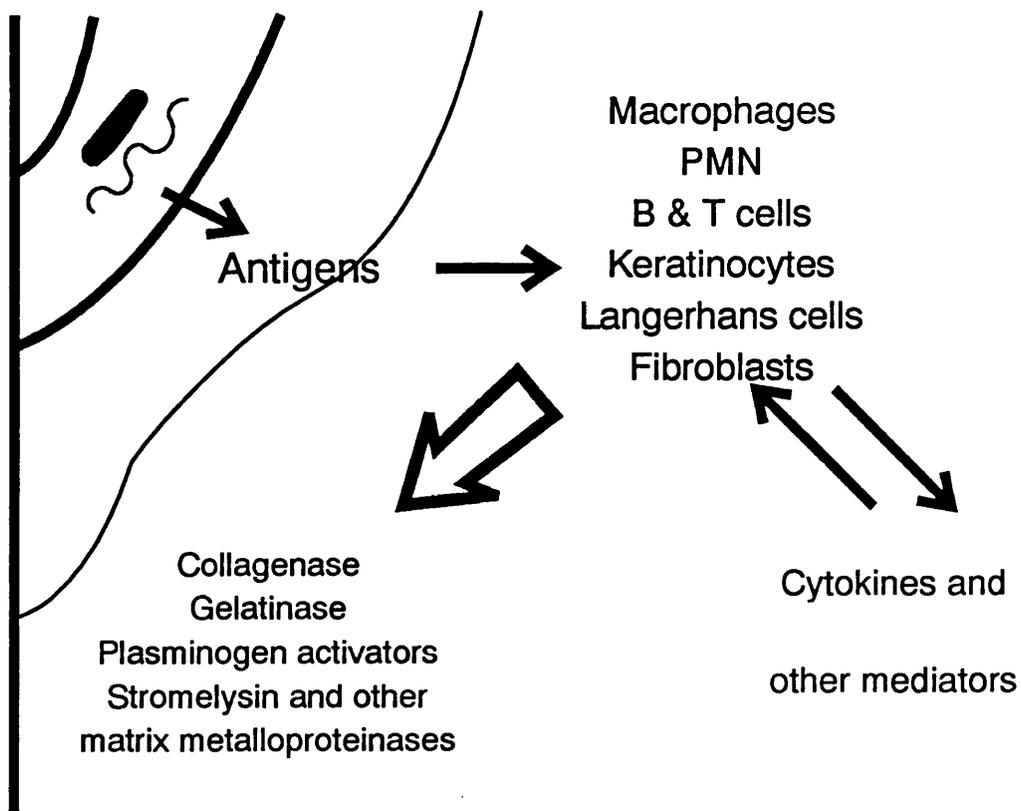
As shown in Table 1.5 collagenase is one of the most extensively studied proteinases in relation to periodontal disease due to its obvious implication in its pathogenesis via the degradation of collagen. The fibroblast and PMN type collagenases share the unique property of being able to cleave type I, II and III collagens in the helical domain. The PMN collagenase has a higher molecular weight than the fibroblast type and cleaves preferentially type I collagen in contrast again to the fibroblast type collagenase which prefers type III collagen (Mallya et al.,

**Table 1.6** Properties of endopeptidases (proteinases) detected in the gingival crevice.

<b>Class</b>	<b>Proteinase</b>	<b>Function</b>	<b>Reference</b>
<b>Cysteine</b>	Cathepsins B,H,L	Collagen & proteoglycan degradation	Maciewicz & Etherington, 1988; Nguyen et al., 1990
		Latent collagenase activation	Beckhout & Vaes 1977
		Degradation of bone organic matrix	Delaisee et al., 1980 Everts et al., 1988
<b>Serine</b>	Elastase	Elastin, collagen & immunoglobulin degradation	Janoff & Scherer, 1968 Starkey et al., 1977 Solomon et al., 1978
		Cleavage of complement components	Venge, 1978
		Latent collagenase activation	Cawston et al., 1981
	Cathepsin G	Collagen degradation	Starkey et al., 1977
		Cleavage of complement components	Venge, 1978
		Latent collagenase activation	Sorsa et al., 1990
	Mast cell tryptase	Latent collagenase activation	Gruber et al., 1988
		Cleavage of complement components	Schwartz et al., 1983
		Plasminogen activator	Collagenase activation via plasmin generation
	<b>Aspartic</b>	Cathepsin D	Degradation of collagen breakdown products
<b>Metallo</b>	Collagenase	Native collagen degradation	Ohlsson, 1978
		General protein substrates	Fields et al., 1990
	Gelatinase	IV/V collagen and basement membrane degradation	Wilhelm et al., 1989 Hibbs et al., 1985

1990). Interstitial collagenases also show activity against general proteinase substrates (Fields et al., 1990). Gingival fibroblasts have been shown to produce interstitial type collagenase (Heath et al., 1982) and this ability is also shared by periodontal ligament fibroblasts (Overall & Sodek, 1987) and is induced (together with gelatinase activity), at least as far as gingival fibroblasts are concerned, by IL-1 (Richards & Rutherford, 1988; Meikle et al., 1989), TNF- $\alpha$  (Meikle et al., 1989) and concanavalin A (Overall, Wrana & Sodek, 1991). Interstitial type collagenases are also produced by macrophages, endothelial cells and keratinocytes (Fig. 1.2). Many of the proteinases released in the gingival crevice, as well as being able to induce direct damage to the host may also activate interstitial collagenases inducing further periodontal damage (Table 1.6). Particularly important in this process seems to be plasminogen activator, which due to its ability to transform plasminogen into the active enzyme plasmin, activates latent collagenases.

The implication of proteases of bacterial origin in periodontal destruction cannot be excluded and several putative periodontal pathogens including *P. gingivalis* and *A. actinomycetemcomitans* demonstrate proteolytic/collagenolytic activity (Slots, 1981; Robertson et al., 1982; Birkedal-Hansen et al., 1988; Scheinken, 1988). Whether such enzymes could penetrate the periodontal



**Figure 1.2** Antigens or inflammatory mediators may induce resident cells of the periodontium to release metalloproteinases (from Kinane 1992).

PMN: polymorphonuclear leucocyte.

tissues and maintain sufficient concentrations therein to contribute to periodontal destruction is questionable (Kelstrup & Theilade, 1974; Birkedal-Hansen *et al.*, 1988). Moreover, recent studies have shown that the predominant collagenases in the gingival crevice are of mammalian origin, evidenced by the breakdown of collagen into three and one quarter collagen fragments, which is characteristic of the function of vertebrate collagenases (Villela *et al.*, 1987; Sorsa *et al.*, 1990).

## **1.7 Protease inhibitors**

### **1.7.1 Introduction**

Modulation of proteinase activity in biological fluids and tissues is accomplished by protease inhibitors either locally produced or circulating in plasma. The major plasma protease inhibitors are  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M),  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT) and  $\alpha$ 1-antichymotrypsin.  $\alpha$ 2-M and  $\alpha$ 1-AT account for approximately 90% of serum's protease inhibiting capacity (Ohlsson, 1978a) and consequently these particular protease inhibitors have received most attention in periodontal research. In addition, specific metalloproteinase activity inhibition within the tissues is accomplished by the family of the tissue inhibitors of metalloproteinases (TIMP).

$\alpha$ 2-M is a proteinase inhibitor with outstanding properties (reviewed in Starkey & Barrett, 1977). It is a tetramer of identical subunits (182 kDa) which are covalently linked forming half-molecules, which in turn are non-covalently associated to form the native molecule (725 kDa).  $\alpha$ 2-M has a very wide range of inhibitory function and is active against endopeptidases of all the catalytic types (serine-, cysteine-, aspartic- and metallo-). Only active enzymes react with  $\alpha$ 2-M and cleave the tetramer in the bait region producing a conformational change of the molecule which then irreversibly entraps the endopeptidase and inactivates it. The enzyme, however, although entrapped in the  $\alpha$ 2-M molecule can retain its activity against low molecular weight peptides like chromogenic and fluorogenic substrates (Starkey & Barrett, 1977).  $\alpha$ 2-M proteinase complexes, once formed, are rapidly removed from the circulation (half-life of approximately 10 min) (Ohlsson & Laurell, 1976) or tissues by macrophages via receptor mediated endocytosis (Feldman *et al.*, 1983). The free and bound form of the inhibitor are referred to as the 'slow' and 'fast' form respectively, based on their electrophoretic mobility. This altered mobility of the inhibitor is probably due to its conformational change when reacting with an endopeptidase (Starkey & Barrett, 1977). In addition, the change in  $\alpha$ 2-M isoelectric point from 5.0 to 6.0, associated with complex formation, could be used for the demonstration of the free and bound form of the inhibitor in complex biological fluids (Ohlsson & Skude, 1976).

$\alpha$ 1-AT has a much lower molecular weight (approximately 55 kDa) than  $\alpha$ 2-M and is active mainly against serine proteinases, elastase being the typical example (Kueppers, 1973). It is also called  $\alpha$ 1-proteinase inhibitor as it has been suggested that the former name may be misleading, because trypsin inhibition is functionally much less important than the inhibition of other serine proteases like elastase (Travis & Salvesen, 1983). The inhibition of the protease is performed, in contrast to  $\alpha$ 2-M, by binding of the active site of the enzyme (1:1 molar ratio). The enzyme is then transferred to  $\alpha$ 2-M and eliminated (Ohlsson, 1971a,b). The half life of the complex in the circulation is considerably longer (half life 1h) than that of  $\alpha$ 2-M (Ohlsson & Laurel, 1976).

$\alpha$ 1-AT is a genetically polymorphic protein, the phenotypes characterised by different electrophoretic mobilities. The normally migrating M variant is the commonest in man whereas the slow S and Z gene products are associated with reduced plasma concentration of the inhibitor.  $\alpha$ 1-AT deficiency (S and Z phenotypes) has been associated with chronic inflammatory disorders characterised by uncontrolled proteolysis such as pulmonary emphysema (Kueppers, 1973).

Both  $\alpha$ 2-M and  $\alpha$ 1-AT are mainly serum derived glycoproteins, their serum concentrations being approximately 2mg/ml (Johansson, 1979). Their main site of production is the

liver.  $\alpha 1$ -AT is regulated as an acute-phase reactant whereas  $\alpha 2$ -M is a serum acute-phase protein in rat and rabbit but not in man (Starkey & Barrett, 1977). In addition, both  $\alpha 1$ -AT and  $\alpha 2$ -M can be locally produced at the site of inflammation.  $\alpha 1$ -AT is produced by monocytes/macrophages (Perlmutter et al., 1985; Bauer, Ganter & Gerok, 1988) and there is evidence of its production by human polymorphonuclear leucocytes and storage within their primary granules (Mason et al., 1991).

$\alpha 2$ -M is also produced at the cellular level by monocytes and macrophages (Hovi, Mosher & Vaheri et al., 1977; White, Janoff & Godfrey, 1980; Bauer et al., 1988) as well as fibroblasts (Budek et al., 1986). It has recently been suggested that the transition of human monocytes into macrophages *in vitro* is accompanied by a strong induction in the production and secretion of  $\alpha 2$ -M (Bauer et al., 1988) and this may be important *in vivo*.

In addition to their well known protease inhibiting properties, it has been suggested that both  $\alpha 2$ -M and  $\alpha 1$ -AT could modulate several inflammatory and immunological processes. As reviewed recently by James (1980, 1990) *in vitro* evidence suggests that  $\alpha 2$ -M could modulate/inhibit immune responses via several pathways. This could possibly occur through inhibition of proteolytic dependent reactions, binding of important effector molecules like mitogens and cytokines and blocking of cell receptors. Suppression of immunological and inflammatory mechanisms

has also been ascribed to  $\alpha$ 1-AT. Experimental evidence implicates the latter inhibitor in the regulation of lymphocyte responses, complement activation and neutrophil migration (reviewed by Breit & Penny, 1980). It has, for example, been shown that both  $\alpha$ 2-M and  $\alpha$ 1-AT could inhibit natural killing and antibody dependent cell mediated cytotoxicity and that  $\alpha$ 2-M subunits may be particularly effective (Ades et al., 1982; Gravagna et al., 1982; Dickinson et al., 1985).  $\alpha$ 1-AT has been recently shown to inhibit FMLP induced neutrophil chemotaxis and this is probably related to its protease inhibitory capacity (Stockley et al., 1990).  $\alpha$ 2-M has also been found to inhibit lymphocyte responses to several lectins (Miyanaga et al., 1982) and to bind recombinant interleukin-1b (Borth et al., 1990). The evidence reviewed by James (1980, 1990) and Breit and Penny (1980), similarly to the above mentioned individual studies is, however, based on *in vitro* observations and should therefore be viewed with care (Munck-Petersen, 1990). The implication however of  $\alpha$ 2-M and  $\alpha$ 1-AT in immunological reactions cannot be excluded and further research in the field may elucidate their *in vivo* relevance to inflammatory and immunological processes.

### **1.7.2 Protease inhibitors in the gingivae**

In contrast to proteases, protease inhibitors have been investigated less extensively in relation to periodontal

disease and some conflicting reports exist in the literature.

$\alpha$ 2-M and  $\alpha$ 1-AT in the gingival tissues are derived from the circulation as well as from monocytes/macrophages and gingival fibroblasts. Production of  $\alpha$ 2-M by gingival fibroblasts which were cultured to confluence from gingival explants has been demonstrated by Condacci *et al.* (1988). The synthesis of  $\alpha$ 2-M was demonstrated by incorporation of a radiolabelled amino acid into newly synthesized protein. It was suggested by the authors that local production may be an important mechanism for the control of proteolysis in the gingival tissues and the crevice, as this high molecular weight inhibitor would be readily available in the area. In a more recent report from the same group (Giannopoulou *et al.*, 1990), an attempt was made to provide *in vivo* evidence of the local production of  $\alpha$ 2-M using the experimental gingivitis model. GCF was used as the study material and the excretion coefficient ( $\alpha$ 2-M to albumin ratio in GCF corrected for the  $\alpha$ 2-M to albumin ratio in serum) to test for local  $\alpha$ 2-M production. Although the authors conclude that local production occurs, this study suffers from the lack of sufficient statistical analysis, the use of crude means for calculation of the ratios and the assumption that increase/decrease in the  $\alpha$ 2-M/albumin ratio would be due to changes in  $\alpha$ 2-M levels only, whereas albumin consumption in the crevice is also quite possible. Nevertheless, local *in vivo*  $\alpha$ 2-M production is likely to

occur, as suggested from the *in vitro* studies (Hovi et al., 1977; White et al., 1980; Budek et al., 1986; Bauer et al., 1988; Condacci et al., 1988) and in addition to gingival fibroblasts (Condacci et al., 1988), mature macrophages may also contribute (Bauer et al., 1988).

In addition to the major serum protease inhibitors,  $\alpha$ 2-M and  $\alpha$ 1-AT, specific regulation of metalloproteinase activity within the tissues is also accomplished by the family of tissue inhibitors of metalloproteinases (TIMP). These are a family of low molecular weight inhibitors (29 kDa) and fibroblasts (Heath et al., 1982) as well as mononuclear phagocytes (Campbell et al., 1991) are probably responsible for TIMP production within the tissues.

TIMP production by gingival fibroblasts is upregulated by transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) and suppressed by concanavalin A, a lectin which may mimic physiological pathways of cellular activation (Overall et al., 1989; Overall et al., 1991). In addition to suppressing TIMP production by gingival fibroblasts at the transcriptional level concanavalin A also induces collagenase production (Overall et al., 1989; Overall et al., 1991). Therefore, interactions between cytokines, potent inducers of cellular differentiation /activation, and resident cells of the periodontium, may determine whether repair or destruction of periodontal tissues will occur. However, although TIMP is present within the tissues, it has been shown that

collagenase binds preferentially to  $\alpha 2$ -M (Cawston & Mercer, 1986) and this fact taken together with the ability of several serine proteinases, including elastase, to destroy TIMP's protease inhibitory capacity (Okada *et al.*, 1988), emphasises the importance of  $\alpha 2$ -M as a protease inhibitor within the tissues (Docherty & Murphy, 1990). In addition the mechanisms inducing/suppressing TIMP production would also apply to  $\alpha 2$ -M and  $\alpha 1$ -AT because, as mentioned above, differentiation of blood monocytes to tissue macrophages is followed by a strong induction of the production of these inhibitors (Bauer *et al.*, 1988). This would result in the availability of  $\alpha 2$ -M and  $\alpha 1$ -AT within the tissues through local production as well as serum.

As discussed in section 1.6 release of proteases in the gingivae and the crevicular area promotes inflammatory reactions and contributes to connective tissue damage via several pathways, the metalloproteinases playing a significant role. In contrast, protease inhibitors would serve as modulators of protease function in the area and together with their possible immune suppressive properties would hamper the inflammatory process. All the host derived endopeptidases shown to be released in the gingival crevice can be inhibited by the combined function of  $\alpha 2$ -M and  $\alpha 1$ -AT. In fact, gingival collagenase inhibition by  $\alpha 2$ -M has been demonstrated (Birkedal-Hansen *et al.*, 1974) and PMN collagenase is in addition inhibited by  $\alpha 1$ -AT (Ohlsson & Olsson, 1977). Human serum was also shown to inhibit

bacterial collagenase providing indirect evidence that plasma proteinase inhibitors are effective against bacterial proteases (Gibbons & McDonald, 1961).

### **1.7.3 Serum protease inhibitors and periodontal disease status**

When the association between  $\alpha$ 1-AT deficiency and pulmonary emphysema was established an attempt was made to investigate whether a similar association existed in periodontal disease. Peterson and Marsh (1979) investigated  $\alpha$ 1-AT phenotypes in a group consisting of 100 subjects with no signs of periodontal disease and 50 subjects who suffered from periodontal disease of varying severity. Although no correlations could be observed between  $\alpha$ 1-AT serum concentrations and periodontal disease severity, the periodontitis patient group demonstrated higher frequency of  $\alpha$ 1-antitrypsin MZ and MS phenotypes in serum when compared to the control group. The hypothesis that an association between  $\alpha$ 1-AT deficiency and incidence of periodontal disease could exist was investigated further by Sandholm, Saxen and Koistinen (1981) in a group of 19 juvenile periodontitis patients. Juvenile periodontitis was chosen as the clinical entity to be investigated because of its low prevalence, which is similar to that of  $\alpha$ 1-AT deficiency. In contrast to the study of Peterson and Marsh (1979) all patients in the latter study proved to be

of the most common phenotype MM.  $\alpha$ 1-AT and  $\alpha$ 2-M concentration in serum was within the normal range in this group of juvenile periodontitis patients. The authors therefore concluded that juvenile periodontitis is not related to  $\alpha$ 1-AT deficiency. In a more recent study (Zafiropoulos et al., 1988) the findings of the previous investigators (Sandholm et al., 1981) were verified as the concentration of  $\alpha$ 1-AT in serum was not found to differ in juvenile and rapidly progressive periodontitis patients when compared to periodontally healthy controls. This was also the case for  $\alpha$ 2-M serum levels (Zafiropoulos et al., 1988) which had, in addition, been shown to be within the normal range in a group of 25 juvenile periodontitis patients in a previous study (Sandholm & Saxen, 1983). An association has also been sought between  $\alpha$ 2-M serum concentration and periodontal disease status, as determined by mean subject gingival index and probing depths in a group of 86 subjects (Skaleric et al., 1987). Although the authors suggest that a positive causal association exists between periodontal disease and serum  $\alpha$ 2-M levels, the very low, albeit significant, correlation coefficient is a reflection of the great number of subjects used ( $r \leq 0.26$ ,  $p < 0.05$ ), and has weak biological significance.

In conclusion, it seems that little can be gained from examining protease inhibitor levels in serum either for identifying high risk groups or determining periodontal disease status. As protease release is a local event in

periodontal disease, occurring predominately at sites of inflammation and/or periodontal disease activity it would appear more pertinent and fruitful to investigate protease inhibitors in the gingival crevice and tissues.

#### **1.7.4 Protease inhibitors in gingival crevicular fluid**

The most widely investigated inhibitor in the gingival crevice is  $\alpha$ 2-M, although recent reports have also focused on  $\alpha$ 1-AT and its complex to neutrophil elastase.

Probably the first report on the presence of  $\alpha$ 2-M and  $\alpha$ 1-AT in the gingival crevice is that of Ohlsson *et al.* (1974). These authors demonstrated the presence of both inhibitors in healthy and inflamed 'crevicular material' using an electroimmunoassay and reported higher concentrations in the sample eluate from inflamed when compared to healthy gingivae. When examining  $\alpha$ 1-AT using crossed immunoelectrophoresis, these authors (Ohlsson *et al.*, 1974) observed considerable heterogeneity in its migratory pattern and took this as an indication that the protease inhibitor was complexed to proteases. As elastolytic activity was still evident in samples from both healthy and inflamed gingivae, the authors concluded that the protease inhibitory capacity of  $\alpha$ 1-AT was saturated.

In a contemporary study, Scheinken and Genco (1977) reported that the concentration of  $\alpha$ 1-AT and  $\alpha$ 2-M in GCF

from inflamed sites is approximately 70% of that of serum for both inhibitors and a similar ratio, although slightly higher, was obtained in a later study by Tollefsen and Saltvedt (1980). The ratio of  $\alpha$ 1-AT to transferrin in GCF and serum from patients with gingivitis and periodontitis has also been compared and higher mean ratios have been obtained in GCF. This did not, however, correlate with the severity of gingival inflammation (Asman, Bergstrom & Soder, 1981).

Condacci et al. (1982) investigated  $\alpha$ 2-M in gingival crevicular washings during an experimental gingivitis period and in individual GCF samples before and after periodontal treatment. Radial immunodiffusion was used to assess total (bound and unbound)  $\alpha$ 2-M concentration in the gingival washings which was shown to increase with the development of gingival inflammation and to drop gradually after the reinstatement of oral hygiene measures and the resolution of gingivitis. Using an electroimmunoassay, absolute amounts of total  $\alpha$ 2-M (bound and unbound) were shown to drop with treatment and the resolution of gingival inflammation in GCF samples from 11 patients although the specific content ( $\alpha$ 2-M  $\mu$ g/mg of GCF) was shown to increase. The free and bound form of the inhibitor were also assessed in separate GCF samples from the same patients using isoelectric focusing followed by crossed immunoelectrophoresis and although free and bound form was invariably detected prior to treatment, after treatment

only the bound form of  $\alpha 2$ -M was observed. The reason for the presence of free  $\alpha 2$ -M in the inflamed and its absence from the healthy crevice could not be explained by the authors. The presence of free  $\alpha 2$ -M in inflamed sites in this study, could be due to serum contamination of the GCF sample in inflamed sites. This is quite possible as long sampling periods (paper strips 3-5 min) were employed.

In a more recent study Skaleric et al. (1986) reported that GCF  $\alpha 2$ -M concentration is lower in sites with more gingival inflammation and higher proportion of periodontal attachment loss. In contrast, however, to Condacci et al. (1982) no bound  $\alpha 2$ -M was found in GCF from inflamed sites. The authors hypothesize that any bound form of the inhibitor is removed by macrophage receptor mediated endocytosis. However, this is difficult to accept. Significant amounts of proteases are released within the gingival crevice, mainly by degranulating /phagocytosing polymorphonuclear leucocytes. This process would be enhanced by the invasive GCF sampling procedure applied in this study (microcapillary tubes for the collection of  $2\mu\text{l}$  of GCF). In addition, adherence of PMNs on the glass surface of the microcapillary tubes, used in this study for GCF collection, would lead to degranulation and release of lysosomal proteinases. Thus, any free  $\alpha 2$ -M would readily bind to proteases. Mononuclear phagocytes and macrophages are scarce in the gingival crevice (Skapski & Lehner, 1976) and therefore the mechanism of removal of these complexes

suggested by the authors does not apply. The small number of patients/sites (n=14) participating in this study and the use of an invasive GCF sampling technique dictate further research if the bound/free  $\alpha$ 2-M ratio in the gingival crevice is of interest.

Sengupta, Lamster and Khocht (1988) also assessed the effect of treatment on absolute amounts of GCF  $\alpha$ 2-M levels using ELISA methodology. Similarly to Condacci *et al.* (1982) they reported a significant reduction in  $\alpha$ 2-M absolute amounts after treatment. This was also the case in a more recent study of the same group (Lamster *et al.*, 1990). The latter investigators (Lamster *et al.*, 1990) also sought to establish within subject correlations (intraclass correlations) for GCF  $\alpha$ 2-M as well as other GCF constituents of the local inflammatory response including lysosomal enzymes and immunoglobulins. The high intraclass correlations obtained for  $\alpha$ 2-M and immunoglobulins in GCF led the authors to conclude that although these proteins might be locally produced their levels seem to be highly influenced by systemic factors, most probably their serum concentration levels.

Sibraa *et al.* (1991) have attempted to devise a method which, with modification, could be used for chairside GCF acute phase protein level assessment, including  $\alpha$ 2-M and  $\alpha$ 1-AT. Direct and indirect immunodot were evaluated using either  $^{125}\text{I}$  or enzyme labelled antibody/colorimetrics as a

marker.  $\alpha$ 1-AT was not readily detectable in GCF samples using this methodology.  $\alpha$ 2-M demonstrated lower concentrations in diseased sites.

Two recent reports (Zafiropoulos et al., 1991; Huynh et al., 1992) have focused on  $\alpha$ 1-AT/elastase complex assessment in GCF using a commercially available kit (Merck), based on enzyme linked immunosorbent assay (ELISA) methodology. This ELISA methodology reflects elastase concentration in GCF, which avidly binds to  $\alpha$ 1-AT. In contrast, the inhibitor will also bind to other available serine proteinases like Cathepsin G and plasminogen activator and metalloproteinase like collagenase (Ohlsson & Olsson, 1977). Thus assessment of the  $\alpha$ 1-AT/elastase complex in GCF via ELISA methodology will only partly reflect the bound form of the inhibitor and will not provide information on the true bound/free  $\alpha$ 1-AT ratio. Nevertheless, these reports add a different dimension to  $\alpha$ 1-AT research.

Zafiropoulos et al. (1991) demonstrated a positive association between GCF  $\alpha$ 1-AT/elastase complex concentration and several black pigmented Bacteroides species with *P. gingivalis* (formerly *B.gingivalis*) showing the highest association with the complex. The association of GCF  $\alpha$ 1-AT/elastase complex with bacterial species associated with periodontal disease may reflect higher PMN activity at sites colonised with higher numbers of putative

periodontal pathogens. Clinical parameters however demonstrated weak correlations with GCF  $\alpha$ 1-AT/elastase complex concentration.

Huynh *et al.* (1992) reported higher GCF concentration of  $\alpha$ 1-AT/elastase complex in stable gingivitis, adult periodontitis and rapidly progressive periodontitis when compared to healthy sites, where in most cases the levels of the complex were below the detectability limit of the assay.  $\alpha$ 1-AT/elastase levels were higher, when compared to both healthy and stable sites, in an additional 11 sites where progressive attachment loss during the two month observation period was detected.

Perhaps the only recent report on simultaneous assessment of  $\alpha$ 2-M and  $\alpha$ 1-AT in GCF is that of Giannopoulou *et al.* (1992) who assessed these protease inhibitors during experimental gingivitis. Although this study mainly focuses on determining whether bound or free elastase exists in GCF, some interesting information appears in relation to the two inhibitors. Both absolute amounts and concentrations of  $\alpha$ 2-M and  $\alpha$ 1-AT were shown to increase with the development of gingival inflammation. This is in contrast to previous reports (Skaleric *et al.*, 1986; Sibraa *et al.*, 1991) including one from the same group (Condacci *et al.*, 1982) who have shown that GCF  $\alpha$ 2-M concentration decreased in more diseased sites. This could be due to the different methods of GCF collection employed: Condacci *et*

*al.* (1982) collected GCF using filter paper strips for 3 to 5 min; *Sibraa et al.* (1991) used filter paper strips for an undefined period of time (or until 1/3 of the paper strip was visibly wet); finally, *Skaleric et al.* (1986) sampled using microcapillary tubes and collected 2 $\mu$ l of GCF. These methods of GCF collection are thought to irritate the gingival tissues (*Cimasoni, 1983; Persson & Page, 1990*) and could thus increase vascular permeability in the area which could adversely affect GCF  $\alpha$ 2-M concentration in an uncontrollable manner. In contrast, *Giannopoulou et al.* (1992), collected GCF using a much less invasive technique (paper strips for 30s) and thus concentration of GCF constituents in GCF samples obtained in this study probably reflect better the true situation in the crevice.

In conclusion, although several attempts have been made to assess  $\alpha$ 2-M and  $\alpha$ 1-AT in GCF and to associate them with periodontal disease status at specific sites, few reports have focused on the simultaneous assessment of both inhibitors in GCF. Such data would provide information on the protease inhibitory potential at specific sites. In addition inconsistencies exist as far as  $\alpha$ 2-M is concerned regarding its concentration in GCF and its association with periodontal disease status. Finally, with the exception of the study of *Huynh et al.* (1992) no reports of a longitudinal nature exist in the literature which would elucidate the relationship of protease inhibitor levels

with active periodontal destruction.  $\alpha$ 2-M and  $\alpha$ 1-AT have important regulating functions on the activity of destructive proteases and in several processes of both inflammatory and immunological relevance such that further research in the area is justified (see Aims; section 1.9).

## **1.8 Iron-binding proteins: The transferrins**

### **1.8.1 Introduction**

The transferrins are a group of iron-binding proteins involved in iron transport and include transferrin (TF) and lactoferrin (LF).

TF and LF are glycoproteins with a molecular weight of approximately 80kDa. TF is mainly serum derived with a plasma concentration of approximately 2mg/ml (Johansson, 1979). The main site of production of TF is the liver although monocytes/macrophages (Stecher & Thorbecke, 1967) and T lymphocytes (Lum *et al.*, 1986) may also contribute in TF synthesis within the tissues. Plasma TF is regulated as a negative acute-phase reactant, its concentration decreasing during the acute phase of inflammation. LF is a highly cationic protein, structurally similar to TF (Metz-Boutigue *et al.*, 1984), but immunologically distinct. LF is mainly a protein of external secretions and it is present in milk, tears as well as saliva (Masson, Heremans

& Dive, 1966). It is also contained, specifically and in abundance, within PMN secondary granules (Spitznagel et al., 1974) but not in other leucocytes (Bennet & Kokocinski, 1978) and only in trace amounts in serum (Hetherington, Spitznagel & Quie, 1983). LF is produced at the promyelocyte stage (Rado et al., 1984) while mature PMNs lose their ability to synthesise LF.

TF and LF have high affinity for iron and bind two ferric ions per molecule. LF binds more avidly to iron than TF (Aisen & Leibman, 1972) and retains its iron binding ability even at low pH (up to approximately pH 2) (Masson & Heremans, 1968) whereas TF's affinity for iron falls with even a moderate pH drop.

The main function of TF is the transport of iron between sites of absorption, storage, utilization and excretion (Aisen, 1980). It is now well established that all cells requiring iron, express a TF receptor and transport of iron into the cells is performed via receptor mediated endocytosis. Upon iron donation apo-TF (iron free) is released to the environment via a reverse mechanism (Dautry-Varsat, 1986). One of the very early recognised functions of LF is its ability to inhibit bacterial growth by binding iron and creating an iron limiting environment. This may be particularly important at sites of inflammation where the lowered pH may limit the affinity of iron to TF. It has also been shown that LF may have bactericidal

properties independent of iron deprivation (Arnold *et al.*, 1982). A recent report suggests that LF and lysozyme may act in synergy to induce killing of Gram negative bacteria and this is associated with the ability of LF to bind to bacterial lipopolysaccharide (Ellison & Giehl, 1991). Lack of LF seen in individuals with PMN secondary granule deficiency, is associated with increased incidence of infections (Breton-Gorius *et al.*, 1980). Finally, both TF and LF have been shown to bind to oral isolates (Beighton *et al.*, 1992; Ellison & Giehl, 1991). In addition, LF by binding to bacteria may reduce their hydrophobicity, and diminish their ability to adhere and readhere, thus rendering them susceptible to phagocytosis (Wilton, 1986).

During the past two decades a number of additional properties of both TF and LF have been investigated and their implication in immunological and inflammatory mechanisms is now recognised (Brock, 1989). TF is implicated in lymphocyte transformation and subsequent proliferation (Brock & Rankin, 1981) and although it has been proposed that TF *per se* may mediate these events (Brock & Mainou-Fowler, 1983), it is more probable that TF functions as an iron donor (Brock, Mainou-Fowler & Webster, 1986). TF production by activated helper T-cells has been shown and it has been suggested that TF could function in an autocrine regulatory manner (Lum *et al.*, 1986). LF has been shown to bind to macrophages, some investigators claiming through a specific receptor (Bartal, Padeh &

Passwell, 1987; Birgens, 1991), and to increase their proliferation rate in mice (Oria et al., 1988). Moreover, there is evidence showing that LF may inhibit the production of prostaglandins and interleukin-1 by monocytes/macrophages (Zucali et al., 1989; Pelus et al., 1979; Bartal et al., 1987) and may enhance PMN adhesiveness and modulate their chemotaxis (Oseas et al., 1981; Gallin, 1984). LF has also been implicated in the regulation of myelopoiesis by suppressing the production of granulocyte-macrophage colony stimulating factor (GM-CSF), probably by its ability to inhibit interleukin-1 production (Zucali et al., 1989). Both LF and TF may act as iron scavengers at sites of inflammation and thereby inhibit the production of free hydroxyl radical formation which could otherwise cause damage to the host tissues (Britigan et al., 1989). LF due to its affinity for iron even at low pH, may be more effective in this process particularly in infectious and diseased tissue where pHs are variable. TF in contrast is more suited, by its narrow pH iron-binding range, to function in the pH regulated plasma and tissues.

### **1.8.2 Iron-binding proteins in periodontal disease**

Despite the evidence reviewed in the previous section both LF and TF have received very limited attention from investigators of periodontal disease.

In an early experimental gingivitis study Norman and coworkers (1979) were not able to demonstrate any significant change in serum TF, compared to baseline, 3 and 21 days after cessation of all oral hygiene measures in eight healthy volunteers.

The presence of TF in GCF was initially demonstrated by Brill and Bronnestam (1960) using immunoelectrophoresis. Schenkein and Genco (1977) assessed TF levels in GCF from diseased sites using radial immunodiffusion and reported that it occurred at levels of approximately 70% of that of serum which are similar to those reported for  $\alpha$ 2-M and  $\alpha$ 1-AT by the same investigators. This is in contrast to the findings of Asman et al. (1981) who reported that the dilution of  $\alpha$ 1-AT in GCF is higher than that of TF when compared to serum.

The importance of PMNs in the pathogenesis of periodontal disease has already been discussed in section 1.5 together with PMN granule characteristics and the stimuli for their release. LF has been used as a marker when investigating possible mechanisms of PMN secondary granule release in response to whole dental plaque, Gram positive, as well as Gram negative isolates (Baehni et al., 1977; Baehni et al., 1978; Tsai et al., 1978). In an interesting *in vitro* study (Baehni et al., 1979b), primary and secondary granule release from polymorphonuclear leucocytes was assessed following stimulation from autologous whole dental plaque

collected during a 21 day experimental gingivitis episode. PMNs responded in a dose dependent manner when incubated with increasing amounts of dental plaque. LF release from secondary granules was significantly higher when PMNs were incubated with day 21 compared to day 3 plaque, whereas myeloperoxidase release, indicating primary granule exocytosis, did not demonstrate the same pattern. Moreover, it has been shown that PMN phagocytosis of *Treponema denticola* leads to LF release (Hurlen et al., 1984).

As LF is an exclusive constituent of PMN secondary granules, and is contained only in trace amounts in serum, it has been suggested that GCF LF could prove a useful marker of crevicular PMNs (Fine & Mandel, 1986; Curtis et al., 1989). In addition, it has been suggested that it could act in synergy with TF to produce an iron limiting environment which would inhibit bacterial growth (Curtis et al., 1989).

Despite the above evidence LF has only been assessed in one study to date by Friedman et al. (1983). These investigators reported higher LF concentration in pooled GCF from gingivitis, periodontitis and juvenile periodontitis patients when compared to healthy controls. No reports exist in the literature on the presence of LF in discrete GCF samples and its association with periodontal disease status/activity at specific sites.

In conclusion, information on GCF iron-binding proteins is scarce. In view of their possible combined function and implication in the inflammatory process quantification of GCF iron binding proteins may provide information on their role in periodontal inflammation. In addition the possibility of LF acting as an exclusive PMN marker in the gingival crevice renders its assessment in GCF particularly pertinent (see Aims; section 1.9).

## 1.9 Aims

The objectives of the series of investigations undertaken in this thesis are:

### 1) Preliminary experiments:

To establish new, or modify existing, methods for the quantification of protease inhibitors ( $\alpha$ 2-macroglobulin and  $\alpha$ 1-antitrypsin) and iron-binding proteins (transferrin and lactoferrin) in microamounts of gingival crevicular fluid. The development of techniques to permit simultaneous quantification of these proteins in the same GCF sample was the ultimate objective.

### 2) Clinical studies:

#### I) Cross sectional studies:

a) To examine whether lactoferrin could act as a crevicular polymorphonuclear leucocyte marker.

b) To test the ability of GCF protease inhibitors and iron-binding proteins to distinguish between healthy, gingivitis and periodontitis sites.

## II) Experimental gingivitis study:

To establish the dynamics of GCF protease inhibitors and iron-binding proteins during experimentally induced gingivitis and to provide a profile of their changes as inflammation develops.

## III) Longitudinal study of periodontitis patients on maintenance care:

To investigate whether incipient periodontal disease breakdown could be associated with GCF protease inhibitor or iron-binding protein levels. In addition, the potential of clinical conditions to act as predictors/indicators for significant attachment level change was investigated.

## **CHAPTER 2**

**Subjects, clinical methods,  
design and statistical analysis**

## **2.1 Subjects**

Prior to the commencement of each of the following studies ethical approval was granted from the local ethical committee. Individuals participating in these studies were informed of the protocol and consent was obtained.

These individuals had no history of systemic conditions which could influence the course of periodontal disease or were they on medications that could affect its manifestations, such as chronic antibiotic use, phenytoin and other calcium channel blockers.

### **2.1.1 Cross-sectional studies on gingival crevicular fluid and gingival crevicular washings**

Subjects attending the Periodontal Department of the Glasgow Dental Hospital were enrolled in these studies. GCF samples were collected from a total of 62 patients (27 males, 35 females) with an age range of 20 to 57 years. Forty one of these individuals suffered from advanced periodontal disease as determined by clinical and radiographic examination (APD category), whereas the remaining 21 subjects could be classified as periodontally healthy with no attachment loss, pocket depths  $\leq$  3mm and no significant gingival inflammation (H category). An additional twenty one individuals (7 males; 14 females) with an age range of 20 to 52 years participated in the

study on GCWs. Sixteen of these individuals belonged to the APD category whereas the remaining five were periodontally healthy.

### **2.1.2 Experimental gingivitis study**

Six healthy dental students (5 males, 1 female; age range of 22 to 23 years) were enrolled in this study. These students demonstrated no clinically detectable periodontal attachment loss or pocketing >3mm in any site. Dental students were preferred because of their high degree of dental awareness and compliance.

### **2.1.3 Longitudinal study of periodontitis patients on maintenance phase of therapy**

Thirty eight periodontitis patients on a maintenance phase of therapy (17 males; 21 females), with an age range 26 to 68 years, attending the Periodontal Department of the Glasgow Dental Hospital consented to participate in this study. Study inclusion criteria were that these patients:

- a) had been diagnosed in the past as suffering from advanced periodontal disease (CPITN scores of 4 in at least four sextants) and had received a full course of periodontal treatment in the Glasgow Dental Hospital;
- b) were on maintenance for at least 1 year;

c) were still showing signs of persisting periodontal disease, defined as residual pocketing of  $\geq 4$ mm and bleeding on probing in at least 4 non-adjacent sites. Pocketing was measured, during a recruiting appointment, with a PC12 (Prisma, UK) periodontal probe and recorded using a standard periodontal pocket chart. These patients were followed over a period of three months.

## **2.2 Clinical methods**

### **2.2.1 Clinical indices**

One examiner (EA), with the help of an assistant, was used throughout these studies to record all clinical indices.

#### **2.2.1.1 The Modified Gingival Index**

The Modified Gingival Index (MGI) (Lobene *et al.* 1986) was used to assess gingival inflammation and is described below:

0 Absence of inflammation.

1 Mild inflammation; slight change in colour, little change in texture of any portion but not the entire marginal or papillary gingival unit.

- 2 mild inflammation; criteria as above but involving the entire marginal or papillary gingival unit.
- 3 Moderate inflammation; glazing, redness, oedema, and/or hypertrophy of the marginal or papillary gingival unit.
- 4 Severe inflammation; marked redness, edema and or hypertrophy of the marginal or papillary gingival unit, spontaneous bleeding, congestion or ulceration.

This index differs from the commonly used Gingival Index (Løe & Silness, 1963) in that it eliminates the bleeding upon probing/pressure component, and provides increased sensitivity in the lower region of the scoring scale. The MGI was, therefore, preferred because it permits non-invasive evaluation of the severity of gingival inflammation, and thus facilitates GCF sampling. In the experimental gingivitis study whole mouth MGI scores (WMGI) refer to the average MGI of all sites assessed within a subject. In all other studies, unless otherwise stated, site scores refer to a single MGI recording.

#### **2.2.1.2 The Plaque Index**

The plaque index (PI) of Silness and Løe, (1964) was used for recording plaque accumulation. The scoring system for this index is as follows:

- 0 No plaque in the gingival area.
- 1 A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may only be recognised by running a probe across the tooth surface.
- 2 Moderate accumulation of soft deposits within the gingival crevice, on the gingival margin and/or adjacent tooth surface, which can be seen by the naked eye.
- 3 Abundance of soft matter within the gingival crevice and/or on the gingival margin and adjacent tooth surface.

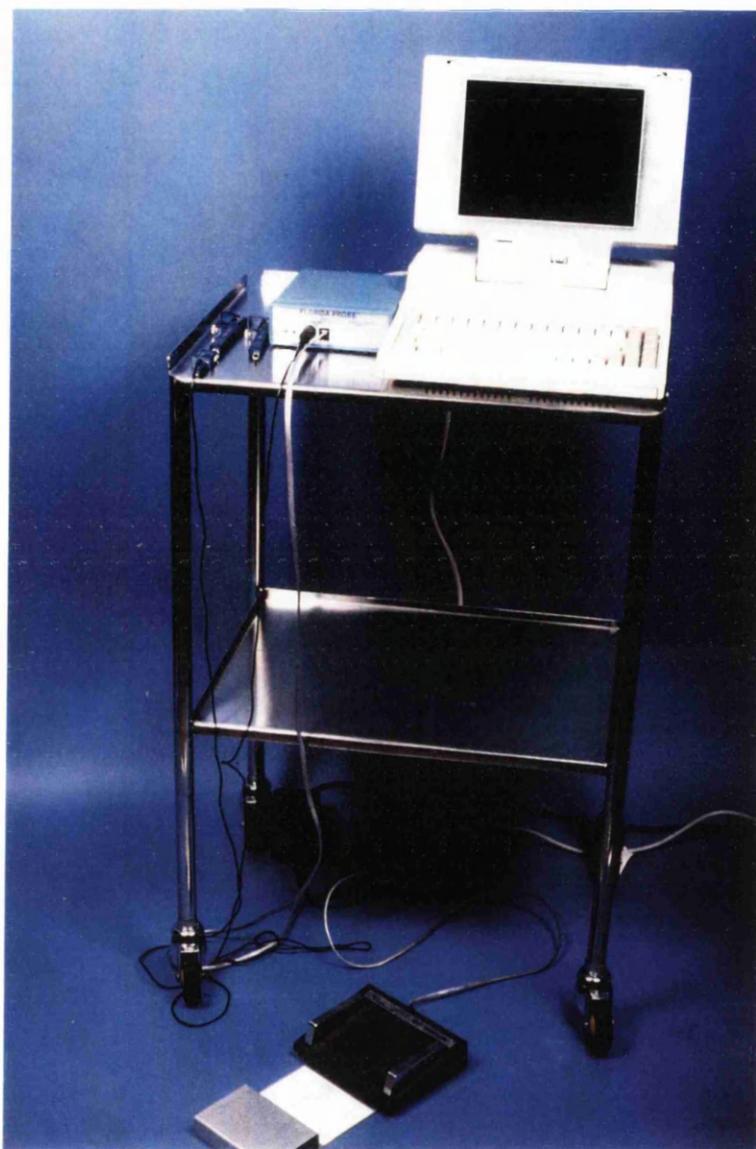
In the experimental gingivitis study whole mouth PI scores (WPI) refer to the average PI of all surfaces assessed within a subject. In all other studies, unless otherwise stated, site scores refer to a single site PI recording.

#### **2.2.1.3 Probing depth and attachment level measurements**

In the cross-sectional studies on GCF and GCWs, probing/pocket (PD) depth was recorded to the nearest mm using a Williams periodontal probe.

The Florida Probe (Florida Probe Corporation, Florida, USA) (Gibbs et al., 1988) was used for probing depth and attachment level (AL) recording in the longitudinal study of periodontitis patients on maintenance care. This is an electronic pressure sensitive probe set at 20g. The Florida Probe records PD/AL to the nearest 0.2mm. The system consists of a pocket depth and an attachment level ('stent') handpiece, a foot switch, computer interface and portable personal computer (Fig. 2.1). The measurements of PD and AL are made electronically, using the 'pocket depth' (Fig. 2.2) and 'attachment level, stent' (Fig. 2.3) handpieces respectively, and transferred automatically to the computer when the foot switch is pressed. Recordings are displayed on the computer screen which was turned away from the operator in order to allow 'blind' measurements. In addition, measurements were recorded on paper by the assistant. The probe tip has a diameter of 0.4mm, no visible graduations along its length and it reciprocates through a sleeve. The edge of the sleeve is the reference from which measurements are recorded.

A fixed reference point is required for attachment level measurements. The Florida Probe 'stent' handpiece has a 2mm diameter disc at the edge of its sleeve which, during attachment level measurement, is seated on a custom made soft acrylic stent covering the crowns of the teeth. These stents were constructed for both upper and lower arches for each patient from a 2mm thick silicone layer using a



**Figure 2.1** The Florida Probe: the system consists of a pocket depth and attachment level ('stent') handpiece, a foot switch, a computer interface and a portable personal computer.

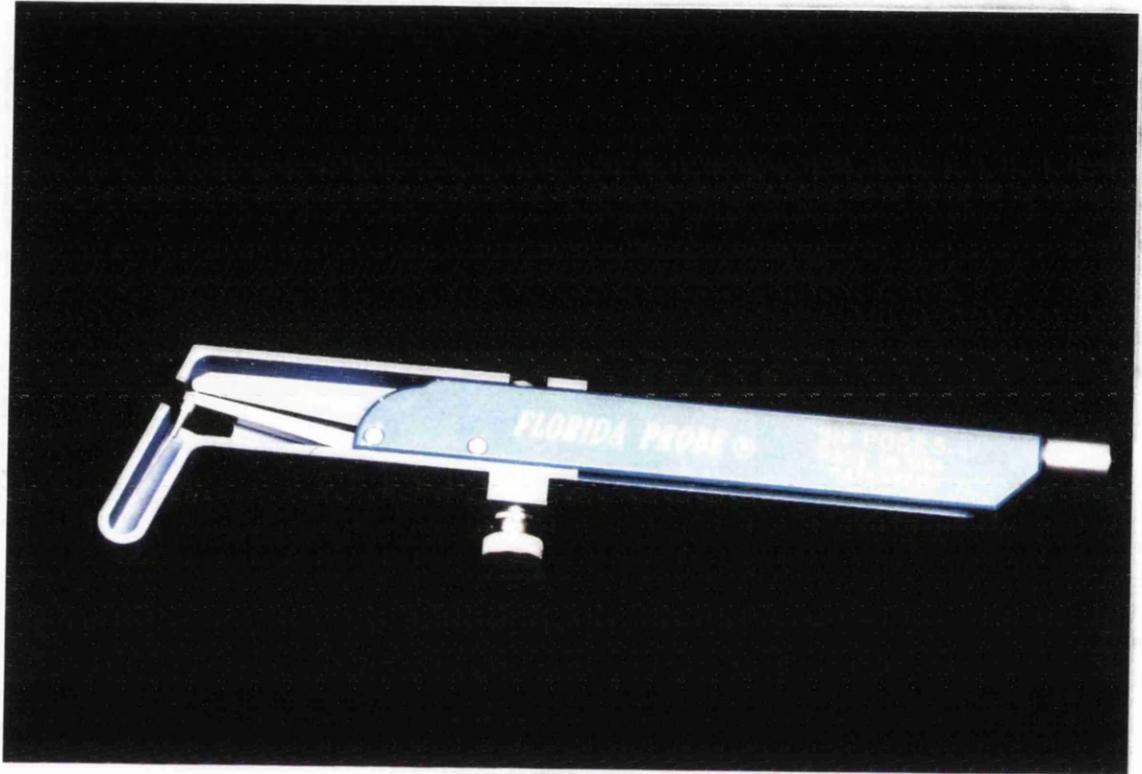


Figure 2.2 The Florida Probe 'pocket depth' handpiece.

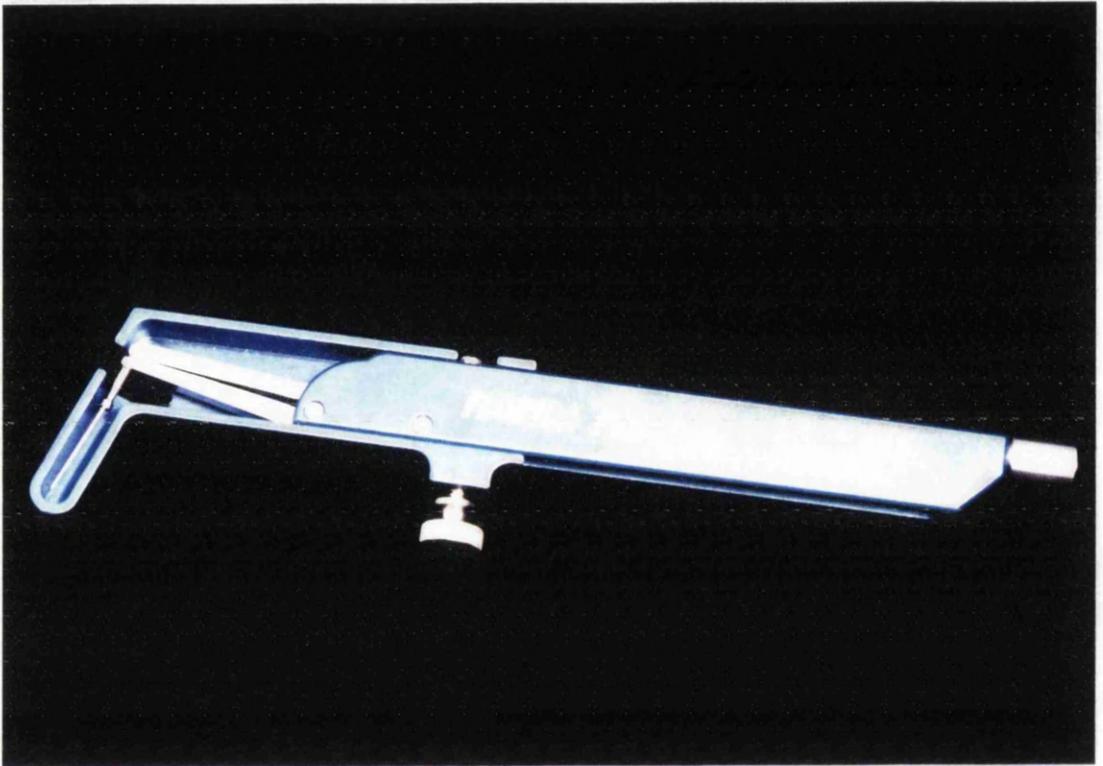
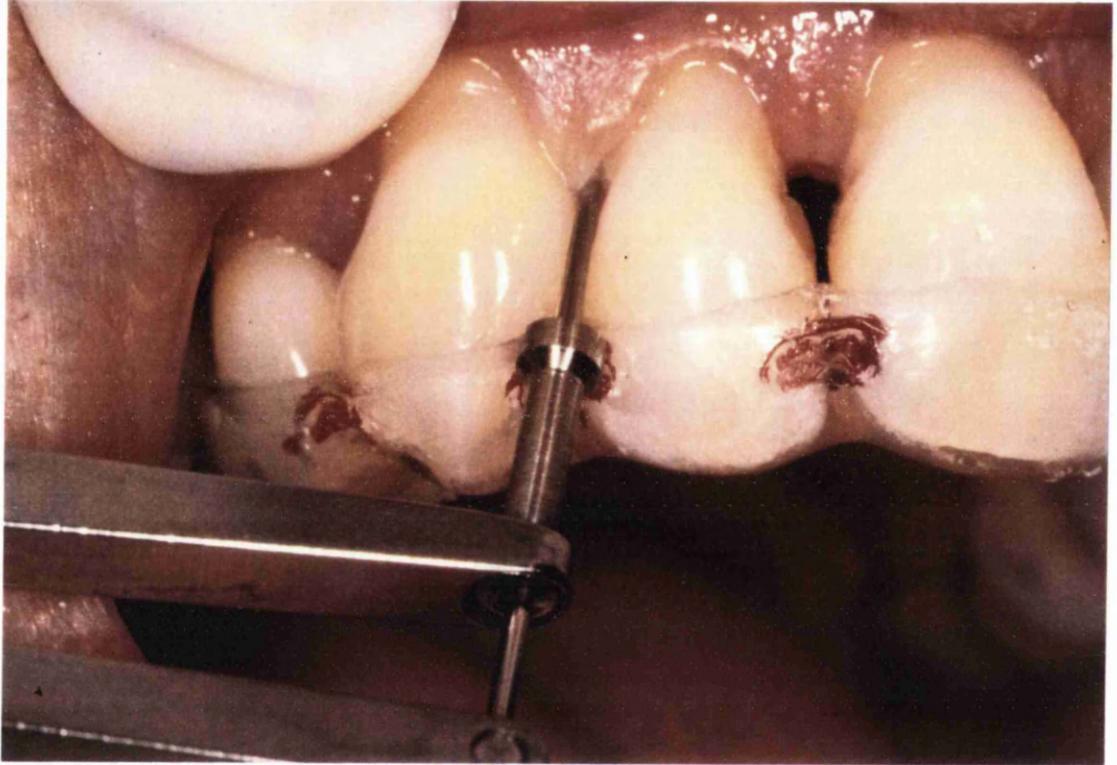


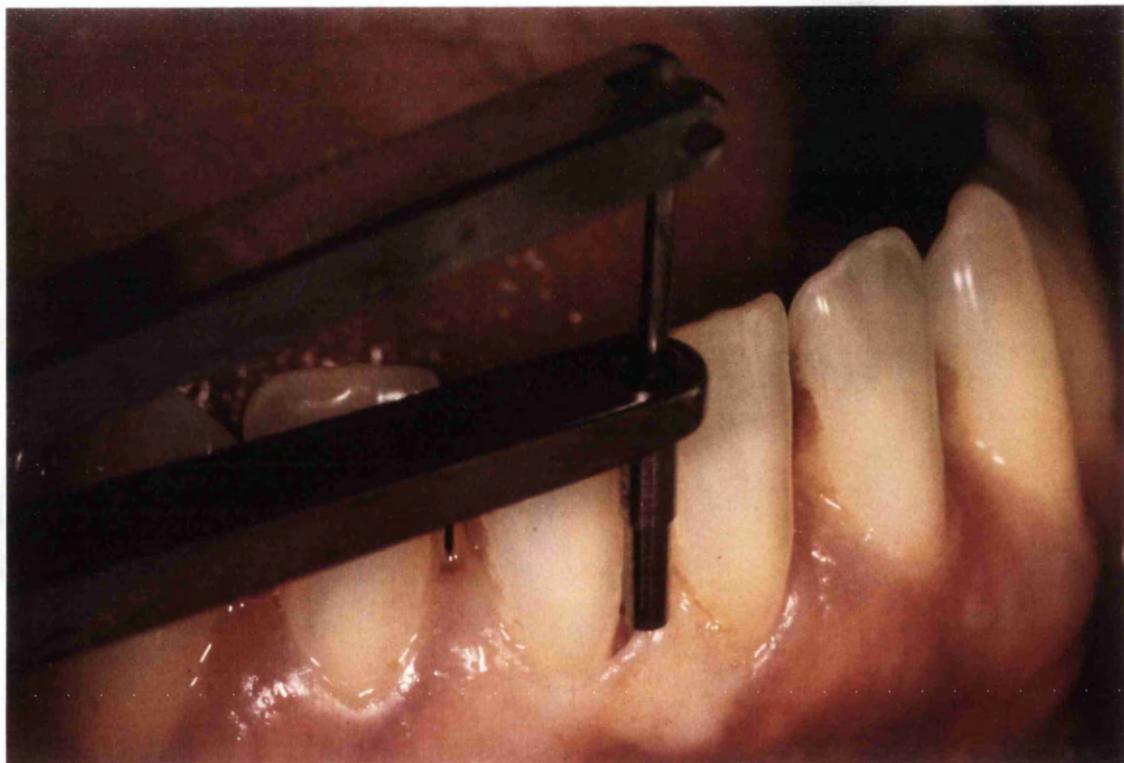
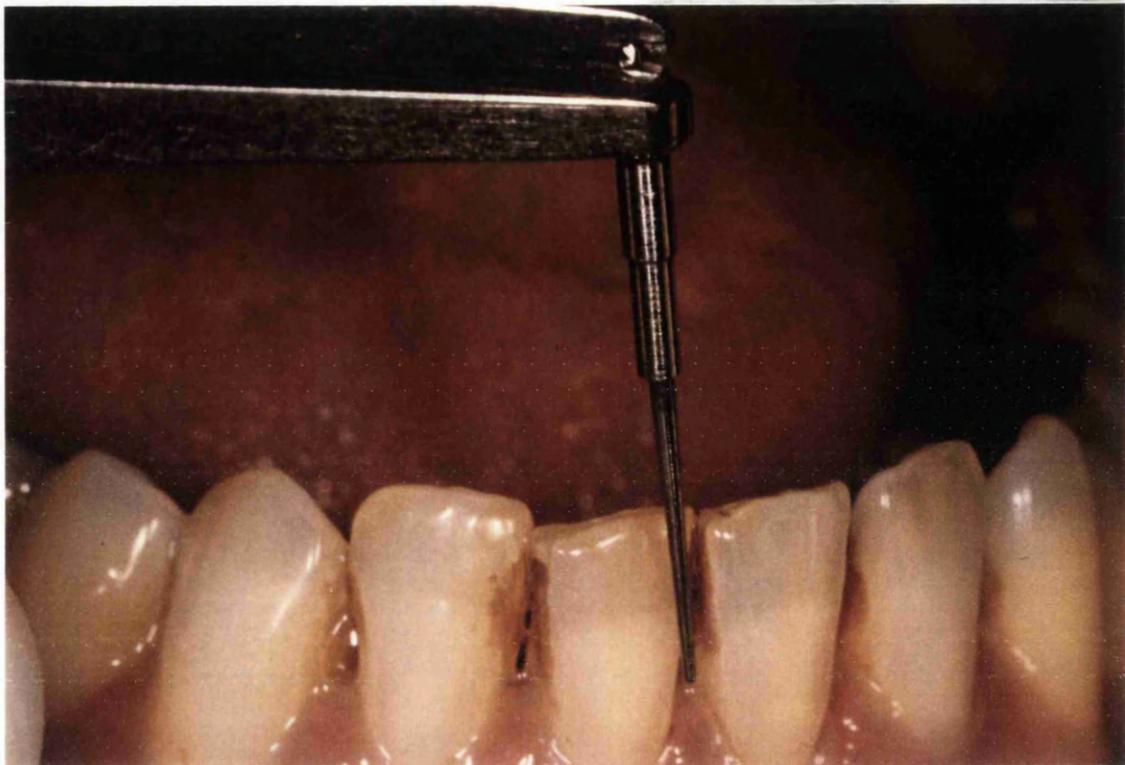
Figure 2.3 The Florida Probe 'stent' handpiece.

modification of the method described by Isidor, Karring and Attström, (1984). The stents were made on individually cast models using a vacuum forming unit. The stent was then trimmed to cover approximately the coronal one third of the crowns of the teeth at the sites selected for the study. At other sites as much as possible of the stent was preserved in order to assist location and retention. Grooves approximately 1mm deep, 2mm wide and 1mm high were cut, about 1mm away from the apical edge of the stent, at the sites to be used in the study, using an inverted cone bur and a low speed handpiece. The grooves were then marked with a permanent pen for ease of identification. The disc of the probe handpiece was located in these grooves during AL measurements. Attachment level was assessed relative to the point at which the probe handpiece disc was seated on the stent (Fig. 2.4). In this way, attachment level measurements do not rely on careful location of the cemento-enamel junction (CEJ). The identification of the CEJ is often complicated by its subgingival location or the presence of restorations. Buccal/lingual AL measurements were taken at the tooth's largest curvature. Interproximal AL recordings were taken at the mid point between the contact point and the adjacent line angle. The grooves on the stent were located relatively to these points.

PD is recorded when the probe sleeve is brought into contact with the gingival margin (Fig. 2.5). Care was



**Figure 2.4** Attachment level measurement using the Florida Probe 'stent' handpiece.



**Figure 2.5** Probing depth measurement using the Florida Probe 'pocket depth' handpiece.

taken for the probe tip to be parallel to the long axis of the tooth. PD measurements were taken at the same points as AL recordings.

#### **2.2.1.4 Change in attachment level**

Attachment level change between two points in time was assessed using a modification of the tolerance method of Haffajee *et al.*, (1983b). Duplicate AL measurements at each time point provide a measure of examiner error. The standard deviation of duplicate measurements of a site, both at the first and second time points, were pooled to produce the 'site standard deviation'. Differences between all duplicate measurements for all sites selected for the study within a patient, were used to compute the mean difference of duplicate measurements for each individual. The standard deviation of the mean difference was termed the 'patient standard deviation'. Patient standard deviations were averaged to derive a 'population standard deviation'. For the mean of the second pair of AL measurements to be considered significantly different from the mean of the first pair, attachment level change:

- a) had to exceed the 'population threshold' (which was 2 population standard deviations);
- b) had to exceed the 'patient threshold' (which was 3 patient standard deviations); and finally

c) had to be greater or equal to the 'site threshold' (which was 3 site standard deviations).

Significant attachment level gain or loss was assessed retrospectively on completion of the study using the above method.

#### **2.2.1.5 Attachment level grading**

An attachment level grading system was produced. This represents the direction and extent of attachment level change at specific sites during the longitudinal study of periodontitis patients on maintenance care. Individual sites were classified in 1 of 5 categories (grades) of attachment level change. Sites demonstrating significant attachment gain or loss over the observation period, using the tolerance method, were classified as grade 1 and 5 respectively. A much less stringent criterion of AL change of  $\geq 1\text{mm}$  was also used. Sites demonstrating AL gain or loss between baseline and three months (recall) of  $\geq 1\text{mm}$ , without any other criterion being considered (section 2.2.1.4), were classified as grade 2 and grade 4 sites respectively. All other sites were classified as grade 3 sites. In this way a range of AL change grades, varying from 1 (most improvement) to 5 (most deterioration), was produced. The above grading system is an adaptation of the method described by Jenkins, MacFarlane and Gilmour, (1988).

## **2.2 1.6 Bleeding on probing**

Bleeding upon probing (BOP) was scored dichotomously using the Florida Periodontal Probe. Scoring was performed within 30s of probing.

### **2.2.1.7 Suppuration**

Presence or absence of suppuration was recorded after applying gentle pressure on the gingival wall of the pocket at an apico-coronal direction using a ball burnisher.

## **2.2.2 Sample collection**

### **2.2.2.1 Gingival crevicular fluid sampling**

Whatman grade 4 (Whatman Labsales Ltd., Maidstone, Kent) paper strips (2x13mm) were used for GCF collection. Although Whatman grade 3 chromatography paper is recommended by some authors (Griffiths, Curtis & Wilton, 1988), this type of paper could not be used, as it was impossible to obtain a zero reading with the Periotron 6000 when the strips were dry. The Whatman grade 4 paper strips were cut manually using a steel ruler and scalpel. Rubber gloves were worn during their preparation in order to avoid contamination of the strips by substances from the operators hands. A line was drawn on each strip at 8mm, indicating the length of the paper strip to be inserted

between the Periotron jaws and that part of the strip to be used for GCF sampling. The strips were then placed in glass universal bottles for autoclaving and storage.

In the clinic, GCF was sampled after the PI and MGI scores were taken but prior to any other clinical recordings which could cause irritation of the tissues and serum contamination of the sample. Such recordings included PD or AL measurements, BOP, suppuration etc. The individual crevicular site was gently air-dried in an apico-coronal direction and any visible supragingival plaque was removed. The area was carefully isolated with cotton wool rolls and saliva ejector to avoid saliva contamination of the samples. The paper strip was introduced into the crevice until mild resistance was felt, whilst care was taken to avoid mechanical injury of the tissues (Fig. 2.6). The strip was left in the crevice for 30s, and then transferred to the chairside located Periotron 6000 (Harco Electronics, Winnipeg, Canada) (Fig. 2.7) for volume determination. It was then stored in labelled individual sterile 1ml microcentrifuge tubes, and placed on ice until all sampling was completed. The strips were subsequently transported to the laboratory and stored frozen at  $-30^{\circ}\text{C}$  (in initial studies) or  $-70^{\circ}\text{C}$ , until further processing.

In the longitudinal study on maintenance periodontitis patients the procedure was slightly modified. A pencil dot was drawn on the sampling part of the strip at 1mm length



**Figure 2.6** Gingival crevicular fluid sampling.



Figure 2.7 The Periotron.

from its edge indicating the length to be introduced in the pocket. In this way a standardised length of the paper strip was inserted in the crevice/pocket.

#### **2.2.2.2 Gingival crevicular washing sampling**

Gingival crevicular washings were obtained using a modification of the Skapski and Lehner method (1976). The teeth were isolated with saliva ejector and cotton rolls and any visible supragingival plaque was removed. Aliquots of 20 $\mu$ l of sterile phosphate buffered saline (PBS) were ejected and reaspirated in the crevice 3 times using a 20  $\mu$ l micropipette, fitted with a flat ended pipette tip (Labsystems, UK), resting interdentally on the surface of the tooth (Fig. 2.8). The gingival washings were then stored in labelled, individual, sterile 1ml microcentrifuge tubes, labelled, and placed in ice until completion of GCW sampling. GCWs were transferred to the laboratory and stored at 4°C until further processing.

#### **2.2.2.3. Blood sampling**

In the experiments requiring human serum, a 10ml venous blood sample was collected, from the antecubital fossa, in glass bottles. Blood samples were transferred to the laboratory and stored at 4°C until centrifuging at 3000rpm (12min) for serum recovery.



Figure 2.8 Gingival crevicular washing.

### **2.2.3 Selection of periodontal sites**

#### **2.2.3.1 Cross-sectional studies on gingival crevicular fluid and gingival crevicular washings**

Sites to be sampled were selected by clinical inspection and the use of pre-existing pocket charts and/or radiographs. In this way, periodontal probing, which could cause irritation of the tissues and serum contamination of the sample, was avoided.

#### **2.2.3.2 Experimental gingivitis study**

GCF samples were obtained from the mesial aspect of the upper first premolars. These teeth did not demonstrate any attachment loss and were free of any dental restorations.

#### **2.2.3.3 Longitudinal study of periodontitis patients on maintenance care**

A conventional pocket chart, constructed on a recruiting appointment using a PC12 periodontal probe, was used for site selection in this study. All non-adjacent sites, (accessible to GCF sampling and attachment level recording) exhibiting pocketing  $\geq 4\text{mm}$  on the pocket chart were selected for this study. Interproximal sites were preferred to mid-buccal or mid-lingual sites due to their higher chance of demonstrating attachment loss (Haffajee et

al., 1983a). Mesial sites were preferred to distal sites as they are more accessible. On the upper posterior molars, palatal sites were preferred to buccal sites and on the lower teeth buccal/labial sites were preferred to lingual sites as these sites are less prone to saliva contamination of the GCF sample.

## **2.3 Clinical design**

### **2.3.1 Cross-sectional studies on gingival crevicular fluid and gingival crevicular washings**

In all cross-sectional studies, as the term implies, samples were obtained and clinical recordings made at one time point. Clinical indices used in these studies were the MGI, PD and GCF volume. In order to establish the levels of GCF protease inhibitors and iron-binding proteins in relation to the clinical presentation of the sites sampled, sites were categorised into one of three clinical groups: 'healthy', 'gingivitis' and 'periodontitis'. Sites with MGI scoring of 0 or 1 and PD not exceeding 2mm were categorised as 'healthy'. MGI between 2 and 4, and PD less than or equal to 3mm were the criteria for the 'gingivitis' sites, whereas sites with PD exceeding 3mm were designated 'periodontitis' sites.

### **2.3.2 Experimental gingivitis study**

The experimental gingivitis model introduced by Loe, Theilade and Jensen (1965) was adapted for use in these studies. Subjects were enrolled in the trial 10 days prior to the commencement of the experimental gingivitis period (day -10), when their MGI and PI were recorded. Oral hygiene instruction and prophylaxis were then given. Ten days later (day 0/baseline) all oral hygiene procedures were ceased for 3 weeks. Clinical indices were recorded and GCF was sampled on days 0, 7, 14 and 21 of the experimental gingivitis period and 1 and 2 weeks after the reinstatement of oral hygiene measures. Clinical indices used in this study were the MGI, PI and GCF volume. Whole mouth MGI and PI recordings were also made. GCF was collected from the upper first premolars.

### **2.3.3 Longitudinal study of periodontitis patients on maintenance care**

All patients in this study were seen at three appointments: i) initial recruitment appointment; ii) baseline appointment (0 months); and iii) recall appointment (3 months). No instrumentation was carried out during the course of this study. At the initial recruitment appointment a medical history form was completed by the patient and the pocket charting, to be used for site selection, was constructed. An alginate impression was

taken and from the resulting cast, a soft acrylic stent for attachment level measurements was constructed (section 2.2.1.3). Subsequently, the patient was recalled for the baseline appointment. During this appointment and in the following order:

- a) the consent form was signed by the patient and the clinician in charge, and the medical history form was confirmed;
- b) PI and MGI recordings were taken first;
- c) GCF samples were then taken from the preselected sites and GCF Periotron readings recorded;
- d) the first set of AL measurements at the sites sampled was subsequently made. During this procedure bleeding on probing and suppuration were also registered. The stent was then removed;
- e) after changing patient position and reapplying the stent the duplicate set of AL measurements was made;
- f) Finally PD was assessed;

The same procedure was followed at three months.

#### **2.4 Statistical Analysis**

All statistical analyses were performed on an IBM PC computer using the Minitab or SPSS/PC statistical package.

In the cross-sectional study on GCWs, associations between PMN numbers or LF in the washings and the clinical indices (MGI, PD and GCF volume) were determined by the non-parametric Spearman rank correlation coefficient. The association between LF levels and PMN numbers in GCWs was assessed by the Pearson correlation coefficient on the  $\log_{10}$  transformed data.

In the cross-sectional studies on GCF the site was considered as the statistical unit.  $\alpha 2$ -M,  $\alpha 1$ -AT, TF and LF levels in GCF were markedly skewed and required logarithmic transformation in order to satisfy their distributional requirements prior to statistical analysis. Due to this skewed distribution, the geometric mean and 95% confidence intervals are shown rather than the standard error or standard deviation. Significant differences in  $\alpha 2$ -M,  $\alpha 1$ -AT, TF and LF levels among the clinical groups were determined by one way analysis of variance and two sample t-tests. As three comparisons were made among clinical groups when using the two sample t-tests, significance level was set at 0.025 ( $0.05/\sqrt{3} \approx 0.025$ , where 3=number of comparisons) in order to compensate for multiple comparisons (Brown & Swanson Beck, 1988).

In the experimental gingivitis study data was analyzed using repeated measures multivariate analysis of variance and the MANOVA procedure on the SPSS/PC statistical package (Norusis, 1990). Both subject and site were considered as

the experimental unit. When the subject was considered as the unit, GCF constituent levels from the two sites were averaged within each subject. In order to satisfy the distributional requirements of the data for the tests a  $\log_{10}$  transformation was required ( $\log_{10}(1+x)$ ,  $x$ =original data value). In an attempt to investigate which days demonstrated significantly elevated GCF protease inhibitor and iron-binding protein levels since baseline, individual paired t-tests were employed on the  $\log_{10}$  transformed data, comparing baseline levels of each protein to each subsequent day, considering the subject and site as the experimental unit. Significance level for the paired t-tests was set at 0.02 ( $0.05/\sqrt{5}$ , where 5=number of comparisons made).

A further transformation of the data to z scores was used in this study. The z scores for each protein are produced by subtracting each data value from the grand mean of the protein and dividing by its standard deviation. This results in a new variable for each protein which has a grand mean of 0 and standard deviation of 1. In this way differences in overall level among the four proteins are removed and focusing on the pattern of change is allowed.

In the longitudinal study of periodontitis patients on maintenance care, analysis of the clinical findings was performed considering both subject and site as the experimental unit. When the subject was considered as the

experimental unit, clinical indices within the subject were averaged, and one score for each clinical index (MGI, PI, GCF volume, AL change, BOP and S) produced per patient. BOP and S scores (0 = absence or negative and 1 = presence or positive) averages produced scores, ranging from 0 to 1, with the decimal digits being equivalent to the percentage of bleeding or suppurating sites per subject. Subjects were ranked according to their mean attachment level scores (rank 1= most improvement or maximum mean attachment gain; rank 38= most deterioration or maximum mean attachment loss), as well as by their mean baseline MGI, PI, PD, BOP, GCF volume and S scores, and Spearman rank correlation was employed to assess associations between clinical indices and attachment level change. In order to examine whether true associations between the clinical indices and attachment level change were being masked by the inclusion of a large number of stable sites the two best (maximum attachment gain) and two worst sites (maximum attachment loss) per patient were compared.

Baseline clinical indices scores, including MGI PD and GCF volume, for the two best and two worst sites per patient were averaged and paired tests (t-test for GCF volume; sign tests for the clinical indices) were used to compare for differences between best and worst sites within each subject. When the sites were considered as independent, Spearman rank correlations were used to test for associations between attachment level grade and clinical

indices. Sites demonstrating significant attachment gain were compared with the sites demonstrating significant attachment loss, when considered as independent, using the Mann-Whitney test.

GCF protease inhibitors and iron-binding protein levels were compared between periodontally active and matched stable control sites, within each patient, using paired t-tests on the  $\log_{10}(1+x)$  data both at baseline and recall appointments. Repeated measures MANOVA procedures were also applied, on the baseline and recall appointments separately, in order to test for differences between active and stable sites, simultaneously for all four proteins.

The model was extended and repeated measures MANOVA incorporating the four proteins while testing for site (stable versus active), appointment (baseline versus recall) and site by appointment effects was run on the  $\log_{10}(1+x)$  transformed data. If  $b_1$  and  $b_2$  represent the responses for each protein ( $\alpha_2$ -M,  $\alpha_1$ -AT, TF and LF) at baseline, at the active and stable sites respectively, and  $r_1$  and  $r_2$  similarly at recall, then the null hypotheses for these effects represent:

a) stable versus active:  $(b_1+r_1)-(b_2+r_2)=0$

b) baseline versus recall:  $(b_1+b_2)-(r_1+r_2)=0$

c) site by appointment:  $(b_1-b_2)-(r_1-r_2)=(b_1-r_1)-(b_2-r_2)=0$

Of the above, the most potentially useful would be the site by appointment effect, as it compares the change ( $\delta$ ) between baseline and recall at active versus stable sites.

In order to illustrate the diagnostic potential of a test based on the levels of protease inhibitors and iron-binding proteins in GCF, a 2x2 table based on the data of  $\alpha$ 2-M,  $\alpha$ 1-AT, TF and LF at the recall appointment from active and stable sites was formulated. A combined test based on the values of the four proteins,  $\alpha$ 2-M,  $\alpha$ 1-AT, LF and TF was applied. Thus, the test was considered positive when a site demonstrated  $\alpha$ 2-M,  $\alpha$ 1-AT, TF, and LF levels beyond a designated threshold value. These threshold values represent the geometric means of  $\alpha$ 2-M,  $\alpha$ 1-AT, TF and LF levels (ng/30s) at active sites at the recall appointment. All four conditions should apply simultaneously in order for the test to be considered positive, whereas if the levels of any one of the proteins dropped below the designated threshold values the test was considered negative. Specificity and sensitivity values and positive and negative predictive values were calculated using standard methods as shown in Table 2.1.

**Table 2.1** Diagnostic test characteristics and definitions.

	<b>Disease present</b>	<b>Disease absent</b>	
<u><b>Positive test</b></u>	a True positive	b False positive	<b>Positive predictive value</b> $a/(a+b)$
<u><b>Negative test</b></u>	c False negative	d True negative	<b>Negative predictive value</b> $d/(c+d)$
	<b>Sensitivity</b> $a/(a+c)$	<b>Specificity</b> $d/(b+d)$	

## **CHAPTER 3**

**Materials, experimental methods,  
and preliminary experiments**

### **3.1 Materials**

#### **3.1.1 Buffers and reagents**

All chemicals were analytical grade and were obtained from BDH Chemicals Ltd., Poole, Dorset, England, unless otherwise stated.

##### **3.1.1.1 Phosphate buffered saline (PBS)**

PBS pH 7.4, 10mM phosphate buffer, containing 150mM NaCl.

##### **3.1.1.2 Coating buffer (CB)**

Carbonate-bicarbonate buffer prepared by dissolving 1.59g  $\text{Na}_2\text{CO}_3$ , 2.93g  $\text{NaHCO}_3$  and 0.2g  $\text{NaN}_3$  up to 1 litre of distilled water. The pH was adjusted to 9.6 (titration with 1M HCl).

##### **3.1.1.3 Incubation buffer (IB)**

This was prepared by dissolving 8g NaCl, 0.2g  $\text{KH}_2\text{PO}_4$ , 0.2g KCl, 1.44g  $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$ , 0.5g Tween 20 (SIGMA Chemical Company Ltd., Poole, Dorset), and 1g lyophilised bovine serum albumin (BSA) (SIGMA) up to 1 litre distilled water (pH 7.4).

In the case of the albumin sandwich ELISA, BSA was omitted and replaced by 5% (w/v) powder of proprietary milk (MARVEL) (IB/M).

#### **3.1.1.4 Wash buffer (PBST)**

A stock solution of 10 times concentrated incubation buffer (nil BSA) was prepared. This was diluted 1/10 prior to use.

#### **3.1.1.5 Substrate buffer (SB)**

The substrate buffer was prepared by dissolving 0.95g citric acid and 1.4g  $\text{Na}_2\text{HPO}_4$  anhydrous in 100ml of distilled water (pH 5). 40mg of ortho-phenyldiamine (OPD) (SIGMA Chemical Company Ltd., Poole, Dorset) and 40 $\mu\text{l}$   $\text{H}_2\text{O}_2$  were then added. The substrate buffer was prepared fresh just before use.

### **3.1.2 Stains**

#### **3.1.2.1 White blood cell diluting fluid**

A stock solution was prepared by adding 0.1g crystal violet to 100 ml 1% glacial acetic acid. Prior to use this solution was diluted 1/10 with 1% glacial acetic acid.

### 3.1.2.2 Leishman's stain

Leishman's stain (SIGMA) was prepared at 0.2 % w/v in purified methanol.

### 3.1.3 Purified standards and antisera for enzyme linked immunosorbent assays (ELISA)

Purified  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M), transferrin (TF), and albumin (Alb) were obtained from SIGMA. Lactoferrin (LF),  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT) and polymorphonuclear leucocyte elastase were purchased from Calbiochem (Novabiochem Ltd., Nottingham). All standards came in the form of lyophilised powders and were reconstituted according to the manufacturer's instructions. Goat and rabbit anti- $\alpha$ 2-M, goat anti-TF and rabbit anti-Alb were obtained from SIGMA. Goat anti-Alb and anti-LF as well as rabbit anti-LF and anti-TF were obtained from Nordic Immunological Laboratories (Maidenhead, Berkshire) whereas goat and rabbit anti- $\alpha$ 1-AT were purchased from Calbiochem. The horseradish peroxidase (HRP) conjugated anti-rabbit IgG (goat) was purchased from ICN Immunobiologicals (Lisle, IL, USA). Finally, the sheep anti-TF and the HRP conjugated anti-sheep/goat IgG (donkey) were obtained from the Scottish antibody production unit (SAPU, Carluke, Lanarkshire, Scotland). Of the above antisera the rabbit antisera to  $\alpha$ 2-M,  $\alpha$ 1-AT and Alb, the goat anti-TF and the HRP conjugates were fractionated. All reagents, after

reconstitution (if required), were aliquoted and stored at -20°C until used. Reagents were replaced regularly to avoid deterioration.

### **3.2 Experimental methods and preliminary experiments**

In this section, to aid clarity, methods as well as the preliminary experiments necessary to establish these methods, and the results will be presented and discussed together.

#### **3.2.1 Calibration of the Periotron 6000**

##### **3.2.1.1 Determination of fluid type to be used for the calibration of the Periotron 6000**

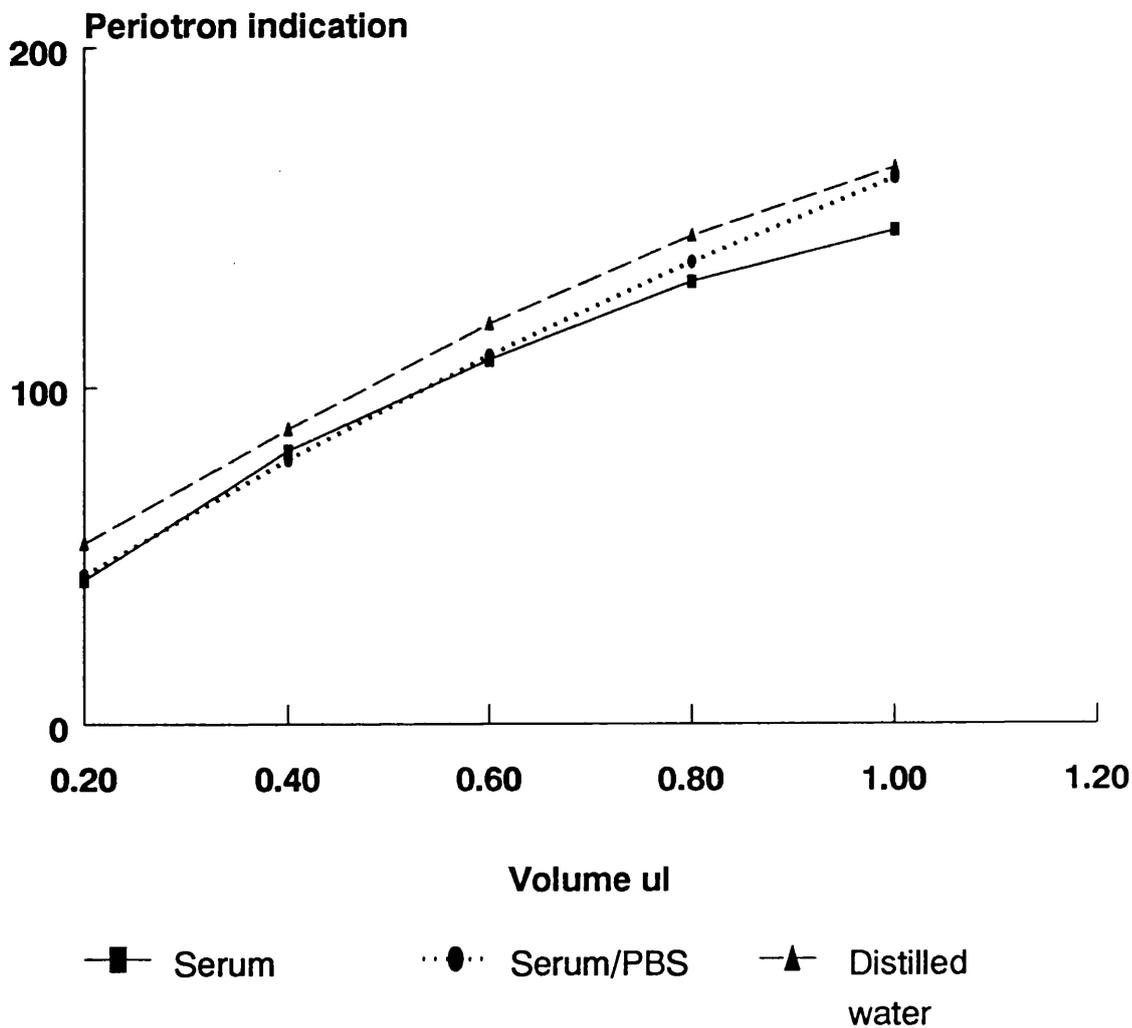
Prior to calibrating the Periotron 6000 for use in clinical trials a simple experiment was conducted in order to select the type of fluid to be used, and to determine the effect of fluid consistency on the digital readouts of the instrument. Three different types of fluid were evaluated: a) serum; b) serum/PBS at 1:1 dilution; and c) distilled water.

Volumes of 0.2 to 1 $\mu$ l in 0.2 $\mu$ l increments were delivered onto Whatman grade 4 paper strips using a Hamilton microsyringe. The strips were inserted between the Periotron jaws up to the 8mm line. Measurements were

performed six times for each volume and fluid type and the Periotron digital indication recorded. The mean of the six measurements was taken and plotted against the volume to obtain a calibration line for each fluid type as shown in Figure 3.1. Consistent with the observations of Van Der Bijl et al., (1986) the serum line was located below that of distilled water. These investigators attributed the lower readings obtained by serum when compared to distilled H<sub>2</sub>O, to its lower dielectric dissipation. Following this experiment serum/PBS 1:1 was selected as the calibrating fluid and was used throughout these experiments, as, with respect to a major part of its constituents, GCF represents a dilution of serum.

#### **3.2.1.2 Calibration of the Periotron 6000 for use in clinical trials**

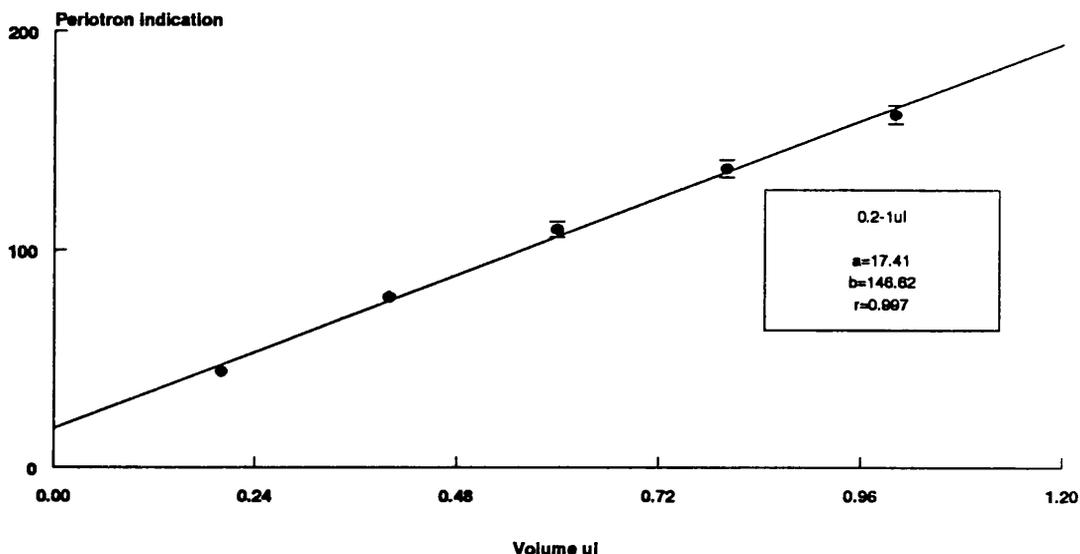
In initial experiments, prior to the commencement of a clinical trial a Periotron calibration curve was constructed, using the protocol described above and serum/PBS 1:1 as the experimental fluid. Linear regression analysis was used to fit the standard curve as shown in Figure 3.2, (a). GCF volumes could then be estimated from Periotron values, using the linear function of the type  $I = a + b \times v$ , where  $I$  = Periotron indication,  $v$  = volume,  $a$  = the intercept and  $b$  = the  $v$  coefficient (Fig. 3.2 (a)). This type of standard curve was used when assessing GCF volumes for the cross-sectional studies. In later experiments,



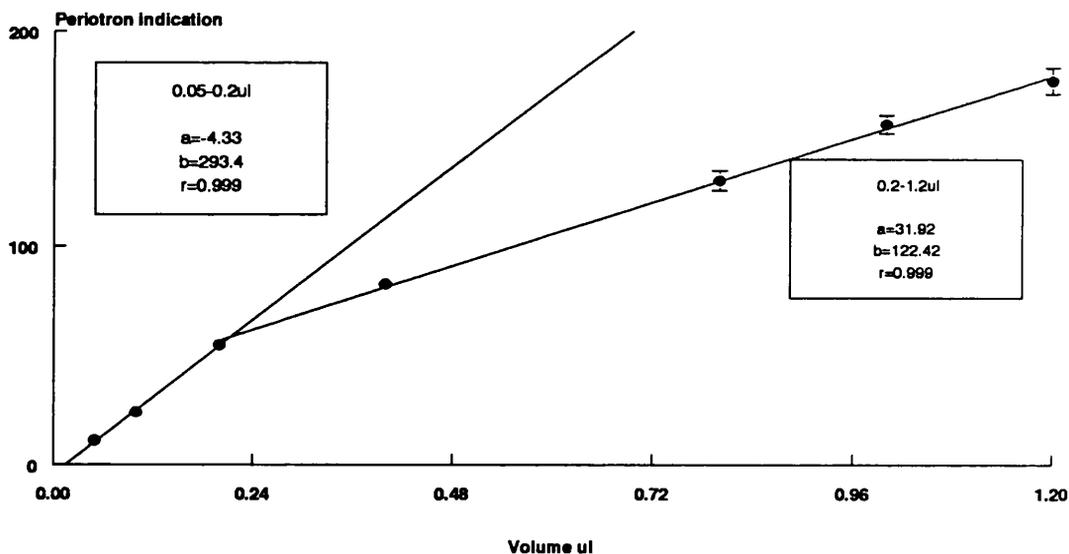
**Figure 3.1** Calibration lines of the Periotron 6000 using serum, serum/PBS at 1:1 dilution and distilled water. Periotron indication is plotted against fluid volume ( $\mu\text{l}$ ). Each point represents the mean of six measurements for each type of fluid. Variation between replicate measurements was very low and thus standard deviations are not shown (see also Fig. 3.2).

PBS: phosphate buffered saline

(a)



(b)



**Figure 3.2** Calibration curves of the Periotron 6000 at the volume range of: (a) 0.2 to 1.0  $\mu$ l and (b) 0.05-1.2  $\mu$ l where data were resolved in two lines, 0.05-0.2  $\mu$ l and 0.2-1.2  $\mu$ l. Each point represents the mean of three measurements ( $\pm$ SD). Linear regression analysis was used to plot the best fitting lines and the regression coefficients are given.

a= the intercept; b= the volume coefficient  
r= Pearson correlation coefficient  
SD= standard deviation

volumes used for the calibration of the instrument were extended to the ultra low range. Calibration curves were thus constructed using Serum/PBS 1:1 at volumes of 0.05, 0.1, 0.2, 0.4, 0.8, 1 and 1.2 $\mu$ l. Measurements for each volume were performed in triplicate. In an effort to reduce daily variability and thus improve accuracy, the instrument was calibrated in the periodontal clinic each day samples were collected. The curve was split into an upper and a lower portion as described by Lamster et al., (1985) and two separate lines fitted by the regression method as shown in Figure 3.2, (b). The lower portion included volumes of 0.05, 0.1 and 0.2 $\mu$ l whereas the upper portion included all the remaining volumes. Determination of GCF volume from given Periotron indications was performed from the corresponding part of the Periotron calibration curve using the appropriate equation. If a sample yielded volume beyond the measuring range of the Periotron 6000, it was arbitrarily assigned a volume of 1.3 $\mu$ l. However, this only occurred for 10 samples (2.6%) in the longitudinal study of periodontitis patients on maintenance care

### **3.2.2 Quantification of GCF constituents**

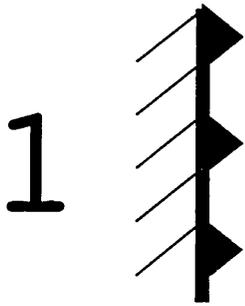
$\alpha$ 2-M,  $\alpha$ 1-AT, TF, LF and Alb, in GCF were quantitated via the use of indirect, competitive or sandwich, solid phase immunosorbent assays (ELISAs). Competitive immunoassays were initially used for the quantitation of GCF  $\alpha$ 2-M and TF

in the cross-sectional studies. Sandwich immunoassays were developed later, in an effort to improve detectability limits, and were used for GCF quantitation of  $\alpha$ 1-AT and LF in all studies, and of  $\alpha$ 2-M and TF in the experimental gingivitis and longitudinal study of maintenance periodontitis patients. A sandwich ELISA was also used for assessing Alb in GCF samples from the longitudinal study of maintenance periodontitis patients.

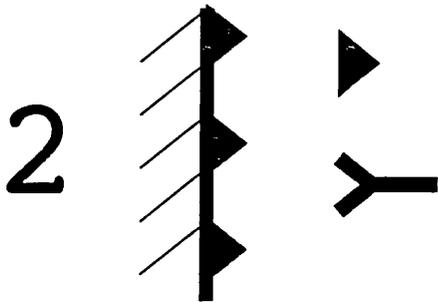
In the cross-sectional studies on GCF, different samples were used for assessing  $\alpha$ 2-M,  $\alpha$ 1-AT, TF and LF. In all other studies analysis for all four proteins was performed on the same GCF sample. In the longitudinal study of maintenance periodontitis patients, in addition to  $\alpha$ 2-M,  $\alpha$ 1-AT, TF and LF, Alb was also assessed in the same GCF sample.

#### **3.2.2.1 Competitive ELISA methodology**

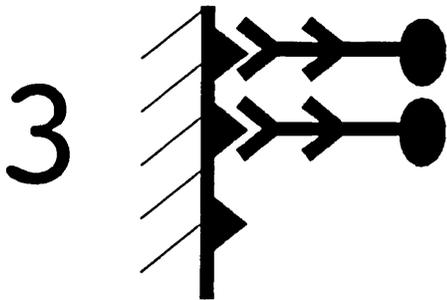
The competitive ELISA methods are modifications of the technique described previously by Altschuh and van Regenmortel (1982). Briefly, as shown in Figure 3.3, the antigen, human  $\alpha$ 2-M or TF, was coated onto the 96-well polystyrene microelisa plate (Immulon I, Dynatech Laboratories, Billinghamurst, Sussex). The experimental samples and the corresponding antibody, goat anti- $\alpha$ 2-M or sheep anti-TF, were then added. The principle of this assay is that the solid and the liquid phase antigen will



Coating with standard antigen



Incubate with standard/sample  
and antiserum



Add HRP anti-sheep/goat IgG

**Figure 3.3** Competitive ELISA: basic principles.

HRP: horseradish peroxidase

compete for the binding of the antibody. This resulted in the sample concentration being inversely proportional to the amount of antibody bound onto the solid phase antigen, which in turn was quantified indirectly by the addition of the HRP conjugated anti-sheep/goat IgG. Visualisation was achieved by incubation with the substrate buffer and stopping the reaction with  $H_2SO_4$ . The plate was read at 490nm using a Dynatech Minireader II plate reader (Dynatech Laboratories, Alexandria, VA) and the optical densities (OD) were obtained.

Each plate included serial two-fold dilutions of purified  $\alpha 2$ -M or TF, resulting in a total of 10 standard solutions, at the concentration range  $2 \times 10^4$  to 39ng/ml. This permitted construction of a standard curve from which sample antigen quantities could be estimated. Only the central wells (60 in total) were used on each plate for standards or samples, which were run in triplicate, in order to avoid the so-called edge-effect (increased variability in the periphery of the plate) (Tijssen, 1985). The peripheral wells were used for running the controls which are as shown in Table 3.1. All controls were assayed in duplicate with the exception of the zero-antigen (number 14; Table 3.1) which was assayed in quadruplicate. A detailed outline of the method is given in Table 3.2.

**Table 3.1** Control tests for competitive and sandwich ELISAs. For volumes and concentrations of reagents refer to tables 3.2 and 3.3 respectively. When the addition of a reagent was omitted the equivalent volume of the respective buffer alone was added. Coating refers to antigen or goat antiserum for the competitive or sandwich ELISAs respectively. Standard was added at  $2^{-5}$  dilution of its starting concentration. In the case of the competitive ELISAs, standard and antiserum were added in a single step. For the sandwich ELISAs antiserum refers to the second antibody (rabbit).

Control	STEP			
	Coating	Standard	Antiserum	HRP Conjugate
1.	-	-	-	-
2.	+	-	-	-
3.	-	+	-	-
4.	-	-	+	-
5.	-	-	-	+
6.	+	+	-	-
7.	+	-	+	-
8.	+	-	-	+
9.	-	+	+	-
10.	-	+	-	+
11.	-	-	+	+
12.	+	+	+	-
13.	+	+	-	+
14.	+	-	+	+
15.	-	+	+	+

**Table 3.2** Steps and reagents for the  $\alpha 2$ -macroglobulin ( $\alpha 2$ -M) and transferrin (TF) competitive ELISAs. The respective standards and antisera were used for each assay. Samples were eluted in 1ml of IB.

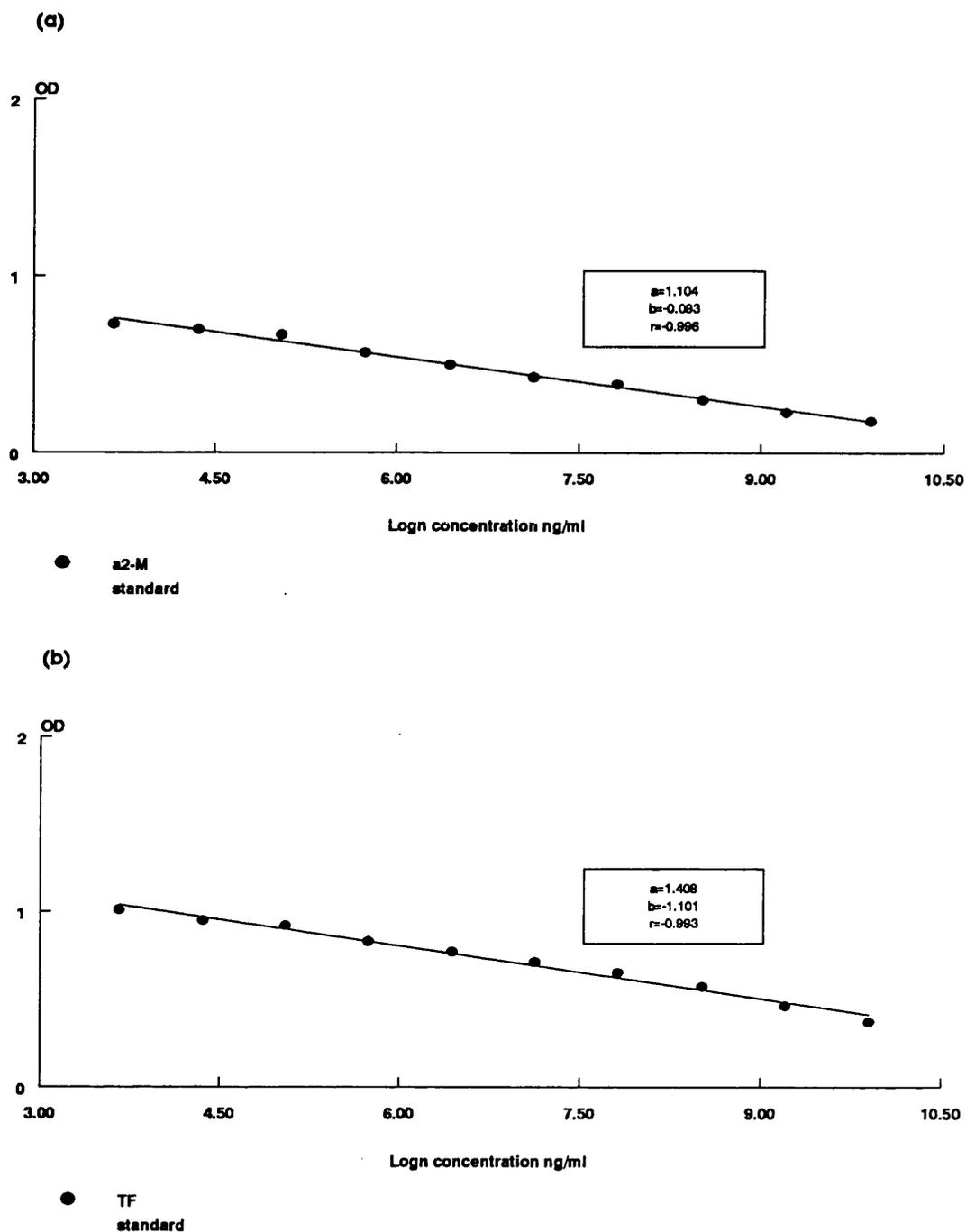
<b>COMPETITIVE ELISAs</b>	
<b>STEPS</b>	<b>REAGENTS</b>
1. Wash with 200 $\mu$ l/well CB thrice and dry.	
2. Coat with 100 $\mu$ l/well standard antigen. Incubate overnight at 4°C.	Standard $\alpha 2$ -M or TF: 1 $\mu$ g/ml in CB.
3. Wash with 200 $\mu$ l/well PBST, 3x6 times and dry. Removal of excess and loosely coated antigen.	
4. Add 50 $\mu$ l/well standard antigen or eluted sample, and 50 $\mu$ l/well specific antiserum. Incubate for 2h at 37°C.	standard $\alpha 2$ -M or TF: 2x10 <sup>4</sup> -39ng/ml in IB antiserum: goat anti- $\alpha 2$ -M or sheep anti-TF 1/(4x10 <sup>4</sup> ) in IB.
5. Wash with 200 $\mu$ l/well PBST, 3x6 times, and dry. Removal of free antigen, excess antibody and liquid phase antigen-antibody complexes.	
6. Add 100 $\mu$ l/well HRP conjugated anti-IgG. Incubate for 90min at 37°C.	HRP anti-sheep/goat IgG (donkey): 1/1000 in IB
7. Wash with 200 $\mu$ l/well PBST, 3x6 times and dry. Removal of excess HRP-anti-IgG.	
8. Add 100 $\mu$ l/well SB. Incubate for approximately 15min at 22°C. Stop colour development with 50 $\mu$ l/well 1M H <sub>2</sub> SO <sub>4</sub> . Read at 490nm.	

CB= coating buffer  
 PBST= wash buffer  
 IB= incubation buffer  
 HRP= horseradish peroxidase

### 3.2.2.2 Preliminary experiments and establishment of the competitive ELISAs

In order to establish the  $\alpha$ 2-M and TF competitive assays, different concentrations of antigen coating (10 $\mu$ g/ml, 1 $\mu$ g/ml and 100ng/ml) as well as antiserum and HRP conjugate (serial chess-board type dilutions) were used. The optimal conditions, as described in the previous section, were thus defined for both assays, bearing in mind, that the specific antiserum should not be in excess of the solid or liquid phase antigen. Overnight incubation at 4°C was used for coating plates with the antigen as this gives more uniform results. Incubation for 2h, 3½h or 4h with the standard/specific antiserum gave similar standard curve resolution and 2h incubation was selected for this step. Finally, incubation with the conjugate at 1/1000 dilution in incubation buffer for 90 min gave low non-specific binding (OD<0.05) in the absence of anti- $\alpha$ 2-M or anti-TF (control 8; Table 3.1) and maximum OD in the lowest standard close to 1 after 15min incubation with the substrate. As non-specific binding in the absence of specific antiserum and antiserum/standard (controls 8 and 13 respectively; Table 3.1) was very low, blocking during incubations with incubation buffer alone (containing 0.1% w/v BSA and 0.05% w/v Tween 20) was considered adequate.

Figures 3.4 (a) and (b) show typical standard curves for the  $\alpha$ 2-M and TF competitive assays respectively, at the



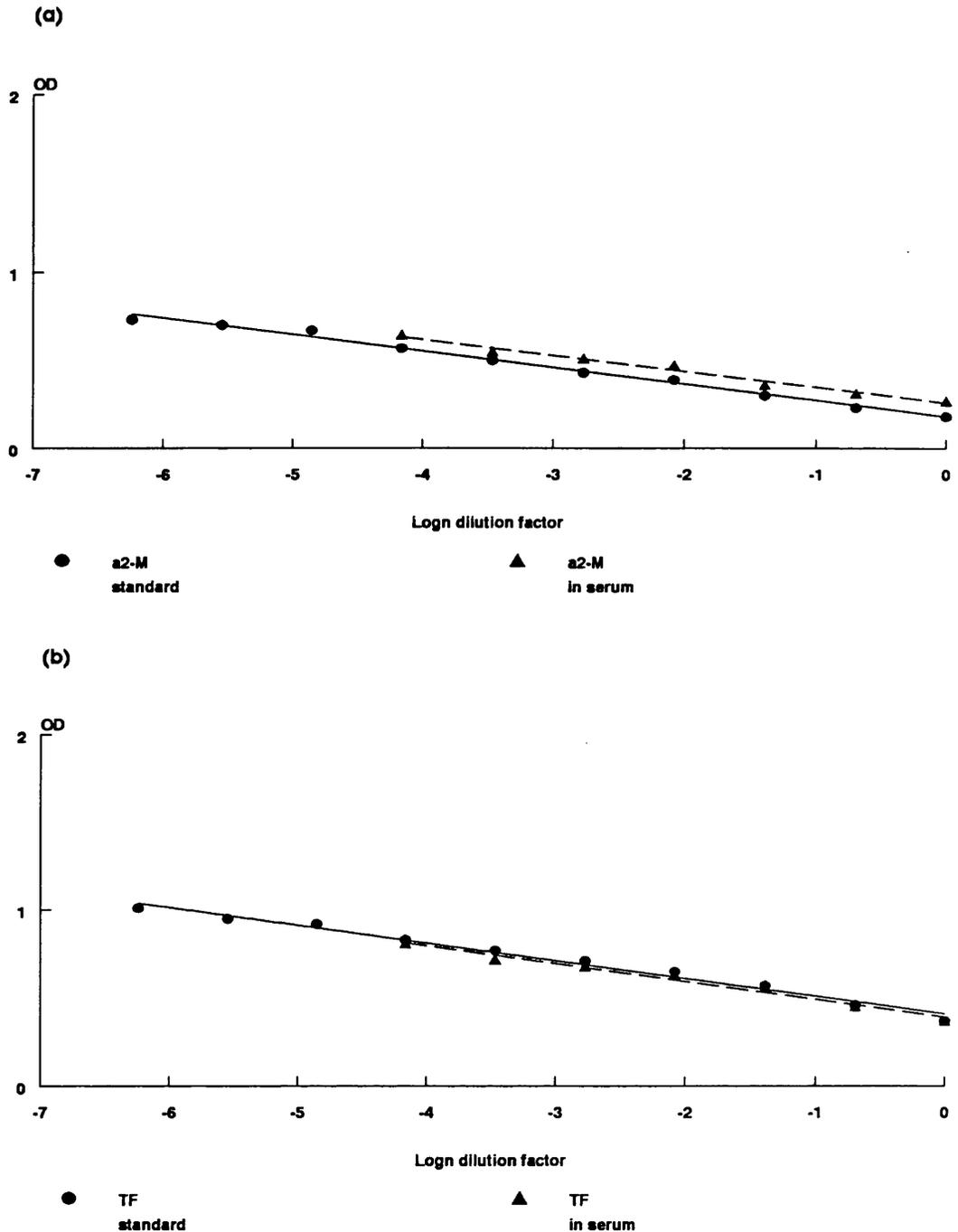
**Figure 3.4** Typical calibration curves for (a)  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M) and (b) transferrin (TF) competitive ELISAs at the concentration range of  $2 \times 10^4$  to 39 ng/ml. ODs (490nm) are plotted against  $\log_n$  concentration (ng/ml). The least squares method was used to plot the best fitting line and the regression coefficients are shown.

OD= optical density  
 a= the intercept  
 b= the  $\log_n$  concentration coefficient  
 r= Pearson correlation coefficient

concentration range of  $2 \times 10^4$  - 39ng/ml. As mentioned in the previous section, in these assays the amount of specific antiserum reacting with the solid phase antigen is inversely proportional to the liquid-phase antigen concentration, resulting in high ODs for low antigen standard or sample concentrations (Fig. 3.4). The detectability limit of the assay, defined as the lowest concentration of the standard antigen yielding ODs differing significantly from the buffer blank (control 14; Table 3.1) was beneath our lowest standard. Below or above however the quoted concentration range, experimental error became significant due to flattening of the curve.

Linear regression analysis was used to plot the best fitting line ( $\alpha 2$ -M:  $r^2 = -0.991$ ; TF:  $r^2 = -0.986$ ) and produce the linear function  $OD = a + b \times (\log_n c)$ , where OD= optical density,  $c$ = concentration,  $a$ = the intercept,  $b$ = the  $\log_n c$  coefficient from which sample antigen quantities with known ODs could be estimated (Fig. 3.4). A similar standard curve was produced, and a new line fitted, each time an assay was run.

Figures 3.5 (a) and (b), depict curves obtained by standard antigen and serum serial two fold dilutions for the  $\alpha 2$ -M and TF competitive assays respectively. Concentrations of standard  $\alpha 2$ -M and TF ranged from  $2 \times 10^4$  to 39ng/ml in incubation buffer. Serum dilution range was 1/100 to 1/6400 in incubation buffer for both assays. As serum



**Figure 3.5** Standard and serum dilution curves for (a)  $\alpha 2$ -macroglobulin ( $\alpha 2$ -M) and (b) transferrin (TF) competitive ELISAs. Starting concentration of standard for both assays was  $2 \times 10^4$  ng/ml and of serum 1/100 in IB. 10 serial two fold dilutions of the initial solution were assayed for standard and 7 for serum. Initial dilution factor was arbitrarily termed 1 and two-fold dilution factors thereafter,  $2^{-1}$  to  $2^{-9}$  for standard and  $2^{-1}$  to  $2^{-6}$  for serum. ODs are plotted against  $\log_n$  dilution factor. Linear regression was used to plot the best fitting lines.

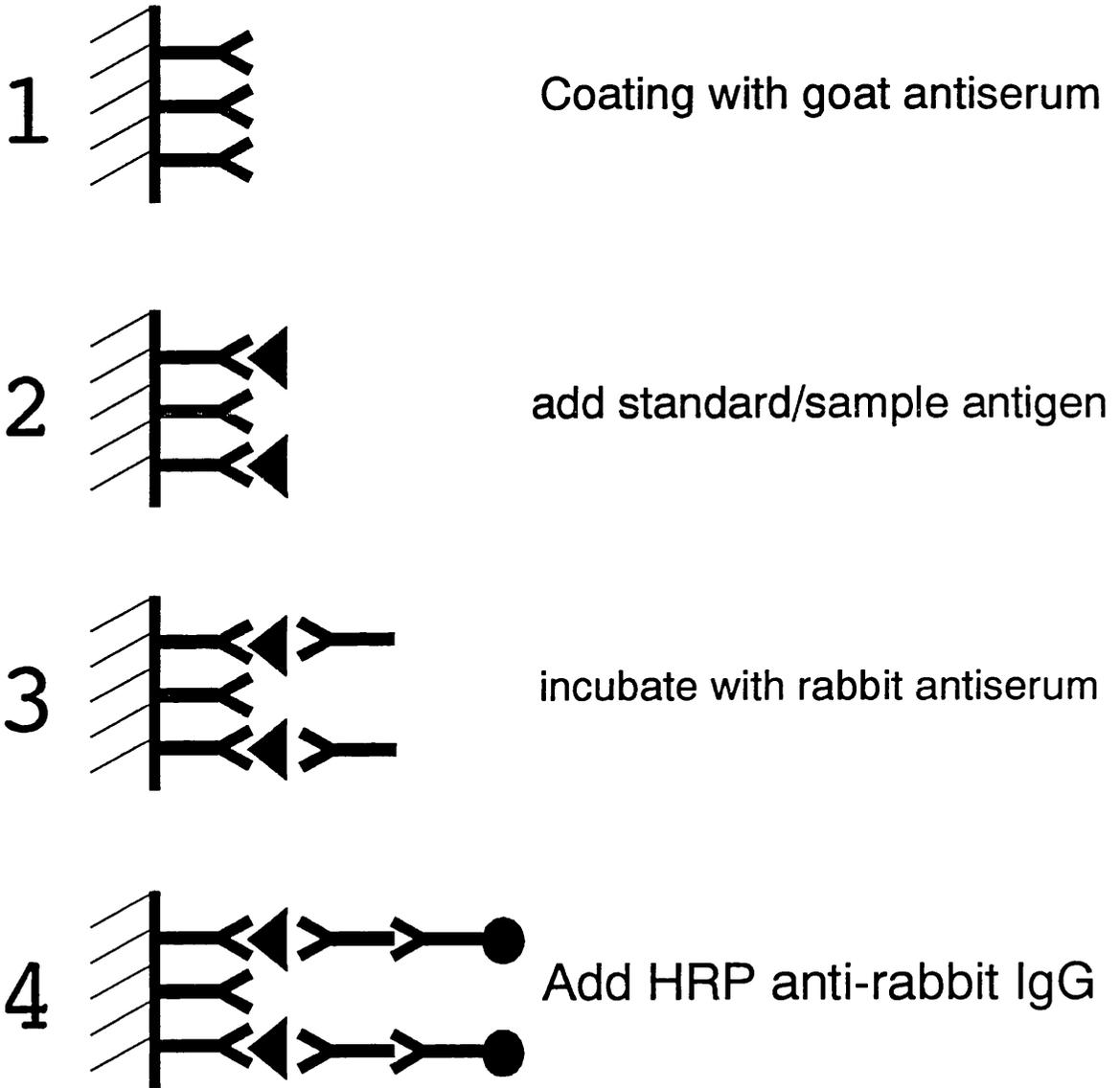
OD= optical density; IB= incubation buffer

antigen ( $\alpha$ 2-M and TF) concentration was not known, initial dilution factor for standard and serum was arbitrarily termed 1 and two fold dilution factors thereafter  $2^{-1}$  to  $2^{-9}$  for the standard, and  $2^{-1}$  to  $2^{-6}$  for serum. ODs of standard and serum antigen were plotted against  $\log_n$  dilution factor and linear regression was used to plot the best fitting lines (Fig. 3.5). As depicted in Figure 3.5 serial two-fold dilutions of serum gave curves parallel to the highly purified standard for both  $\alpha$ 2-M and TF competitive assays. This demonstrates that these proteins in serum, respond in a similar way to the highly purified standard, and that no interference by other serum components occurs.

Finally, a number of GCF samples eluted in 1ml of incubation buffer were assayed and yielded concentrations within the range of the competitive assays, and 1ml was therefore established as the elution volume for GCF in the studies where the competitive assays were used. This resulted in a detection limit of total  $\alpha$ 2-M and TF in GCF samples of 39 ng.

### 3.2.2.3 Sandwich ELISA methodology

The five sandwich ELISAs ( $\alpha$ 2-M,  $\alpha$ 1-AT, TF, LF and Alb) are based on the technique described by Hetherington *et al.*, (1983). In summary (Fig. 3.6), the 96-well polystyrene microplate (Immulon IV, Dynatech Laboratories, Billinghamurst, Sussex) was coated with the first antibody,



**Figure 3.6** Sandwich ELISA: basic principles.

HRP: horseradish peroxidase

a goat antiserum specific to the antigen to be quantified. The eluate of the sample was then added and any antigen present was captured by the immobilized antibody. Incubation with the second specific antiserum, developed in rabbit, followed. Finally, the HRP conjugated anti-rabbit IgG (goat) was added. Visualisation was achieved by incubation with the substrate and stopping the reaction with  $H_2SO_4$ . The plate was read at 490nm, and ODs obtained using the Dynatech Minireader II.

In the sandwich assays the amount of antigen present in the sample is directly proportional to the amount of the second antiserum (rabbit), which was quantified indirectly by the addition of the HRP conjugated anti-rabbit IgG, resulting in high ODs for high antigen concentrations. The method is described in detail in Table 3.3. Plates included 10 serial two-fold dilutions of purified antigen for the construction of a standard curve. The working range for each assay is given in Table 3.4. Only the central wells were used when running standards or samples (in triplicate) in an effort to avoid the edge-effect. The peripheral wells were used for assaying the controls which were as shown in Table 3.1. All controls were run in duplicate except for the zero-antigen (control 14; Table 3.1) which was run in quadruplicate.

**Table 3.3** Steps and reagents for the transferrin (TF), lactoferrin (LF),  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M),  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT) and albumin (Alb) Sandwich ELISAs. The respective standards and antisera were used for each assay. Standard concentration range is given in table 3.4.

<b>SANDWICH ELISAs</b>	
<b>STEPS</b>	<b>REAGENTS</b>
1. Wash with 200 $\mu$ l/well CB thrice and dry.	
2. Coat with 100 $\mu$ l/well specific antiserum. Incubate overnight at 4°C.	goat antisera to: TF, LF, $\alpha$ 2-M and Alb 1/6000 in CB; $\alpha$ 1-AT 1/3000 in CB.
3. Wash with 200 $\mu$ l/well PBST, 3x6 times, and dry. Removal of excess and loosely coated antibody.	
4. Add 100 $\mu$ l/well standard antigen or eluted sample. Incubate for 2h at 37°C.	standard TF, LF, $\alpha$ 2-M, Alb or $\alpha$ 1-AT in IB <sup>a</sup> .
5. Wash with 200 $\mu$ l/well PBST, 3x6 times, and dry. Removal of free and loosely bound antigen.	
6. Incubate with 100 $\mu$ l/well second specific antiserum overnight at 4°C.	rabbit antisera to: TF, LF, $\alpha$ 2-M, Alb or $\alpha$ 1-AT 1/4000 in IB <sup>a</sup> .
7. Wash with 200 $\mu$ l/well PBST, 3x6 times, and dry. Removal of free and loosely bound antigen.	
8. Add 100 $\mu$ l/well HRP conjugated anti-IgG. Incubate for 90min at 37°C.	HRP anti-rabbit IgG (goat): 1/4000 in IB <sup>a</sup>
9. Wash with 200 $\mu$ l/well PBST, 3x6 times and dry. Removal of excess HRP-anti-IgG.	
10. Add 100 $\mu$ l/well SB. Incubate at 22°C ( $\alpha$ 2-M, $\alpha$ 1-AT and TF assay for ~15min. LF assay ~20 min) and stop colour development with 50 $\mu$ l/well 1M H <sub>2</sub> SO <sub>4</sub> . Read at 490nm.	

<sup>a</sup> In the case of the Alb ELISA, IB was replaced by IB/M  
CB= coating buffer, PBST= Wash buffer  
IB= incubation buffer, IB/M= IB nil BSA, plus 5% MARVEL,  
HRP= horseradish peroxidase

**Table 3.4** Working range of the standard antigen for the  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M),  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT), transferrin (TF), lactoferrin (LF) and albumin (Alb) sandwich ELISAs. This was produced by serial two fold dilutions of the standard antigen in the appropriate buffer, resulting in 10 standard solutions for the construction of a standard curve.

<b>Assay</b>	<b>Working range ng/ml in IB<sup>a</sup></b>
<b><math>\alpha</math>2-M</b>	200 - 0.39
<b><math>\alpha</math>1-AT</b>	100 - 0.19
<b>TF</b>	50 - 0.09
<b>LF</b>	125 - 0.24
<b>Alb</b>	500 - 0.98

<sup>a</sup> In the case of the Albumin ELISA, IB was replaced by IB/M  
 IB= incubation buffer  
 IB/M= IB nil BSA, plus 5% MARVEL

### 3.2.2.4 Preliminary experiments and establishment of the sandwich ELISAs

#### (a) Selection of microtitre plate type for the sandwich ELISAs

Five different types of Immulon (Dynatech Laboratories) microelisa plates (one of each: I, M129 (A)/G, M129 (B)/G, III and IV) were tested in order to check if they conform with the claims of the manufacturer. These types of plates are supposed to have low variability among their wells and therefore be suitable for quantitative ELISAs.

The plates were washed with 200 $\mu$ l/well coating buffer thrice and dried. They were then incubated with whole goat serum (SAPU) at 1/10<sup>4</sup> in coating buffer (100 $\mu$ l/well), for 2h at 37°C. Only the 60 inner wells were used, whereas the peripheral wells were treated with coating buffer alone. Following this coating step, each plate was washed with 200 $\mu$ l/well PBST, (3 sets of 6 washes each), and dried. All plates were then incubated with HRP conjugated anti-sheep/goat IgG at 1/1000 dilution in incubation buffer (100 $\mu$ l/well) for 90min at 37°C and subsequently washed as above with PBST. The substrate buffer (100 $\mu$ l/well) was then added and the plates incubated at room temperature for a suitable time depending on the plate being tested. The reaction was stopped with 50 $\mu$ l/well 1M H<sub>2</sub>SO<sub>4</sub> and the plate read at 490 nm.

The coefficient of variation ( $CV \% = SD \times 100 / \text{mean OD}$ ) was then calculated for each plate. This represents a measure of variability among repeated tests (in this case the OD readings obtained for the 60 wells of the plate). As shown in Table 3.5, Immulon III gave the highest  $CV = 25.3\%$ , whereas all the other plates gave CVs ranging between 3.3 to 7.3%. The exceptionally high CV obtained with the Immulon III plate was surprising as this plate is advertised by the manufacturer as having certified CV of below 6%. This plate was, therefore, retested at two different concentrations of goat serum ( $1/10^4$  and  $1/(5 \times 10^4)$  in coating buffer) and overnight incubation at  $4^\circ\text{C}$  for the coating step. Although overnight coating at  $4^\circ\text{C}$  and a higher dilution of goat serum improved the CV for the Immulon III plate (19.2% and 11% at the  $1/10^4$  and  $1/(5 \times 10^4)$  dilutions respectively) it still remained much higher than that obtained with all other types of plates. Following this experiment Immulon IV plates were selected for the sandwich ELISAs.

(b) Establishing the  $\alpha 2\text{-M}$ ,  $\alpha 1\text{-AT}$ , TF and LF sandwich ELISAs

A series of experiments were conducted in order to establish the best conditions for each of the four sandwich ELISAs. The standard chess-board type dilutions were used for defining the optimal goat, rabbit and HRP conjugated

**Table 3.5** Coefficient of variation (CV) obtained with 5 different Immulon plates (Dynatech Laboratories). Mean OD  $\pm$  SD of the central 60 wells for each plate is given. % CV is defined as (SD x 100)/ mean OD.

Immulon Plate type	OD $\pm$ SD	CV
I/ M129	1.229 $\pm$ 0.09	7.3%
M129 A/(G)	1.188 $\pm$ 0.06	5.0%
M129 B/(G)	1.656 $\pm$ 0.07	4.2%
III	0.831 $\pm$ 0.21	25.3%
IV	1.21 $\pm$ 0.04	3.3%

OD= optical density  
SD= standard deviation

antiserum dilutions and these were as discussed in section 3.2.2.3, Table 3.3.

Overnight incubation at 4°C was used to coat the plates as it was shown to reduce within plate variation. Incubation for 2h at 37°C, or overnight at 4°C, after the antigen or rabbit antiserum was added, did not significantly alter the resolution of the standard curve. Overnight incubation at 4°C with the rabbit antiserum, however, lowered the background in the absence of the antigen (control 14; Table 3.1), and was preferred. In all assays, incubation with the HRP anti-rabbit IgG at 1/4000 for 90min was shown to give maximum ODs between approximately 1 and 1.8 (depending on the assay), and very low non-specific binding ( $OD < 0.05$ ) in the absence of the rabbit antiserum or the antigen ( $OD \leq 0.08$  depending on the assay) (controls 13 and 14 respectively; Table 3.1). Therefore, blocking with incubation buffer only, (containing 0.1% w/v BSA and 0.05% w/v Tween 20) during incubations, was judged to be sufficient.

The precision ranges of each of the four sandwich ELISAs are shown in Table 3.6. Precision range was defined as the linear part of the working range (Table 3.4). A prerequisite was that the OD of the lowest standard (mean  $\pm$  SD) of the precision range differed significantly from that of the zero antigen control (mean  $\pm$  SD) (control 14; Table 3.1). Outwith the precision range, experimental

**Table 3.6** Precision range of the standard antigen for the  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M),  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT), transferrin (TF) and lactoferrin (LF) sandwich ELISAs. Precision range was defined the linear part of the working range (table 3.4).

<b>Assay</b>	<b>Precision range ng/ml in IB</b>
<b><math>\alpha</math>2-M</b>	100 - 1.56
<b><math>\alpha</math>1-AT</b>	25 - 0.78
<b>TF</b>	25 - 0.78
<b>LF</b>	31.25 - 0.49

IB= incubation buffer

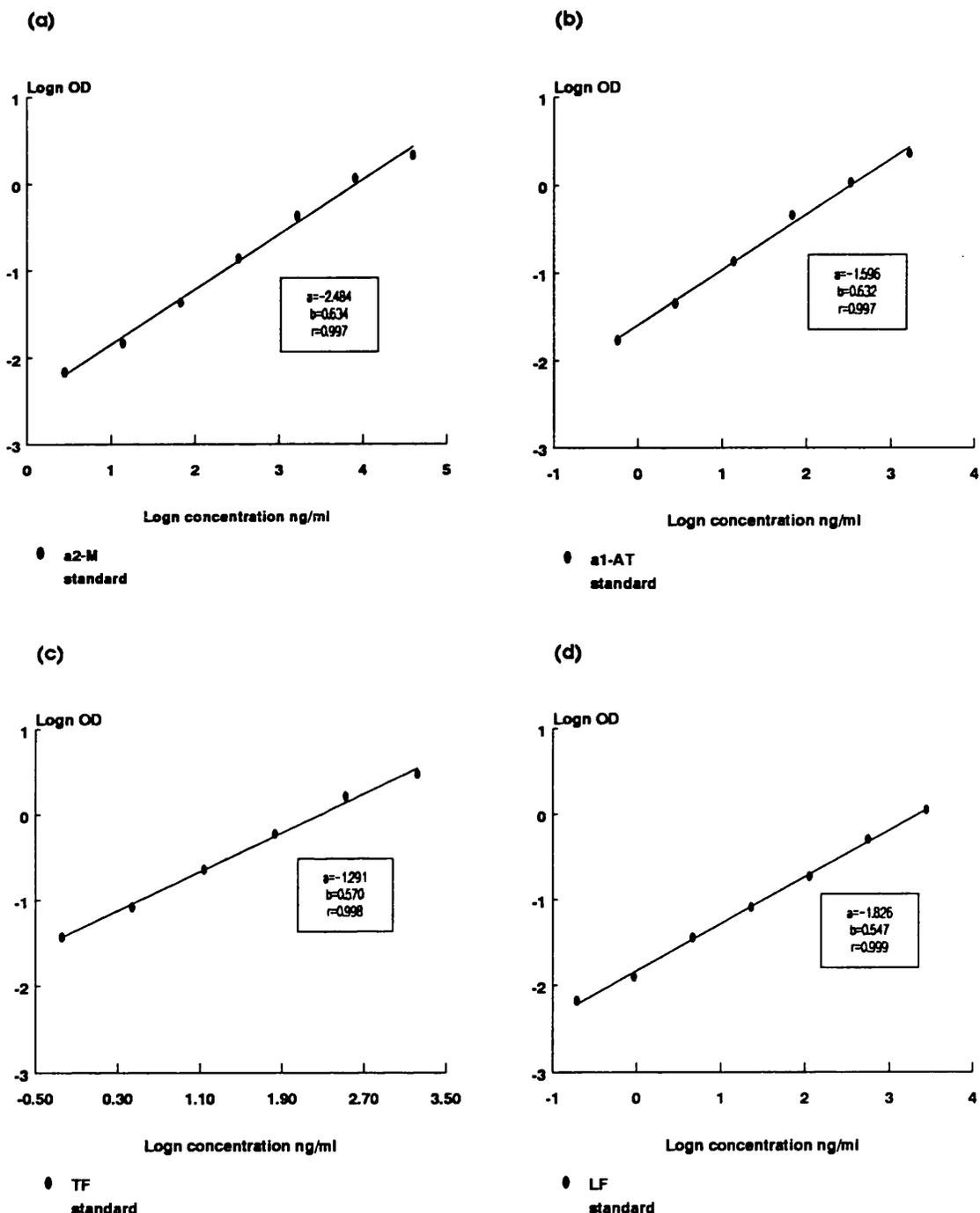
error increased due to the typical flattening of the curve.

Figure 3.7 (a to d) shows typical standard curves (precision ranges) for the  $\alpha 2$ -M,  $\alpha 1$ -AT, TF and LF sandwich ELISAs. The least squares method was used to plot the best fitting line and  $r^2 > 0.99$  were obtained for all assays. Linear functions of the type  $\log_n OD = a + b \times (\log_n c)$ , where OD= optical density, c= concentration, a= the intercept and b= the  $\log_n c$  coefficient were thus produced, and used for the determination of sample antigen quantities. A similar standard curve was generated and a new line fitted each time an assay was run.

In an attempt to investigate if serum and standard antigen responded in a similar way, serial two-fold dilutions of serum in incubation buffer were assayed in each of the four sandwich ELISAs in the following dilution ranges (depending on the assay):

- i)  $\alpha 2$ -M assay:  $1/(4 \times 10^4)$  to  $1/(256 \times 10^4)$ ;
- ii)  $\alpha 1$ -AT assay:  $1/(8 \times 10^4)$  to  $1/(256 \times 10^4)$ ;
- iii) TF assay:  $1/(16 \times 10^4)$  to  $1/(512 \times 10^4)$ ; and
- iv) LF assay:  $1/24$  to  $1/1536$ .

The initial dilution was arbitrarily termed 1 for both standard and serum antigen and the two fold dilution factors thereafter,  $2^{-1}$  to  $2^{-6}$  for the  $\alpha 2$ -M and LF assays

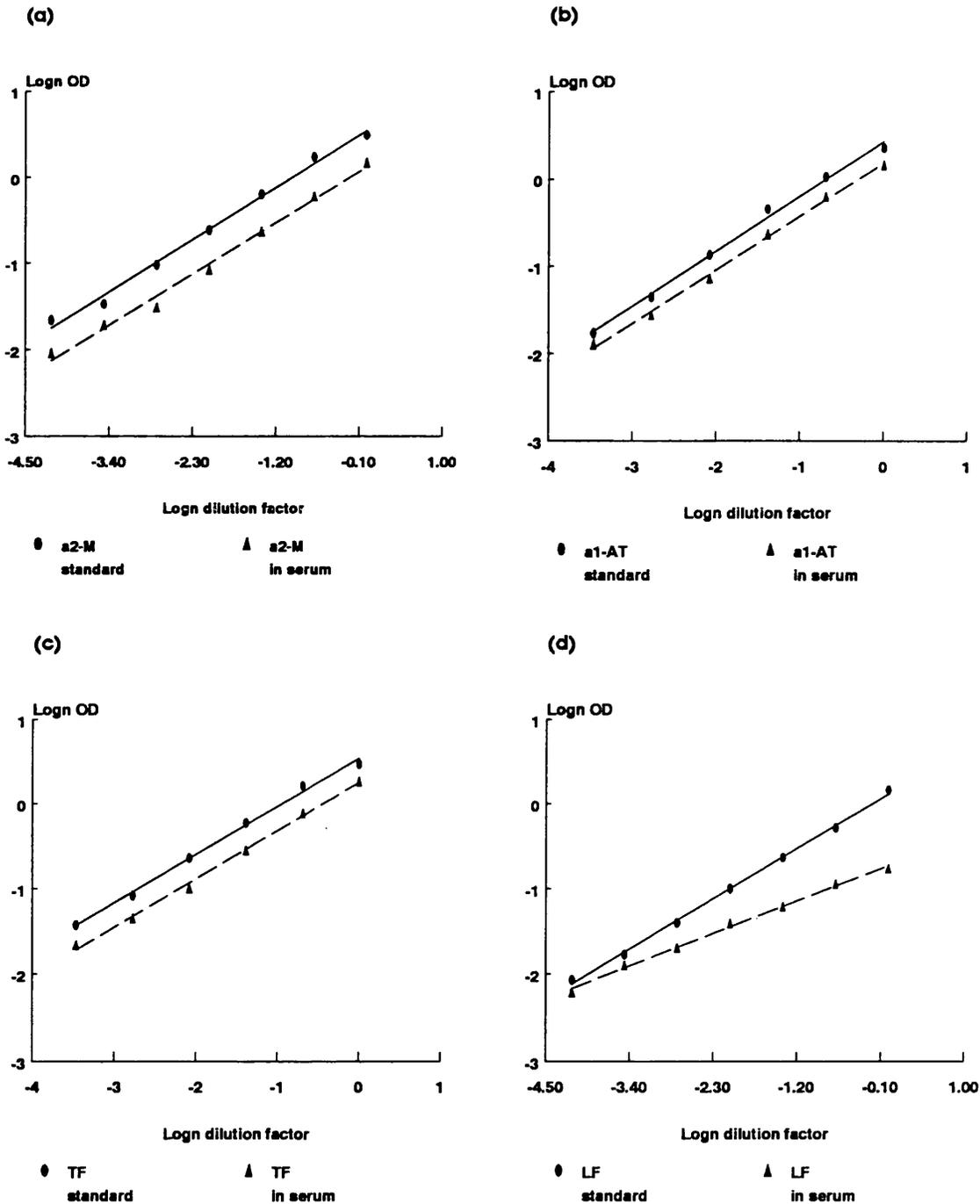


**Figure 3.7** Typical calibration curves for the (a)  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M), (b)  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT), (c) transferrin (TF) and (d) lactoferrin (LF) sandwich ELISAs (precision range; Table 3.6).  $\text{Log}_n$  ODs (490nm) are plotted against  $\text{log}_n$  concentration (ng/ml). The least squares method was used to plot the best fitting line and the regression coefficients are shown.

OD= optical density; a= the intercept  
 b= the  $\text{log}_n$  concentration coefficient  
 r= Pearson correlation coefficient

and  $2^{-1}$  to  $2^{-5}$  for  $\alpha 1$ -AT and TF.  $\log_n$  ODs were plotted against  $\log_n$  dilution factors for both standard and serum and linear regression was used to plot the best fitting lines (Fig. 3.8). As shown in Figure 3.8 serum and standard dilution curves were parallel at least as far as  $\alpha 2$ -M,  $\alpha 1$ -AT and TF were concerned, illustrating that serum and standard respond in a similar way for these proteins (Fig. 3.8 (a) to (c)). This was not, however, the case for LF as shown in Figure 3.8 (d). LF is present in only very low concentrations ( $\sim 300$ ng/ml) in serum (Oloffson et al., 1977). It, therefore, required a much lower starting serum dilution (only 1/24 in incubation buffer compared to  $1/(4 \times 10^4)$  to  $1/(16 \times 10^4)$  used for the other proteins). This could account for the interference observed, as other serum components would be in much higher concentrations than LF in the eluate. TF, which is structurally related to LF and occurs in much higher concentrations in serum ( $10^4$  fold higher than LF), could also be responsible for a degree of the interference observed.

In order to investigate if serum components would interfere when assaying GCF LF, and thus influence the reliability of the assay, serum at 1/1000 dilution in incubation buffer was added in the LF standards and the resolution of the standard curve examined. The 1/1000 dilution of serum was used for the following reasons: a) GCF samples are eluted in a minimum of 1ml of incubation buffer; b) assuming a maximum of approximately 1 $\mu$ l of GCF and a consistency



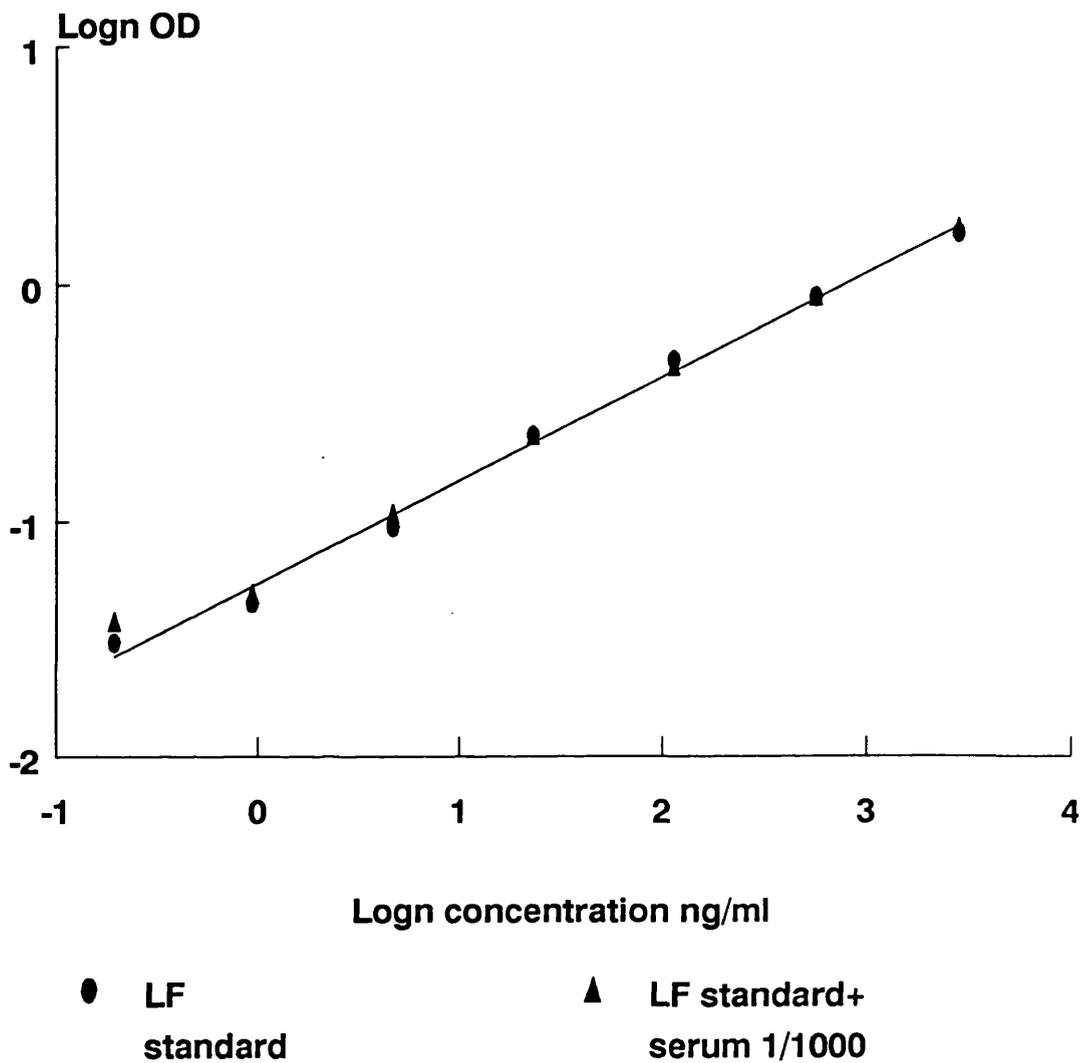
**Figure 3.8** Standard and serum dilution curves for the (a)  $\alpha 2$ -macroglobulin ( $\alpha 2$ -M), (b)  $\alpha 1$ -antitrypsin ( $\alpha 1$ -AT), (c) transferrin (TF) and (d) lactoferrin (LF) sandwich ELISAs. Standards were assayed at the precision range (Table 3.6). Starting dilution of serum was  $1/(4 \times 10^4)$  for  $\alpha 2$ -M,  $1/(8 \times 10^4)$  for  $\alpha 1$ -AT,  $1/(16 \times 10^4)$  for TF and  $1/24$  for LF. 7 serial two fold dilutions for the  $\alpha 2$ -M and LF assays and 6 for the  $\alpha 1$ -AT and TF assays, for standard and serum were tested.  $\text{Log}_n$  ODs are plotted against  $\text{log}_n$  dilution factor. Linear regression was used to plot the best fitting lines.

OD= optical density; IB= incubation buffer

similar to that of serum, the maximum concentration of serum components in the experimental eluate would be 1/1000 of their original concentrations in serum. As shown in Figure 3.9 addition of 1/1000 serum in our standards did not influence the resolution of the standard curve for LF. Moreover, when TF and LF were substituted for each other in their sandwich ELISAs, they gave results similar to the no antigen control for the whole width of their dilution ranges.

Finally, GCF samples ( $\alpha 2$ -M,  $\alpha 1$ -AT, TF: n=5; LF: n=4) originally eluted in 1ml of incubation buffer were diluted a further 1/5 in incubation buffer and two serial two-fold dilutions in incubation buffer produced which were assayed for  $\alpha 2$ -M,  $\alpha 1$ -AT, TF and LF. As shown in Table 3.7, acceptable dilution profiles were obtained for each one of the proteins in all the samples. This confirmed that the four proteins in GCF respond in a similar way to the highly purified standard and that the assays developed could be used for their quantitation in GCF.

The above experiment provided some information on the elution/dilution procedure required for GCF which was to be used for analysis of  $\alpha 2$ -M,  $\alpha 1$ -AT, TF and LF by the sandwich ELISAs. A larger number of GCF samples were then tested and the elution/dilution procedure for GCF was established. It was observed that a dilution of GCF samples of approximately  $1/(25 \times 10^3)$  to  $1/(5 \times 10^4)$  for  $\alpha 2$ -M,  $1/(5 \times 10^4)$



**Figure 3.9** Standard curve for the lactoferrin (LF) sandwich ELISA (precision range; 31.25 to 0.49 ng/ml) with and without the addition of serum (final concentration of serum in standard solutions, 1/1000 of its original concentration).  $\text{Log}_n$  ODs are plotted against  $\text{log}_n$  concentration of standard.

OD= optical density

**Table 3.7**  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M),  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT), transferrin (TF) and lactoferrin (LF) as determined in serial dilutions of GCF eluate. Samples were initially eluted in 1 ml of IB and further diluted in IB as shown below. Results are expressed in ng/ml of the resulting eluate. 'Low' or 'over' refers to samples that yielded ODs below or above the precision range of the respective assay.

Sample	GCF $\mu$ l	Dilution factor	Assay			
			$\alpha$ 2-M	$\alpha$ 1-AT	TF	LF
Concentration in eluate (ng/ml)						
1.	0.192	1/5	5.2	14.8	12.3	6.1
		1/10	2.8	6.2	5.4	3.4
		1/20	1.6	2.6	2.6	1.9
2.	0.107	1/5	1.9	5.8	5.8	5.3
		1/10	low	2.4	2.7	3.0
		1/20	low	1.4	1.2	1.6
3.	0.476	1/5	31.0	over	over	5.6
		1/10	15.3	15.4	18.9	3.2
		1/20	7.3	7.2	10.5	1.6
4.	0.464	1/5	17.5	over	over	12.1
		1/10	9.1	19.0	22.3	7.1
		1/20	4.5	8.2	12.5	3.9
5.	0.350	1/5	52.5	over	over	*
		1/10	24.9	20.0	21.7	*
		1/20	11.6	9.2	12.7	*

GCF= gingival crevicular fluid  
 IB= incubation buffer  
 OD= optical density  
 \*= not done

to  $1/10^5$  for  $\alpha 1$ -AT and LF, and  $1/10^5$  to  $1/(2 \times 10^5)$  for TF would yield concentrations in the eluate within the precision range of each ELISA. In order to achieve these dilutions, GCF samples were eluted in 1ml (or equivalent\*) of incubation buffer and then further diluted in incubation buffer depending on their original volumes as shown in Table 3.8. However, if a GCF sample, yielded ODs below the precision range of the assay, it was re-run at the minimum of 1/2.5 dilution of the eluate for  $\alpha 2$ -M and 1/5 for  $\alpha 1$ -AT, TF and LF. This resulted in a similar detectability limit of total protein in GCF samples for all assays. This was 3.9ng for  $\alpha 2$ -M,  $\alpha 1$ -AT and TF and 2.45ng for LF. Comparing this to the competitive ELISAs, there was a 10-fold improvement in the detection limit of total  $\alpha 2$ -M and TF. This was particularly advantageous and the main reason for developing the sandwich ELISAs. The competitive assays, although suitable for assessing  $\alpha 2$ -M and TF in GCF samples obtained from diseased sites, failed in many cases to detect  $\alpha 2$ -M and TF in GCF samples obtained from healthy sites during the experimental gingivitis study when the samples were eluted in the standard way (1ml of incubation buffer). This was very important as it made the quantitation of these proteins in health and during the initial stages of inflammation impossible. The sandwich assays proved to be more sensitive and to have lower detectability limits allowing the standard elution

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GCF elution procedure was slightly modified in the longitudinal study of periodontitis patients on maintenance; refer to section 3.2.3.1.

**Table 3.8** GCF sample dilution procedure prior to assaying by the  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M),  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT), transferrin (TF) and lactoferrin (LF) sandwich ELISAs. Samples were originally eluted in 1ml of IB and further diluted, depending on their original GCF volume (v), as shown below in IB, to yield a final dilution of approximately at the range of: a)  $1/(25 \times 10^3)$  to  $1/(5 \times 10^4)$  for  $\alpha$ 2-M; b)  $1/(5 \times 10^4)$  to  $1/10^5$  for  $\alpha$ 1-AT and LF; and c)  $1/10^5$  to  $1/(2 \times 10^5)$  for TF.

GCF volume (v) in $\mu$ l	Assay		
	$\alpha$ 2-M	$\alpha$ 1-AT and LF	TF
	Dilution factor		
$v \leq 0.1$	1/2.5	1/5	1/10
$0.1 < v \leq 0.2$	1/5	1/10	1/20
$0.2 < v \leq 0.4$	1/10	1/20	1/40
$0.4 < v \leq 0.8$	1/20	1/40	1/80
$0.8 < v$	1/40	1/80	1/160

GCF= gingival crevicular fluid

IB= incubation buffer

v= GCF volume

procedure of GCF samples to be maintained and in addition less eluate volume to be used, thus preserving material for other assays.

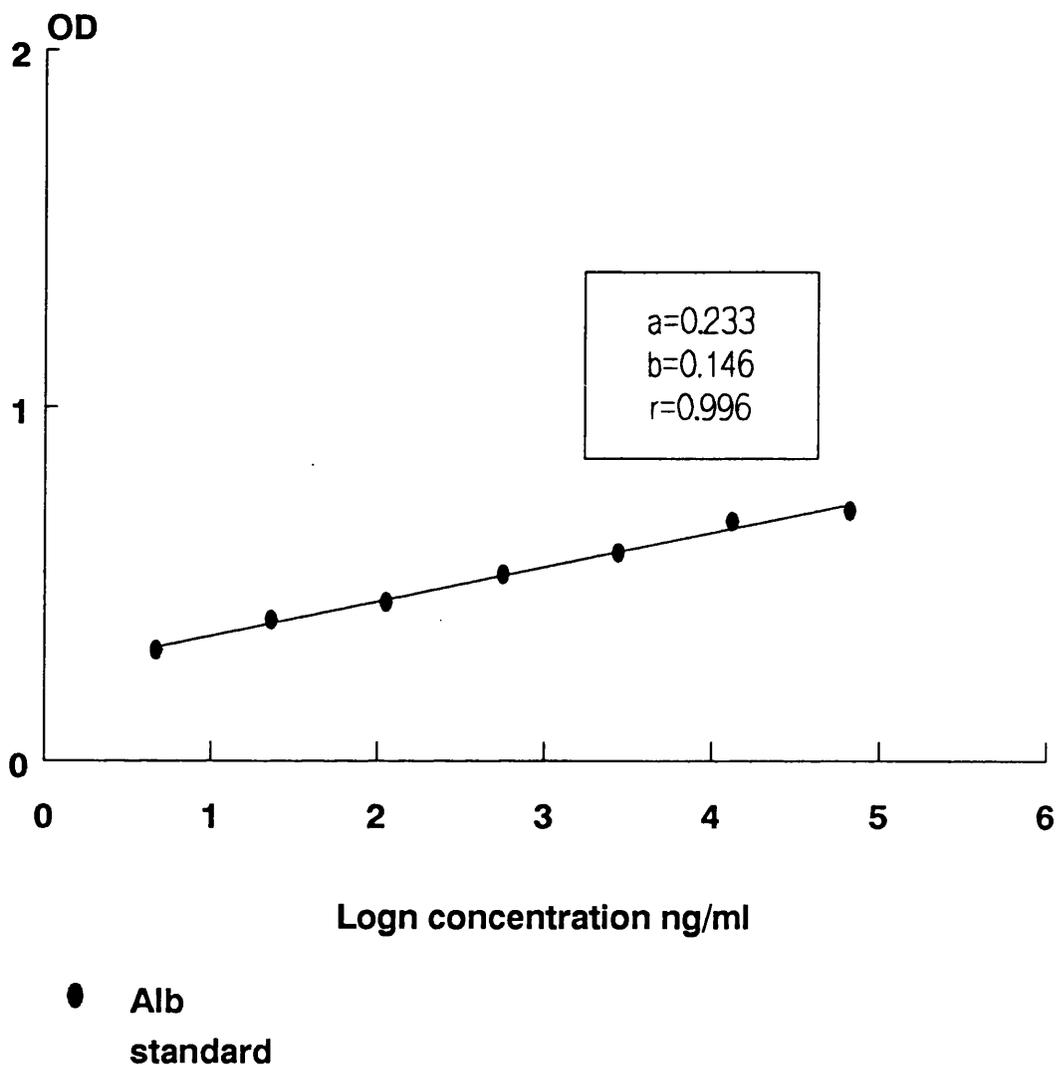
(c) Establishing the albumin sandwich ELISA

The standard chess board type dilutions were used to determine the best conditions for the goat, rabbit and HRP conjugated antisera as well as the standard for the Alb sandwich ELISA and these are as shown in section 3.2.2.3, Table 3.3. The standard incubation times, as used for the other sandwich ELISAs, were examined and were found to be satisfactory and hence used for the Alb sandwich ELISA. Incubation of standard, rabbit antiserum and conjugate in incubation buffer containing 0.1% w/v BSA had to be modified due to the cross-reactivity of the anti-human Alb antisera (rabbit and goat) with BSA. Incubations in PBST alone containing nil BSA were inadequate, as background OD was very high and no differentiation between serial standard Alb dilutions could be achieved. Incubation in IB/M (incubation buffer containing 5% w/v MARVEL) lowered the background sufficiently (OD~0.3) and enhanced curve resolution. A separate 1h blocking step with IB/M or gelatin at 37°C did not lower the background further and was thus omitted.

The precision range of the Alb assay, defined as the linear part of the working range (Table 3.4), was 125 to 1.95ng/ml.

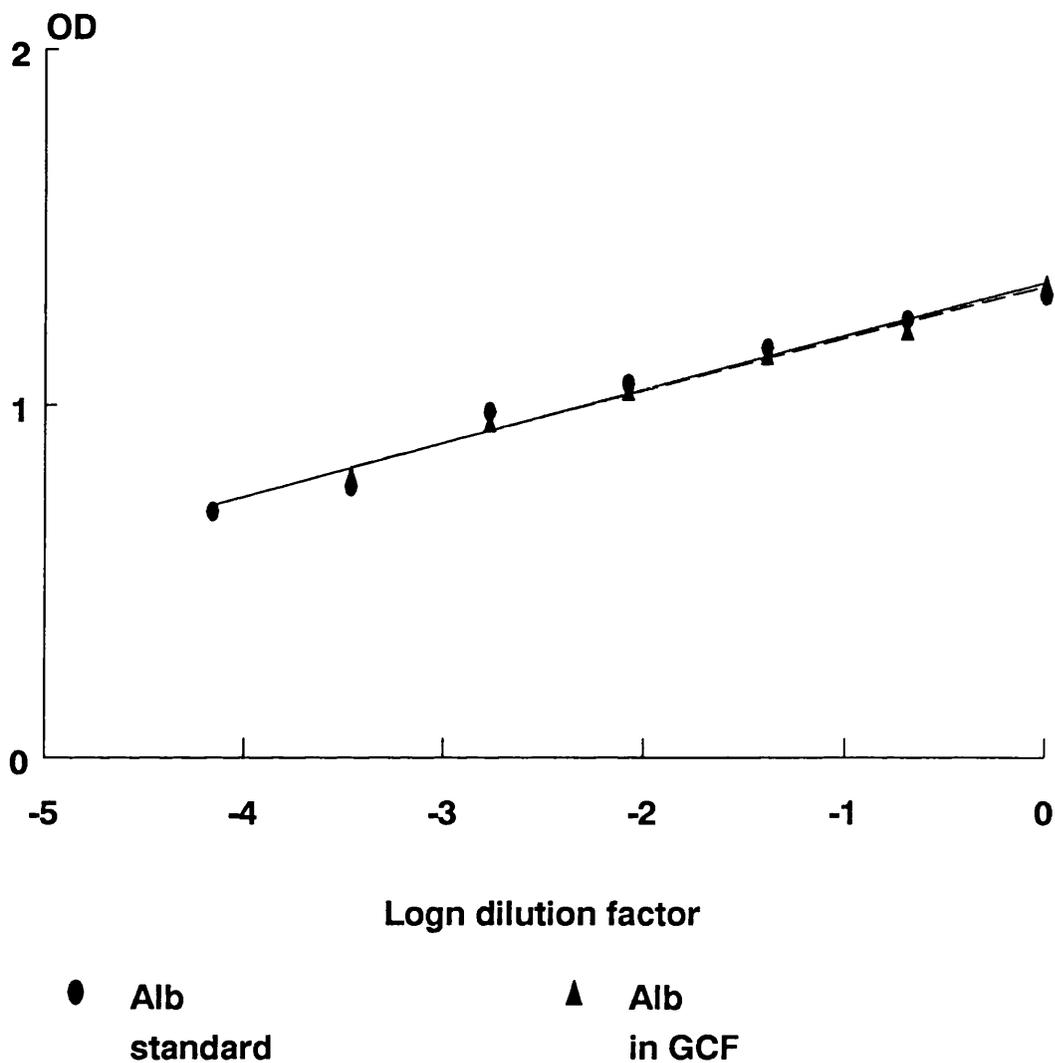
Figure 3.10 shows a typical standard curve for the Alb sandwich ELISA. The least squares method was used to plot the best fitting line and produce the linear function of the type  $OD = a + b \times (\log_n c)$ , where OD= optical density,  $c$ = concentration,  $a$ = the intercept and  $b$ = the  $\log_n c$  coefficient which was used for the determination of sample antigen quantities. A similar standard curve was generated and a new line fitted each time an assay was run.

In order to investigate if Alb in GCF and purified Alb standard responded in a similar way, GCF in the dilution range of 1/40 to 1/1280 in incubation buffer was assayed by the Alb sandwich ELISA. Initial dilution of standard (125ng/ml) and GCF was termed 1 and two fold dilution factors thereafter  $2^{-1}$  to  $2^{-6}$  for serum and  $2^{-1}$  to  $2^{-5}$  for GCF and ODs were plotted against  $\log_n$  concentration. Linear regression was then used to plot the best fitting line (Fig. 3.11). As shown in Figure 3.11, standard albumin and albumin in GCF gave very similar dilution profiles (superimposed), providing evidence that this assay can be reliably used for the quantitation of albumin in GCF.



**Figure 3.10** Typical calibration curve for the albumin (Alb) sandwich ELISA (precision range; 125 to 1.95ng/ml). ODs (490nm) are plotted against  $\log_n$  concentration. The least squares method was used to plot the best fitting line and the regression coefficients are shown.

OD= optical density  
 a= the intercept  
 b= the  $\log_n$  concentration coefficient  
 r= Pearson correlation coefficient



**Figure 3.11** Standard and gingival crevicular fluid (GCF) dilution curves for the albumin (Alb) sandwich ELISA. Starting concentration of standard was 125ng/ml in IB/M. GCF was eluted in 0.5ml of IB/M and further diluted starting at 1/40 in IB/M. 7 serial two fold dilutions of standard and 6 of GCF in IB/M were assayed. Initial dilution factor of standard and GCF was arbitrarily termed 1 and two-fold dilution factors thereafter,  $2^{-1}$  to  $2^{-6}$  for standard and  $2^{-1}$  to  $2^{-5}$  for GCF. ODs are plotted against  $\log_n$  dilution factor. Linear regression was used to plot the best fitting lines.

IB/M= IB nil BSA, plus 5% MARVEL; OD= optical density

GCF samples were used to establish the dilution procedure prior to assaying for albumin. Samples eluted in 0.5ml of IB (nil BSA) were further diluted in IB/M as shown in Table 3.9 depending on their original GCF volume. If a sample yielded ODs below the lower limit of the precision range of the assay, it would be retested at 1/50 dilution. This resulted in a detection limit of total Alb in GCF of 49ng.

### **3.2.2.5 $\alpha$ 2-macroglobulin and $\alpha$ 1-antitrypsin binding to elastase: effect on their detection by sandwich ELISAs**

This experiment was conducted in order to determine if variation of  $\alpha$ 2-M or  $\alpha$ 1-AT molar ratio to a protease (in this case elastase) in the experimental solution would affect their detection by the sandwich ELISAs used in these studies.

Six solutions of  $\alpha$ 2-M/elastase in IB were prepared.  $\alpha$ 2-M at a final concentration of 200ng/ml and elastase ranging from 260ng/ml to 8ng/ml were used. In this way a total of six solutions of elastase/ $\alpha$ 2-M were obtained, at molar ratios of 16:1, 8:1, 4:1, 2:1, 1:1, and 1:2. An additional solution of  $\alpha$ 2-M in IB at a concentration of 200ng/ml and no elastase was prepared. All the above solutions were incubated for 2h at 37°C, in order to achieve complex formation, and then frozen at -30°C. All six solutions were thawed and assayed (in 1/16 dilution in IB) by the  $\alpha$ 2-

**Table 3.9** GCF sample dilution procedure prior to assaying by the albumin sandwich ELISAs. Samples were originally eluted in 0.5ml of PBST and further diluted, depending on their original GCF volume (v), as shown below in IB/M, to yield a final dilution of approximately at the range of  $1/10^6$  to  $1/(2 \times 10^6)$

GCF volume (v) in $\mu$ l	Assay
	Albumin
	Dilution factor
$v \leq 0.1$	1/50
$0.1 < v \leq 0.2$	1/100
$0.2 < v \leq 0.4$	1/200
$0.4 < v \leq 0.8$	1/400
$0.8 < v$	1/800

PBST= wash buffer= incubation buffer nil BSA  
 IB/M= incubation buffer nil BSA, 5% MARVEL  
 v= GCF volume

M ELISA. Percentage recovery was calculated relative to the  $\alpha$ 2-M solution containing no elastase.

A similar procedure was used for  $\alpha$ 1-AT. Six 1ml solutions of  $\alpha$ 1-AT/elastase in IB were prepared.  $\alpha$ 1-AT at a final concentration of 100ng/ml and elastase ranging from 1000ng/ml to 25ng/ml were used. In this way six solutions of elastase/ $\alpha$ 1-AT at molar ratios ranging between 20:1, 10:1, 5:1, 2.5:1, 1:1 and 1:2 were obtained. An additional solution of 100 ng/ml  $\alpha$ 1-AT in IB, containing no elastase was prepared. The resulting seven solutions were incubated at 37°C for 2h, in order to achieve complex formation, and then frozen at -30°C. Prior to assaying by the  $\alpha$ 1-AT ELISA method the solutions were thawed, and a further 1/32 dilution in IB was employed. Percentage recovery was calculated using the  $\alpha$ 1-AT solution containing no elastase as a standard (Table 3.10).

Binding of elastase to  $\alpha$ 2-M did not significantly affect its detection (recovery rate; Table 3.10) in this experiment. This is probably due to the nature of the  $\alpha$ 2-M/ protease interaction. Only active enzymes react with  $\alpha$ 2-M, which cleave the tetramer in the bait region resulting in a conformational change of the molecule which then irreversibly entraps the endopeptidase and inactivates it. As  $\alpha$ 2-M is a relatively big molecule its antigenicity is seemingly not affected. The  $\alpha$ 2-M ELISA therefore assesses the total amount of the inhibitor present in the

**Table 3.10** Recovery rate of  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M) and  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT) (sandwich ELISAs) after binding to elastase. Prior to assaying  $\alpha$ 2-M and  $\alpha$ 1-AT were incubated for 2h at 37C° with elastase, at the molar ratios shown below. Percentage recovery was calculated versus standard  $\alpha$ 2-M or  $\alpha$ 1-AT which received the same treatment.

$\alpha$ 2-macroglobulin		$\alpha$ 1-antitrypsin	
elastase: $\alpha$ 2-M	recovery	elastase: $\alpha$ 1-AT	recovery
16:1	101%	20:1	49%
8:1	97%	10:1	53%
4:1	86%	5:1	54%
2:1	110%	2.5:1	57%
1:1	100%	1:1	84%
1:2	116%	1:2	101%

crevice irrespective of the amount of proteases. In contrast, binding of elastase to  $\alpha$ 1-AT resulted in approximately 50% reduction of its recovery rate by the sandwich ELISA when molar ratios of elastase: $\alpha$ 1-AT ranging from 20:1 to 2.5:1 were used.  $\alpha$ 1-AT has a molecular weight of 55kDa which is much lower than that of  $\alpha$ 2-M. Binding of the inhibitor to elastase could cause masking of some epitopes of the  $\alpha$ 1-AT molecule and this could result in a reduction of the antigenicity of  $\alpha$ 1-AT. However, at elastase: $\alpha$ 1-AT ratios of 1:1 and 1:2 detection of  $\alpha$ 1-AT returned to 84% and 101% respectively. Thus, it is possible that under experimental conditions the amount of  $\alpha$ 1-AT detected by the sandwich ELISA is approximately 50% of the total amount present in the crevice.

### **3.2.3 Gingival crevicular fluid elution**

#### **3.2.3.1 Gingival crevicular fluid elution technique**

GCF samples were eluted in 1ml of incubation buffer for 1h at room temperature. The strips were then discarded and the eluate was aliquoted (200 $\mu$ l) and stored in microcentrifuge tubes at -70°C. In the longitudinal study of periodontitis patients on maintenance care the procedure was slightly modified. Samples were eluted in 0.5ml of PBST (incubation buffer with no BSA) for 1h at 22°C. 100 $\mu$ l aliquot of the eluate was stored separately at -70°C for the subsequent quantitation of GCF Alb. The remaining

0.4ml were diluted 1/2 in incubation buffer containing 0.2% BSA (instead of the usual 0.1%). The resulting eluate was aliquoted and stored at -70°C. In this way the standard dilution procedure of GCF samples for assaying for  $\alpha$ 2-M,  $\alpha$ 1-AT, TF and LF could be maintained (Table 3.8) while obtaining material suitable for assessing GCF Alb.

### **3.2.3.2 Recovery rate of $\alpha$ 2-macroglobulin, $\alpha$ 1-antitrypsin, transferrin and lactoferrin from Whatman grade 4 paper strips**

With the development of the sandwich ELISAs it was possible to quantify all four proteins in the same GCF sample. It therefore became imperative to determine their recovery rate when absorbed on paper strips as this would influence the determination of their relative levels in GCF.

To achieve this, the following protocol was followed for  $\alpha$ 2-M,  $\alpha$ 1-AT and TF; 1 $\mu$ l of serum was delivered by a Hamilton microsyringe either:

a) on Whatman grade 4 paper strips (n=7) which were subsequently eluted in 1ml of incubation buffer for 1h at 22°C using a rotatory mixer; or

b) directly (n=3) in 1ml of incubation buffer which was also rotated for 1h at 22°C.

The eluate was diluted further 1/250 in incubation buffer prior to assaying for  $\alpha$ 1-AT and TF and 1/125 for  $\alpha$ 2-M, in order to achieve concentrations in the eluate within the working ranges of the sandwich ELISAs. The concentration of each of the above proteins in the original 1ml eluate of the strips was compared against that in the 1ml eluate when direct delivery in buffer was used and the % recovery calculated (Table 3.11).

For LF, 1 $\mu$ l of 5mg/ml LF stock solution was delivered on paper strips (n=7) and eluted as above in 1ml of incubation buffer. The same stock solution was also delivered directly in 1ml incubation buffer (n=3) and rotated, like the strips, for 1h at 22°C. The resulting eluate was diluted further 1/200 in incubation buffer and assayed by the sandwich ELISA. The LF concentration in the 1ml eluate of the strips was compared against the concentration of LF when direct delivery of the stock solution in the buffer was used, and the percentage LF recovery from the strips calculated (Table 3.11).

Finally, an experiment was performed in order to ensure that paper strips were not contaminated by any of the proteins to be tested, prior to their use on patients. Sterile paper strips (n=10), were eluted in the standard procedure and the 1ml eluate tested by the sandwich ELISAs for  $\alpha$ 2-M,  $\alpha$ 1-AT, TF and LF. None of the strips yielded

**Table 3.11** Recovery rate of  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M),  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT), transferrin (TF) and lactoferrin (LF) from paper strips. For  $\alpha$ 2-M,  $\alpha$ 1-AT and TF 1 $\mu$ l of serum and for LF 1 $\mu$ l of standard (5mg/ml) was delivered, either: a) on paper strips (n=7) which were subsequently eluted in 1ml IB; or b) directly in 1ml of IB (n=3). The mean concentration in the 1ml eluate ( $\pm$  SD), resulting from both methods of delivery, is given together with % recovery.

Analyte	Concentration in 1ml eluate ng/ml		Recovery rate
	strip (n=7)	IB (n=3)	
$\alpha$ 2-M	1520 $\pm$ 52	1527 $\pm$ 53	99.5%
$\alpha$ 1-AT	1266 $\pm$ 72	1273 $\pm$ 41	99.4%
TF	2230 $\pm$ 100	2480 $\pm$ 82	89.9%
LF	3763 $\pm$ 232	4509 $\pm$ 81	83.4%

IB= incubation buffer  
SD= standard deviation

positive results at the lowest detectability limits of the assays.

### 3.2.3.3 Gingival crevicular sample elution for the recovery of polymorphonuclear leucocytes

Recently a method has been described for the approximate determination of PMN numbers in discrete GCF samples, collected with paper strips (Cimasoni & Giannopoulou, 1988). This technique would appear to permit assessment of PMN constituent levels like LF, and PMN numbers from the same GCF sample.

A pilot study was, therefore, performed on a limited number of GCF samples in order to investigate if PMNs could be recovered from GCF samples collected with paper strips. Nine GCF samples with variable volumes were obtained in the periodontal clinic using the standard method (Whatman grade 4 paper strips for 30s). These were divided into two groups and were processed as follows:

a) the first group (n=5) were eluted, as described by Cimasoni and Giannopoulou (1988), by vortexing for 30s in 150 $\mu$ l of PBST;

b) the second group (n=4) were processed as described by Ebersole *et al.*, (1984) for the recovery of GCF antibodies. A microcentrifuge tube cup was fitted at the upper third of

a similar tube (400µl). The inner cup was pierced to allow the passage of fluid through it, but not the passage of a paper strip. The paper strips were then set on top of this inner cup, soaked with 50µl of PBST and microcentrifuged at 3000rpm for 5s. This procedure was repeated twice resulting in a total elution volume of 150µl.

50µl of the eluate obtained by both methods was then cytocentrifuged at 70xg (Shandon Scientific Limited, U.K.) and stained by the Leishman method. No cells could be detected in any of the 9 samples. For this reason, we resorted to the semiquantitative method of GCWs for the determination of PMNs in the crevice and to obtain their relationship with crevicular LF.

### **3.2.4 Processing of gingival crevicular washings**

#### **3.2.4.1 Quantitation of lactoferrin in gingival crevicular washings**

One µl aliquot of the washing, obtained using a Hamilton Microsyringe, was eluted in 1ml of incubation buffer and the eluate was stored separately at -70°C for subsequent quantitation of GCW LF. This was performed by the LF sandwich ELISA. No further dilution of the sample was required.

### 3.2.4.2 Total and differential white cell counts in gingival crevicular washings

Within thirty minutes of GCW collection a total cell count was performed on 5 $\mu$ l of the sample. The sample was diluted 1/2 with white blood cell diluting fluid and the total count performed on a Neubaur chamber. The total number of white blood cells per  $\mu$ l GCW was thus assessed. A differential count of 200-300 cells into polymorphonuclear and mononuclear cells was carried out after cytocentrifuging the rest of the sample at 70g (Shandon Scientific Limited, U.K.) and staining the cells by the Leishman method. The % PMNs in the GCW was obtained. The number of PMN/ $\mu$ l GCW was finally calculated using the formula: (total cell count x % PMNs)/100.

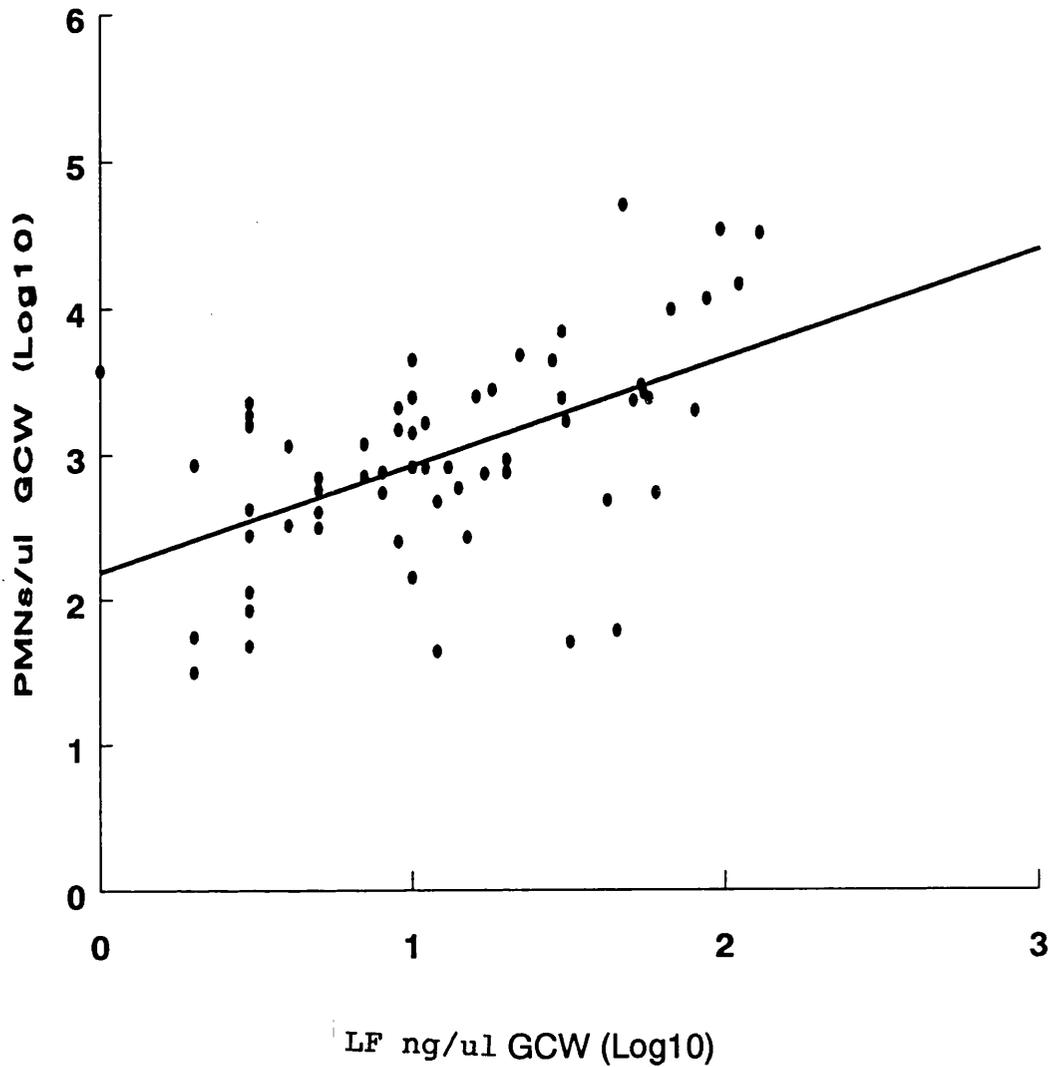
## **CHAPTER 4**

### **Results**

#### 4.1 Lactoferrin as a marker of polymorphonuclear leucocytes in the gingival crevice: a cross-sectional study on gingival crevicular washings

This study was performed to examine if lactoferrin (LF) in the crevice correlated with the number of crevicular polymorphonuclear leucocytes (PMNs) and thus, has potential as a marker of PMN migration into the crevice.

Gingival crevicular washings (GCWs) were obtained from a total of 63 sites. The mean MGI and PD of the sites sampled was 1.87 (SD:1.28) and 3.19mm (SD:2.2) respectively. Both LF levels (ng/ $\mu$ l GCW) and PMN numbers (PMNs/ $\mu$ l GCW) were determined in each sample. The relationship between GCW LF levels and PMN numbers was examined, the Pearson correlation coefficient was determined, and the least squares method used to plot the best fitting line as shown in Figure 4.1. LF demonstrated a positive and significant association ( $r=0.531$ ,  $p<0.001$ ,  $n=63$ ) with PMN numbers in the crevice. The association between both GCW LF levels and GCW PMN numbers and the clinical indices was also assessed (Table 4.1) using the non-parametric Spearman rank correlation coefficient. GCW LF levels and PMN numbers correlated positively and significantly with both MGI and PD, but LF demonstrated consistently higher correlation coefficients than PMNs with both clinical indices (Table 4.1).



**Figure 4.1** Polymorphonuclear leucocytes (PMNs/ $\mu$ l GCW) as function of lactoferrin levels (LF ng/ $\mu$ l GCW) in gingival crevicular washings (GCW). Both variables are transformed to  $\log_{10}$ . The Pearson correlation coefficient ( $r=0.531$ ,  $p<0.001$ ,  $n=63$ ) was determined and the best fitting line plotted by the least squares method (regression analysis).

**Table 4.1** Spearman rank correlation coefficients (*r*) between clinical indices and lactoferrin (LF) levels and polymorphonuclear (PMN) numbers in gingival crevicular washings (GCW) from 63 sites. Probability levels are shown in parenthesis.

<b>Parameter</b>	<b>LF (ng/<math>\mu</math>l GCW)</b>	<b>PMNs (PMNs/<math>\mu</math>l GCW)</b>
<b>MGI</b>	r=0.452 (p<0.001)	r= 0.279 (p<0.030)
<b>PD</b>	r=0.513 (p<0.001)	r= 0.388 (p<0.003)

MGI= modified gingival index  
 PD= pocket depth

## **4.2 Protease inhibitors and iron-binding proteins at healthy, gingivitis and periodontitis sites: cross-sectional studies on gingival crevicular fluid**

These studies were undertaken in order to determine the ability of protease inhibitors ( $\alpha$ 2-macroglobulin and  $\alpha$ 1-antitrypsin) and iron-binding proteins (transferrin and lactoferrin) in gingival crevicular fluid (GCF) to distinguish between healthy, gingivitis and periodontitis sites. Different GCF samples were used for each protein. Results were expressed as total amounts (ng/30s) as well as concentrations (ng/ $\mu$ l GCF). One way analysis of variance (ANOVA) and individual two-sample t-tests were employed on the  $\log_{10}$  transformed data in order to determine significant differences in GCF levels of each protein among the three clinical groups. The significance level for the t-tests was set at 0.025 in order to compensate for multiple comparisons (section 2.4).

### **4.2.1 $\alpha$ 2-macroglobulin at healthy, gingivitis and periodontitis sites**

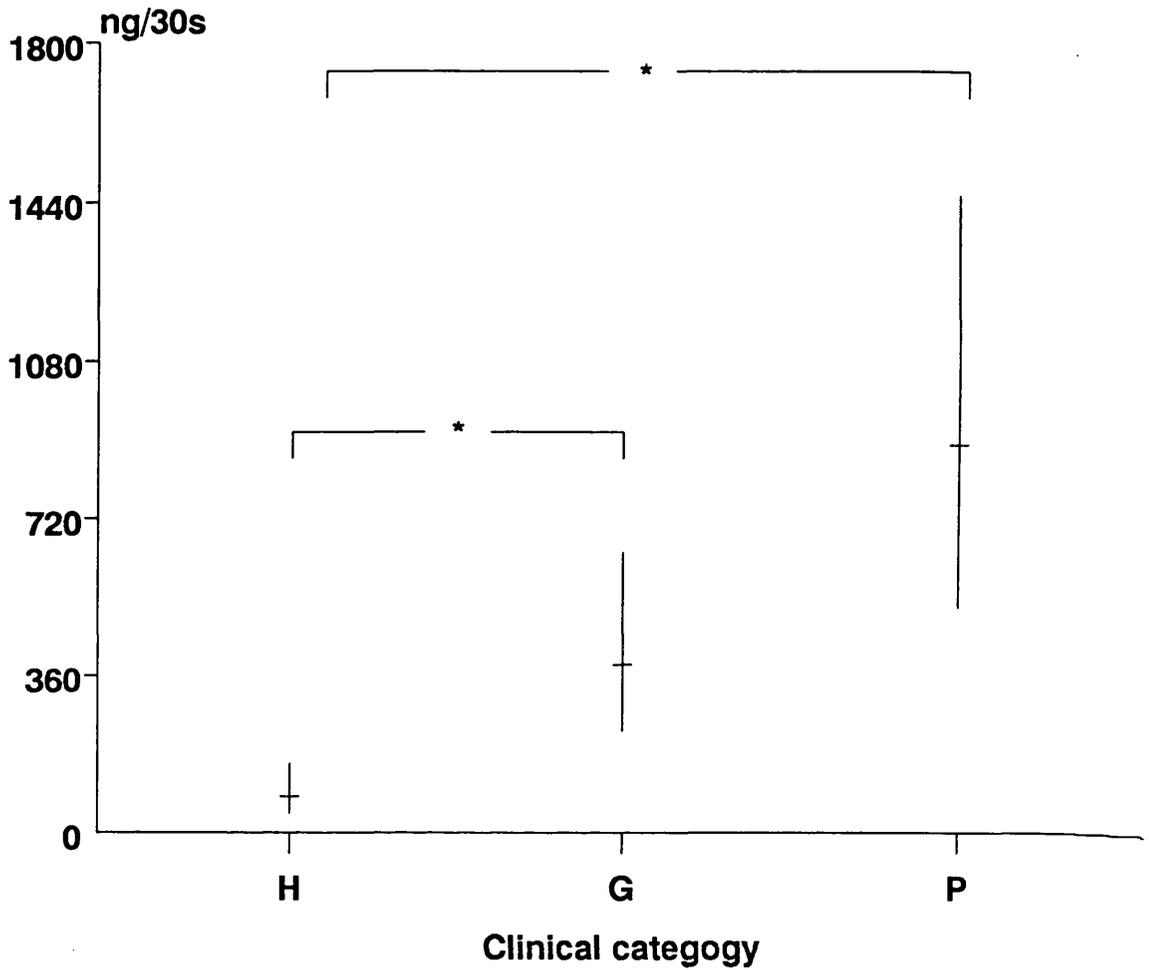
A total of 78 sites were sampled for the determination  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M) levels in GCF. These sites were allocated into three clinical groups, healthy, gingivitis and periodontitis, based on their MGI and PD values and according to the criteria outlined in section 2.3.1. In

healthy sites MGI ranged from 0 to 1 whereas PD between 1 and 2mm. At gingivitis sites MGI ranged between 2 and 4, and PD between 1 and 3mm. Finally at periodontitis sites MGI ranged between 2 and 4 and PD between 4 and 10mm. The sample size, clinical indices and GCF volumes (mean  $\pm$  SE) for each of the three clinical groups are shown in Table 4.2. Figures 4.2 and 4.3 demonstrate the geometric mean and 95% confidence intervals for GCF  $\alpha$ 2-M in the three clinical groups, expressed as absolute amounts and concentrations respectively. One way ANOVA demonstrated that significant differences existed within the three clinical groups using both methods of expressing the results (absolute amount and concentration) ( $p < 0.001$ ). Further analysis, using two-sample t-tests, revealed significantly higher absolute amounts of  $\alpha$ 2-M (ng/30s) in GCF samples from gingivitis and periodontitis when compared to healthy sites ( $p < 0.001$ ). There was no significant difference ( $p = 0.09$ ) however, in  $\alpha$ 2-M levels (ng/30s) between gingivitis and periodontitis sites although the latter demonstrated higher mean total  $\alpha$ 2-M (Fig. 4.2). When results were expressed on a concentration basis (ng/ $\mu$ l GCF),  $\alpha$ 2-M showed a statistically significant increase at gingivitis and periodontitis over healthy sites ( $p < 0.01$ ; two sample t-tests) but again no significant difference could be demonstrated in  $\alpha$ 2-M concentration in GCF between gingivitis and periodontitis sites (Fig. 4.3).

**Table 4.2** Mean clinical indices ( $\pm$  SE) and gingival crevicular fluid (GCF) volume, at sites used for GCF  $\alpha$ 2-macroglobulin determination (see also Figs 4.2 & 4.3). Sample size is given under each clinical category in parenthesis.

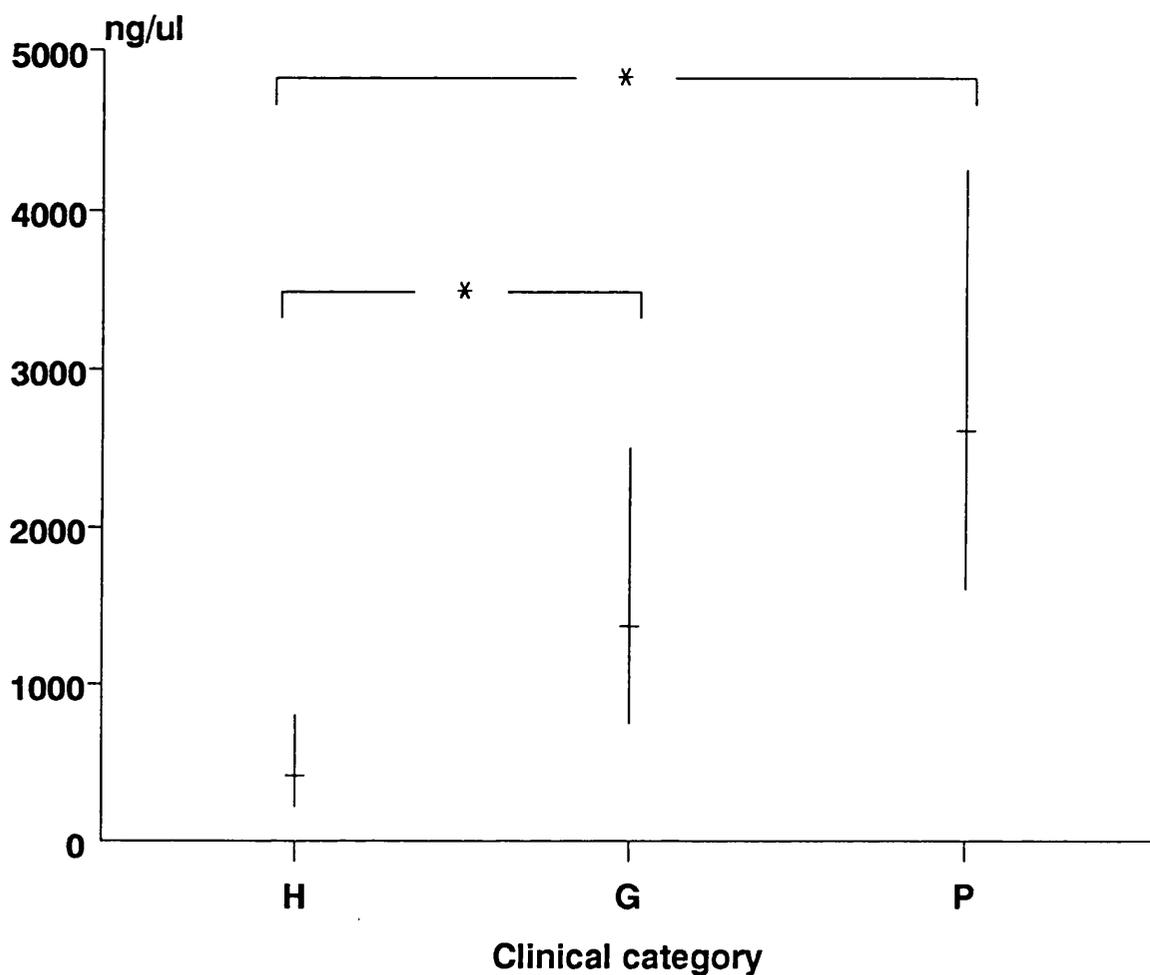
$\alpha$ 2-macroglobulin			
Parameter	Site		
	Healthy (n=25)	Gingivitis (n=25)	Periodontitis (n=28)
MGI	0.28 $\pm$ 0.09	2.64 $\pm$ 0.14	3.39 $\pm$ 0.13
PD	1.12 $\pm$ 0.07	2.28 $\pm$ 0.15	4.89 $\pm$ 0.28
GCF ( $\mu$ l)	0.28 $\pm$ 0.03	0.35 $\pm$ 0.04	0.43 $\pm$ 0.04

MGI= modified gingival index  
 PD= pocket depth



**Figure 4.2** Total  $\alpha$ 2-macroglobulin (ng/30s) in gingival crevicular fluid from healthy (H, n=25), gingivitis (G, n=25) and periodontitis (P, n=28) sites. Geometric mean and 95% confidence intervals are shown.

\* Significantly different from healthy sites  $p < 0.001$



**Figure 4.3**  $\alpha$ 2-macroglobulin concentration (ng/ $\mu$ l) in gingival crevicular fluid from healthy (H, n=25), gingivitis (G, n=25) and periodontitis (P, n=28) sites. Geometric mean and 95% confidence intervals are shown.

\* Significantly different from healthy sites  $p < 0.01$

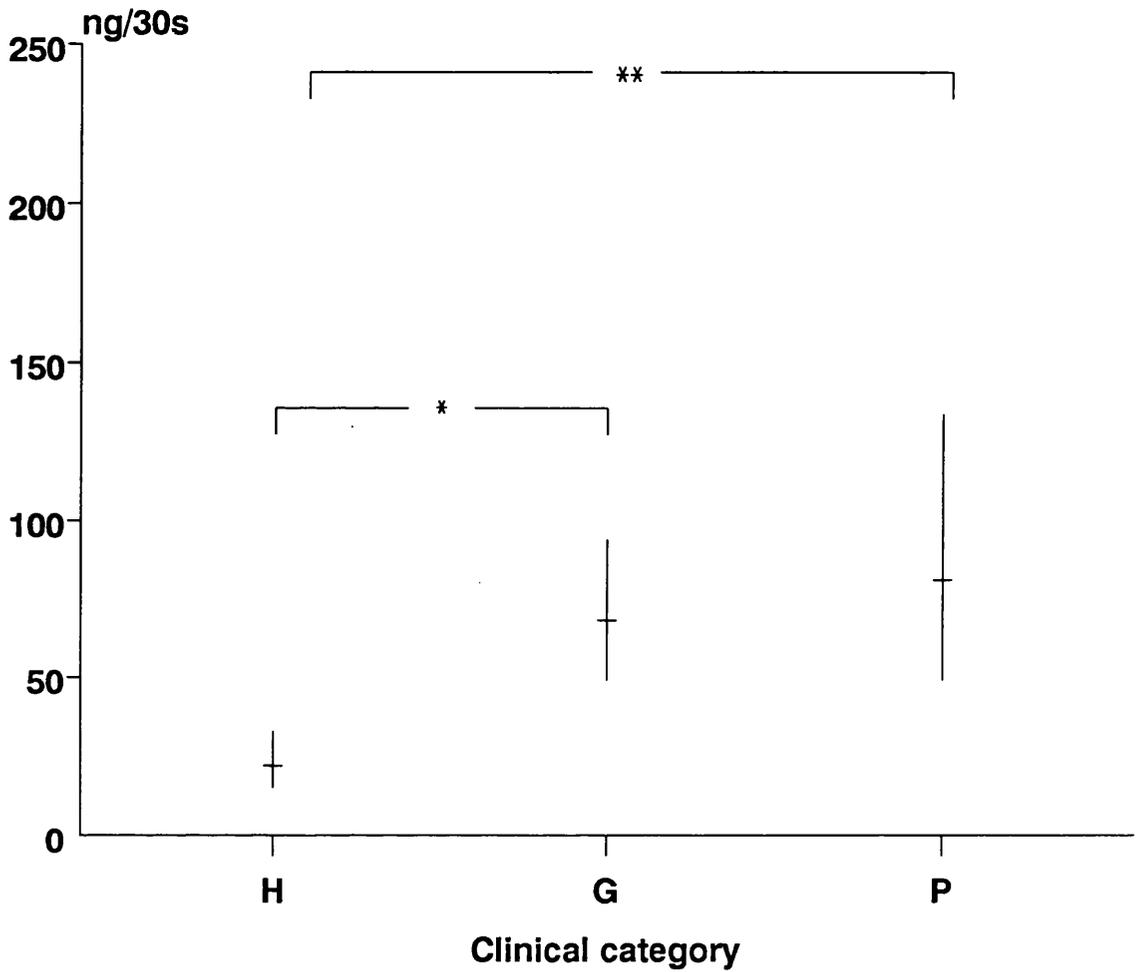
#### 4.2.2 $\alpha$ 1-antitrypsin at healthy, gingivitis and periodontitis sites

Sixty eight GCF samples were assayed for  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT). These samples were obtained from sites which were allocated into the three clinical groups i.e. healthy, gingivitis and periodontitis. MGI ranged between 0 and 1 in the healthy group, 2 and 4 in the gingivitis group and 1 and 4 in the periodontitis group. PD ranged between 1 and 2mm in the healthy group, between 2 and 3mm in the gingivitis group and 4 and 10mm in the periodontitis group. Mean ( $\pm$ SE) clinical indices and GCF volumes, and sample size for each of the three clinical groups are shown in Table 4.3. Figure 4.4 demonstrates total GCF  $\alpha$ 1-AT at healthy, gingivitis and periodontitis sites whereas Figure 4.5, depicts GCF  $\alpha$ 1-AT concentrations (geometric means and 95% confidence intervals). One way ANOVA demonstrated that significant differences existed among the three clinical groups when results were expressed as absolute amounts (ng/30s) ( $p < 0.001$ ) but not as concentrations ( $p = 0.183$ ). As shown in Figure 4.4, gingivitis and periodontitis sites demonstrated higher total GCF  $\alpha$ 1-AT than healthy sites and this increase was statistically significant ( $p < 0.005$  and  $p < 0.001$  respectively; two sample t-tests). Although periodontitis sites demonstrated higher mean levels than gingivitis sites, this increase was not statistically significant.

**Table 4.3** Mean clinical indices ( $\pm$  SE) and gingival crevicular fluid (GCF) volume at sites used for GCF  $\alpha$ 1-antitrypsin determination (see also Figs 4.4 & 4.5). Sample size is given under each clinical category in parenthesis.

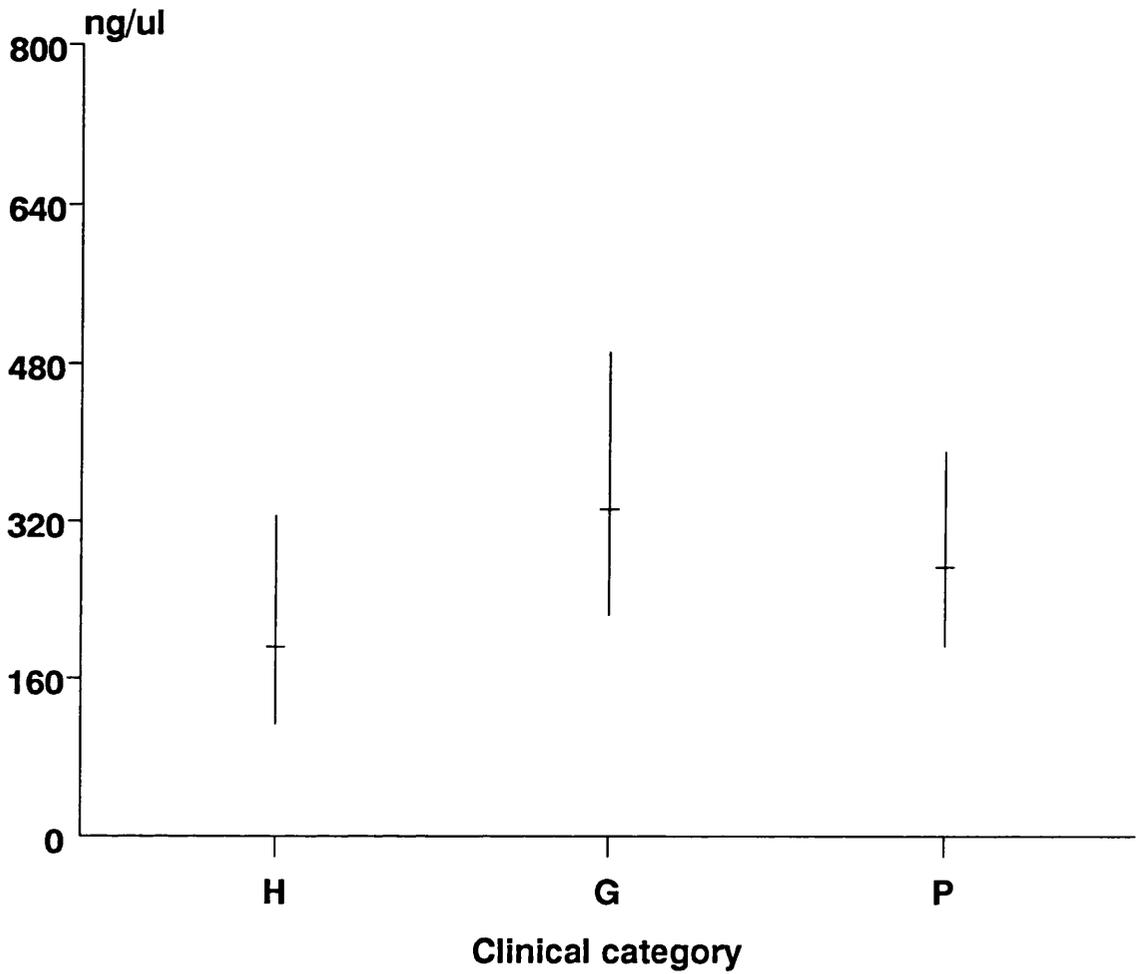
$\alpha$ 1-antitrypsin			
Parameter	Site		
	Healthy (n=24)	Gingivitis (n=22)	Periodontitis (n=22)
MGI	0.17 $\pm$ 0.08	2.64 $\pm$ 0.12	3.14 $\pm$ 0.17
PD	1.04 $\pm$ 0.04	2.32 $\pm$ 0.10	5.02 $\pm$ 0.30
GCF ( $\mu$ l)	0.18 $\pm$ 0.03	0.27 $\pm$ 0.04	0.37 $\pm$ 0.07

MGI= modified gingival index  
 PD= pocket depth



**Figure 4.4** Total  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT) (ng/30s) in gingival crevicular fluid from healthy (H, n=24), gingivitis (G, n=22) and periodontitis (P, n=22) sites. Geometric mean and 95% confidence intervals are shown.

\* Significantly different from healthy sites  $p < 0.005$   
 \*\* Significantly different from healthy sites  $p < 0.001$



**Figure 4.5**  $\alpha$ 1-antitrypsin concentration (ng/ $\mu$ l) in gingival crevicular fluid from healthy (H, n=24), gingivitis (G, n=22) and periodontitis (P, n=22) sites. Geometric mean and 95% confidence intervals are shown.

Consistent with the results of the one way ANOVA, no significant differences could be demonstrated when two sample t-tests were employed on the GCF  $\alpha$ 1-AT concentration data of the three clinical groups, although there was a tendency for increasing  $\alpha$ 1-AT GCF concentration at gingivitis and periodontitis over healthy sites (Fig. 4.5).

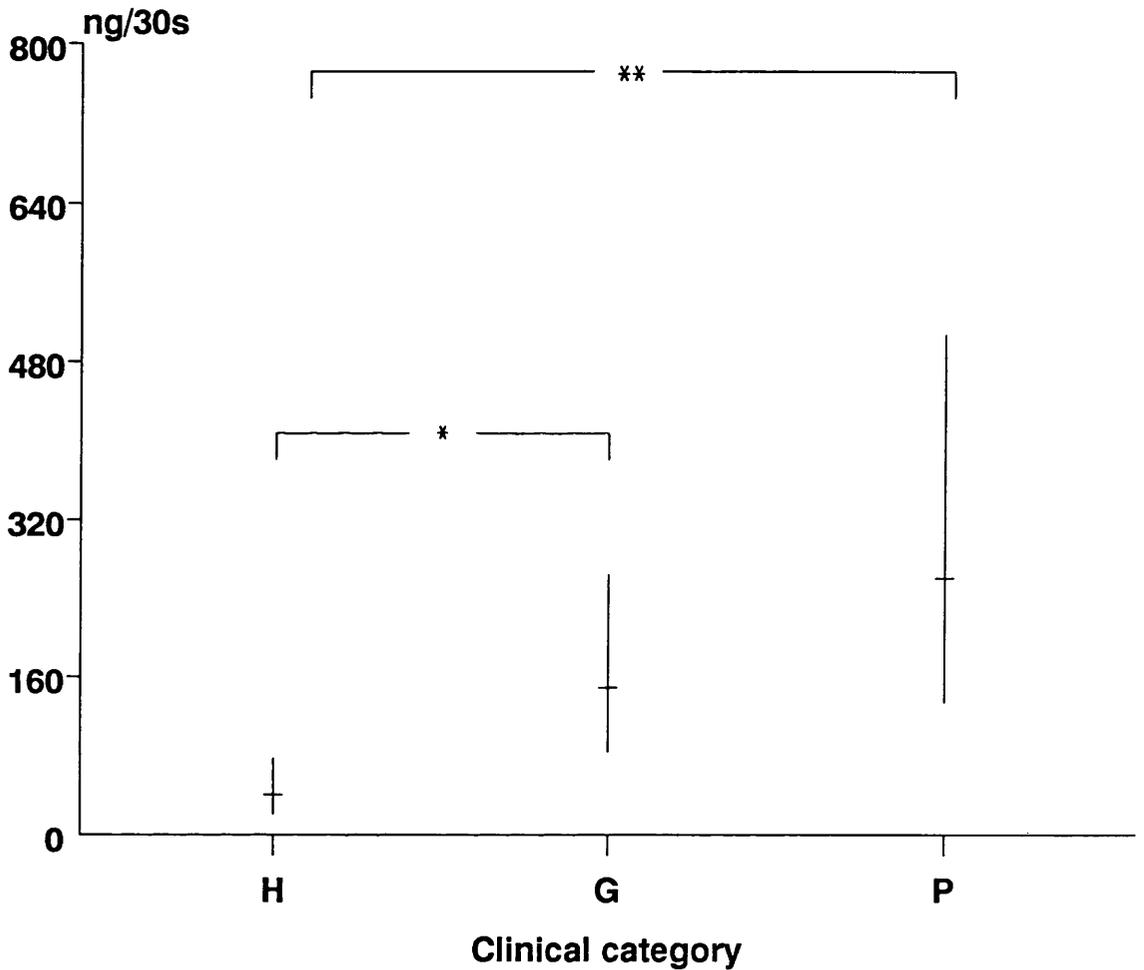
#### **4.2.3 Transferrin at healthy, gingivitis and periodontitis sites**

Transferrin (TF) was assayed in a total of 92 GCF samples from discrete sites which were allocated into the healthy, gingivitis and periodontitis groups. In the healthy group MGI ranged between 0 and 1, and PD between 0 and 2mm. In the gingivitis group MGI ranged between 2 and 4, and PD between 1 and 3mm. Finally, in the periodontitis group MGI ranged between 2 and 4, and PD between 4 and 12mm. Table 4.4 demonstrates the mean ( $\pm$  SE) clinical indices and GCF volumes, and sample sizes in each one of the three clinical groups. Figures 4.6 and 4.7 demonstrate the levels of TF in GCF (geometric mean and 95% confidence intervals) in each of the three clinical categories for both methods of expressing the results (total and concentration respectively). Similarly to  $\alpha$ 2-M and  $\alpha$ 1-AT, one way ANOVA demonstrated that the clinical grouping of the site had a significant effect ( $p < 0.001$ ) when total GCF TF was examined. When individual groups were tested for

**Table 4.4** Mean clinical indices ( $\pm$  SE) and gingival crevicular fluid (GCF) volume at sites used for GCF transferrin determination (see also Figs 4.6 & 4.7). Sample size is given under each clinical category in parenthesis.

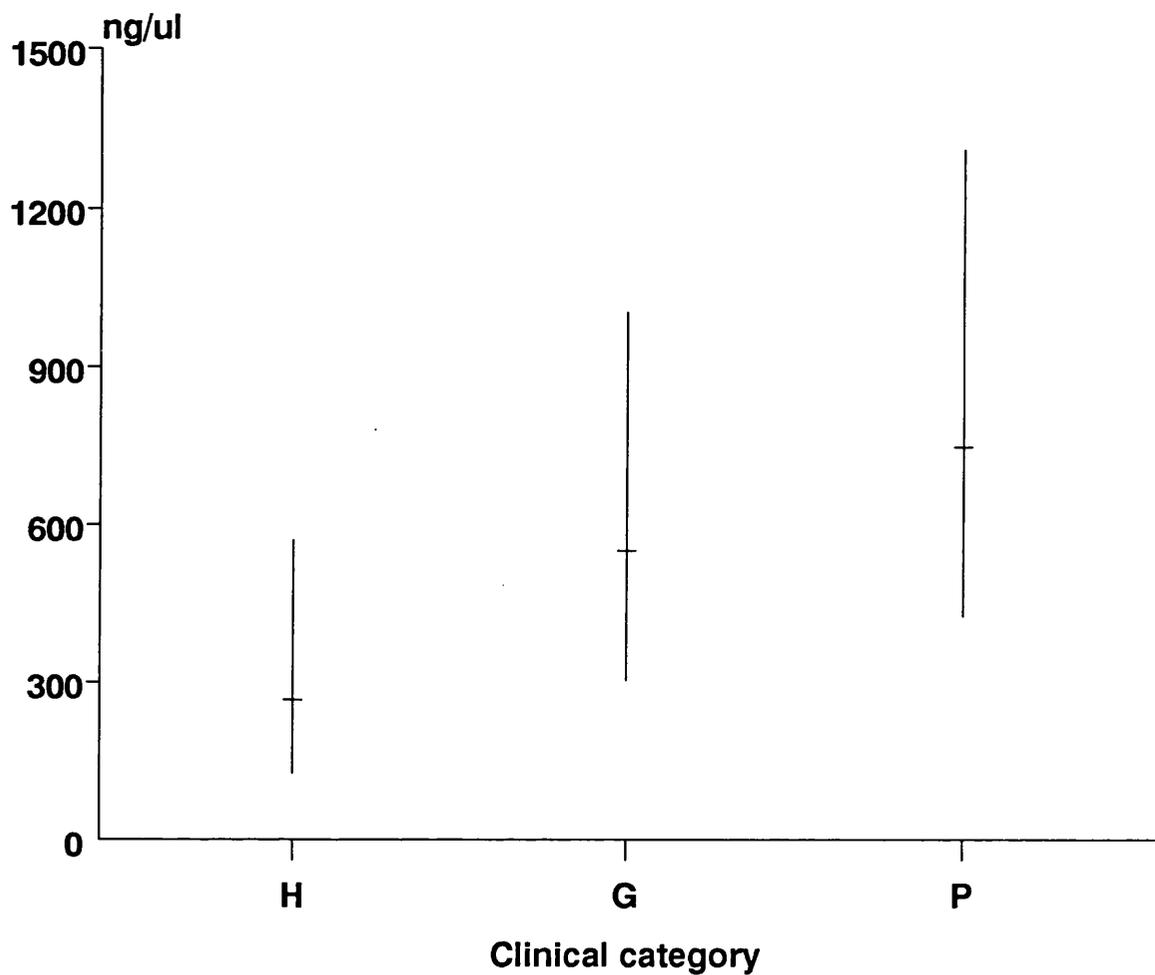
<b>Transferrin</b>			
<b>Parameter</b>	<b>Site</b>		
	<b>Healthy (n=29)</b>	<b>Gingivitis (n=31)</b>	<b>Periodontitis (n=32)</b>
<b>MGI</b>	0.45 $\pm$ 0.09	2.61 $\pm$ 0.12	3.34 $\pm$ 0.11
<b>PD</b>	1.13 $\pm$ 0.08	2.39 $\pm$ 0.13	5.30 $\pm$ 0.36
<b>GCF (<math>\mu</math>l)</b>	0.27 $\pm$ 0.03	0.38 $\pm$ 0.04	0.45 $\pm$ 0.04

MGI= modified gingival index  
 PD= pocket depth



**Figure 4.6** Total transferrin (TF) (ng/30s) in gingival crevicular fluid from healthy (H, n=29), gingivitis (G, n=31) and periodontitis (P, n=32) sites. Geometric mean and 95% confidence intervals are shown.

\* Significantly different from healthy sites  $p < 0.005$   
 \*\* Significantly different from healthy sites  $p < 0.001$



**Figure 4.7** Transferrin concentration (ng/ $\mu$ l) in gingival crevicular fluid from healthy (H, n=29), gingivitis (G, n=31) and periodontitis (P, n=32) sites. Geometric mean and 95% confidence intervals are shown.

differences, using two sample t-tests, gingivitis and periodontitis sites demonstrated significantly elevated levels over healthy sites ( $p < 0.005$  and  $p < 0.001$  respectively). Once again no significant difference could be demonstrated between gingivitis and periodontitis sites as far as total GCF TF was concerned, although mean total TF was higher at the latter (Fig. 4.6). When results were expressed as concentrations one way ANOVA failed to demonstrate significant differences ( $p = 0.067$ ) among the clinical groups and this was also the case when two-sample t-tests were performed on the data. The increase however of GCF TF concentration at periodontitis over healthy sites almost reached significance at the Bonferroni corrected significance threshold of 0.025 level ( $p = 0.03$ ; two sample t-test) (Fig. 4.7).

#### **4.2.4 Lactoferrin at healthy, gingivitis and periodontitis sites**

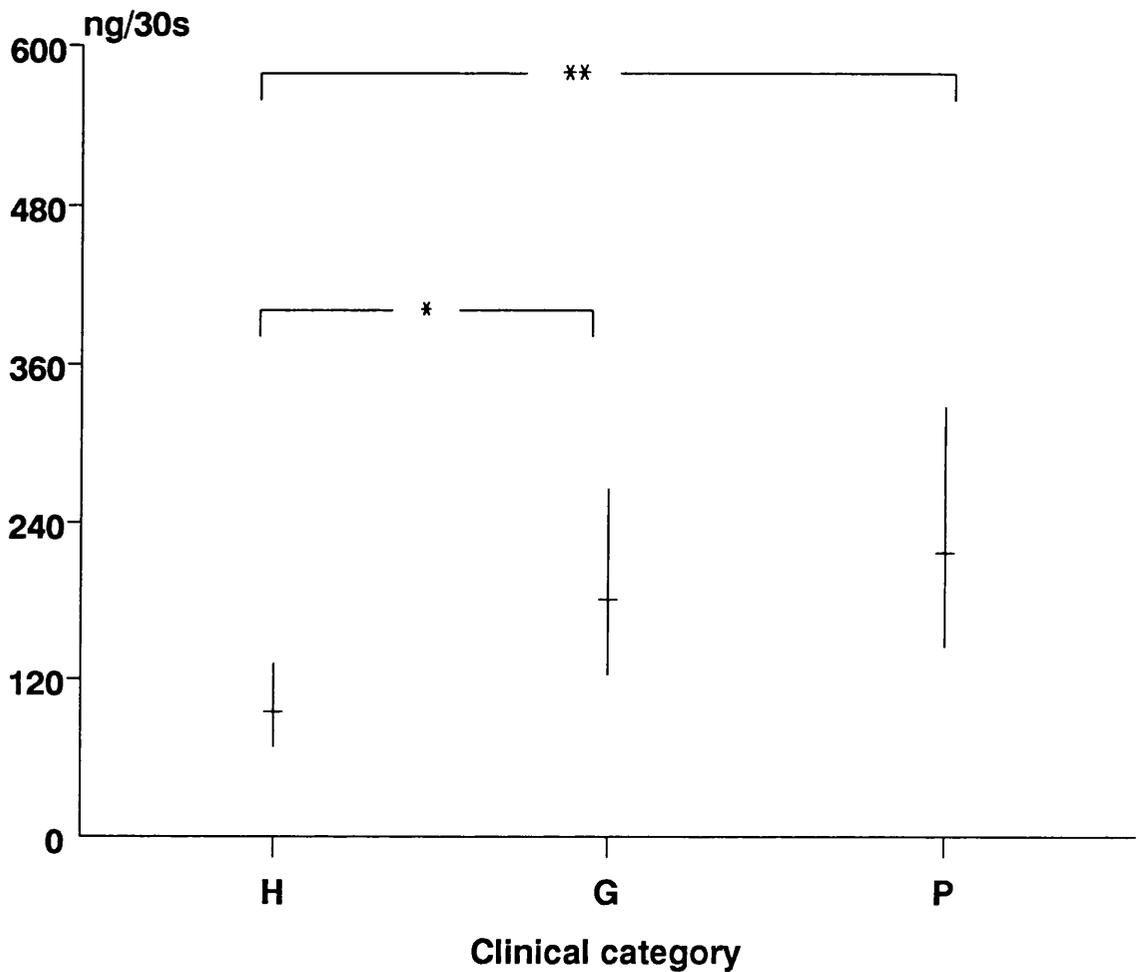
Lactoferrin (LF) was examined in GCF samples from 71 sites which were designated into the three clinical groups (healthy, gingivitis and periodontitis). MGI scores ranged between 0 and 1 in the healthy group, between 2 and 3 in the gingivitis group, and 1 and 4 in the periodontitis group. PD ranged between 1 and 2mm in the healthy group, between 2 and 3mm in the gingivitis group and between 4 and 9mm in the periodontitis group. Sample sizes and mean ( $\pm$ SE) clinical indices and GCF volumes for each of the

three clinical categories are shown in Table 4.5. Figure 4.8 depicts total LF at healthy, gingivitis and periodontitis sites while Figure 4.9 demonstrates LF concentration (geometric mean and 95% confidence intervals). One way ANOVA demonstrated that significant differences existed ( $p=0.004$ ) among healthy, gingivitis and periodontitis sites as far as total GCF LF was concerned. As shown in Figure 4.8 total LF was significantly higher at gingivitis ( $p=0.012$ ) and periodontitis ( $p=0.0023$ ) when compared to healthy sites (two sample t-tests). Consistent with the other proteins, periodontitis sites exhibited higher mean total LF than gingivitis sites but this was not statistically significant. In contrast, however, to GCF  $\alpha 2$ -M,  $\alpha 1$ -AT and TF, mean GCF LF concentration appeared to drop at diseased (gingivitis and periodontitis) compared to healthy sites (Fig. 4.9), although this was just outwith statistical significance when tested by one way ANOVA ( $p=0.055$ ). When two sample t-tests were performed to compare GCF LF concentration between clinical groups, according to the one way ANOVA results, the drop between GCF LF at healthy and gingivitis or periodontitis sites, although close, failed to reach significance at the Bonferroni corrected 0.025 level ( $p=0.049$  and  $p=0.031$  respectively).

**Table 4.5** Mean clinical indices ( $\pm$  SE) and gingival crevicular fluid (GCF) volume at sites used for GCF lactoferrin determination (see also Figs 4.8 & 4.9). Sample size is given under each clinical category in parenthesis.

<b>Lactoferrin</b>			
<b>Parameter</b>	<b>Site</b>		
	<b>Healthy (n=26)</b>	<b>Gingivitis (n=21)</b>	<b>Periodontitis (n=24)</b>
<b>MGI</b>	0.38 $\pm$ 0.10	2.57 $\pm$ 0.11	2.46 $\pm$ 0.17
<b>PD</b>	1.54 $\pm$ 0.10	2.71 $\pm$ 0.10	5.04 $\pm$ 0.29
<b>GCF (<math>\mu</math>l)</b>	0.09 $\pm$ 0.02	0.32 $\pm$ 0.06	0.35 $\pm$ 0.06

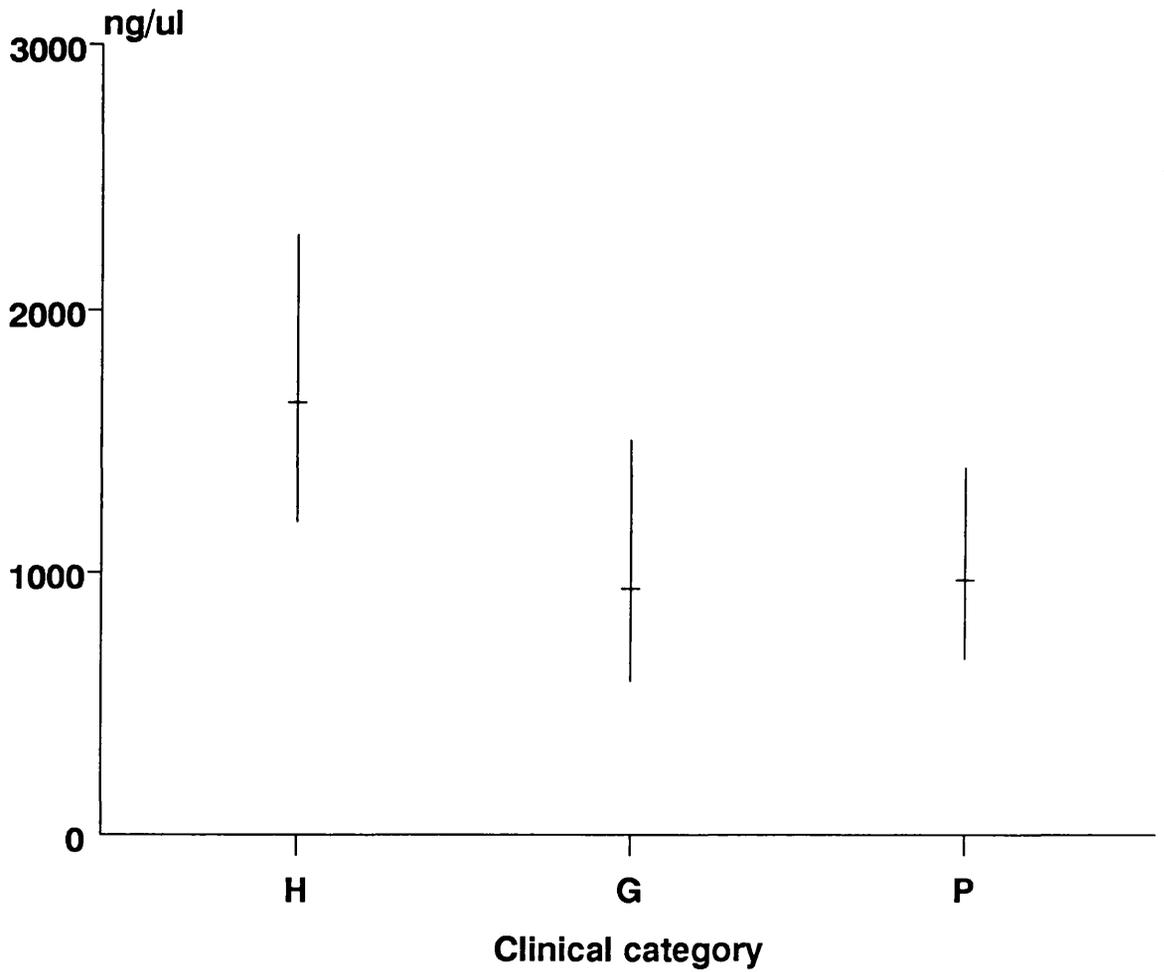
MGI= modified gingival index  
 PD= pocket depth



**Figure 4.8** Total lactoferrin (ng/30s) in gingival crevicular fluid from healthy (H, n=26), gingivitis (G, n=21) and periodontitis (P, n=24) sites. Geometric mean and 95% confidence intervals are shown.

\* Significantly different from healthy sites  $p < 0.02$

\*\* Significantly different from healthy sites  $p < 0.003$



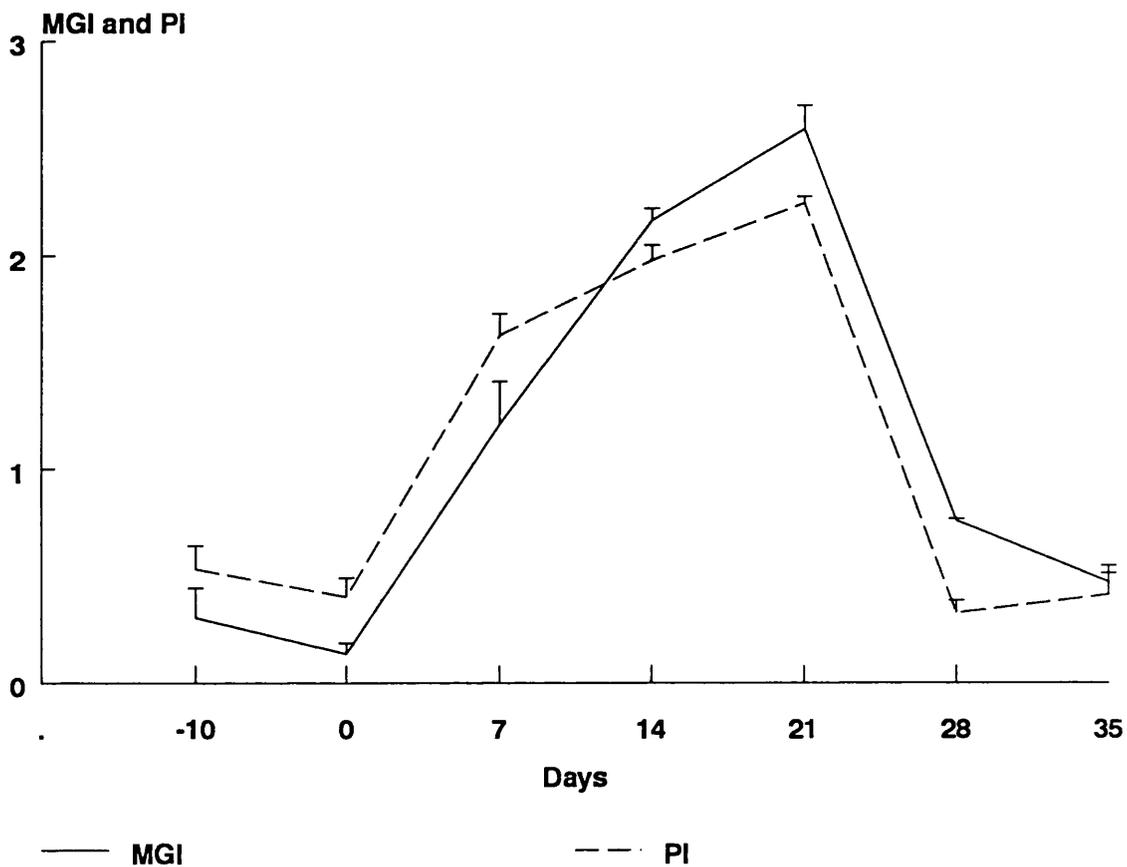
**Figure 4.9** Lactoferrin concentration (ng/ $\mu$ l) in gingival crevicular fluid from healthy (H, n=26), gingivitis (G, n=21) and periodontitis (P, n=24) sites. Geometric mean and 95% confidence intervals are shown.

### **4.3           Gingival crevicular fluid protease inhibitors and iron-binding proteins during the development of gingival inflammation: an experimental gingivitis study**

This study was undertaken to examine the changes of protease inhibitors ( $\alpha$ 2-M and  $\alpha$ 1-AT) and iron-binding proteins (TF and LF) in GCF during experimentally induced gingival inflammation. In this study  $\alpha$ 2-M,  $\alpha$ 1-AT, TF and LF levels were assessed in the same GCF sample and results were expressed as total amounts in ng/30s.

#### **4.3.1       Clinical Indices**

Figure 4.10 illustrates the changes of the whole mouth MGI (WMGI) and PI (WPI) scores of the six subjects, during the experimental gingivitis trial. At the initial appointment, 10 days prior to the commencement of the experimental gingivitis study, the WMGI and WPI were low. Subjects were then given oral hygiene instruction and prophylaxis which resulted in a slight drop in the WMGI and WPI at baseline (day 0) of the experimental gingivitis trial (Fig. 4.10). After the withdrawal of oral hygiene measures, plaque accumulated rapidly as shown by the steep increase of the WPI whereas WMGI lagged behind. Both WMGI and WPI peaked on day 21 of the experimental gingivitis trial. With the reinstatement of oral hygiene measures WPI dropped rapidly and the WMGI followed, remaining however, slightly higher



**Figure 4.10** Whole mouth MGI and PI scores of the 6 subjects during the experimental gingivitis trial. The mean and standard error at each time point are shown.

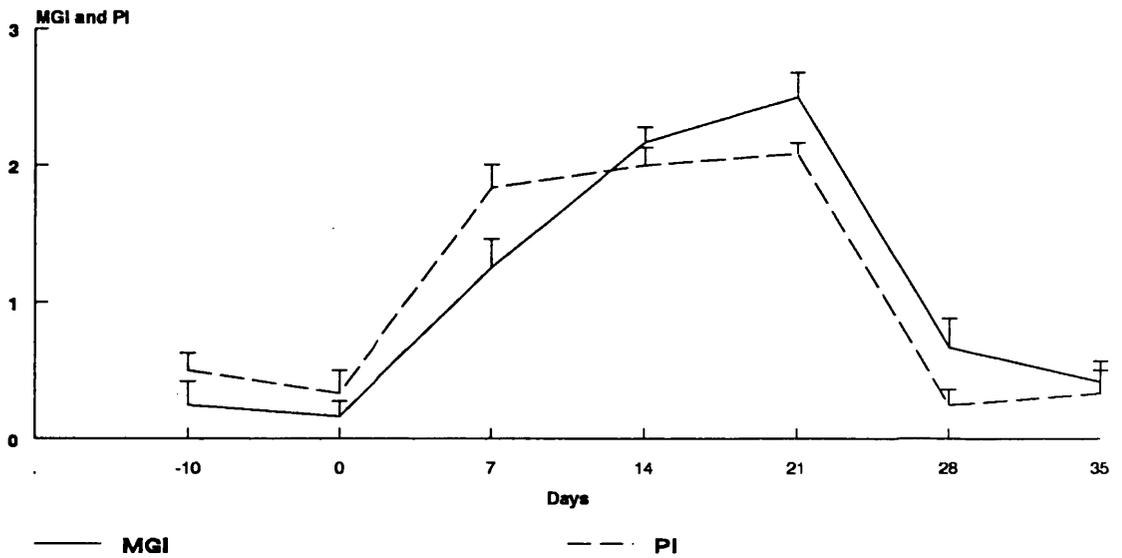
MGI= modified gingival index  
 PI= plaque index

than baseline levels at day 35 of the experimental gingivitis trial. The changes in clinical indices for the 6 subjects at the sites sampled for GCF (n=6 subjects) are shown in Figure 4.11. Clinical indices at these sites behaved in the same way as the full mouth scores, with the PI rising first and the MGI lagging behind. GCF volume followed the initial changes of the clinical indices (Fig. 4.11) and rose with the accumulation of plaque and the development of gingival inflammation. GCF volume reached its peak on day 14 of the experimental gingivitis episode and remained higher than baseline after the reinstatement of oral hygiene measures.

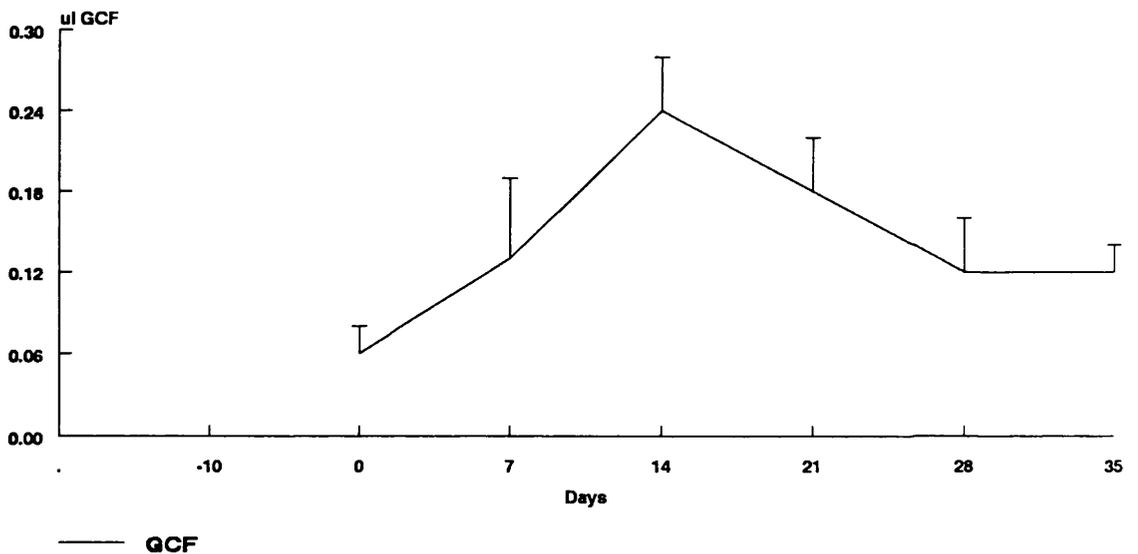
#### **4.3.2 GCF protease inhibitors and iron-binding proteins during the experimental gingivitis trial**

As mentioned in section 2.4 repeated measures analysis of variance (MANOVA) was used to determine significant differences in  $\alpha$ 2-M,  $\alpha$ 1-AT, TF and LF levels over time during the experimental gingivitis trial. Each protein was tested separately. Analysis was performed on the  $\log_{10}$  transformed data considering both the site and subject as the experimental unit. When the subject was considered as the unit, sites within each subject were averaged and one score was produced per subject (section 2.4). In an attempt to investigate which days demonstrated significantly elevated GCF levels of protease inhibitors

(a)



(b)

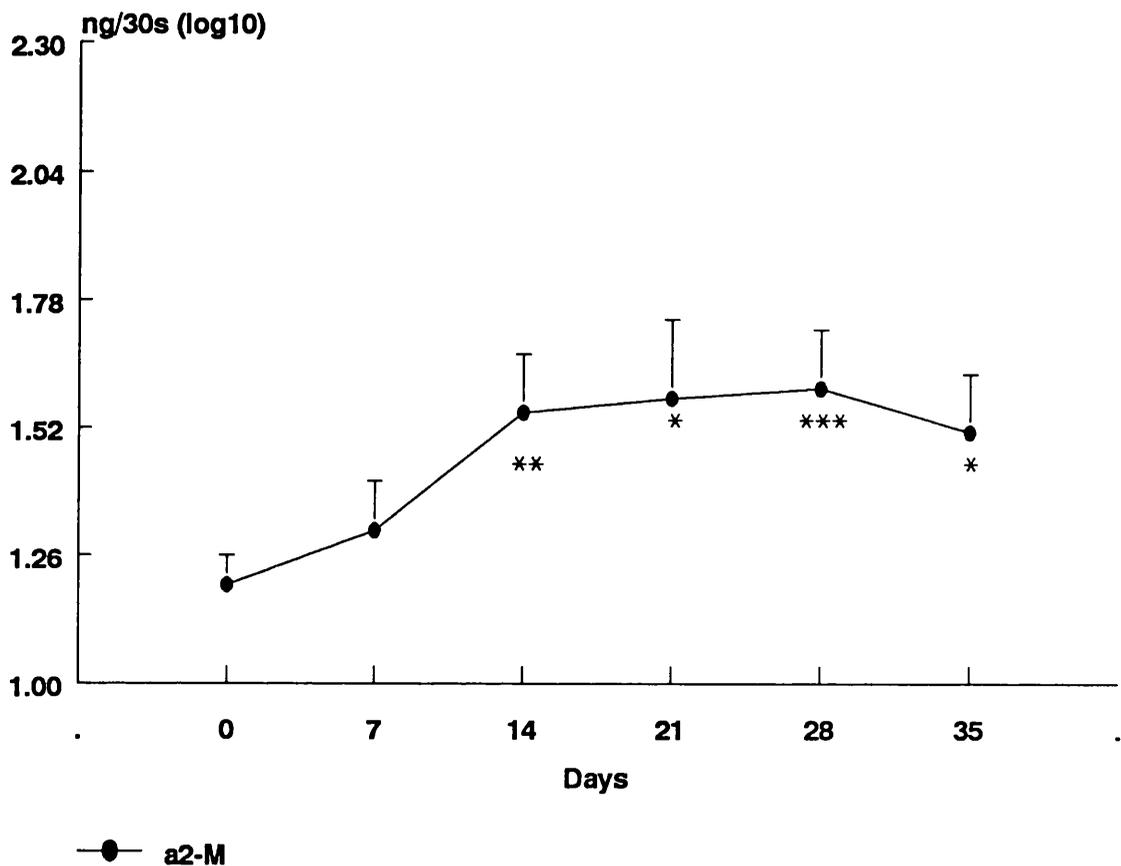


**Figure 4.11** (a) MGI, PI and (b) GCF volume at the sites sampled during the experimental gingivitis trial. The mean and standard error (n=6 subjects) at each time point are shown.

MGI= modified gingival index  
PI= plaque index  
GCF= gingival crevicular fluid

and iron-binding proteins since baseline, individual paired t-tests were employed on the  $\log_{10}$  transformed data, comparing baseline levels of  $\alpha 2$ -M,  $\alpha 1$ -AT, TF and LF to each subsequent day (n=6 subjects). The accepted significance level for the t-tests was set at 0.02 (Bonferroni correction; section 2.4).

GCF  $\alpha 2$ -M level changes over time for the 6 subjects enrolled in the experimental gingivitis trial are depicted in Figure 4.12 (mean  $\pm$  SE). Repeated measures MANOVA revealed that GCF  $\alpha 2$ -M levels changed over time during the experimental gingivitis episode both when the subject (p=0.015) and site (p=0.033) were considered as the experimental unit. When GCF  $\alpha 2$ -M levels on individual days were tested for differences from baseline using paired t-tests (n=6 subjects),  $\alpha 2$ -M was significantly elevated on day 14 of the experimental gingivitis episode (p=0.013). On day 21 although mean GCF  $\alpha 2$ -M was higher than baseline (Fig. 4.12) the increase failed to reach statistical significance at the stringent Bonferroni corrected 0.02 level (p=0.042). Interestingly, GCF  $\alpha 2$ -M was significantly elevated compared to baseline, even during the recovery period, on days 28 and 35 of the experimental gingivitis trial (Fig. 4.12), that is one and two weeks after the reinstatement of oral hygiene measures. More precisely, although on day 28 GCF  $\alpha 2$ -M was significantly higher than baseline (p=0.009), on day 35 mean GCF  $\alpha 2$ -M levels were still higher than baseline but this was not significant at the Bonferroni corrected



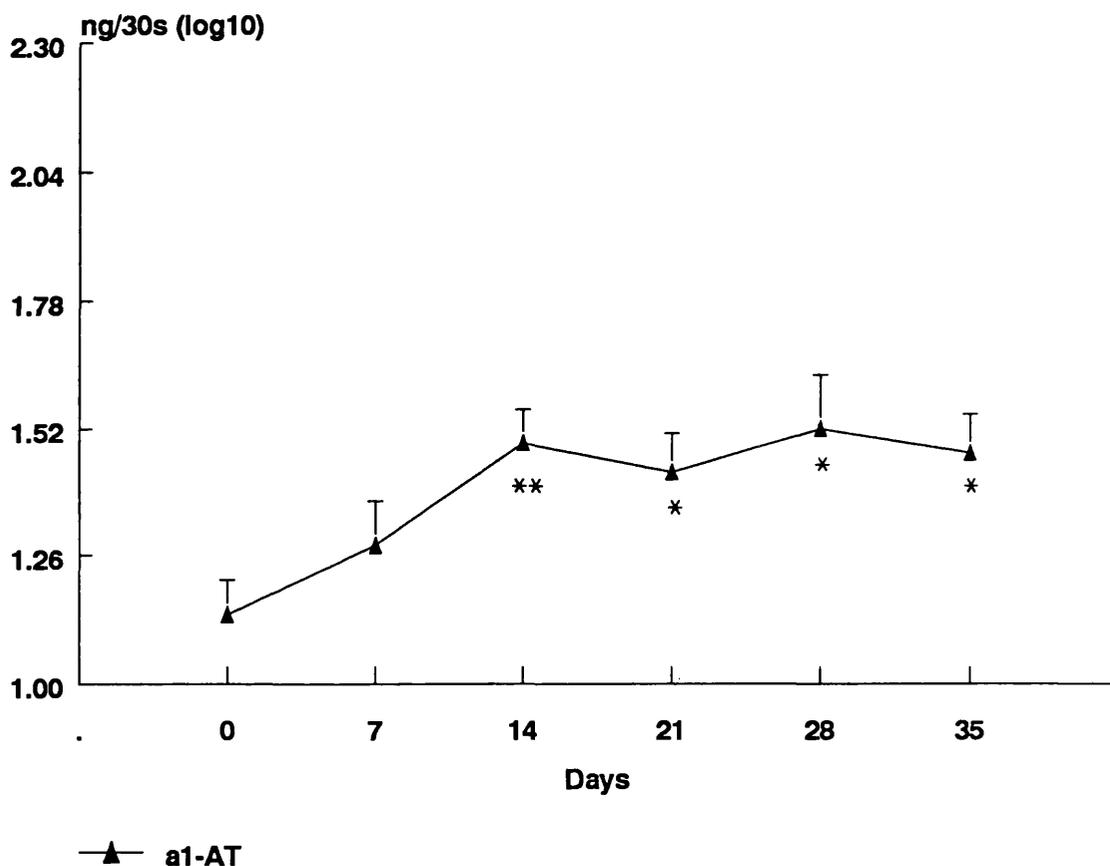
**Figure 4.12** Total  $\alpha_2$ -macroglobulin ( $\alpha_2$ -M) changes in gingival crevicular fluid, during the experimental gingivitis trial. The mean and standard error (n=6 subjects) at each time point are shown.

- \*\*\* Significantly different from baseline  $p < 0.01$
- \*\* Significantly different from baseline  $p < 0.02$
- \* Difference from baseline failed to reach significance at  $p < 0.02$  but  $p < 0.05$

0.02 significance level ( $p=0.026$ ). Similar but significant results were obtained when the site was considered as the experimental unit for the paired t-tests, although significance was in most cases stronger ( $p\leq 0.01$  on days 14 and 21) and thus the null hypothesis for difference between days 0 and 21 could be rejected at the 0.02 level.

Figure 4.13 demonstrates GCF  $\alpha 1$ -AT changes (mean  $\pm$  SE) for the 6 subjects during the experimental gingivitis study. Repeated measures MANOVA revealed that a significant day effect existed on GCF  $\alpha 1$ -AT levels when both the subject and site were considered as the experimental unit ( $p=0.012$  and  $p=0.018$  respectively). When GCF  $\alpha 1$ -AT levels on individual days were compared to baseline (paired tests,  $n=6$  subjects) day 14 demonstrated significantly elevated GCF  $\alpha 1$ -AT levels ( $p=0.002$ ), whereas on days 21, 28 and 35 the increase in GCF  $\alpha 1$ -AT just failed to reach significance at the Bonferroni corrected 0.02 significance level ( $p=0.034$ ,  $p=0.039$  and  $p=0.04$  respectively) (Fig. 4.13). Similar results were obtained when the site was considered as the experimental unit for the paired t-tests ( $p=0.001$  and  $p=0.008$  for days 14 and 21 respectively).

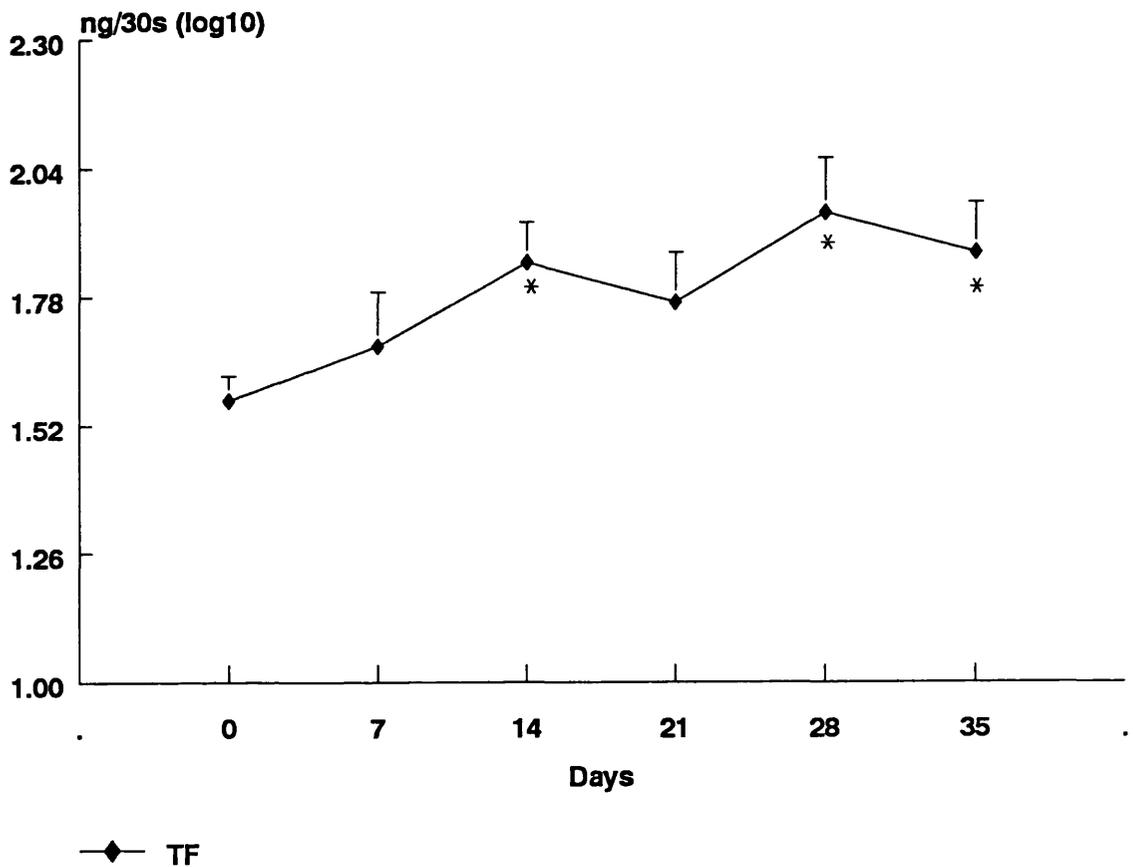
GCF TF mean response for the 6 subjects during the experimental gingivitis study demonstrated a similar picture to  $\alpha 2$ -M and  $\alpha 1$ -AT as shown in Figure 4.14 (mean  $\pm$  SE). Repeated measures MANOVA on GCF TF levels considering the subject as a unit demonstrated that significant



**Figure 4.13** Total  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT) changes in gingival crevicular fluid during the experimental gingivitis trial. The mean and standard error (n=6 subjects) at each time point are shown.

\*\* Significantly different from baseline  $p < 0.02$

\* Difference from baseline failed to reach significance at  $p < 0.02$  but  $p < 0.05$

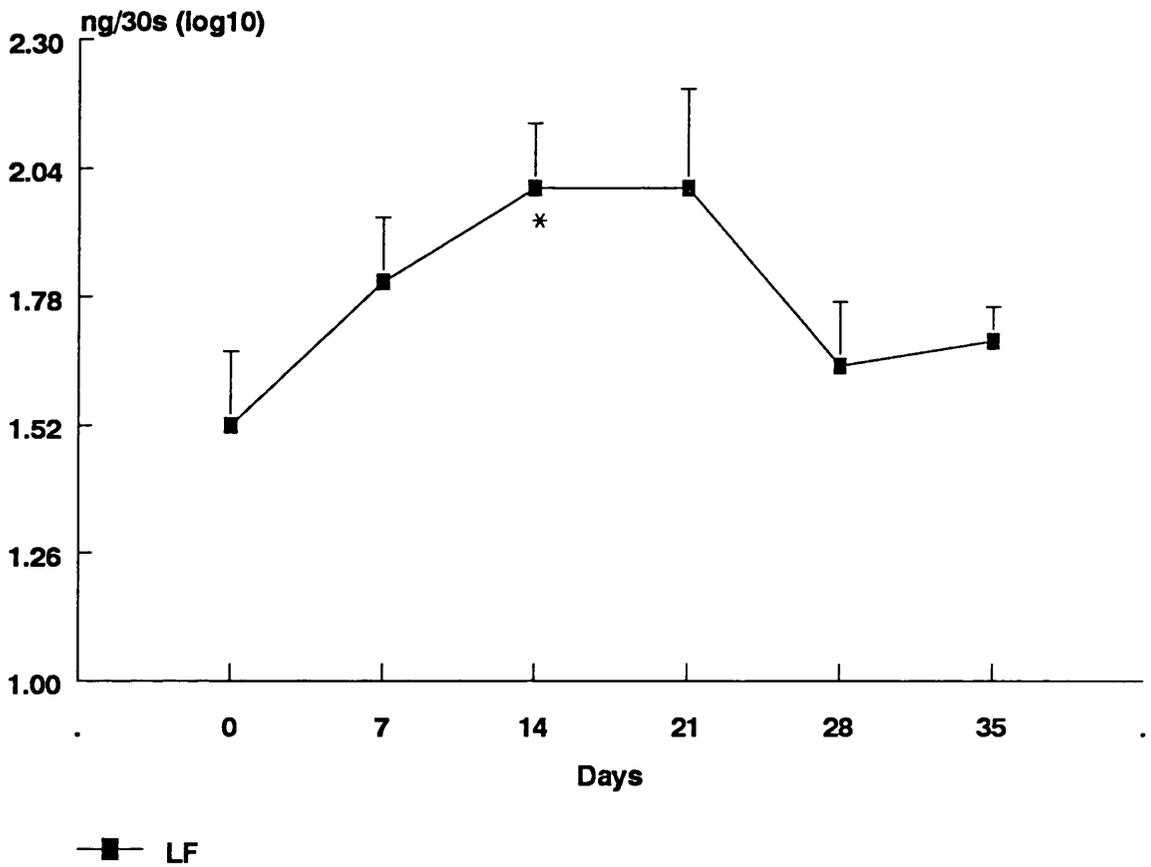


**Figure 4.14** Total transferrin (TF) changes in gingival crevicular fluid during the experimental gingivitis trial. The mean and standard error (n=6 subjects) at each time point are shown.

\* Significantly different from baseline  $p < 0.02$

differences existed among days ( $p=0.02$ ). Interestingly however, when the site was considered as the experimental unit, repeated measures MANOVA failed to detect differences in GCF TF levels among days ( $p=0.264$ ), demonstrating that overall variability was somewhat greater when results were analysed in this way. On the follow-up analysis, GCF TF on individual days was compared against baseline (paired  $t$ -tests,  $n=6$  subjects) and as shown in Figure 4.14, GCF TF appeared significantly elevated compared to baseline on days 14 ( $p=0.012$ ), 28 ( $p=0.014$ ) and 35 ( $p=0.019$ ). Again although mean GCF TF was higher than baseline on day 21 of the experimental gingivitis episode, this increase failed to reach statistical significance ( $p=0.082$ ). When results were analysed on the site basis slightly lower significance levels were obtained when GCF TF on days 14, 21 and 35 was compared to baseline and these differences did not reach significance at the 0.02 level (but  $p<0.05$ ).

GCF LF mean response by time for the 6 subjects demonstrated a different picture (Fig. 4.15) compared to the previously described GCF constituents ( $\alpha 2$ -M,  $\alpha 1$ -AT and TF). LF rises and similarly to the other proteins plateaus between days 14 and 21 of the experimental gingivitis episode. One week after the reinstatement of oral hygiene measures however, LF drops to almost baseline levels. When repeated measures MANOVA was applied to the  $\log_{10}$  transformed data, it demonstrated that a significant day effect existed on GCF LF levels, when both the subject and

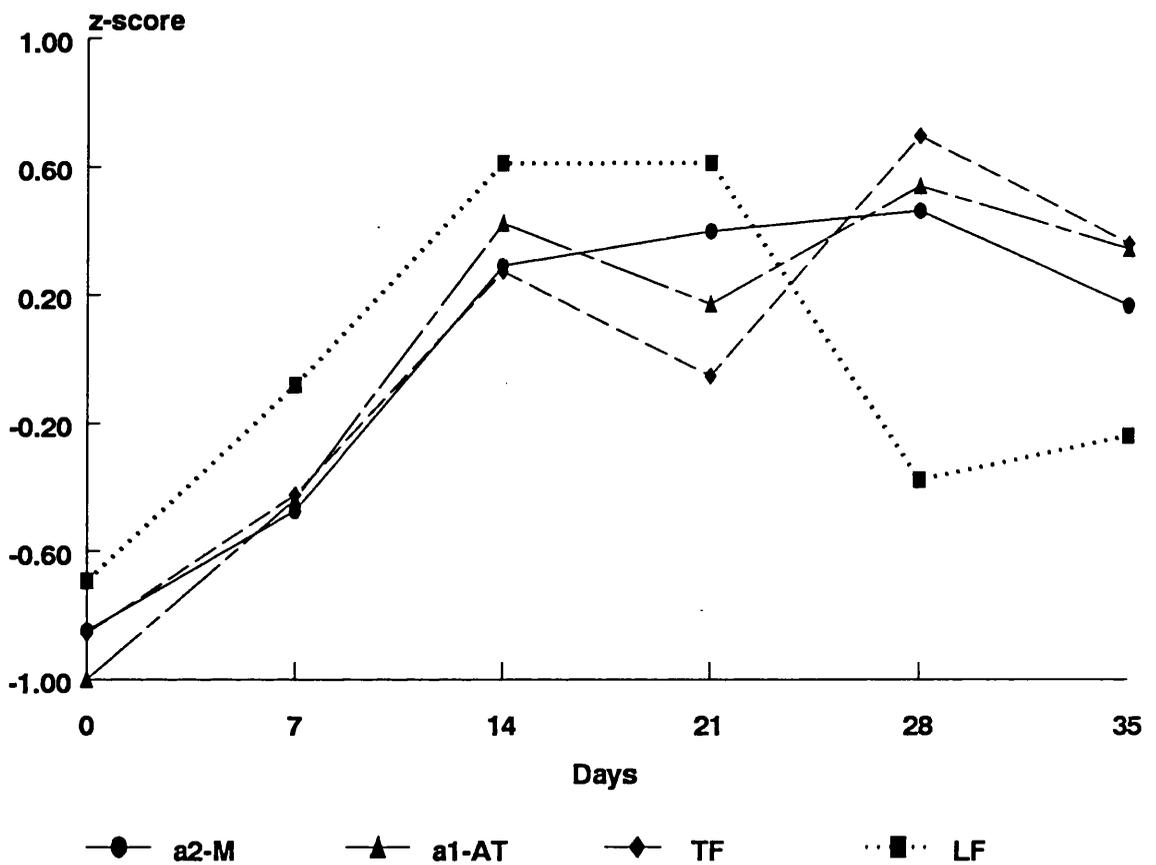


**Figure 4.15** Total lactoferrin (LF) changes in gingival crevicular fluid during the experimental gingivitis trial. The mean and standard error (n=6 subjects) at each time point are shown.

\* Significantly different from baseline  $p < 0.02$

site were treated as the experimental unit ( $p=0.001$  and  $p=0.003$  respectively). Subsequent analysis, using paired t-tests and the subject as the experimental unit, revealed that GCF LF levels on day 14 were significantly elevated compared to baseline  $p=0.009$  whereas on day 21, although GCF LF was still higher than baseline this was just outwith statistical significance ( $p=0.062$ ). On days 28 and 35 GCF LF mean levels had dropped and demonstrated no difference from baseline ( $p>0.3$ ). When the site was considered as the experimental unit, significant differences since baseline were, similarly, obtained on days 14 ( $p=0.001$ ) and 21 ( $p=0.001$ ).

The pattern of the mean response for the six subjects is clearer when the z scores of  $\alpha 2$ -M,  $\alpha 1$ -AT, TF and LF are plotted against time (Fig. 4.16). The z scores for each protein are produced by subtracting each data value from the grand mean of the protein and dividing by its standard deviation (section 2.4). In this way differences in overall level among the four proteins are removed and focusing on the pattern of change is allowed. As shown in Figure 4.16  $\alpha 2$ -M,  $\alpha 1$ -AT and TF present a similar pattern rising and remaining high until at least day 28, one week after the reinstatement of oral hygiene measures. In contrast, the changes in LF levels over time are different, demonstrating a drop on day 28, soon after subjects resumed oral hygiene procedures.



**Figure 4.16** Total  $\alpha 2$ -macroglobulin ( $\alpha 2$ -M),  $\alpha 1$ -antitrypsin ( $\alpha 1$ -AT), transferrin (TF) and lactoferrin (LF) changes in gingival crevicular fluid; average (n=6 subjects) z-scores of the  $\log_{10}$  transformed data during the experimental gingivitis trial.

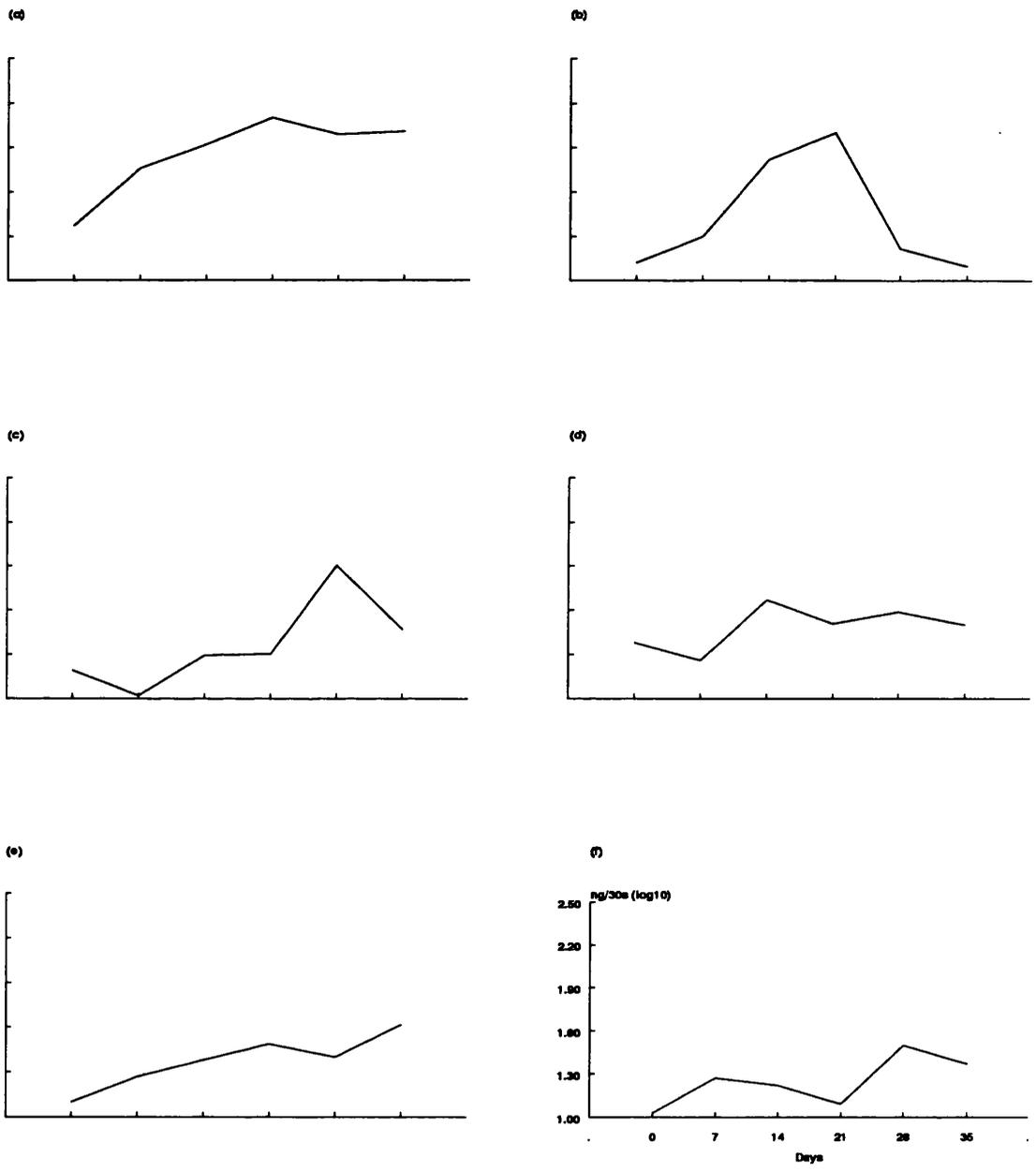
Figures 4.12 to 4.16 focused on the mean response of the 6 subjects by time for each of the protease inhibitors ( $\alpha$ 2-M and  $\alpha$ 1-AT) and iron-binding proteins (TF and LF) during the trial. Significant variability however existed among subjects. This is illustrated in Figures 4.17 through to 4.20 (a to f) which show each individual's response by time during the experimental gingivitis episode (each point represents the average of two sites per subject).

#### **4.4 GCF protease inhibitors, iron-binding proteins and clinical parameters in relation to periodontal attachment loss: a longitudinal study of periodontitis patients on maintenance care**

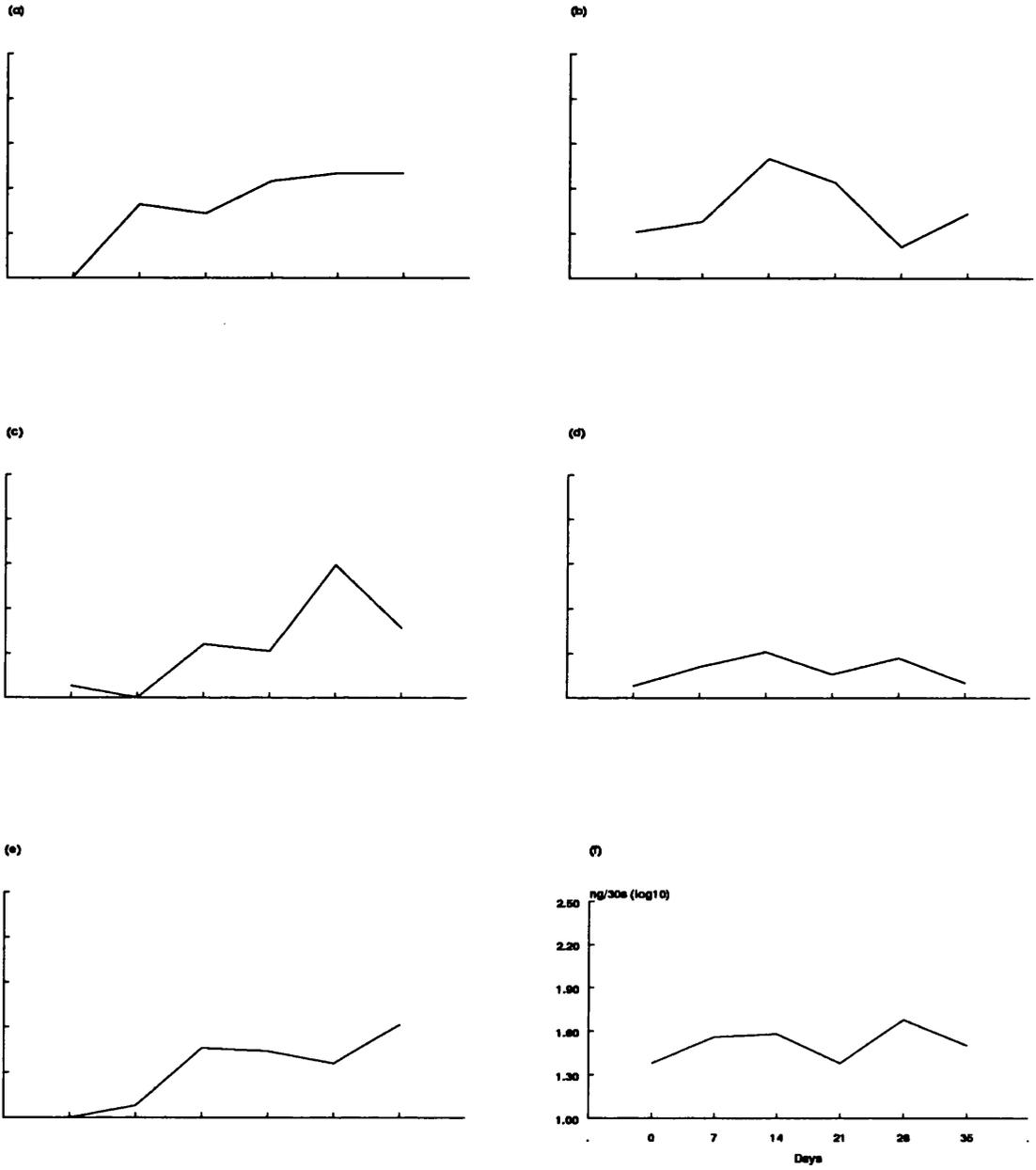
The main aim of this study was to investigate whether incipient periodontal breakdown could be related to changes in protease inhibitor ( $\alpha$ 2-M and  $\alpha$ 1-AT) and iron-binding protein (TF and LF) levels in GCF. In addition, the association of clinical parameters with attachment level change was examined.

##### **4.4.1 Clinical findings**

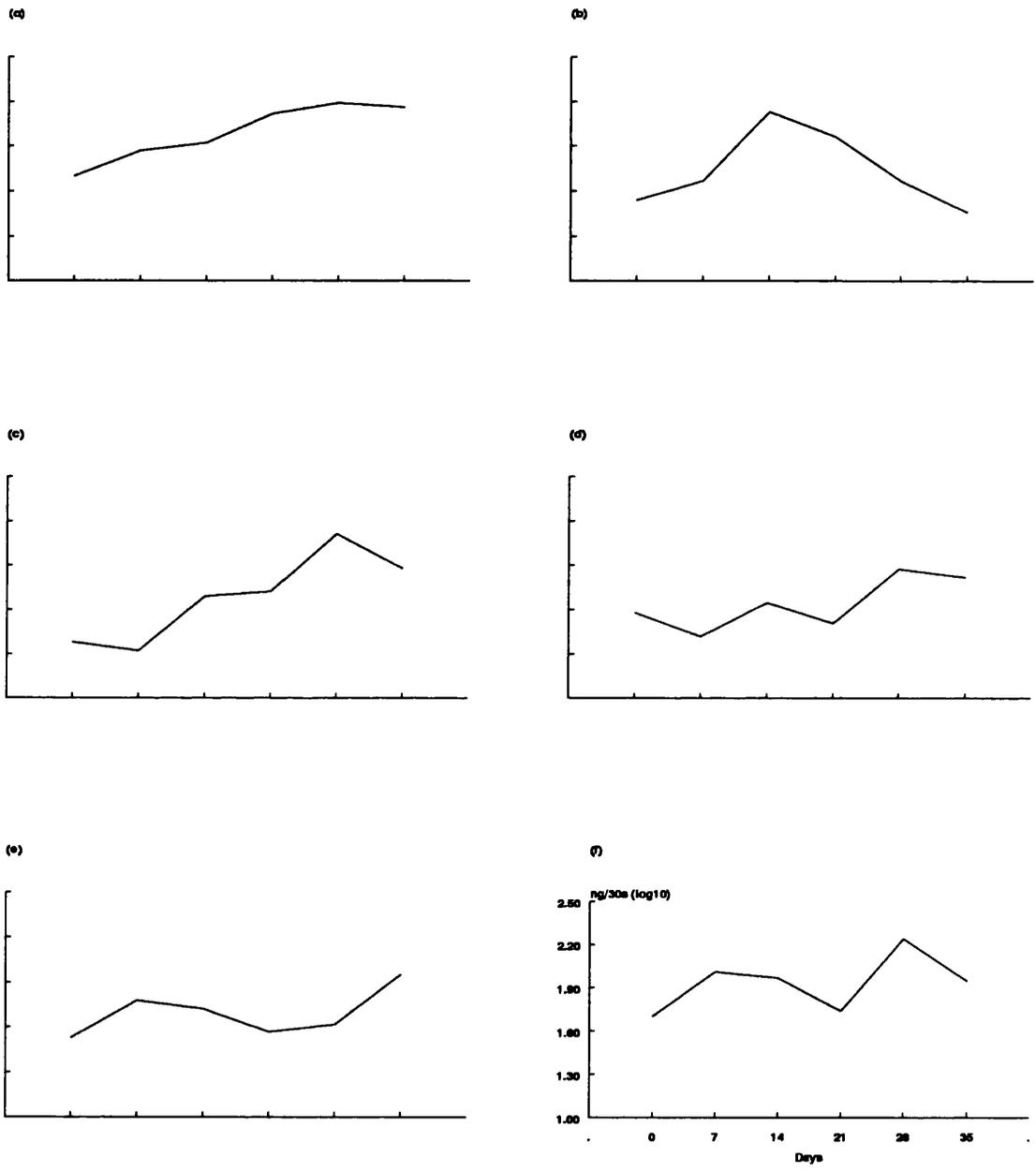
The 38 maintenance periodontitis patients enrolled in this study yielded a total of 384 sites. Clinical indices were recorded and GCF sampled at the baseline and recall appointments. A minimum of 6 and a maximum of 15 non-



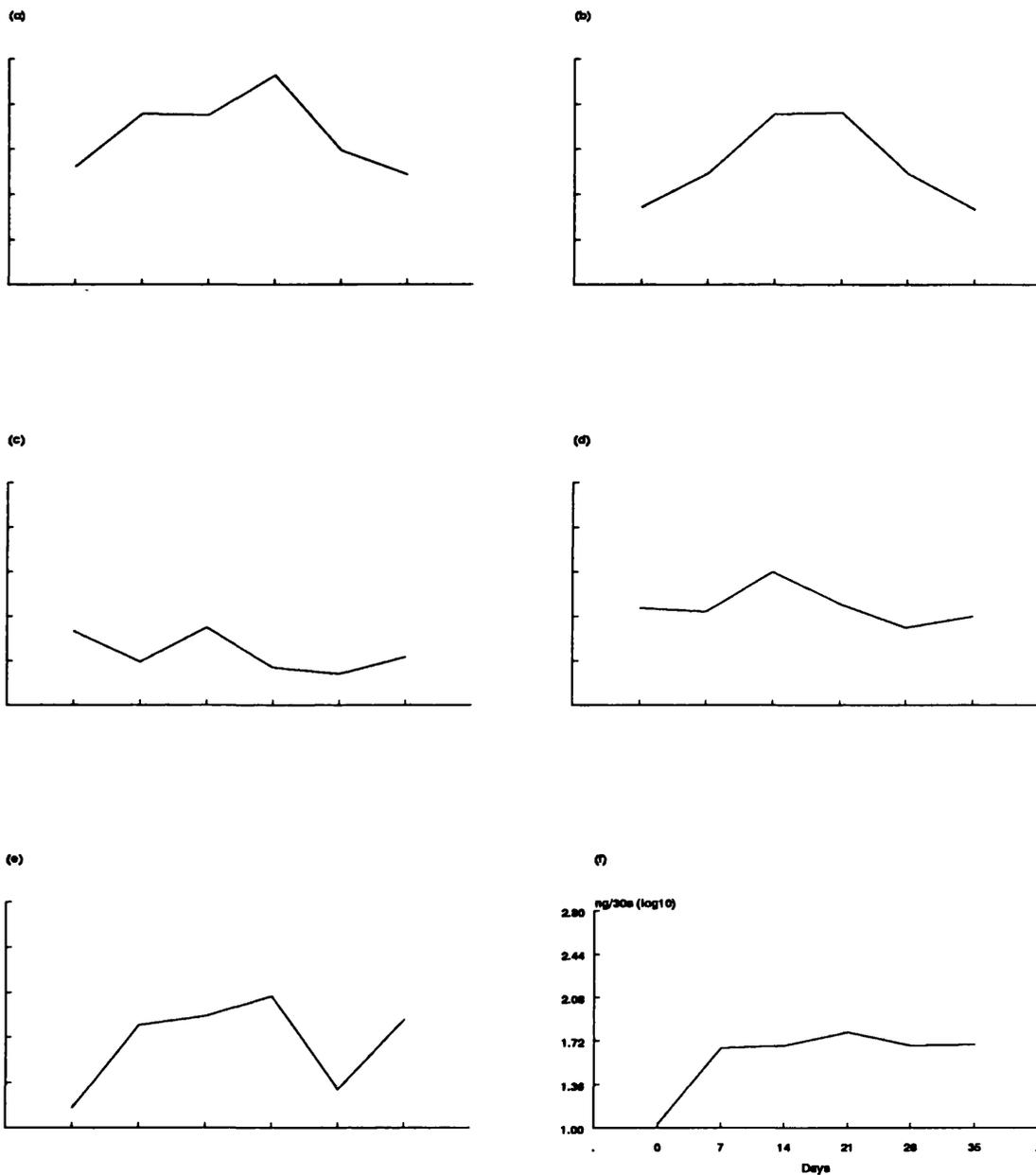
**Figure 4.17** (a) to (f): Total  $\alpha_2$ -macroglobulin changes in gingival crevicular fluid for each of the six subjects during the experimental gingivitis trial.



**Figure 4.18** (a) to (f): Total  $\alpha$ -1-antitrypsin changes in gingival crevicular fluid for each of the six subjects during the experimental gingivitis trial.



**Figure 4.19** (a) to (f): Total transferrin changes in gingival crevicular fluid for each of the six subjects during the experimental gingivitis trial.



**Figure 4.20** (a) to (f): Total lactoferrin changes in gingival crevicular fluid for each of the six subjects during the experimental gingivitis trial.

adjacent sites (mean 10 sites) were obtained per subject participating in this study.

The baseline mean clinical indices of the sites sampled are presented in Table 4.6. The frequency distribution of the different scores of MGI and PI at baseline are given in Figures 4.21 (a, b). Mean MGI was low (2.05) (Table 4.6) and as shown in Figure 4.21 most sites yielded scores between 1 and 3. No sites demonstrated MGI score of 4. PI was also low and approximately 72% of all sites demonstrated scores of 0 or 1 (Fig 4.21). Most sites bled on probing (60%) whereas the incidence of suppuration was low (3.4%). One of the prerequisites for sites to be selected for this study was that they had to exhibit pocketing  $\geq 4\text{mm}$  when measured using a PC12 periodontal probe on a pre-baseline recruiting appointment (section 2.2.3.3). Pocketing assessed using the Florida Probe was generally less than conventional probe readings.

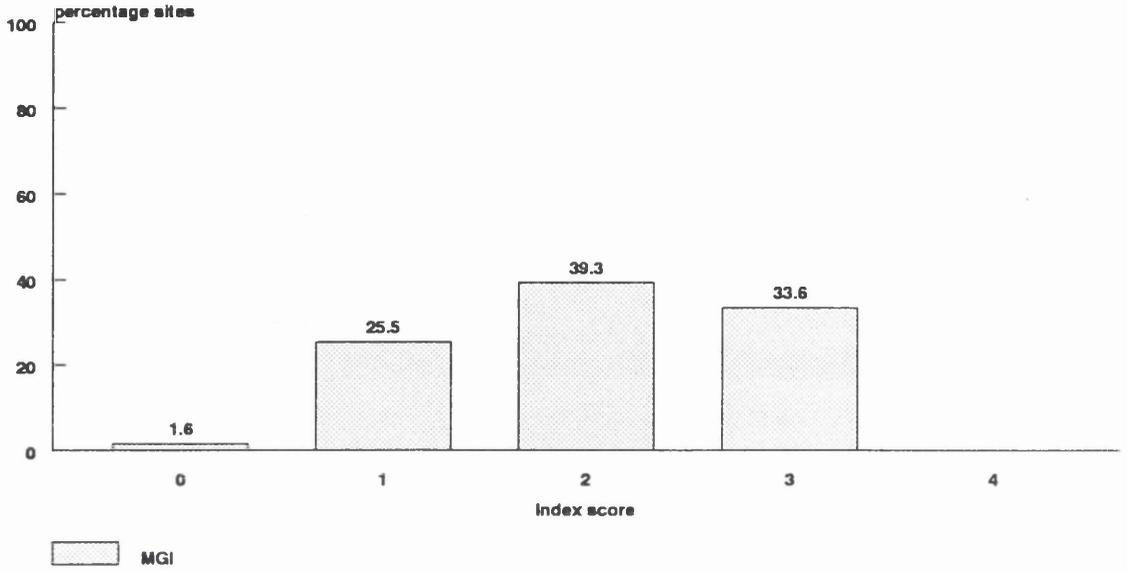
Two attachment level measurements were taken at each time point from each of the 384 sites. This experimental design was used to provide a measure of examiner error during the study and also provide the appropriate measures for the use of the tolerance method for detecting attachment level changes (method detailed in section 2.2.1.4). Standard deviation values of the differences of replicate measurements for the 38 subjects studied, varied from

**Table 4.6** Baseline clinical indices at the 384 sites selected for the longitudinal study on maintenance periodontitis patients. The mean and standard deviation (SD) of all sites is given.

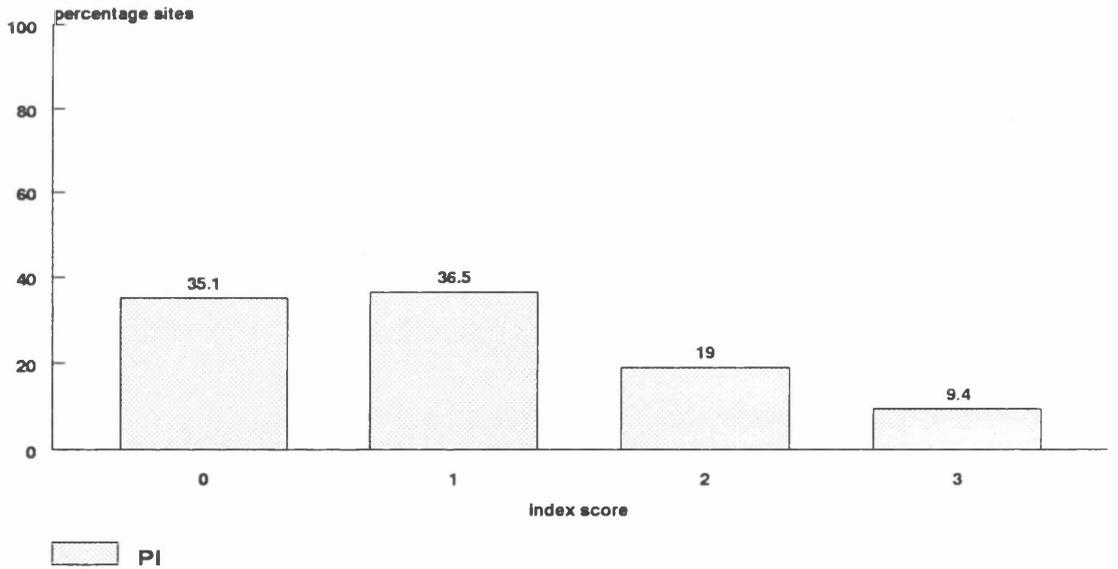
<b>Clinical indices in n=384 sites</b>		
<b>Parameter</b>	<b>Mean</b>	<b>SD</b>
<b>MGI</b>	2.05	0.81
<b>PI</b>	1.03	0.96
<b>PD (mm)</b>	4.44	1.35
<b>GCF (<math>\mu</math>l)</b>	0.31	0.29

MGI= modified gingival index  
 PI= plaque index  
 PD= pocket depth  
 GCF= gingival crevicular fluid

(a)



(b)



**Figure 4.21** Frequency distribution of baseline (a) MGI and (b) PI scores at the 384 sites examined during the longitudinal study of periodontitis patients on maintenance.

MGI= modified gingival index  
PI= plaque index

0.196 to 0.608 with a mean of 0.423mm. The population threshold was therefore set at 0.85mm (2 x Mean SD).

During the study, 15 sites (3.9%) demonstrated significant attachment loss, taking into account all three thresholds, and were classified as grade 5 sites (most deterioration; section 2.2.1.5). These sites were widely distributed, 13 sites occurring each in different individuals, whereas 2 sites only were obtained in the same individual (14 individuals in total). Attachment loss at these sites ranged from 0.9mm to 3.8mm (mean 1.91mm). The site demonstrating the minimum attachment loss of 0.9mm was the only site exhibiting significant attachment loss below 1mm, although exceeding all three thresholds (site, subject and population). All other sites exhibited attachment loss of  $\geq 1.2$ mm. Another 13 sites (3.4%) demonstrated significant attachment gain as defined by the tolerance method and were classified in the grade 1 category (most improvement; section 2.2.1.5). Attachment gain in these sites ranged between 1.3 and 2.5mm (mean 1.82mm). Ten of these thirteen attachment gain sites were from 3 subjects (2, 3 and 5 sites from each) and the remaining 3 sites were obtained from different individuals.

When a much less stringent threshold for determining attachment level change, specifically of  $\geq 1$ mm between the two appointments without any other thresholds being taken into account (section 2.2.1.4), was applied, another two

grades of attachment level change were produced. 17 sites (4.4%) were classified as grade 2 (gain  $\geq 1\text{mm}$ ) and 23 sites (6%) as grade 4 (loss  $\geq 1\text{mm}$ ). All other sites ( $n=316$ ; 82.3%) which were considered to be stable were classified as grade 3 sites (section 2.2.1.5). The frequency distribution and percentages of attachment level grading for all sites are shown in Table 4.7.

#### 4.4.2 Clinical parameters in relation to attachment level changes

In order to investigate if baseline clinical parameters could be associated with attachment level changes when considering the subject as a unit, mean clinical indices (MGI, PI, PD), GCF volume, bleeding on probing (BOP) and suppuration (S), were obtained for all sites within each subject (section 2.4). Subjects were ranked according to their mean attachment level scores (rank 1= most improvement= mean attachment gain 0.80mm; rank 38= most deterioration=mean attachment loss 0.76mm) as well as their mean baseline MGI, PI, PD, BOP and S scores and rank correlations obtained as shown in Table 4.8. With the exception of subject mean initial PD which showed a positive and significant association ( $r=0.380$ ;  $p=0.019$ ) with mean attachment level changes, all other correlations were low and failed to reach statistical significance. In order to examine if true associations were being masked by the inclusion of a large number of stable sites the two

**Table 4.7** Distribution and percentages of attachment level (AL) change grading in n=384 sites. Grading ranged from 1 (significant attachment gain) to 5 (significant attachment loss) as described in section 2.2.1.5.

<b>Attachment level grade frequency distribution</b>		
<b>Grade</b>	<b>Frequency</b>	<b>Percentage</b>
<b>1 significant AL gain</b>	13	3.4
<b>2 gain <math>\geq</math> 1mm</b>	17	4.4
<b>3 stable</b>	316	82.3
<b>4 loss <math>\geq</math> 1mm</b>	23	6.0
<b>5 significant AL loss</b>	15	3.9
<b>Total</b>	<b>384</b>	<b>100</b>

**Table 4.8** Spearman rank correlation coefficients (r) between baseline subject mean clinical indices and attachment level change in 38 patients. The value of r and significance level (p-values) are given.

Parameter	Mean attachment level change (n=38 subjects)	
	r	p-value
MGI	0.136	p=0.416
PI	0.084	p=0.615
PD	0.380	p=0.019
BOP	0.083	p=0.620
S	0.004	p=0.981
GCF ( $\mu$ l)	0.120	p=0.472

MGI= modified gingival index  
 PI= plaque index; PD= pocket depth  
 BOP= bleeding on probing; S= suppuration  
 GCF= gingival crevicular fluid

**Table 4.9** Clinical indices (mean  $\pm$  SE) for best and worst sites for all subjects (n=38). Clinical indices for the 2 best (most improvement; greatest attachment level gain) and 2 worst sites (most deterioration; greatest attachment level loss) were averaged for each subject and used to compute mean best and worst scores within each individual. Significance levels (p-values) of paired tests to compare best and worst sites are shown.

Parameter	Baseline Clinical Indices		
	mean $\pm$ SE, n=38 subjects		paired tests
	best sites	worst sites	p-value
MGI	2.065 $\pm$ 0.10	2.056 $\pm$ 0.11	0.839 <sup>a</sup>
PI	1.011 $\pm$ 0.14	1.018 $\pm$ 0.11	1.000 <sup>a</sup>
PD (mm)	4.704 $\pm$ 0.16	4.329 $\pm$ 0.17	0.405 <sup>a</sup>
GCF ( $\mu$ l)	0.279 $\pm$ 0.03	0.232 $\pm$ 0.02	0.229 <sup>b</sup>

<sup>a</sup> paired sign test; <sup>b</sup> paired t-test  
 MGI= modified gingival index  
 PI= plaque index; PD= pocket depth  
 GCF= gingival crevicular fluid

best and two worst sites per patient were compared. The baseline scores MGI, PI, PD and GCF volume, for the two best and two worst sites per patient were averaged and paired tests (t-test for GCF volume; sign tests for clinical indices) were used to compare for differences between best and worst sites within each subject. The mean scores of the 38 patients for the clinical indices of their 2 best and 2 worst sites are shown in Table 4.9. None of the clinical indices differed significantly between best and worst sites (Table 4.9). These results were left unchanged when Wilcoxon sign paired rank tests were used instead of sign tests to test for differences in MGI, PI and PD for best and worst sites.

This lack of association was confirmed when the sites were considered as independent (n=384) and rank correlations run between attachment level grading and the clinical indices (MGI, PI and PD) and GCF volume. As shown in Table 4.10 all correlations were very low and none was statistically significant despite the large number of sites used to test for associations. Moreover, bleeding on probing did not demonstrate significant association with attachment level grading (chi-square= 7.957; p=0.09). Finally, grade 1 (most improvement) and grade 5 (most deterioration) sites were treated assuming independence and compared using the Mann-Whitney test. Table 4.11 shows the clinical indices at grade 1 and grade 5 sites. No statistically significant differences (Mann-Whitney test; Table 4.11) were observed

**Table 4.10** Spearman rank correlation coefficients (r) between baseline clinical indices and attachment level grade in n=384 sites. The value of r and significance level (p-value) are given.

Parameter	Attachment level grade (n=384 sites)	
	r	p-value
MGI	0.021	p=0.685
PI	0.034	p=0.509
PD	-0.078	p=0.127
GCF ( $\mu$ l)	0.010	p=0.420

MGI= modified gingival index  
 PI= plaque index  
 PD= pocket depth  
 GCF= gingival crevicular fluid

**Table 4.11** Baseline clinical indices (mean  $\pm$  SE) at grade 1 and grade 5 sites (significant attachment gain and loss respectively). Sample size is given under each category in parenthesis. Differences in clinical indices between the two groups were tested by the Mann-Whitney test. Significance levels (p-values) are given.

Parameter	Baseline Clinical Indices		
	mean $\pm$ SE		Mann-Whitney
	Grade 1 sites (n=13)	Grade 5 sites (n=15)	p-value
MGI	1.92 $\pm$ 0.24	2.27 $\pm$ 0.18	0.269
PI	0.85 $\pm$ 0.22	1.20 $\pm$ 0.22	0.261
PD	5.2 $\pm$ 0.33	4.64 $\pm$ 0.40	0.230
GCF ( $\mu$ l)	0.26 $\pm$ 0.07	0.28 $\pm$ 0.08	0.695

MGI= modified gingival index  
 PI= plaque index  
 PD= pocket depth  
 GCF= gingival crevicular fluid

for any of the clinical indices between grade 1 and grade 5 sites. In addition, no association could be demonstrated between BOP and classification of a site as grade 1 or grade 5 (chi-square= 0.817; p=0.366).

#### **4.4.3 GCF protease inhibitors and iron-binding proteins at periodontally active versus stable sites**

A total of 15 sites demonstrated significant attachment loss (tolerance method) during the 3 month observation period (section 4.4.1). Two of these active sites were observed in the same individual, and the worst site only (most deterioration) was selected for GCF analysis. In this way, a total of 14 active sites in 14 individuals were enrolled in this study and were analysed for GCF protease inhibitors and iron-binding proteins. In this way a balanced design was obtained facilitating statistical analysis. The site excluded from subsequent analysis was also the site demonstrating attachment loss of 0.9mm, the minimum significant attachment loss obtained during the three month observation period (section 4.4.1). Each active site was paired, within each individual, to a non-progressive stable site demonstrating comparable baseline MGI and PD. Results were expressed in two ways: i) as total amounts in ng/30s and ii) corrected for the levels of albumin (Alb) in GCF (ng/ $\mu$ g Alb).

Table 4.12 shows the mean clinical indices at active and stable sites. No significant differences were obtained when paired tests were used to compare active and stable sites at baseline as far as MGI and PD are concerned, and even when PI and GCF volume were compared (paired sign test: MGI, PI, PD; paired t-test: GCF volume).

The levels (geometric mean and 95% confidence intervals) of  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M),  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT), transferrin (TF) and lactoferrin (LF) (ng/30s) at active and stable sites at baseline are given in Table 4.13. Active versus stable sites were compared using paired t-tests on the  $\log_{10}$  transformed data. No significant differences were obtained for any of the proteins under investigation, between active and stable sites at baseline (Table 4.13).

The levels of protease inhibitors and iron-binding proteins in GCF (ng/30s) from active and stable sites were also assessed at the recall appointment. No significant differences could be detected when paired t-tests were used (on the  $\log_{10}$  transformed data) to test for differences between active and stable sites at the recall appointment as far as their GCF protease inhibitor and iron-binding protein levels were concerned (Table 4.14).

Similar findings were obtained when these protein levels were corrected for albumin in GCF (ng/ $\mu$ g Alb) and compared

**Table 4.12** Clinical indices (mean  $\pm$  SE) at active and stable sites (n=14) used for the assessment of GCF  $\alpha$ 2-macroglobulin,  $\alpha$ 1-antitrypsin, transferrin and lactoferrin levels. Differences in clinical indices were tested using paired tests. Significance levels (p-values) are given.

<b>Baseline Clinical Indices</b>			
<b>Parameter</b>	<b>mean <math>\pm</math> SE (n=14)</b>		<b>paired tests</b>
	<b>Active sites</b>	<b>Stable sites</b>	<b>p-value</b>
<b>MGI</b>	2.21 $\pm$ 0.19	2.00 $\pm$ 0.21	0.375 <sup>a</sup>
<b>PI</b>	1.14 $\pm$ 0.23	1.21 $\pm$ 0.24	1.000 <sup>a</sup>
<b>PD</b>	4.54 $\pm$ 0.42	4.89 $\pm$ 0.24	0.267 <sup>a</sup>
<b>GCF (<math>\mu</math>l)</b>	0.291 $\pm$ 0.08	0.306 $\pm$ 0.10	0.819 <sup>b</sup>

<sup>a</sup> paired sign test

<sup>b</sup> paired t-test

MGI= modified gingival index

PI= plaque index; PD= pocket depth

GCF= gingival crevicular fluid

**Table 4.13** Baseline  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M),  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT), transferrin (TF) and lactoferrin (LF) levels (ng/30s) in gingival crevicular fluid (GCF) from periodontally active and stable sites. Sample size, geometric mean and 95% confidence intervals are given. Paired t-tests were performed to compare for differences between active and stable sites. Significance levels (p-values) are shown.

<b>Baseline GCF <math>\alpha</math>2-M, <math>\alpha</math>1-AT, TF and LF levels (ng/30s)</b>			
<b>Parameter</b>	<b>mean (95% confidence interval) (n=14)</b>		<b>paired t-test</b>
	<b>Active sites</b>	<b>Stable sites</b>	<b>p-value</b>
<b><math>\alpha</math>2-M</b>	38 (20-72)	31 (14-69)	0.556
<b><math>\alpha</math>1-AT</b>	81 (41-157)	87 (51-147)	0.831
<b>TF</b>	54 (22-130)	47 (15-139)	0.754
<b>LF</b>	135 (64-281)	107 (49-232)	0.468

**Table 4.14** Recall  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M),  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT), transferrin (TF) and lactoferrin (LF) levels (ng/30s) in gingival crevicular fluid (GCF) from periodontally active and stable sites. Sample size, geometric mean and 95% confidence intervals are given. Paired t-tests were performed to compare for differences between active and stable sites. Significance levels (p-values) are shown.

<b>Recall GCF <math>\alpha</math>2-M, <math>\alpha</math>1-AT, TF and LF levels (ng/30s)</b>			
<b>Parameter</b>	<b>mean (95% confidence intervals) (n=14)</b>		<b>paired t-test</b>
	<b>Active sites</b>	<b>Stable sites</b>	<b>p-value</b>
<b><math>\alpha</math>2-M</b>	39 (22-70)	34 (21-55)	0.591
<b><math>\alpha</math>1-AT</b>	86 (53-139)	80 (52-124)	0.772
<b>TF</b>	90 (43-186)	56 (31-101)	0.213
<b>LF</b>	141 (78-254)	128 (74-220)	0.761

between active and stable sites at both baseline and recall appointments. Total albumin in  $\mu\text{g}/30\text{s}$  in GCF at active ( $n=14$ ) and stable sites ( $n=14$ ) on baseline was 1.7 (0.5-2.9) and 1.5 (0.7-2.3) respectively (geometric mean and 95% confidence intervals). Similarly, on recall total albumin at active and stable sites was 1.7 (0.6-2.8) and 1.1 (0.4-1.8)  $\mu\text{g}/30\text{s}$  respectively. Tables 4.15 and 4.16 give the levels of  $\alpha 2\text{-M}$ ,  $\alpha 1\text{-AT}$ , TF and LF expressed in  $\text{ng}/\mu\text{g Alb}$  (geometric mean and 95% confidence intervals) and the results of the paired tests (run on the  $\log_{10}$  transformed data) to compare between active and stable sites at baseline and recall appointments respectively. None of the differences were statistically significant.

These results were confirmed when multivariate repeated measures analysis of variance (repeated measures MANOVA on the  $\log_{10}$  transformed data) was used to test for all four proteins ( $\alpha 2\text{-M}$ ,  $\alpha 1\text{-AT}$ , TF and LF) simultaneously for differences between active and stable sites at the baseline and recall appointments for both ways of expressing the results ( $\text{ng}/30\text{s}$  Table 4.17;  $\text{ng}/\mu\text{g Alb}$  Table 4.18). Two separate MANOVA procedures were run to test for differences at the baseline and recall appointments.

Finally, repeated measures MANOVA and a model incorporating the four proteins while testing simultaneously for site (stable versus active), appointment (baseline versus recall) and site by appointment effects was run on the  $\log_{10}$

**Table 4.15** Baseline  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M),  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT), transferrin (TF) and lactoferrin (LF) levels (ng/ $\mu$ g Alb) in gingival crevicular fluid (GCF) from periodontally active and stable sites. Sample size, geometric mean and 95% confidence intervals are given. Paired t-tests were performed to compare for differences between active and stable sites. Significance levels (p-values) are shown.

<b>Baseline GCF <math>\alpha</math>2-M, <math>\alpha</math>1-AT, TF and LF levels (ng/<math>\mu</math>g Alb)</b>			
<b>Parameter</b>	<b>mean (95% confidence interval) (n=14)</b>		<b>paired t-test</b>
	<b>Active sites</b>	<b>Stable sites</b>	<b>p-value</b>
<b><math>\alpha</math>2-M</b>	47 (31-71)	30 (16-57)	0.240
<b><math>\alpha</math>1-AT</b>	98 (65-150)	80 (54-120)	0.450
<b>TF</b>	58 (26-129)	45 (17-120)	0.505
<b>LF</b>	163 (89-301)	98 (47-206)	0.161

**Table 4.16** Recall  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M),  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT), transferrin (TF) and lactoferrin (LF) levels (ng/ $\mu$ g Alb) in gingival crevicular fluid (GCF) from periodontally active and stable sites. Sample size, geometric mean and 95% confidence intervals are given. Paired t-tests were performed to compare for differences between active and stable sites. Significance levels (p-values) are shown.

<b>Recall GCF <math>\alpha</math>2-M, <math>\alpha</math>1-AT, TF and LF levels (ng/<math>\mu</math>g Alb)</b>			
<b>Parameter</b>	<b>mean (95% confidence intervals) (n=14)</b>		<b>paired t-test</b>
	<b>Active sites</b>	<b>Stable sites</b>	<b>p-value</b>
<b><math>\alpha</math>2-M</b>	43 (35-53)	42 (31-56)	0.870
<b><math>\alpha</math>1-AT</b>	94 (66-135)	98 (70-138)	0.826
<b>TF</b>	98 (70-137)	69 (50-95)	0.099
<b>LF</b>	154 (83-286)	156 (98-248)	0.959

**Table 4.17** Multivariate repeated measures analysis of variance (MANOVA) for  $\alpha$ 2-macroglobulin,  $\alpha$ 1-antitrypsin, transferrin and lactoferrin (ng/30s) in GCF from active versus stable sites for the baseline and recall appointments. Multivariate results (Pillai's test) for each appointment are shown. The value of the test (value), the approximate F (Appr. F), the hypothesis and error degrees of freedom (Hyp. DF and Error DF respectively) and the significance level (p) are given.

<b>Repeated Measures MANOVA: Pillai's test</b>					
	<b>Value</b>	<b>Appr. F</b>	<b>Hyp. DF</b>	<b>Error DF</b>	<b>p</b>
<b>Effect:</b>					
<b>active versus stable</b>					
<b>Baseline</b>	0.286	1.001	4	10	0.451
<b>Recall</b>	0.170	0.514	4	10	0.727

**Table 4.18** Multivariate repeated measures analysis of variance (MANOVA) for  $\alpha$ 2-macroglobulin,  $\alpha$ 1-antitrypsin, transferrin and lactoferrin (ng/ $\mu$ g Alb) in GCF from active versus stable sites for the baseline and recall appointments. Multivariate results (Pillai's test) for each appointment are shown. The value of the test (value), the approximate F (Appr. F), the hypothesis and error degrees of freedom (Hyp. DF and Error DF respectively) and the significance level (p) are given.

<b>Repeated Measures MANOVA: Pillai's test</b>					
	<b>Value</b>	<b>Appr. F</b>	<b>Hyp. DF</b>	<b>Error DF</b>	<b>p</b>
<b>Effect:</b>					
<b>active versus stable</b>					
<b>Baseline</b>	0.280	0.972	4	10	0.464
<b>Recall</b>	0.224	0.722	4	10	0.596

transformed data (section 2.4). Of the above, the most interesting is the site by appointment effect, as it compares the change ( $\delta$ ) in protease inhibitor and iron-binding protein levels (ng/30s) between baseline and recall at active versus stable sites (section 2.4). As shown in Table 4.19 (ng/30s) and 4.20 (ng/ $\mu$ g Alb) none of these effects were significant, confirming that in this study no difference could be detected in GCF levels of  $\alpha$ 2-M,  $\alpha$ 1-AT, TF and LF levels between periodontally active and stable sites when expressing the results either as ng/30s or ng/ $\mu$ g Alb.

The above results suggest that it is not possible to differentiate periodontally active from stable sites using a diagnostic test based on the levels of protease inhibitors or iron-binding proteins in GCF. In order to illustrate this, data from the recall appointment (ng/30s; Table 4.14) were used to assess specificity, sensitivity as well as predictive values. A combined test based on the values of the four proteins  $\alpha$ 2-M,  $\alpha$ 1-AT, LF and TF was used. Thus, the test was considered positive when a site demonstrated  $\alpha$ 2-M  $\geq$  39ng/30s,  $\alpha$ 1-AT  $\geq$  86ng/30s, TF  $\geq$  90ng/30s and LF  $\geq$  141ng/30s. These threshold values represent the geometric means of  $\alpha$ 2-M,  $\alpha$ 1-AT, TF and LF levels (ng/30s) at active sites, as shown in Table 4.14. All four conditions should apply simultaneously in order for the test to be considered positive, whereas if the levels of any one of the proteins dropped below the

**Table 4.19** Multivariate repeated measures analysis of variance (MANOVA) for  $\alpha$ 2-macroglobulin,  $\alpha$ 1-antitrypsin, transferrin and lactoferrin (ng/30s) in GCF from active versus stable sites at baseline and recall appointments. Multivariate results are shown. The value of the test (value), the approximate F (Appr. F), the hypothesis and error degrees of freedom (Hyp. DF and Error DF respectively) and the significance level (p) of the main effects and their interactions are given.

<b>Repeated Measures MANOVA: Pillai's test</b>					
<b>Effect</b>	<b>Value</b>	<b>Appr. F</b>	<b>Hyp. DF</b>	<b>Error DF</b>	<b>p</b>
<b>Appointment: Baseline versus recall</b>	0.162	0.483	4	10	0.748
<b>Site: Active versus stable</b>	0.246	0.817	4	10	0.542
<b>Appointment by site</b>	0.129	0.371	4	10	0.824

**Table 4.20** Multivariate repeated measures analysis of variance (MANOVA) for  $\alpha$ 2-macroglobulin,  $\alpha$ 1-antitrypsin, transferrin and lactoferrin (ng/ $\mu$ g Alb) in GCF from active versus stable sites at baseline and recall appointments. Multivariate results are shown. The value of the test (value), the approximate F (Appr. F), the hypothesis and error degrees of freedom (Hyp. DF and Error DF respectively) and the significance level (p) of the main effects and their interactions are given.

<b>Repeated Measures MANOVA: Pillai's test</b>					
<b>Effect</b>	<b>Value</b>	<b>Appr. F</b>	<b>Hyp. DF</b>	<b>Error DF</b>	<b>p</b>
<b>Appointment: Baseline versus recall</b>	0.151	0.445	4	10	0.774
<b>Site: Active versus stable</b>	0.230	0.746	4	10	0.582
<b>Appointment by site</b>	0.160	0.478	4	10	0.752

designated values the test was considered negative. As shown in Table 4.21 sensitivity was low (28%), whereas specificity reached approximately 86%. Positive and negative predictive values were low. In 33% of the cases a positive test would have misclassified an inactive site as being active. Similarly, in 46% of the cases a negative test would have misclassified an active site as being inactive.

**Table 4.21** Characteristics of a diagnostic test based on the levels (ng/30s) of  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M),  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT), transferrin (TF) and lactoferrin (LF) at the recall appointment. Sensitivity, specificity and positive and negative predictive values are shown.

	Active site	Stable site	
<u>Positive test</u> $\alpha$ 2-M $\geq$ 39ng/30s & $\alpha$ 1-AT $\geq$ 86ng/30s & TF $\geq$ 90 ng/30s & LF $\geq$ 141 ng/30s	4	2	<b>Positive predictive value</b>  <b>67%</b>
<u>Negative test</u> $\alpha$ 2-M < 39ng/30s or $\alpha$ 1-AT < 86ng/30s or TF < 90 ng/30s or LF < 141 ng/30s	10	12	<b>Negative predictive value</b>  <b>54%</b>
	<b>Sensitivity</b>	<b>Specificity</b>	
	<b>28%</b>	<b>86%</b>	

## **CHAPTER 5**

### **Discussion**

## **5.1 Considerations on methodology**

### **5.1.1 Quantification of gingival crevicular fluid iron-binding proteins and protease inhibitors**

The use of enzyme linked immunosorbent assays (ELISA) for the assessment of constituents in biological fluids is common practice today and it has replaced methods such as radial immunodiffusion and radioimmunoassays. The advantages of the ELISA methodology are considerable. It provides precision and low detectability limits in conjunction with eliminating radiation dangers associated with the use of radioimmunoassays. Preliminary experiments and results for the establishment of the ELISAs used in this thesis have been presented and discussed in chapter 3. The methods were validated and proven suitable for the quantification of protease inhibitors and iron-binding proteins in GCF.

The use of ELISA methodology, however, for the quantification of protease inhibitors in GCF could be criticised for its inability to distinguish between bound and free forms of the inhibitor. GCF is rich in enzymes which are excreted mainly from degranulating PMNs (chapter 1; Table 1.5). Thus, it is highly likely that protease inhibitors reaching the crevice would readily bind to proteases. Within the tissues any binding of a protease to  $\alpha 2$ -M or  $\alpha 1$ -AT results in the rapid removal of the complex

by monocytes/ macrophages (Ohlsson & Laurell, 1976; Feldman et al., 1983). Thus, inhibitor /proteinase complexes within the gingivae would be present for only very short periods of time and would not, therefore, be readily detected. In contrast, in the gingival crevice where macrophages/monocytes are scarce (Skapski & Lehner, 1976), proteinase/inhibitor complexes would persist for longer time periods. Some investigators (Condacci et al., 1982; Skaleric et al., 1986) have suggested that studying the free/bound inhibitor ratio in the crevice would provide information on the protease inhibiting potential of GCF and, therefore, be of more relevance to periodontal disease status. However, if one is interested in the situation within the gingival tissues, then the total amount of the inhibitors in GCF would be more representative due to the considerations mentioned above.

The total (free and bound) amounts of  $\alpha 2$ -M could in addition reflect the numbers of mature macrophages within the gingival tissues, as it has been shown that transition of monocytes into macrophages is followed by a strong induction in  $\alpha 2$ -M production (Bauer et al., 1988). As reviewed recently by Page (1991), mature macrophages are one of the key cells contributing to periodontal tissue damage by release, upon activation, of their potent proteases within the tissues (Chapter 1; Fig. 1.1 & 1.2). Total GCF  $\alpha 2$ -M could perhaps reflect monocyte/macrophage functional state within the gingivae.

In addition to the previous considerations, techniques used for the assessment of the free and bound form of  $\alpha$ 2-M and  $\alpha$ 1-AT have the following disadvantages:

a) as far as  $\alpha$ 2-M is concerned, the use of isoelectric focusing and crossed immunoelectrophoresis for the assessment of its free and bound form (Ohlsson & Skude, 1976) requires large GCF sample volumes and excludes the simultaneous assessment of other constituents in the same sample. For example Condacci *et al.* (1982) and Skaleric *et al.* (1986), in order to obtain sufficient GCF sample volumes, employed long sampling times (paper strips for 3-5 min) and the use of microcapillaries for the collection of GCF respectively. Such procedures could, however, cause irritation of the gingival tissues and induce serum leakage from the subepithelial microvasculature thus altering GCF constituents (Cimasoni, 1983; Persson & Page, 1990). In addition, in order to compensate for the lack of sensitivity of the technique, the whole GCF sample was required for the assessment of free and bound form of the inhibitor, despite the large volumes obtained when using the above mentioned sampling protocols. Thus, no other GCF components could be investigated.

b) examining  $\alpha$ 1-AT/elastase complexes by ELISA methodology as performed by Huynh *et al.* (1992) and Zafiroopoulos *et al.* (1991) represents elastase concentration in GCF. This is because elastase will bind avidly to  $\alpha$ 1-AT whereas the

inhibitor will, in addition, bind to other available serine proteinases like Cathepsin G and plasminogen activator, or metalloproteinases like collagenase (Ohlsson & Olsson, 1977). Thus assessment of  $\alpha$ 1-AT/elastase complexes in GCF will only partly reflect the bound form of the inhibitor and will not provide information on the true bound/free  $\alpha$ 1-AT ratio.

### 5.1.2 Gingival crevicular fluid data reporting

Traditionally, constituent activities/amounts present in biological fluids have been expressed as concentrations (activity or amount per unit volume). As Lamster *et al.* (1986) initially pointed out, this method may not be applicable to GCF. GCF is an inflammatory exudate or proinflammatory transudate and is unique in properties, differing from most other biological fluids. Microamounts of GCF are produced in an open environment, the gingival crevice, and are constantly cleared towards the oral cavity. Thus, in contrast to most other biological fluids a large pool of fluid does not exist. Usually, sampling means that the total or most of the amount of fluid present in the gingival crevice is removed. If sampling time is standardised then expression of the results as total amounts per time may be preferable. Reporting GCF constituents as total amounts has additional advantages as it avoids the calculation of GCF volume in its determination. GCF volumes are usually below 1 $\mu$ l rendering

their precise measurement particularly difficult. Although the Periotron is considered accurate in the assessment of GCF volume, minimal errors in the estimation of the latter would result in disproportionately large alterations in the concentration of GCF constituents (Lamster *et al.*, 1988a). In the ultra low range of GCF volumes, losses from evaporation could easily occur both during sampling and transfer of the sample to the Periotron, even if the instrument is located chairside. Most investigators seem to agree that expression of GCF constituent data as total amounts is preferable (Lamster *et al.*, 1986, 1988a; Wolff *et al.*, 1988; Cao & Smith, 1989; Persson & Page, 1990; Page, 1992; Smith & Geegan, 1991) although others seem to have reservations and usually report results both as concentrations and total amounts (Huynh *et al.*, 1992; Eley & Cox, 1992; Cox & Eley, 1992a, 1992b; Giannopoulou *et al.*, 1992).

### **5.1.3 Gingival crevicular washings**

Gingival crevicular washings (GCW) were used in order to sample crevicular LF and PMNs and investigate the relationship between them. Recently a method has been described for the approximate determination of PMN numbers in discrete GCF samples, collected with paper strips (Cimasoni & Giannopoulou, 1988). This technique would appear to permit assessment of LF levels and PMN numbers from the same GCF sample. However, in a pilot study

performed in our clinics and laboratories on a limited number of GCF samples (section 3.2.3.3), no PMNs could be recovered from the strips, either when vortexing (Cimasoni & Giannopoulou, 1988) or centrifuging the sample (Ebersole *et al.*, 1984). Thus, we resorted to the semiquantitative method of gingival crevicular washings (GCWs) to determine numbers of PMNs in the crevice and their relationship with LF. LF levels and PMN numbers in gingival crevicular washings were expressed as concentrations (ng/ $\mu$ l GCW, PMNs/ $\mu$ l GCW respectively), which are directly proportional to their absolute amounts present within the crevice.

## **5.2 Cross-sectional studies**

### **5.2.1 Lactoferrin as a marker of polymorphonuclear leucocytes in the gingival crevice**

This study was performed to investigate whether lactoferrin (LF) could act as a marker of crevicular polymorphonuclear leucocytes (PMN) in the crevice. The reasoning behind the choice of LF as a PMN marker includes the following: a) LF is contained specifically and in abundance in PMN secondary granules (Spitznagel *et al.*, 1974) but not in other leucocytes (Bennett & Kokocinski, 1978) and only in trace amounts in serum (Hetherington *et al.*, 1983); b) PMN secondary granules are more numerous than primary granules (Falloon & Gallin, 1986) and their contents are released earlier and more readily during *in vitro* PMN stimulation

(Bentwood & Henson, 1980); c) PMN secondary granules have been shown to be preferentially released, over primary granules, during both adherence *in vitro* and migration through epithelium *in vivo* (Wright & Gallin, 1979); d) finally, Wilton (1986) summarised the stimuli for PMN degranulation in the crevice (section 1.5.2) and concluded that PMN secondary granule release is more likely *in vivo*. GCF LF, therefore, is a good candidate as a marker of PMN emigration in the crevice (Fine & Mandel, 1986, Curtis et al., 1989).

When the relationship between PMN numbers and LF levels in gingival crevicular washings from discrete sites was examined a positive ( $r=0.531$ ) and significant ( $p<0.001$ ) association was obtained (section 4.1). The moderately strong relationship between LF levels and crevicular PMNs could be due to the LF contribution by degranulating PMNs within the subepithelial gingival connective tissue. The connective tissue or intra-epithelial PMNs would not have been accounted for in our assessment of PMNs, as gingival crevicular washings would only harvest leucocytes already present in the crevice.

When associations between clinical indices and LF concentration or PMN numbers in gingival crevicular washings from specific sites were examined positive correlations were obtained (section 4.1). PMN numbers increase in the gingival crevice with the development of

experimental gingivitis (Kowashi et al., 1980; Thurre et al., 1984) and more PMNs are found in periodontitis compared to control sites (Thurre et al., 1984). Therefore, a positive association of PMN numbers and LF levels with clinical indices is expected.

The correlation of LF in gingival crevicular washings with the clinical indices was higher than that obtained for crevicular PMNs. This may reflect lower errors in the measurement of LF than PMN numbers in the crevice. In addition, as LF may reflect activated PMNs in the area, it suggests that measuring PMN activation may be more pertinent when assessing disease status. LF may, therefore, prove a better test of periodontal disease status than crevicular PMNs at a specific site. This effect would, however, be optimised if care is taken to separate the cellular components of the GCW/GCF sample prior to freezing or eluting and subsequent assaying for LF in order to avoid disruption of the PMNs present in the sample.

In conclusion, our data support the view that LF may prove a useful marker of PMN emigration and/or activation in the crevice. LF determination in GCF is more accurate and simpler to perform than the enumeration of PMNs using GCWs or styroflex strips and may provide a simple and effective marker of crevicular PMNs.

## 5.2.2 Protease inhibitors and iron-binding proteins at healthy, gingivitis and periodontitis sites

In these studies,  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M),  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT), transferrin (TF) and lactoferrin (LF) were assessed in different GCF samples. The data from these studies demonstrate that higher absolute amounts (ng/30s sample) of protease inhibitors ( $\alpha$ 2-M,  $\alpha$ 1-AT) and iron-binding proteins (TF, LF) are present in gingival crevicular fluid from diseased sites (gingivitis and periodontitis), than from clinically healthy sites (section 4.2). As mean MGI, PD and GCF volume were higher in diseased sites (gingivitis and periodontitis) the influence each factor has on the levels of these proteins is unclear. However,  $\alpha$ 2-M,  $\alpha$ 1-AT and TF are mainly serum derived and, therefore, increased amounts in diseased sites could be accounted for by increased crevicular fluid flow as a result of increased vascular permeability following gingival inflammation. This is strongly suggested by the higher GCF volume readings obtained from diseased sites. However, the possibility of increased local production within the gingival tissues can not be excluded. *In vitro* studies have shown that these proteins could be produced from cells residing within the periodontal tissues. Gingival fibroblasts are capable of producing  $\alpha$ 2-M (Condacci et al., 1988) and monocytes/macrophages can produce  $\alpha$ 2-M (Hovi et al., 1977; White et al., 1980; Bauer et al., 1988),  $\alpha$ 1-AT (Perlmutter et al., 1985, Bauer et al., 1988) and TF

(Stecher & Thorbecke, 1967). In addition,  $\alpha$ 1-AT is produced and stored within PMN primary granules (Mason et al., 1991) whereas inducer T-lymphocytes may contribute to TF synthesis within the tissues (Lum et al., 1986).

The increase in LF levels (ng/30s sample) in diseased when compared to healthy sites is probably governed by different mechanisms. As LF is contained in only trace amounts in serum (Hetherington et al., 1983), its higher levels in GCF from gingivitis and periodontitis sites cannot be accounted for solely by increased vascular permeability. Migration of PMNs in the gingival crevice and degranulation therein must be the major factor contributing to increased GCF LF levels. As mentioned previously, PMN numbers increase in the gingival crevice with the development of experimental gingivitis (Kowashi et al., 1980; Thurre et al., 1984) and more PMNs are found in periodontitis compared to control sites (Thurre et al., 1984). The higher PMN numbers in the crevice at diseased sites would, therefore, lead to higher LF levels. However, a proportion of LF may be released by the PMN infiltrate present within the gingival tissue underlying the junctional epithelium area and may be passively transferred into the crevice by GCF flow.

The mean absolute amounts of protease inhibitors ( $\alpha$ 2-M,  $\alpha$ 1-AT), and iron-binding proteins (TF, LF) were not found to differ significantly between gingivitis and periodontitis sites. There was, however, a marked tendency for increased

levels at periodontitis over gingivitis sites. In this cross-sectional study probing depths were the differentiating factor between gingivitis and periodontitis sites. Probing depth measurements taken at one point in time should, however, be primarily regarded as history of previous periodontal destruction. In addition, they provide a measure of the inflammatory status of the supracrestal connective tissue due to deeper probe penetration at more inflamed sites (Armitage *et al.*, 1977; Caton *et al.*, 1981). Thus, it should be realised that increased probing depths do not reflect active periodontal destruction and this was considered to have a bearing on the inability to distinguish between gingivitis and periodontitis sites.

We could not detect the same statistically significant differences when results were expressed on a concentration basis. This is a reflection of the higher variability observed when the results are expressed in this way and supports the views of previous investigators that total amounts may be a preferable way of expressing GCF constituent data (section 5.1.2).  $\alpha$ 1-AT exhibited a tendency for increasing values from health to disease (gingivitis and periodontitis), although none of the differences were significant and this was also the case for TF. The increase in TF concentration in periodontitis over healthy sites almost reached significance at 0.025 level ( $p=0.03$ ) and could be associated with increased numbers of

helper T-lymphocytes at periodontitis sites (Lum et al., 1986; Malberg et al., 1992). In contrast, GCF  $\alpha$ 2-M concentration was significantly increased at both gingivitis and periodontitis sites when compared to healthy sites. As mentioned above, in addition to monocytes/macrophages,  $\alpha$ 2-M is produced by gingival fibroblasts (Condacci et al., 1988), one of the most abundant cells of the gingivae, and this could contribute to the significant elevation in GCF  $\alpha$ 2-M concentration in diseased sites. Alternatively, this increase could simply reflect lower variability in GCF  $\alpha$ 2-M concentration in comparison to the other proteins.

A different overall picture, compared to  $\alpha$ 2-M,  $\alpha$ 1-AT and TF was observed when GCF LF results were expressed as concentrations. Although just outwith statistical significance ( $p=0.065$ ), GCF LF concentration demonstrated a clear drop with increasing disease severity, from healthy to gingivitis and periodontitis sites. The different pattern in GCF LF concentration changes with increasing disease severity, reflects the different origin of this protein in GCF when compared to  $\alpha$ 2-M,  $\alpha$ 1-AT and TF. As mentioned above LF is locally produced, solely by degranulating PMNs (Spitznagel et al., 1974). Serum contribution would be minimal due to its very low LF concentration (Hetherington et al., 1983). A closer look at our results reveals, that although both total LF and GCF volume increases in diseased crevices, the latter increases

by approximately a factor of 3 to 4 whereas LF increases only by a factor of 2. This is in contrast to the response of the primarily serum derived TF which has a similar molecular weight (80 kDa). Although different GCF samples were used for the quantification of the two iron-binding proteins, in these studies, it is clear that TF increased proportionately to GCF crevicular fluid volume (both increased by approximately a factor of 2). As LF correlates with PMN numbers in the crevice, it seems that a disproportionate increase in GCF volume compared to PMN numbers, results in dilution of the locally produced LF. This is consistent with the observation of Kowashi et al. (1980) who demonstrated that crevicular PMN numbers in gingival washings increased by a factor of 2 after 21 days of plaque accumulation, during experimental gingivitis. During the same period, GCF flow demonstrated an increase by approximately a factor of 5. PMN migration is regulated by chemotactic factors whereas GCF flow induction, by vasoactive substances and although they could be observed simultaneously they are clearly under different control. Decreasing concentration of gingival crevicular fluid lysosomal enzymes with increasing periodontal disease severity has been reported by some investigators (Lamster et al., 1986), the majority of reports have, however, demonstrated increased levels with the severity of periodontal pathology (Chapter 1; Table 1.5). However, none of the enzymes investigated is contained specifically

in PMNs and other cells of the periodontium could contribute and thus change the overall picture.

Of the GCF proteins examined in this study,  $\alpha$ 2-M is the one studied most extensively in the past. Our results are consistent with previous investigations on the absolute amounts of  $\alpha$ 2-M. Thus, Condacci *et al.* (1982) and Sengupta *et al.* (1988) demonstrated that the absolute amounts of  $\alpha$ 2-M increased at sites with higher degrees of gingival inflammation. When, however, Condacci *et al.* (1982), Skaleric *et al.* (1986) and, in a more recent report, Sibraa *et al.* (1991) expressed their results on a concentration basis, they reported, in contrast to our results, that the specific content of the inhibitor decreased at sites with increased periodontal pathology. Thus, an apparent inconsistency exists, between our study, demonstrating that the specific content of the inhibitor increases at diseased sites, and the above mentioned reports. This could be due to the different methods of GCF collection employed: Condacci *et al.* (1982) collected GCF using filter paper strips for 3 to 5 min; Sibraa *et al.* (1991) used filter paper strips for an undefined period of time (or until 1/3 of the paper strip was visibly wet); finally Skaleric *et al.* (1986) sampled using microcapillary tubes and collected 2 $\mu$ l of GCF. These methods of GCF collection are thought to irritate the gingival tissues (Cimasoni, 1983; Persson & Page, 1990) and could thus increase vascular permeability in the area which could adversely affect GCF  $\alpha$ 2-M

concentration in an uncontrollable manner. In our study we used a less invasive method of GCF collection (paper strips for 30s) that minimises irritation and microleakage of the subepithelial microvasculature and reflects better the GCF constituent concentrations. Our findings are consistent with recent investigations (Giannopoulou *et al.*, 1990; Giannopoulou *et al.*, 1992), where a GCF collection protocol identical to ours was employed. Although these reports refer to experimental gingivitis studies and did not examine sites with increased probing depths, they do confirm that GCF  $\alpha$ 2-M concentration increases at sites with higher degree of gingival inflammation. This demonstrates how easily different methodologies of GCF collection could affect concentrations of its constituents, and again supports the view that reporting results as absolute amounts with standardised sampling times is preferable.

Few investigations exist on GCF  $\alpha$ 1-AT (Ohlsson *et al.*, 1974; Scheinken & Genco, 1977; Tollefsen & Saltvedt, 1980; Asman *et al.*, 1981; Zafiroopoulos *et al.*, 1991; Huynh *et al.*, 1992), TF (Scheinken & Genco, 1977; Asman *et al.*, 1981) and LF (Friedman *et al.*, 1983). As detailed in sections 1.7 and 1.8 these studies differ substantially in methodology to the present study and, therefore, comparison of the results is difficult. Some of these reports are comparative studies between serum and GCF (Scheinken & Genco, 1977; Tollefsen & Saltvedt, 1980; Asman *et al.*, 1981). In other cases either pooled samples were used

(Friedman *et al.*, 1983) or 'crevicular material' was sampled (Ohlsson *et al.*, 1974).

As mentioned previously, the recent reports by Zafiroopoulos *et al.* (1991) and Huynh *et al.* (1992) have focused on the detection of  $\alpha$ 1-AT/elastase complexes and, therefore, are more representative of elastase levels in GCF rather than  $\alpha$ 1-AT (section 5.1.1).

The present cross-sectional studies on GCF  $\alpha$ 2-M,  $\alpha$ 1-AT, TF and LF clearly indicate that their absolute amounts increase at sites with periodontal pathology. However, cross-sectional studies can offer little information on the involvement and dynamic changes of the above GCF constituents during the development of gingival inflammation or probing attachment loss. Longitudinal studies were, thus, conducted to investigate these issues.

### **5.3 Experimental gingivitis study**

#### **5.3.1 Clinical findings**

Whole mouth clinical indices (WPI and WMGI) demonstrated the standard pattern (section 4.3.1). WPI rose first and WMGI lagged behind, indicating that plaque accumulation precedes clinically detectable gingival inflammation. Clinical indices at the teeth sampled for GCF followed the same pattern, demonstrating that these teeth were

representative of the whole mouth. The increase in GCF volume during the trial is consistent with early experimental gingivitis studies designed to investigate the relationship of GCF flow with gingival inflammation (Løe & Holm-Pedersen, 1965; Egelberg & Attström, 1973). After the reinstatement of oral hygiene GCF volumes dropped slightly remaining, however, higher than baseline.

### **5.3.2 Protease inhibitors and iron-binding proteins in gingival crevicular fluid during the development of gingival inflammation**

The sandwich ELISA methodology applied in this study permitted the quantification of protease inhibitors ( $\alpha$ 2-M,  $\alpha$ 1-AT) and iron-binding proteins (TF, LF) in the same GCF sample. This offered the major advantage of allowing to follow the changes of these proteins simultaneously. This served well the aim of this investigation which was to determine the dynamics of protease inhibitors and iron-binding proteins in GCF during the development of experimental gingivitis.

In this study results were reported as absolute amounts in ng/30s sample. The rationale for expressing results of GCF constituents as total amounts (ng/30s) has been already discussed in section 5.1.2. When data from the cross-sectional studies were expressed as absolute amounts and concentrations, a much greater variability was observed

with the latter method. Moreover, GCF volumes collected during the experimental gingivitis trial were in the ultra low range, thus supporting the view that minimal errors in GCF volume assessment could create disproportionate flaws in concentrations of constituents (Lamster *et al.*, 1988a).

$\alpha$ 2-M,  $\alpha$ 1-AT and TF demonstrated a very similar pattern during the experimental gingivitis period reflecting their common origin in GCF (section 4.3.2). Their levels rose in the crevice with the development of experimental gingivitis. This rise could be attributed to increased vascular permeability and is supported by the simultaneous rise of GCF volume. Accumulation of plaque in the gingival crevice stimulates an inflammatory response in the region, which in turn results in increased vascular permeability. In addition, as mentioned previously (section 5.2.2), increased local production could also contribute to the higher amounts of these proteins in the crevice. Thus, monocytes/ macrophages could be responsible for the combined production of  $\alpha$ 2-M,  $\alpha$ 1-AT and TF within the tissues (Stecher & Thorbecke, 1967; Hovi *et al.*, 1977; White *et al.*, 1980; Perlmutter *et al.*, 1985; Bauer *et al.*, 1988). The proportion (and absolute numbers) of activated macrophages in the gingivae increases during the development of experimental gingivitis (Topoll *et al.*, 1989), and this might be of significance for the production of  $\alpha$ 2-M,  $\alpha$ 1-AT and TF. In addition, it has been shown that the percentage of inducer/helper T-lymphocytes increases

with the degree of gingival inflammation in gingival biopsies from periodontitis patients (Malberg *et al.*, 1992) and these cells could contribute to higher amounts of TF in the crevice (Lum *et al.*, 1986).

The dynamics of  $\alpha$ 2-M,  $\alpha$ 1-AT and TF during the healing period, after the reinstatement of oral hygiene measures, reveals a similar pattern still. Despite the drop in MGI and PI (section 4.3.1) the levels of these proteins remain high after the reinstatement of oral hygiene measures (section 4.3.2). This finding suggests that the resolution of gingival inflammation occurs faster at the clinical when compared to the biochemical level as far as  $\alpha$ 2-M,  $\alpha$ 1-AT and TF are concerned. It has recently been shown that clinical improvement precedes the histological resolution of gingival inflammation after 21 days of plaque accumulation (Brex *et al.*, 1988b). In the present study vascular permeability was maintained after the reinstatement of oral hygiene measures, and this is depicted by the fact that GCF volume demonstrated only a slight drop after the reinstatement of oral hygiene. Vascular permeability would have contributed in the maintenance of the levels of  $\alpha$ 2-M,  $\alpha$ 1-AT and TF in the crevice. In addition, resident cells of the periodontium which are induced to produce  $\alpha$ 2-M,  $\alpha$ 1-AT and TF may continue to do so, after the reinstatement of oral hygiene measures. In the case of protease inhibitors lower degradation rates by microorganisms in the crevice (Carlsson *et al.*, 1984) or lower clearance rates (after

binding to proteases) by macrophages/monocytes within the tissues, would have also contributed to the sustained levels of these proteins in the crevice.

GCF LF demonstrated a different overall pattern. The PMN derived LF follows closely the changes in the clinical indices, rising with the development of gingival inflammation and dropping after the reinstatement of oral hygiene measures (section 4.3.2). The rise in GCF LF levels with the development of gingival inflammation could be attributed to increasing PMN emigration and degranulation within the crevice rather than increased exudation from serum. LF drops rapidly after the reinstatement of oral hygiene measures. This probably reflects the removal of chemotactic agents (dental plaque) (Hellden & Lindhe, 1973) which results in a drop in PMN emigration rates and thus LF in the crevice.

The above discussion is based on the pattern of change of the mean response of protease inhibitors and iron-binding proteins in GCF. As in any clinical trial, and more likely so during experimental gingivitis, considerable variability existed among subjects. This is demonstrated by the relatively weak overall significance levels obtained (section 4.3.2) and is more clearly depicted when one examines protease inhibitor and iron-binding protein responses in GCF for each subject individually (Fig. 4.17 to 4.20; section 4.3.2) during the experimental gingivitis

trial. Although most subjects maintained the pattern conveyed by the mean responses it is obvious that some did not. Individual variability is a drawback of the experimental gingivitis model and it was recognised as early as the first experimental gingivitis trial ever reported (Løe et al., 1965). Løe and coworkers (1965) had clearly stated that clinical signs of gingival inflammation are visible only 10 days after cessation of oral hygiene measures in some subjects, whereas others require 15 to 21 days of plaque accumulation until inflammation develops.

Experimental gingivitis studies on GCF constituents are relatively scarce, and this is probably due to the difficulty of obtaining volunteers. In a recent report, which used similar methodology to the present study, GCF  $\alpha$ 2-M and  $\alpha$ 1-AT absolute amounts were shown to increase with the development of experimental gingivitis (Giannopoulou et al., 1992). In addition, elastase, a PMN primary granule constituent, also rose. In a fashion resembling total LF changes in the present study, elastase dropped readily to baseline levels after the reinstatement of oral hygiene measures.  $\alpha$ 2-M and  $\alpha$ 1-AT demonstrated a more gradual drop, but in contrast to the present study, 8 days after the reinstatement of oral hygiene had returned to baseline levels. The authors also reported that GCF volume had similarly dropped to baseline levels 8 days after the reinstatement of oral hygiene measures. Thus, vascular permeability was controlled earlier in the study of

Giannopoulou and coworkers (1992) than in the present report and this may account for the discrepancy observed.

No investigations have been performed on the longitudinal changes of iron-binding proteins in GCF during experimentally induced gingivitis.

Collagen loss is one of the early events during experimental gingivitis (Page & Schroeder, 1976; Payne et al., 1975) and is mediated by the release of proteases in the gingival tissues. As mentioned above, LF is contained within PMN secondary granules (Spitznagel et al., 1974) which also contain lysozyme and collagenase (Baggiolini et al., 1978). Collagenase is a potent proteinase which is released in the crevice simultaneously with LF during PMN degranulation and once activated could contribute to the development of inflammation and cause surrounding tissue damage. In addition, proteases contained within PMN primary granules could be released during adherence of PMNs on non-phagocytosable surfaces, regurgitation during feeding and after cell death. Such enzymes include the Cathepsins (G, B, D) and elastase which could, simultaneously with inducing direct tissue damage, activate secreted PMN and fibroblast type collagenase and result in specific collagen breakdown (chapter 1; Table 1.6). Thus, although LF may have properties beneficial to the host (section 1.8), simultaneous release of other PMN constituents may cause damage to the tissues. Moreover, LF

could directly cause damage to host cells due to its strongly cationic charge.

$\alpha$ 2-M and  $\alpha$ 1-AT are the major protease inhibitors and their presence in the crevice demonstrates their involvement in the regulation of proteases including those referred to above. For damage to occur, a localised imbalance of proteinase/ inhibitor levels is required. Numerous recent reports have demonstrated protease activity in the gingival crevice both during experimental gingivitis and in cross-sectional studies (section 1.6; Table 1.5). However, in many of these reports low molecular weight substrates were used in order to assess protease activity (Eisenhauer et al., 1983; Cox & Eley, 1989a & b; Cox & Eley, 1992; Beighton & Life, 1989; Beighton et al., 1990; Eley and Cox, 1992a & b). Such substrates would not distinguish between free and bound form of the enzyme, as proteases bound to  $\alpha$ 2-M would retain their activity against low molecular weight peptides (Starkey & Barrett, 1977). One would not therefore be able to conclude with certainty, based only on the above quoted reports, that protease/inhibitor imbalance occurs in the crevice. In addition, a recent report has shown that at least during experimental gingivitis no free elastase can be detected in the crevice and that the enzyme is totally inactivated by the combined function of  $\alpha$ 2-M and  $\alpha$ 1-AT (Giannopoulou et al., 1992). Nevertheless, other studies which assessed collagenase activity using native collagen as substrate clearly demonstrate the presence of

active collagenase in the crevice (Kowashi *et al.*, 1980; Larivee *et al.*, 1986). The presence of active collagenase in GCF indicates that a relative imbalance of protease/inhibitor amounts is possible in the crevice. This imbalance could be due to disproportionate release of enzymes by degranulating PMNs, increased degradation of protease inhibitors by bacteria (Carlsson *et al.*, 1984), and finally inactivation of the protease inhibitors by oxidative mechanisms (Matheson *et al.*, 1979; Carp & Janoff, 1980).

The mechanisms leading to protease/inhibitor imbalance and subsequent periodontal damage within the tissues could be different. If inhibitors were bound to proteases within the gingival tissues they should be readily removed (Ohlsson & Laurell, 1976; Feldman *et al.*, 1983) by cells of the monocyte/macrophages lineage. The increase of protease inhibitors in the crevice during the development of experimental gingivitis suggests that their exudation/production rate within the tissues and through to the crevice is higher than their clearance rate after binding to proteases. It seems, therefore, that protease inhibitor levels within the tissues are in excess of proteases during the development of experimental gingivitis. Whether, the ratio of proteases to inhibitors in the tissues, is suitable for optimal protease inhibition is, however, unknown. In addition, PMN derived proteinases may display their destructive function, when the PMN is closely

attached to its substrate (host-tissue surfaces). The close contact would inhibit the accessibility of substances of molecular weight greater than 50 kDa (Wright & Silverstein, 1984).  $\alpha$ 2-M (Mwt: 725 kDa) would thus be completely excluded from the site of activity, whereas even if small amounts of  $\alpha$ 1-AT (Mwt:55 kDa) could reach, they could be readily inactivated by oxidative mechanisms (Wilton 1986; Matheson et al., 1979; Carp & Janoff, 1980). In this context protease inhibitors within the tissues may act as homeostatic regulators, functioning when PMNs release their contents in open interphases, preventing excessive tissue damage.

The present study has provided information on protease inhibitor and iron-binding protein changes in GCF during the development of experimentally induced gingivitis in humans. The following stage in this investigation was to examine the changes of the same proteins during the development of periodontal attachment loss.

#### **5.4 Longitudinal study of periodontitis patients on maintenance care**

##### **5.4.1 Clinical findings**

In the present study, probing measurements of pocket depth and attachment level were performed using an electronic pressure sensitive periodontal probe, the Florida Probe

(Gibbs et al., 1988). The Florida probe was originally described as having a force of 25g (Gibbs et al., 1988) but this was recently adapted to 20g which seemed to be the tolerance limit of the patients. Van der Velden (1979) suggested that a force of 0.75 N is optimal for the detection the most coronal dentogingival fibres when a probe tip diameter of 0.63mm is used. This is equivalent to approximately 30g when using a probe tip of 0.4mm in diameter. However, in a recent study a force of 0.25N was found to be optimal when used with a probe tip of 0.4mm diameter (Karayannis et al., 1992) and this is just slightly higher than the probing force applied in this study when using the Florida probe.

Initial selection of the sites in the present study was performed using a conventional manual probe (PC12). On subsequent assessment with the Florida probe, during the course of the study, probing pocket depths were in general shallower. This is in accordance with the observation of Magnusson et al. (1988b). Both the manual PC12 and Florida probes have a diameter tip of 0.4mm. Deeper probing readings, therefore, when using the conventional PC12 probe compared to the Florida Probe, may reflect that higher probing forces than 20g, were applied when the PC12 probe was used.

Sites selected for this study demonstrated low to moderate MGI scores. This probably reflects the fact that patients

participating had already received an extensive course of periodontal treatment and thus marginal gingival inflammation was controlled. This is also suggested by the low suppuration frequency. The 60% bleeding on probing incidence was somewhat unexpected as more sites bled on probing during the initial conventional pocket charting for the selection of the sites. This together with the deeper probing depths obtained when using the manual probe suggests, as already mentioned above, that manual probing forces greater than 20g were applied during conventional pocket charting. The low PI scores may simply reflect good oral hygiene practices just prior to the oral examination appointment. This is suggested by the persistence of some degree of gingival inflammation in most of the sites selected.

The use of precise electronic measurement, constant force, and an occlusal stent in the present study minimised probing errors (reviewed in section 1.3.4) and thus provided greater chance of detecting significant attachment level changes over a short period of time. Thus, the standard deviation of the difference of repeated measurements reported in this study was 0.423mm. This offered an approximately 50% improvement on standard deviations previously reported in the literature when using a manual probe and either an occlusal stent or the cemento-enamel junction for assessment of probing attachment level (Haffajee *et al.*, 1983b; Badersten *et al.*, 1984).

However, this value is higher than that previously reported by the manufacturers of the Florida probe. Magnusson et al. (1988a) reported 0.33mm standard deviation of attachment level duplicate measurements using the Florida probe 'stent' handpiece. Duplicate measurements were taken on different appointments by a periodontist on subjects with minimal signs of periodontal disease. In the same study and when measurements were taken on subjects with advanced periodontal disease during the same appointment an even lower standard deviation (0.20mm) was reported. This was surprising as lower reproducibility would have been expected at sites with greater periodontal pathology (Haffajee et al., 1983b) and suggests that the lower variability was due to the fact that measurements were taken during the same appointment. In the present study, duplicate measurements were also taken during the same appointment. However, care was taken to change patient position and remove and reapply the stent thus simulating the effect of different appointments. This together with the relative inexperience of the author could have contributed to the slightly higher variability observed between this and the study of Magnusson and coworkers (1988a). Simulation of different appointments was required because the variance of the duplicate attachment level measurements (taken on the same study appointment) would dictate whether the change of attachment level measurements between baseline and three months (that is on different appointments) exceeded measurement error. Underestimation

of the variance of duplicate measurements would result in an increased false positive rate for detection of true attachment level change.

The variability observed between duplicate measurements in the present study is similar to that reported (0.46mm) for another newly developed periodontal probe, the Toronto Probe (Birek *et al.*, 1987; Karim *et al.*, 1990). An electronic probe which detects the cemento-enamel junction has, however, been described to perform substantially better compared to both the Florida or the Toronto probe, rendering a standard deviation of differences of replicate measurements of 0.17mm (Jeffcoat & Reddy, 1991). Nevertheless, detection of the cemento-enamel junction may be difficult if this is obstructed by restorations or calculus, even when this instrument is used.

The tolerance method was used in this study to detect significant attachment level change. The tolerance method offers the advantage of being sensitive to changes occurring over a short period of time (Haffajee *et al.*, 1983b). In addition, it is the only method applicable when the design of the study allows for measurements only at two points in time. As detailed in section 2.2.1.4, the tolerance method takes into account the variance of duplicate measurements in the population, the subject and site. Therefore, the threshold to be exceeded in order for the investigator to declare significant attachment level

change differs among individuals and sites. The lower variability offered by using the Florida Probe compared to the manual probe meant that an attachment level change of as little as 0.9mm could be declared as significant, although most of the attachment level changes in the present study were  $\geq 1.2$ mm. During the three month observation period, 3.9% of the 384 sites demonstrated significant attachment loss, whereas 3.4% demonstrated significant attachment gain. The sites demonstrating attachment level gain represent sites that are in a state of remission whereas those losing attachment were in a state of exacerbation. When a much less stringent criterion of attachment level change of  $\geq 1$ mm was applied 8.8% of the sites demonstrated attachment gain whereas 9.9% demonstrated attachment loss. In a recent 6 month duration longitudinal study Jeffcoat and Reddy (1991) reported approximately 15% incidence of attachment loss when a cumulative sum (cumsum; see section 1.3.5) threshold of change of 0.8mm was used. Despite the fact that the threshold in the present study refers to attachment level change whereas in the above mentioned report represents cumsum threshold, the percentage of sites demonstrating attachment loss is comparable. This suggests that many more sites than originally suspected (Lindhe *et al.*, 1983, Haffajee *et al.*, 1983b, Jenkins *et al.*, 1988) may actually lose attachment over shorter time periods. Reduction of the error of probing attachment level measurement is crucial in order to identify such sites.

With the exception of mean subject probing pocket depth, other baseline clinical parameters failed to predict future attachment loss when the site or subject were used as the experimental unit. Neither could attachment loss sites be differentiated from those that gained attachment within the three month observation period on the basis of their baseline clinical indices. This confirms previous reports which have demonstrated the inability of clinical criteria to predict periodontal disease activity (Haffajee *et al.*, 1983a; Jenkins *et al.*, 1989; Badersten *et al.*, 1990) and which have been reviewed in section 1.2.6. The association of mean probing depth with future attachment loss is weak and the significance level obtained has not been adjusted for the effect of multiple comparisons. It is, however, in agreement with a recent investigation where it has been shown that mean probing depth of a subject could act as a risk indicator of future attachment loss (Haffajee *et al.*, 1991).

In conclusion, the Florida Probe substantially reduced the variability of repeated measurements and thus reduced the threshold of attachment level change required to be exceeded in order to detect significant changes. In agreement with previous reports, baseline clinical indices could not be reliably used to predict future attachment loss.

#### 5.4.2 Gingival crevicular fluid protease inhibitors and iron-binding proteins at periodontally active versus stable sites

This study was conducted to investigate whether protease inhibitor ( $\alpha$ 2-M,  $\alpha$ 1-AT) and iron-binding protein (TF, LF) levels in GCF could predict or diagnose incipient probing attachment loss at specific sites within an individual. No studies have been reported in the literature on the ability of GCF protease inhibitors ( $\alpha$ 2-M,  $\alpha$ 1-AT) and iron-binding proteins (TF and LF) to differentiate between periodontally active versus stable sites.

As mentioned above, the tolerance method was used to identify active/loser sites. The tolerance method provides a stringent criterion for the detection of attachment level change, leading to the detection of a small number of sites demonstrating attachment loss. This is desirable, however, as a high threshold for attachment level change provides a low false positive rate for declaring true attachment loss. On the other hand, sites used as controls (stable) were in every case well below the crude 3 standard deviation threshold (1.2mm). This minimised the chance of false negatives and, therefore, the inclusion of loser sites in the group of stable sites. Stable sites were derived from the same individual as the active sites, thus negating subject effects, and were paired for baseline scores of gingival inflammation and probing depth. Pairing was

successful as no significant differences could be detected for these indices at baseline, between active and stable sites (section 4.4.3). In addition, active and stable sites were similar as far as GCF volume and PI are concerned. In this way the effect of the parameter of interest, namely probing attachment loss, was investigated in relation to GCF protease inhibitor and iron-binding protein levels while controlling for the possible confounding effects of other clinical parameters.

An additional innovation in this study was that GCF constituent levels were reported as absolute amounts and as ng of constituent per  $\mu\text{g}$  albumin. In this way the serum contribution to GCF protease inhibitor and iron-binding protein levels was taken into account. This method offers the advantage of assessing specific amounts of GCF constituents relative to albumin. This avoids reliance on accurate assessment of GCF volume and thus avoids errors associated with its calculation.

GCF protease inhibitor ( $\alpha 2\text{-M}$ ,  $\alpha 1\text{-AT}$ ) and iron-binding protein (TF, LF) levels failed to differentiate between active and stable periodontal sites. This was true both prior to, and after the attachment loss episode. In addition, longitudinal changes in  $\alpha 2\text{-M}$ ,  $\alpha 1\text{-AT}$ , TF and LF during the three month observation period did not differ between active and control sites. Earlier studies presented in this thesis have demonstrated that although

periodontitis sites always have higher total amounts of these proteins than gingivitis sites this difference is not statistically significant. It was then hypothesised that the inability of these proteins to differentiate between gingivitis and periodontitis sites could be due to the inclusion of both active and inactive periodontitis sites. In addition, it has been shown that during the development of gingival inflammation the total amounts of  $\alpha$ 2-M,  $\alpha$ 1-AT, TF and LF increase. The fact that probing attachment loss does not influence the levels of these proteins in the crevice suggests that gingival inflammation may be relatively more important in governing their dynamics. It could, therefore, be suggested that at least as far as the serum derived  $\alpha$ 2-M,  $\alpha$ 1-AT and TF are concerned, vascular permeability due to gingival inflammation is the major factor affecting their GCF levels. Localised changes of  $\alpha$ 2-M,  $\alpha$ 1-AT and TF levels at sites of probing attachment loss may be masked by the influx of these proteins in the area from the circulation. This is suggested by a recent study by Lamster and coworkers (1990) who reported high intraclass correlations of  $\alpha$ 2-M and immunoglobulins in GCF. The authors suggested that although these proteins might be locally produced their levels seem to be highly influenced by systemic factors, most probably their serum concentration. In addition, as mentioned previously, periodontal damage may occur at areas of close contact of the effector cells (PMNs, macrophages) with their substrate (host-tissue structures) (Wright & Silverstein, 1984), and

where naturally occurring inhibitors like  $\alpha 2$ -M and  $\alpha 1$ -AT would not have direct access. In this case, as mentioned previously, protease inhibitors present in the area of periodontal damage would act as homeostatic regulators, and their levels would not necessarily reflect active tissue destruction in the area.

The inability of LF to differentiate stable and active periodontal sites was in a sense unexpected as it has been shown that PMN primary granule constituents are elevated in GCF samples from active periodontitis sites (Lamster et al., 1988b; Huynh et al., 1992; Palcanis et al., 1992). These authors suggested that exuberant PMN activity is related to probing attachment loss. Our findings suggest that this is not the case when PMN secondary granule constituents are examined. Perhaps primary granule release and their array of different proteases may be responsible for direct tissue damage. In addition, the requirement of such proteases for the activation of PMN secondary granule collagenase suggests that PMN secondary granule release alone would not be sufficient to cause periodontal damage. Thus PMN secondary granule release may be related to the protective PMN function whereas PMN primary granule release may be indicative of PMN related tissue destruction (Wilton, 1986).

The inability of  $\alpha 2$ -M,  $\alpha 1$ -AT, TF and LF to effectively differentiate active from stable periodontal sites was

demonstrated by the low positive and negative predictive values of a combined test based on the recall (three month) appointment data (section 4.4.3). The sensitivity of the test was also very low (only 28%) demonstrating a very high rate of false negatives. The relatively higher specificity value is probably due to the low overall number of positive tests and is probably an artifact.

Despite the inability of  $\alpha 2$ -M,  $\alpha 1$ -AT, TF and LF to differentiate active and stable sites in the present study, it should be realised, that longitudinal studies of periodontal disease, aiming to identify attachment loss, rely upon repeated measurements (usually of probing attachment level) spaced at appropriate time intervals. Periodontal destruction may, however, occur during exacerbation episodes of small duration, accompanied by changes in protease inhibitor and iron-binding protein levels. The duration of such episodes may be much shorter than the interval at which attachment level measurements were taken in the present study and, therefore, the disease activity episode could have occurred at any point in time during the three month observation period. In this case any alterations in the local GCF  $\alpha 2$ -M,  $\alpha 1$ -AT, TF and LF levels may have been missed. This is more likely for LF which is a local, PMN derived, acute phase reactant and whose changes have been shown to resolve readily during the healing phase of experimental gingivitis (section 4.3.2). In addition, the relatively low number of sites

demonstrating attachment level change compromises the possibility to detect significant differences.

## 5.5 Future suggestions

The above studies have provided information on the changes of GCF protease inhibitors and iron-binding proteins in relation to periodontal disease status. However, studies aiming to identify mechanisms controlling their dynamics in the crevice are required.

Recent studies investigated the regulation of TIMP production by cytokines or mitogens like concanavalin A (Overall et al., 1989; Overall et al., 1991). The production of  $\alpha$ 2-M by gingival fibroblasts has also been demonstrated (Condacci et al., 1988). It would be of interest to investigate whether  $\alpha$ 2-M,  $\alpha$ 1-AT and TF production by resident cells of the periodontium is under similar control.

Investigating the relative contribution of primary and secondary granules towards crevicular protease activity *in vivo* would help clarify the protective and/or destructive function of periodontal PMNs. It has been suggested that PMN primary granule release may be associated with periodontal damage whereas secondary granule release may be related to the protective PMN function (Wilton, 1986). Although some initial attempts have been made to identify

stimuli from the gingival crevice leading to PMN primary or secondary granule release further research may provide information on the conditions leading to preferential release of each type of granule. The use of specific markers like PMN elastase and LF for primary and secondary granule release respectively, would facilitate this investigation. Moreover, immunolocalisation of LF and elastase in gingival biopsies may provide information on the circumstances under which primary and secondary granule release occurs within the gingival tissues.

Immunolocalisation could in addition be used to investigate the distribution of protease inhibitors and proteases in the gingival tissues. This would clarify whether protease release and periodontal damage actually occurs at closed interphases where naturally occurring protease inhibitors do not have direct access.

The Florida Periodontal probe is a powerful tool in the hands of the researching clinician. Studies of periodontal disease progression using instruments with higher accuracy than the conventional periodontal probe will allow for lower thresholds to be used for declaring significant attachment level change. Such studies are already underway (Jeffcoat & Reddy, 1991) and will provide a more precise picture of the pattern of periodontal disease progression to that obtained when conventional probing devices were used.

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## LIST OF PUBLICATIONS

The following publications include material presented as part of this thesis:

Adonogianaki, E., Mooney J. and Kinane D.F. (1992) The ability of gingival crevicular fluid acute phase proteins to distinguish healthy, gingivitis and periodontitis sites. *Journal of Clinical Periodontology*, 19, 98-102.

Adonogianaki, E., Moughal, N.A. and Kinane D.F. (1992) Lactoferrin in the gingival crevice as a marker of polymorphonuclear leucocytes in periodontal diseases. *Journal of Clinical Periodontology*, 19, in press.

Kinane, D.F., Adonogianaki, E., Moughal, N., Winstanley, F.P. Mooney, J. and Thornhill, M.H. (1992) Immunocytochemical characterization of cellular infiltrate, related endothelial changes and determination of GCF acute-phase proteins during experimental gingivitis. *Journal of Periodontal Research*, 26, 286-288.

The following publications are indirectly related to this thesis:

Kinane, D.F., Winstanley, F.P., Adonogianaki, E. and Moughal, N.A. (1992) Bioassay of interleukin 1 (IL-1) in human gingival crevicular fluid during experimental gingivitis. *Archives of Oral Biology*, 37, 153-156.

Moughal, N.A., Adonogianaki, E. and Kinane, D.F. (1992) Langerhans cell dynamics in human gingiva during experimentally induced inflammation. *Journal de Biologie Buccale*, 20, in press.

Moughal, N.A., Adonogianaki, E., Thornhill, M.H. and Kinane D.F. (1992) Endothelial cell leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression in gingival tissue during health and experimentally induced gingivitis. *Journal of Periodontal Research*, 27, in press.